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Scribble Regulates Morphogenesis and Cell Fate Decisions During Mammary Gland Development and Neoplasia

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Abstract of the Dissertation

Scribble Regulates Morphogenesis and Cell Fate Decisions During Mammary Gland Development and Neoplasia

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A unique aspect of epithelial cells in glandular tissues is their definitive vectoral organization or polarity. Studies in model organisms such as worms and flies have identified several genes that regulate the establishment and maintenance of cell polarity in epithelia. Among the genes, the polarity gene Scribble not only regulates epithelial architecture but also regulates tissue growth in *Drosophila*. It is thought that polarity genes such as Scribble are regulators of epithelial morphogenesis in mammals. However, what role Scribble plays during mammalian epithelial morphogenesis and tumorigenesis is largely unexplored. In this thesis, I explore Scribble's role in regulating morphogenesis, cell fate and oncogene-induced tumorigenesis in mammary epithelia.

To explore the role of Scribble during mammary acinar morphogenesis we used two approaches one involving a Scribble RNA-interference (RNAi) and another using a Scribble mutant that inactivates its function by disrupting its membrane localization. Reduced Scribble expression or mislocalization blocked normal cell polarization, apoptosis and lumen formation during morphogenesis of mammary epithelial cells in three-dimensional, organotypic culture. Scribble loss

or mislocalization also blocked apoptosis induced by the c-myc oncogene both in culture and *in vivo*. Furthermore, we found that Scribble expression is reduced or mislocalized in human breast cancer cell lines as well as in primary human breast tumors. These data suggest that Scribble expression levels and localization are critical during normal morphogenesis and during transformation of mammary epithelial cells.

A second aspect of this thesis explored an observation made in normal primary human breast samples where I observed that Scribble expression was restricted to luminal, but not basal, epithelial cells in breast ducts. To determine if there is a causal relationship between Scribble expression and the differentiation of luminal epithelial cell type I downregulated Scribble expression using Scribble RNAi in a multipotent mouse mammary epithelial cell line. Loss of Scribble expression blocked luminal epithelial differentiation. This correlated with an increase in cells with progenitor-like properties as evidenced by increase in the number of cells expressing progenitor markers. Loss of Scribble also increased numbers of secondary mammospheres in culture and improved engraftment efficiency into the cleared mammary fat pad *in vivo*.

The mammary ductal outgrowths derived from cells lacking expression of Scribble, however, did not have the normal bilayered organization and developed ducts that lacked an epithelial layer. This lack of luminal differentiation related to the inability of progenitor cells to undergo asymmetric cell division and activation of Notch signaling pathway. Furthermore, Scribble loss accelerated the tumor formation in breast cancer cell lines and induces development of poorly differentiation tumors in response to transformation by the oncogene ErbB2 demonstrating a role for Scribble during tumor differentiation.

In summary I establish that expression of the polarity regulator, Scribble, plays a tumor suppressive role both via its regulation of apoptotic programs as well as its control of the differentiation status of mammary epithelial cells. I suggest that these two aspects are relevant to different stages of cancer. The regulation of apoptosis being relevant to differentiated epithelia and the regulation of luminal differentiation to tumor initiation and progression. These

results identify Scribble as a novel cell intrinsic regulator of cell survival and differentiation in mammary epithelial cells. It also suggests that Scribble expression may play a central role in initiating and driving the poorly differentiated, basal-like, breast tumors a particularly aggressive and morbid form of breast cancer.

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List of Abbreviations

2D	Two dimensional
3D	Three-dimensional
4-OHT	4-hydroxytamoxifen
ABCG2	ATP-Binding Cassette Transporter G2
ALDH	Aldehyde Dehydrogenase
aPKC	atypical Protein Kinase C
BCRP1	Breast Cancer Related Protein 1
BrdU	Bromo –deoxy Uridine
BSA	Bovine Serum Albuimn
CBF-1/RBP-J	C-promoter-Binding Factor 1 /Recombination signal Binding Protein for immunoglobulin kappa J region
CD	Comma-1D
CK	Cytokeratin
Crb3	Crumbs 3
CRIB	semi-cdc42-Rac1 Interactive Binding
DAPI	4',6-diamidino-2-phenylindole
DLG	Discs Large
EHS	Engelbroth-Holm-Swarm
EMT	Epithelial Mesenchymal Transition
ENU	N-ethyl-N-nitrosourea
ER	Estrogen receptor
FACS	Fluorescence Activated Cell Sorting
FERM	4.1-exrin-radixin-myosin
GEF	Guanine nucleotide Exchange Factor
GMC	Ganglion Mother Cell
Gukh	Guanylate kinase holder
H-SIL	High grade-Squamous Intraepithelial Lesions
HPV	Human Papilloma Virus
JAM-1	Junctional Adhesion Molecule-1
JNK	c-Jun N-terminal Kinase

L-SIL	Low grade-Squamous Intraepithelial Lesions
LAP	LRR and PDZ
LGL	Lethal Giant larvae
LRR	Leucine Rich Repeats
LTR	Long Terminal Repeat
MCP	Mitotic Cell Polarity
MDCK	Madine-Darby Canine Kidney
MMTV	Mouse Mammary Tumor Virus
MRU	Mammary gland Repopulating Unit
NICD	Notch Intracellular Domain
PALS	Protein associated with Lin-7
PATJ	Protein Associated with Tight Junction
Par1	Partitioning defective 1
Par2	Partitioning defective 2
Par3	Partitioning defective 3
Par4	Partitioning defective 4
Par5	Partitioning defective 5
Par6	Partitioning defective 6
PB1	PhoxBem1
PBS	Phosphate Buffered Saline
PDZ	Postsynaptic density-95, Discs large, Zona occludins 1
pERM	phosphorylated-Ezrin/Radixin/Moesin
Pins	Partner of inscutable
PKC-3	Protein Kinase C-3
RNAi	RNA intereference
shRNA	short hairpin RNA
SMA	Smooth Muscle Actin
Scrib	Scribble
TCF/LEF	T Cell Factor/Lymphoid Enhancer Factor
TDLU	Terminal Ductal Lobular Unit
TEB	Terminal End Bud

TGFα	Transforming Growth Factor alpha
TGFβ	Transforming Growth Factor beta
TSHR	Thyrotropin Stimulated Hormone Receptor
UTR	Untranslated Region
VANGL2	Van Goh Like 2
WAP	Whey Acidic Protein
Zeb1	Zinc finger homeodomain Enhancer Binding 1
ZO-1	Zona Occludins 1
ZO-2	Zona Occludins 2

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Chapter 1

Introduction

Many embryonic and adult cell types including neurons, T cells, strereocilliary bundles in the cochlea and epithelial cells exhibit a polarized organization or asymmetry that is critical for its function [1]. While the regulators of apical-basal polarity in epithelial tissues have been well studied in model organisms such as *C. elegans* and *Drosophila* less is known about their roles in mammalian epithelia [2]. The columnar epithelial cells lining some glandular organs such as the breast are exemplified by specialized membrane surfaces. In particular they have a molecularly distinct apical membrane that contacts the lumen and basolateral membranes that are in contact with neighboring cells and the basement membrane [3,4]. Normal secretory epithelial tissues, such as the breast, are comprised of a duct lined by a contiguous monolayer of columnar cells. The apical membrane facing the lumen is specialized to secrete into the lumen. To form these specialized surfaces the cells rely on the polarized distribution of organelles and membrane domains.

An understanding of the normal development of epithelial polarity as it relates to tissue architecture is critical to a deeper understanding of neoplastic processes. In the early stages of hyperplasia epithelial cells become multilayered with many cells losing contact with the lumen and hence losing their apical polarity and thus their distinctive membrane organization. The mechanisms by which the cells loose their ability to maintain a single layer are not well understood. In the following sections I will summarize the current understanding of the molecular processes regulating the epithelial polarity as well as systems in which we can experimentally model polarity. Finally, I will discuss the cellular hierarchy and epithelial differentiation processes that generate the adult mammary gland.

Diversity of Cellular Polarity

All organisms, from single celled organisms such as bacteria and yeast to complex multicellular invertebrates and mammals, maintain molecular and structural asymmetries necessary to perform diverse cellular functions [1]. For example, in unicellular organisms these asymmetries are important for the directional movement of bacteria and to derive daughter cells during yeast division. The increase in the cellular diversity of multicellular organisms utilizes a greater array of cellular asymmetries to give rise to the specialized functions within a tissue. For example, neurons require polarity pathways for the directional movement of signals down an axon as well as for the maintenance of a morphologically distinct cell shape. In epithelial tissues, whose function is directional secretion and absorption, polarity establishes the asymmetric distribution of endocytic and exocytic vesicles. In each of the above cases establishing and maintaining polarized structures is critical to the physiology of the cell or tissue. Intriguingly, a few highly conserved protein complexes regulate all these diverse polarized processes.

Molecular Regulators of Polarity

Epithelia are maintained in a highly organized manner by physical junctions such as gap junctions, desmosomes, adherens junctions, and tight junctions between adjacent cells [5]. These cell-cell contacts are vital to the structure and function of epithelial organs. They are involved in various functions such as cell cohesion, intercellular communication, as a physical diffusion barrier for membrane proteins and as a permeability barrier between the luminal and basolateral spaces [6-8]. In mammalian epithelia the most apical of the junction complexes are belt-like tight junctions composed primarily of claudins. Basal to the tight junctions are the belt-like adherens junctions that are composed of cadherin molecules. Both tight and adherens junctions when established are tethered to the cytoskeleton by large intracellular complexes. These junctions play essential roles during epithelial morphogenesis and differentiation. In fact, genetic screens in model organisms aimed at understanding the molecular

regulators of cell-cell junctions and morphogenesis have identified three genetic/biochemical complexes that regulate epithelial polarity, proper localization of junctions and epithelial morphogenesis. These molecular regulators can be broadly grouped into three conserved complexes: Crumbs complex, Par complex and the Scribble complex [9]. In the sections below, I will provide a brief summary of these three polarity protein complexes and their interactions with each other.

Crumbs Complex

The *crumbs* gene was identified in *Drosophila* as a mutation that resulted in an absent/dispersed cuticle [10,11]. The larvael cuticle is secreted by the underlying epithelium hence this phenotype suggested aberrant epithelial secretion. Overexpression of *crumbs* resulted in the expansion of the apical membrane demonstrating a role for *crumbs* in apical membrane specification [12]. Subsequent to its identification in *Drosophila,* three human homologs were identified as well as homologs in other model organisms (*C. elegans*, *D. rario* and *M. musculus*). All of the Crumbs homologs and isoforms have a highly conserved cytoplasmic tail with variable extracellular domains [13-16].

dCrumbs is an apical transmembrane protein with a large extracellular domain, consisting of 28 epidermal growth factor (EGF)-like repeats, three laminin-G-like repeats, a short cytoplasmic tail with 4.1-exrin-radixin-myosin (FERM) and Postsynaptic density-95, Discs Large, Zona Occludins-1 (PDZ) binding motifs as well as an essential atypical Protein Kinase C (aPKC) phosphorylation site [13,17]. The cytoplasmic domain of Crumbs interacts with several signaling scaffold proteins. The multiprotein interaction is highly conserved suggesting that the Crumbs complex regulates apical polarity by a conserved mechanism. Indeed in mammals the Crumbs complex includes Protein Associated with Lin Seven (PALS); Protein Associated with Tight Junction (PATJ), Partitioning defective-6 (Par6) and aPKC [18-21]. Through its interaction with PALS1 and Crumbs, Par6 recruits aPKC activity to the Crumbs complex [17,21-23]. The interaction of Crumbs with aPKC results in Crumbs

phosphorylation required for the apical restriction of Crumbs [17]. Thus the highly conserved Crumbs complex has been defined as the regulator of apical membrane domains.

Crumbs Complex and Cancer

Deregulation of the Crumbs complex may play an important role in cancer progression. It was found that Crumbs 3 (Crb3) was lost in spontaneously transformed clones of immortalized baby mouse kidney epithelial cells. Re-expression of Crb3 in these cells resulted in decreased metastatic potential of the tumors suggesting that Crb3 loss plays a causal role in transforming these cells [24]. Transcriptional regulators of epithelial-mesenchymal transition (EMT) such as Zinc finger homeodomain enhancer binding 1 (ZEB1) and Snail, and inducers of EMT such as Transforming growth factor β (TGFβ) repress expression of Crb3 [25,26]. Since EMT is frequently associated with metastatic progression, these results suggest that loss of Crb3 not only regulates initiation of tumorigenesis but may also regulate metastatic progression.

Par Complex

The members of the Par complex were discovered in a screen to identify *C. elegans* mutants that exhibit defects during embryonic cell division. The asymmetric positioning of proteins as well as the asymmetric size of the mitotic spindle regulates the first division of the fertilized *C. elegans* embryo. This results in a large anterior daughter cell and a smaller posterior sister cell [27]. A mutagenesis screen identified seven genes that were critical to the regulation of the partitioning process and includes Partitioning defective genes 1 through 6 and Protein Kinase C-3 (PKC3) [28]. During cell division Partitioning defective-3 (Par3), Par6 and PKC3 proteins co-localize to the anterior cortex while Partitioning defective-1 (Par1) and Partitioning defective-2 (Par2) proteins co-localize to the posterior cortex. Partitioning defective-4 (Par4) and Partitioning defective-5 (Par5) are expressed uniformly throughout the cell [29]. In *Drosophila* neuroblasts and epithelium it was demonstrated that Par3, Par6 and aPKC

(homolog of PKC3) form a physical complex necessary for their co-restriction to the apical pole [30,31]. In *C. elegans* and *Drosophila* the highly coordinated regulation of the Par proteins is critical for asymmetric cell division, anterior-posterior axis determinntion and apical-basal polarity.

The partitioning defective genes Par1,3,4,5 and 6 are conserved in vertebrates, and regulate the establishment of apical-basal polarity in mammalian epithelial cells [9,32-35]. The mature Par complex consists of Par3/Par6 and aPKC and is localized to the apical lateral border at tight junctions in polarized mammalian epithelial cells [36,37]. The nucleation of the mature Par complex begins when Par3 is recruited to immature adherens contacts initiated between adjacent epithelial cells. Par-3 is recruited via its PDZ domains to Junctional adhesion molecule-1 (JAM-1) and nectin 1/3 [38,39]. The Par3-JAM-1 interaction is critical for the recruitment of Par6 (comprised of a PDZ, PhoxBem1 (PB1) and semi-cdc42-Rac1 interactive binding (CRIB) domains) [40]. The restriction of the Par complex to the apical-lateral border is dependant on Par3 phosphorylation [34,41,42]. Briefly, Par6 recruits aPKC and the small GTPases Rac1 and cdc42 to Par3 [34]. The binding of Cdc42 to the immature Par complex induces the catalytic activity of aPKC that in turn phosphorylates Par3 [9,43]. The mature complex can now interact with integral tight junction proteins such as Claudins and Occludins to establish mature tight junctions tethered to the cytoskeleton [40]. Thus ordered recruitment of the members of the Par complex is critical for epithelial polarization and functional junction assembly [44].

The proper localization of the Par complex is essential for the correct localization of the basolateral Scribble complex and apical restriction of the Crumbs complex [45]. In addition to the previously discussed phosphorylation of Crumbs by aPKC, the kinase activity of aPKC is also necessary for phosphorylation of lethal giant larvae (Lgl) [46-48]. Phosphorylation of Lgl by aPKC results in a conformational change releasing it from the apical cortex [49]. Lgl release from the complex is necessary for Par6/aPKC to bind to Par3 thus forming the mature Par3/Par-6/aPKC complex [48,50,51]. These data suggest

that regulation of the Par complex is highly dynamic and regulates the localization of the other polarity complexes through complex interactions.

Par complex and cancer

The Par complex is not only dynamic during development but oncogenic cues can also directly regulate the composition of the Par complex in a variety of epithelial tumors. TGFβ activation results in Par6 phosphorylation and subsequent RhoA degradation to induce EMT [52-54]. We demonstrated that activated ErbB2 interacts with Par6/aPKC, and disrupts the Par3/Par6/aPKC complex during the transformation of mammary epithelial cells [55]. Our lab has also shown that Par6b is overexpressed in early stages of human breast cancer [56]. In hepatocellular carcinomas the expression of a Par3 isoform containing the domain required for Par3-aPKC interaction is reduced [57]. Furthermore, aPKC is overexpressed in a number of epithelial cancers including ovarian, breast and lung cancers [58-62]. Thus, disassembly of the Par complex as well as overexpression of members of the Par complex may play important roles in regulating properties associated with the initiation and progression of cancer.

Scribble Complex

Scribble complex as a novel class of tumor suppressors

The *lgl2 (lethal (2) giant larvae)* allele was the first tumor suppressor gene discovered in Drosophila [63]. The ethal giant larvae phenotype consists of extensively overgrown and disrupted imaginal discs, developing brains that fail to undergo metamorphosis and defective germline survival [64,65]. In subsequent *Drosophila* screens the Discs large (*dlg)* and Scribble (*scrib*) genes were both found to phenocopy *lgl* in larvae in that they induced significant disruption of cell architecture and an overgrowth phenotype resulting in lethality [66,67]. Genetic mapping, complementation and sub-cellular localization analyses in *Drosophila* suggested genetic and direct interactions between these three genes and thus established *dlg, lgl and Scrib* as the Scribble complex [67]. The members of the

Scribble complex constitute a novel class of tumor suppressors because they not only resulted in tissue overgrowth but also disrupted epithelial architecture, demonstrating that regulators of cellular organization and tissue architecture can function as tumor suppressors.

In addition to the massive tissue overgrowth, loss of *scrib*, *dlg* or *lgl* cooperated with oncogene activation in *Drosophila* to induce invasive growths. Larvael eye imaginal discs with clonal loss of *scrib* were disorganized and remained undifferentiated. However the clones fail to overgrow and by adulthood were cleared by c-Jun N-terminal Kinase (JNK) mediated apoptosis [68]. Overexpression of oncogenic Ras or Notch in wild type eye discs resulted in moderate tissue overgrowth. In contrast, in combination wit*h lgl, dlg* or *scrib* loss dramatic overgrowth was observed [68-70]. In addition, active Ras cooperates with *scrib* mutants resulting in the invasion and migration of cells from the primary overgrowth in the eye disc to sites throughout the larvae [69]. The cooperation between *scrib* mutants and Ras is dependant on the MAPK signaling downstream of Ras and Jnk activation [68,70]. However, *scrib* mutants did not cooperate with other growth/oncogenic signals such as PI3K, Wingless, Decapentaplegic and hedghog signaling [68]. These data demonstrate that in *Drosophila* epithelial tissues Scribble functions as a tumor suppressor and that Scribble loss in cooperation with Ras/MAPK activation results in the massive overgrowth and invasion of transformed cells.

Scribble as a regulator of adherens junctions and apical restriction

The *scrib* gene was first identified in a *Drosopholia* screen for maternal effect mutants that result in disrupted epithelial morphogenesis [67]. It was demonstrated that maternal Scribble is critical for proper deposition of a smooth larvael cuticle by the underlying epithelia. The *scrib* mutant defects became progressively worse over time with an incomplete layer of epithelial cells separated from the underlying surface because of defective epithelial adhesion. This demonstrates that Scribble regulates epithelial morphogenesis and polarized secretion.

In *Drosophila,* Scribble localized to the septate junctions (the functional analogue of vertebrate tight junctions) basal to adherens junctions. In *scrib* mutants, adherens junctions were not restricted to the apical-lateral border but extended throughout the basolateral membranes and Crumbs, an apical marker, was ectopically expressed throughout the membrane [67]. Hence, Scribble's main role in *Drosophila* epithelia is to restrict the borders of the apical membrane.

Scribble and asymmetric cell division

The *lgl* and *scrib Drosophila* mutants displayed phenotypes in non-epithelial tissues as well. *Drosophila* neuroblasts (neural progenitors) undergo asymmetric mitotic divisions (a form of polarity). This division result in a large apical neuroblast and a daughter cell that differentiates into a small basally located ganglion mother cells (GMC) [71,72]. During prophase and metaphase Dlg, Lgl and Scribble proteins localized to the apical cortex of the neuroblast. However, from anaphase through interphase they are uniformly cortical [73-75]. Other apically enriched proteins include aPKC, Inscutable, Partner of inscrutable (Pins), Par3, Par6 and Gαi [76-78]. Proteins restricted to the basal cortex include the coiled-coil protein Miranda, the transcription factor Prosero, and Numb (the negative regulator of Notch signaling) [79-81]. The enriched apical and basal proteins segregate with their respective cells following cell division and are critical to their future differentiation status. For example, Notch is an important self-renewal signal in *Drosophila* neuroblasts [82]. Hence, its inhibition by Numb is necessary for subsequent GMC differentiation.

In *lgl, dlg* and *scrib* mutants the localization of apical proteins is not altered. However, basal proteins, including Numb, are mislocalized [73,80]. Numb mislocalization blocks neuroblast differentiation and results in an expansion of neuroblasts. Phosphorylation of Numb by aPKC releases its inhibition of Notch driving self-renewal of neuroblasts [80,83]. Expressing dominant negative aPKC in mutant Scribble complex flies rescues the expansion of neuroblasts [76]. Thus Scribble complex proteins are critical for asymmetric division and maintenance of progenitor cells in *Drosophila*.

Scribble structure-function and interacting partners

Scribble functions as a scaffolding protein and its domain structure is highly conserved between *Drosophila* and vertebrates [67,84]. Scribble is a Leucine Rich Repeat (LRR) and PDZ domain protein (or LAP). The LAP family of proteins is only found in Eukaryotes with four known members including Scribble, LANO, Erbin and Densin-180 [85,86]. Scribble contains 16 LRR domains and 4 PDZ domains with two LAP specific domains of unknown function. To determine which domains were critical for Scribble function, structure-function analyses have been carried out in *Drosophila* and mammalian systems. In *Drosophila* epithelia the LRR domain is required for restriction of Scribble to the cell membrane and septate junctions. Mislocalized scribble either due to an LRR deletion or an LRR point mutation failed to rescue the *scrib* phenotype and forced membrane localization of the PDZ domains was not sufficient to rescue polarity or overgrowth defects. However, expression of the LRR domains alone rescued the polarity defect but not the overgrowth defect [87]. Thus the LRR domain is critical for Scribble function at the membrane. In addition in metaphase neuroblasts, the LRR and PDZ2 domains are important for the apical enrichment of Scribble though the LRR domain was sufficient for cortical Scribble localization during interphase [88]. In mammalian cells, a point mutation in LRR domains or its deletion results in a partial Scribble mislocalization from the membrane and is sufficient to phenocopy the migratory defect observed with Scribble loss [89]. In addition the LRR domains and PDZ1 are critical for Scribble overexpression to suppress proliferation in mammalian cells [90]. These data provides some insight into the critical regions of Scribble for it to establish polarity and to assert growth control.

Knowledge of the biochemical interacting partners of Scribble and understanding how they interact has given some but limited insight into the mechanisms through which Scribble exerts control over cell growth and polarity. The Rac Guanine nucleotide exchange factor (GEF) β–Pix has been demonstrated to interact with Scribble via PDZ1 and 2 [91]. A Scribble PDZ

domain interacts with the TVL sequence in the carboxy terminus of the G-protein coupled Thyrotropin Stimulated Hormone Receptor (TSHR). This interaction is important for downregulating activated TSHR receptor in a β-Pix dependant manner [92]. Van gogh-like 2 (Vangl2) and lgl2, two proteins involved in polarity regulation, also interact with Scribble via the LRR domains and PDZ2/3 respectively [93]. Guanylate kinase (Guk) holder (Gukh), a Dlg binding partner, interacts with Scribble via PDZ2 [94]. An interaction has been mapped between the PDZ3 and 4 domains of Scribble and the tight junction associated protein ZO-2 [95].This interaction is important as it suggests a means by which Scribble and Dlg could interact to form a physical complex. These interactions highlight the ability of Scribble to function as a scaffold interacting with proteins involved in diverse cellular functions as well as junction complexes. However, neither the stoichiometry of these interactions nor the functions of these protein complexes are well understood.

Another level of regulation that may impact Scribble function is the control over and expression of alternatively spliced isoforms. In *Drosophila* it is known that several splice variants are expressed in a tissue specific manner [96]. However, though the existence splicing variants in mouse has been reported, their function is not known [97].

Scribble complex in mammalian systems

Members of the Scribble complex are highly conserved in vertebrates. The mammalian homologs of *dlg*, *lgl* and *scrib* can rescue the polarity, overgrowth and neoplastic phenotypes observed in *lgl*, *dlg* and *scrib* mutant flies demonstrating that the protein function is conserved during evolution [98-100].

Similar to that which was observed in its *Drosophila* mutants, Scribble regulates different forms of polarity in mammalian systems. In a polarized epithelial culture model using Madine Darby Canine Kidney (MDCK) cells, cells lacking Scribble grown at low density have defective adherens junctions and cell-cell adhesion demonstrating a role for Scribble in apical-basal polarity [89]. In mammary epithelial cells and in T cells, Scribble loss enhances migration

demonstrating a role for Scribble in migratory polarity [101,102]. In addition, Scribble is required to anchor Crtram, a molecule necessary for T cell polarity in late immune responses required for adaptive immunity [103]. The above data demonstrates that Scribble is necessary for various polarity dependant functions in a mammalian context.

In order to better understand the role of Scribble in mammalian epithelia it is necessary to have a mutant Scribble or knockout mouse models. To date no Scribble knockout models have been reported. However, two *scrib* mutant mouse models are available: the rumpelstilzchen (rumz) and the circletail mice. The *scrib*^{rumz} allele was generated in an ENU mutagenesis screen focused on finding recessive mutations with defective cortical brain development. The rumz mutation is a missense mutation (isoleucine 285 to lysine, I285K) in a LRR domain that results in reduced Scribble protein levels and mislocalization from the membrane [101,104]. The circletail phenotype was observed while generating a mouse line expressing the tyrosinase minigene, [105]. The circletail mutation is not causally related to the transgene and was found to be a single nucleotide insertion in Scribble at codon 947 that results in a truncated protein containing 971 amino acids and is missing the two terminal PDZ domains [84]. The mutation results in a low penetrance semidominant “circletail” (a kinked tail) phenotype in heterozygotes. Homozygous circletail mice as well as the rumz mouse exhibit severe neural tube defects (Closure 1,craniorachischsisis) [84,97,104,105]. In addition, the rumz mouse has a defect in embryonic wound healing, a process dependent on polarized migration, was demonstrated in the skin from rumz mice [101]. Despite these severe phenotypes, neither mutation results in a complete loss of protein. Generation of a conditional knockout mouse model will be necessary to ask important and interesting questions about the role Scribble plays during development.

Scribble and Cancer

Accumulating evidence is beginning to clarify the importance of the Scribble complex in human malignancies. Implications for the role of scribble expression

have been made in cervical, colon and breast cancers [89,106,107]. The E6 protein of high risk Human Papilloma virus (HPV) strains (16 and 18) targets the polarity proteins Scribble and Dlg for proteasomal-mediated degradation by interacting with their PDZ domains and allowing E6AP to ubiquitinate Scribble [108,109]. Furthermore, in HPV driven cervical tumors, loss of Scribble correlates with tumor progression with a modest loss in low-grade squamous intraepithelial lesions (L-SIL) and a significant loss in high-grade squamous intraepithelial lesions (H-SIL) [106]. These data suggest that viral proteins have evolved a way to degrade Scribble as well as other PDZ containing proteins that may play a role in the transformation of epithelial cells [110].

Other mechanisms may play a role in regulating Scribble function in tumors independent of viral proteins. Dlg and Scribble expression is reduced in poorly differentiated adencarcinomas or increased but mislocalized in colorectal adenomas [107,111]. Reduced Scribble expression was observed in 81% of lobular carcinoma *in situ*, a subtype of breast cancer marked by reduced E-cadherin expression [89]. Re-expression of E-cadherin by blocking hypermethylation of its promoter re-localizes Scribble to the membrane in the breast cancer cell line, MDA-MB-231 [89]. In a transgenic model of ocular adenocarcinoma, Scribble, Dlg and Lgl displayed reduced expression patterns and mislocalization [112]. Taken together, these data suggest that tumors may have multiple mechanisms to alter Scribble expression and function.

The other members of the scribble complex have also been implicated in neoplasia. Particularly, Dlg3 and 4 expression is lost in esophageal and gastric cancer [113,114]. Lgl2 expression is lower in many breast, prostate, lung, ovarian cancers as well as melanomas and lymph node positive endometrial cancers [98,115]. This demonstrates that dysregulation of the Scribble complex is observed in neoplasia. However, no causative role has been attributed to Scribble complex deregulation.

In summary, polarity proteins play a role in regulating a plethora of seemingly diverse biological phenomena including the establishment and maintenance of differentiated epithelia. In the histological scoring of neoplasias defective polarity

is critical in the assessment of tumor grade. This suggests that regulators of polarity may play a critical role in tumor formation. This notion is supported by evidence from *Drosophila* as well as correlative data from clinical samples. However, much work is still necessary to discover if indeed polarity plays a role in human cancers and the molecular mechanisms through which polarity is disturbed in neoplasia.

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Modeling Polarity and Morphogenesis

Although many biochemical interactions have been studied in two-dimensional (2D) cell culture, these models may not be reflective of or relevant to i*n vivo* phenomena. In particular, biochemical events related to the developmental program of epithelial morphogenesis cannot be modeled using traditional culture techniques. Growing cells in a three-dimensional (3D) matrix creates organotypic culture conditions and thus has provided us with *in vitro* techniques to recapitulate morphogenesis [116,117]. To date many studies have compared molecular events in 2D versus 3D culture and dramatic shortcomings of 2D systems have been found demonstrating the importance for organotypic models [118].

Growing epithelial cells in a 3D tissue-like context has demonstrated some epithelial cells lines can initiate a morphogenetic program. Growing mammary epithelial cells on reconstituted basement membrane from the Engelbroth-Holm-Swarm (EHS) tumor, has demonstrated that they can undergo a morphogenetic program to form acinar-like structures [119-121]. This technique has now become widely adapted through the use of Matrigel™ or Matrigel™:collagen based culture systems. These systems allow us to study the impact of a gene as well as the role played by microenvironment during morphogenesis and transformation.

A few untransformed cell lines are available for use in 3D models that undergo “normal” morphogenetic programs. Amongst the mammary epithelial cell lines capable of undergoing 3D morphogenesis are untransformed mammary epithelial cell lines such as the human MCF-10A and HMT-3522 (S1) cell lines and the mouse HC11, Scp2 and Eph4 cell line [117,119,122-125]. The typical morphogenesis program in MCF-10A cells consists of an initial phase of proliferation during which a solid sphere of cells forms. Between days six and nine the central cells are cleared by apoptosis resulting in the formation of a hollow lumen. [126]. After day 12, structures with a single layer of polarized epithelial cells are organized around a central lumen and reach a resting phase with low rates of proliferation and apoptosis. Thus growing mammary epithelial

cells in 3D matrix allows us to model acinar morphogenesis in culture and to use such a system to study the interaction and dependence of a genes function on the extracellular matrix.

Adult stem cells in the breast

Breast epithelia is capable of undergoing many cycles of differentiation and involution from its quiescent state in the non-pregnant host to the functional differentiation in the pregnant and lactating state and its involution. This suggested the possibility of an adult epithelial stem or progenitor population capable of giving rise to differentiated luminal epithelial cells and myoepithelial cells as well as undergo self-renewal. Insight into adult stem and progenitor cells was first achieved in the hematopoietic system where the lineage hierarchy was carefully elaborated [127]. Though progress to recreate a similar hierarchy for epithelial stem and progenitor cells has not been achieved, evidence for the existence of such a hierarchy in the breast has been suggested by demonstrations that adult mammary tissue or isolated mammary epithelial cells contain a limited number of cells that can engraft an epithelium-free fat pad of host animals and result in an arborized and differentiated gland [128,129]. Serial transplantations of mammary ductal tissue can be performed successfully over seven generations with each generation producing another functional mammary gland before senescence is achieved [130]. Transplantation studies have demonstrated that mammary gland repopulating units (MRUs) are present throughout the breast though more are present in the developing virgin TEB and fewer in the lactating alveoli [131].

In attempts to identify undifferentiated progenitor cells in the mammary gland as well as their niche, electron microscopic analyses of mouse tissues were performed. These studies revealed an ultrastructurally distinct putative stem cell located in the basal compartment [131,132]. Most luminal epithelial cells are electrondense dark secretory cells because of their extensive secretory machinery. However, there are sporadic small and large light cells with few organelles dispersed throughout the ducts. These cells with few cellular organelles may represent the ultrastructural description of a non-committed progenitor cell [132]. However, electron microscopy studies preclude functional analysis. Thus the cells remain functionally undefined.

Immunohistochemical analysis of markers that are integral to epithelial structure and function provide a method of determining differentiation status throughout the breast. Markers capable of discriminating different cell types as well as their differentiation status include cadherins, cell-type specific cytoskeletal intermediate filaments such as cytokeratins (CK) and transcription factors.

Cadherins are integral membrane proteins that interact with cadherin molecules on neighboring cells in a homotypic manner. In particular, E-cadherin is expressed in the body cells of the developing end bud and throughout the differentiated luminal epithelial cells in the ducts and alveoli of the adult mammary gland [133]. E-cadherin loss-of-function blocks the development of luminal epithelia essential for lactational differentiation [133]. Conversely overexpression of E-cadherin results in precocious alveolar differentiation [134]. In contrast ablation of the myoepithelial specific P-cadherin blocks myoepithelial differentiation [133]. Thus, cadherins not only mark subsets of differentiated epithelia but they also play a critical role in differentiation.

Cytoskeletal proteins are another family of markers of differentiated and undifferentiated epithelial cells. In mouse and humans, cytokeratins constitute a large class of intermediate filaments that cluster as two subtypes: acidic (type I, cytokeratins 9-20) and neutral (type II, cytokeratins 1-8). To form a filament a cytokeratin from each subtype partners to form coiled-coil heterodimers [135]. Different pairs of cytokeratins are expressed in distinct lineages in many epithelial tissues [135]. In the mouse mammary gland basal cells express Cytokeratins 5 and 14 (CK5, CK14) while luminal epithelia express cytokeratins 8/18 (CK8,CK18) [136-138]. Basal cells include undifferentiated cells as well as the differentiated myoepithelial cells and are CK5/14 positive. In addition, the differentiated myoepithelial cells express Smooth muscle actin (SMA). Subpopulations of CK5/14 positive cells have progenitor properties [139-141]. Another cytokeratin of interest is Cytokeratin 6 (CK6). CK6 is strongly expressed during development of the mammary gland in the central cells of the embryonic E16.5 anlagen and the body cells of the TEB [136]. However, in the nulliparous adult gland CK6 positive cells are very rare [136,142]. During the highly

proliferative stages of early pregnancy CK6 + cells expand and in the post-partum breast CK14 and CK6 + cells remain increased and occasionally co-stain [136]. This suggests that CK6 expression marks an undifferentiated cell population in the proliferating mammary gland.

Transcription factors may also be used to distinguish different cell lineages. The p63 nuclear transcription factor marks all basal cells in the mammary epithelium [143]. While all basal cells are p63+ the ΔNp63 isoform is associated with progenitor status in mouse mammary epithelial cells [139]. Together, the CKs and p63 are excellent markers that allow us to identify different cell types *in situ* in the mammary gland.

However, all the above markers are not conducive for isolating live cells to test if they have MRU activity. Therefore, a number of approaches commonly used to identify and purify stem and progenitor cells in other tissues such as the hematopoietic system have been co-opted for the discovery of markers associated with mammary stem cells. These techniques include DNA label retention, dye efflux and separation based on cell surface markers.

A unique property of stem cells is their high proliferative capacity followed by quiescence. Taking advantage of this feature, cells can be labeled *in vitro* or *in vivo* with synthetic nucleosides (i.e., Bromo-deoxyUridine, BrdU). Cells that retain DNA with incorporated synthetic nucleosides following an extended chase period can be identified using microscopy or fluorescence activated cell sorting (FACS). This approach has led to the identification of label retaining cells in the skin, cornea, hair follicle and breast [144-148]. In the breast 5% of cells were identified as label retaining cells and this label retention can be persist even through pregnancy [148-150]. Strikingly, the cells were distributed in the basal niche [147]. Thus, the long label retaining cells exhibit the property of selective DNA segregation that may be associated with the progenitor status of the cell.

Another means of identifying putative progenitor cells is through their ability to pump out dyes. Cells capable of pumping out dyes (Hoechst) are discernable by Fluoresence activated cell sorting (FACS) as cluster of unstained cells constituting a side population distinct stained differentiated cells. The ability to

efflux dye depends on the ATP pump cassette G2 (ABCG2 also known as breast cancer related protein 1 (BCRP1)). This pump is important in multi-drug resistance and is overexpressed in cancer cell lines and tumors following antiproliferative therapy [151]. Cells capable of effluxing dyes from their cytoplasm were first identified and isolated from the bone marrow and when transplanted to hosts were capable of differentiating to form all the lineages required for hematopoiesis thus qualifying them as a stem cell population [152]. Welm *et al* (2002) reported that 2-3% of mammary epithelial cells were capable of dye efflux and that these cells had four-fold more label retaining cells [148]. However, deletion of the side population by interfering with the efflux pumps has no overt phenotype in the breast [153]. Thus while dye efflux may be useful in narrowing down the population of progenitors it does not appear to play an essential role for mammary progenitor cells.

The most robust technique used to identify progenitor sub-populations is the separation of cells using cell surface markers by FACS. By this approach many markers can distinguish between the sub-compartments in the hematopoietic system. Similarly in the breast this approach was applied to work out purification protocols to isolate a pure MRU subset. The markers used individually or in combination can enrich for populations of cells with multipotent or lineage specific properties. Some of the markers include CD10 (common acute lymphocytic leukemia antigen (CALLA)), CD24 (heat stable antigen (HSA)), CD29 (β1 integrin), CD44 (hyalouranate receptor), CD49 (α6 integrin), CD61 (β3 integrin), Stem cell antigen-1 (Sca-1), Mucin-1 (Muc-1 or Epithelial membrane antigen (EMA)) and EpCAM (Epithelial cell adhesion molecule or Epithelial specific antigen (ESA)) [154]. Another marker of progenitor cells is the expression aldehyde dehydrogenase 1 (ALDH1) activity that can be assayed for in live cells by FACS [155,156]. Thus many markers are available that can be used to isolate possible stem/progenitor cells.

Using combinations of the above markers, mouse MRUs were identified at a frequency of 1/60-90 cells in a Lin- $CD24^{med}/CD29^{high}/Sca\text{-}1^{low}/CD49f^{high}$ [140,141]. Initially, this analysis was limited as it failed to isolate 20% of the cells

with progenitor properties. However, improved enrichment has been reported using better dissociation techniques [154].

The molecules that specify the lineage restricted versus multipotent cell subtypes reveals an intriguing pattern. Progenitor cells typically express the basal integrins CD49f and its binding partner CD29 further re-enforcing that the basal compartment of the breast is the progenitor niche [140,141]. This is supported by data from the transgenic depletion of CD29 in CK5 positive cells results in a loss of stem cells [157]. The observation that integrins are integral to mammary stem cell biology suggests that stem cells are dependant on the extracellular signaling for their niche.

The FACS markers can be used to further specify lineage-restricted progenitors capable of forming colonies in low-density culture. Luminal colony forming units are thought to be $CD24^{high}/CD29^{low}/CD49f^{+}/CD14^{+}/CD61^{+}$ and myopeithelial colony forming units are $CD24^{high}/CD29^{low}/CD49f^{+}/CD14^{-}/CD61^{-}$ [140,141].

A cohort of markers were also found that are able to isolate MRUs from human breast epithelia. Human MRUs have been defined by an $EpCAM^{low}/CD49^{high}/MUC\text{-}1^{low}/ALDH^{+}$ [156,158,159]. However, because the mouse fat pad does not provide an adequate microenvironment to test human MRUs, it is more challenging to test a human MRU's potency. Two models have been developed to bypass this problem. One assay humanizes the mouse breast by injecting human fibroblasts into cleared 3 week old fat pads and subsequently engraftment of human cells with outgrowths can be achieved [160]. A second approach is to implant human mammary epithelial cells together with irradiated fibroblasts in a collagen plug under the renal capsule in hormonally supplemented mice. After 4 weeks the plug can be harvested and normal mammary ducts have been observed [161]. However, a possible limitation of these models is their requirement of an immunocompromised host.

Cancer has been thought of as a disease in which progenitor cells are acquire tumorigenic features and are henceforth a tumor-initiating cell [162]. Many of the markers used to identify normal breast progenitors have now been applied to

study tumor-initiating cells. Analysis of tumors using some of these markers has demonstrated that they are also markers of tumor-initiating/stem cells. For example p53-null mouse tumors are enriched in $CD29^{high}/CD24^{high}$ cells with demonstrable tumor initiating characteristics [163]. $EpCAM^{low}/CD24^{low}/CD44^{+}$, a phenotype very similar to $EpCAM^{low}/CD49^{high}/MUC\text{-}1^{-}/ALDH^{+}$ observed in normal human MRUs, were demonstrated to have properties of cancer stem cells such as Wnt-dependant radioresistance [164]. In mouse models of ErbB2 and Wnt driven tumorigenesis Sca-1 was increased and associated with tumor-initiating cells [142,165]. Hence, tumor initiation may begin in cells exhibiting normal stem cell markers.

As I have outlined above we have achieved some understanding of the markers of the progenitor cells of the breast. However, many confusing and conflicting reports remain in the literature that needs clarification. For example, while we have markers that can enrich for populations with increased progenitor properties, we do not have single set of markers that can incontrovertibly identify and isolate stem cells from either mouse or human mammary epithelia. Another large gap in our current understanding is whether many of these markers play a functional role in the development and differentiation of the breast from epithelial progenitors, or are they by-stander markers. For example, while CD29 (β1 integrin) is important for the differentiation program of basal cells, deletion of its binding partner CD49f is not [157,166]. Also, understanding the paracrine factors that regulate stem and progenitor cells in the breast may provide further insight into identifying the stem niche, the functional stem cell and its regulation.

Stem Cell Signaling

The molecular signals regulating stem cell self-renewal and differentiation are highly conserved from *Drosophila* to mammalia [167]. They include a number of well-studied signaling pathways such as the Wnt, Hedghog and Notch pathways. There is mounting evidence that these pathways are important for normal mammary gland development and are deregulated in neoplasia.

The Wnt/β–Catenin signaling pathway was first identified in MMTV driven mammary tumors with MMTV driving aberrant Wnt-1 expression [168]. In the canonical Wnt signaling pathway the frizzled receptors are activated by secreted wnt ligands. β-catenin is stabilized and translocates to the nucleus interacting with T cell factor/lymphoid enhancer factor (TCF/LEF) transactivators to drive target gene transcription [169]. Roles for Wnt in self-renewal in hematopoietic, gut and epidermal cells have been demonstrated [170,171]. In the normal mammary gland loss of Wnt expression impedes development [172]. In contrast, stromal overexpression of Wnt ligands or MMTV-Wnt1 overexpression results in glands with increased numbers of stem cells and tumors with stem cell characteristics including increased side population (dye effluxing cells) and Sca-1^+/$CK6^+$ cells and radioresistance [142,164,169]. These data suggest that Wnt signaling pathways are important in regulating stem cells in the untransformed mammary gland as well driving tumors with progenitor-like properties.

Hedgehog signaling is a paracrine-signaling pathway that involves hedgehog ligands, patched receptors, Smoothened transmembrane effector and the GLI transcription factors. In the absence of hedgehog ligands the Patched receptor represses Smoothened and the GLI proteins are transcriptional repressors. When ligand is present, GLI proteins become transcriptional activators. Enhanced hedgehog signaling results in improved mammosphere formation from human breast epithelial cells [173]. Disruption of patched receptors or overexpression of the smoothened receptor in mice results in defective differentiation (a dramatic increase in $CK6^+$ cells) and dysplastic ducts and alveoli, respectively [174,175]. Furthermore, in breast cancers patched loss is one of the most common changes found among tumor suppressors and Smoothened was frequently expressed ectopically in DCIS [175-177]. These data suggests that hedgehog signaling plays an important role in self-renewal and differentiation and possibly a role in human breast cancers.

Notch signaling is highly conserved in *Drosophila* and mammals. The signaling pathway involves four receptors that receive cell autonomous signals in a heterotypic context from adjacent cells expressing membrane bound ligands

(Delta or Jagged) [178]. Activation of Notch results in its cleavage and internalization and subsequent intramembrane gamma-secretase cleavage releases the notch intracellular domain which enters the nucleus and can interact with transactivators to drive gene expression [179,180]. In mammary progenitors Notch activation is important for self-renewal and differentiation of mammary progenitors and for secondary mammosphere formation [159,181]. Furthermore, MMTV-Notch or WAP-Notch overexpression results in blocked lobuloalveolar differentiation [182,183]. Aberrant Notch signaling also results in mammary tumors with varying degrees of differentiation [183,184]. Thus activation of Notch signaling pathways in the mammary gland results in enhanced self-renewal, blocked differentiation and tumorigenesis.

Tight regulation of the molecular signaling pathways regulating stem cells is critical for maintaining the necessary balance of self-renewal and differentiation in the normal breast. In contrast, any abrogation of these pathways can lead to enhanced self-renewal, clonal overgrowth and neoplasia. All three pathways outlined above are dependant on non-autonomous paracrine signals. Thus understanding their signaling niche is also important.

Direction of Thesis

Regulators of epithelial polarity have been identified and many aspects of their genetic and biochemical interactions have been defined. Drosophila models have attributed a functional importance to these polarity regulators in development and neoplasia. However, the relationship between the changes in polarity pathways and development and the transformation/neoplastic sequelae are currently not well understood. To gain furrther insight into their relationship I have focused on the role played by the baso-lateral polarity protein Scribble in the differentiation and transformation of mammary epithelia.

While we have achieved an understanding of the effects of Scribble mutations on *Drosophila* morphogenesis*,* our understanding of Scribble loss in mammalian epithelial morphogenesis is very limited. In Chapter 2 I will address the following questions: What is the role of Scribble in normal acinar morphogenesis in three-dimensional culture? What is the role of Scribble in Myc-induced tumorigenesis? Is Scribble expression altered in breast cancer cell lines and in primary breast tumors? The knowledge gained through understanding these aspects of Scribble's biology will help us to understand how disruption of epithelial architecture cooperates with oncogenes in mammary epithelial cells.

A second set of questions will seek to address the role of Scribble in mammary gland development/differentiation and tumor initiation. In chapter 3, using *in vitro* cell culture and an *in vivo* transplant models I will address the following questions. Is scribble expression important for the differentiation of mammary progenitors? What is the effect of Scribble loss on differentiation and tumorigenesis? What is the mechanism through which Scribble regulates the differentiation of mammary epithelial cells? Understanding these aspects of Scribble's expression pattern and the developmental outcomes of Scribble loss early in mammary gland development will provide insight into both the progenitor niche as well as into the genesis of undifferentiated/poorly differentiated lesions.

The final Chapter will summarize the impact that my work has had on understanding the role polarity plays in mammary gland development and

neoplasia. It will also highlight areas that warrant further investigation as a result of the work presented in this thesis.

In summary I will establish that Scribble plays a tumor suppressive role both through its regulation of apoptotic programs as well as via its control of the differentiation status of mammary epithelial cells. Heretofore, Scribble has not been implicated in regulation of apoptosis nor has any polarity complex been implicated in mammary gland differentiation The data presented herein will enhance our understanding of how cell architecture shapes the mammary gland via a regulation of mammary progenitors and how proper tissue organization can function as a tumor suppressor.

Chapter 2

Deregulation of Scribble promotes mammary tumorigenesis and reveals a role for cell polarity in carcinoma

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Lixing Zhan performed the experiments presented in for Figures 2.1, 2.2A and C, 2.3, 2.4 and 2.8.

Kenneth C Bergami performed the experiment for Figure 2.2B.

Avi Z. Rosenberg performed the experiments for Figures 2.5, 2.6, 2.7, 2.9 and 2.10]

Introduction

Breast cancer is thought to originate from epithelial cells of the terminal ductal lobular units (TDLU) in the breast [186,187]. Each TDLU has multiple small units referred to as acini that consist of a single polarized layer of luminal epithelial cells surrounding a hollow lumen [187-189]. The establishment and maintenance of a polarized organization is critical for the normal function of mammary epithelial cells *in vivo*. Early during the initiation and progression of carcinoma, epithelial cells loose their ability to maintain a normal polarized organization suggesting a critical role for molecules that regulate cell polarity in breast cancer.

Establishment of apical-basal polarity in mammalian epithelia is coordinated by a set of proteins referred to as polarity regulators [1]. These include the Scribble (Scrib)/Lethal (2) giant larvae (Lgl)/Discs large (Dlg) proteins that direct formation of basolateral membranes. The Crumbs/PALS/PATJ and Partitioning defective 3 (Par3)/Par6/atypical protein kinase C (aPKC) protein complexes that direct establishment of the apical membrane and the apical-basal border, respectively [1,9,190].

In *Drosophila*, loss of function mutations in polarity genes such as *scrib, lgl, and dlg1* result in aberrant proliferation and abnormal cell polarity/architecture, demonstrating a direct relationship between cell polarity regulators and control of cell proliferation [191,192]. Although the mechanistic details of the relationship between polarity and cell proliferation is yet to be well understood, genetic analysis suggests that polarity proteins use separate pathways to regulate cell structure and cell proliferation [65].

Recent studies suggest a role for polarity proteins in human cancers. For example, human *scrib* and *dlg1* are targeted for ubiquitin-mediated proteolysis by the E6 oncoprotein from high-risk strains of human papillomavirus (HPV) [109,193,194]. Genetic alteration in *dlg5* is correlated with inflammatory bowel disease, which predisposes the patients to gastric cancer [195]. Furthermore, loss of Scribble expression is frequently observed in colon and lobular breast cancers [89,107]. In addition, we recently demonstrated that the oncogene ErbB2 requires an interaction with the Par6/aPKC polarity complex to transform three-

dimensional mammary epithelial acini [55]. These findings indicate an important role for polarity proteins during cell transformation. However, neither the mechanism by which polarity regulators control cell transformation nor how they interact with oncogenes are well understood.

To directly investigate how polarity proteins regulate breast cancer progression, we examined the effect of deregulating the polarity protein Scribble by itself or in combination with *c-myc*. Myc was chosen because it not only regulates initiation and progression of breast cancer, but also lacks the ability to disrupt apical-basal polarity of mammary epithelial cells in culture [196]. The latter property of Myc makes it an ideal candidate for investigating interactions between oncogenes and polarity pathways.

Overexpression of *c-myc* is frequently observed in early breast lesions [197] and the *c-myc* locus is rearranged in roughly 5%, and amplified in 15%, of breast tumors [198,199]. Furthermore, Myc protein is overexpressed in approximately 70% of breast tumors and correlates with poor clinical prognosis and disease relapse. Although cells in culture and transgenic mouse models have been used to investigate how Myc transforms mammary epithelial cells, the details of the mechanism is not fully understood [200,201]. Expression of Myc under the control of mouse mammary tumor virus (MMTV) [202], whey acidic protein (WAP) promoter [203] or under a tetracycline-inducible system [204] results in development of mammary tumors with a latency of 7-14 months in 50-80% of virgin glands. The long latency associated with Myc-induced mammary tumorigenesis is likely related to the fact that Myc induces both proliferation and apoptosis in mouse mammary epithelial cells *in vivo* [205]. Coexpression of Myc and growth factors such as transforming growth factor α (TGFα) in transgenic mammary glands shortens the latency by inhibiting apoptosis [206,207]. In addition, overexpression of the anti-apoptotic protein Bcl2 [208], or loss of the pro-apoptotic protein Bax, cooperates with Myc to promote tumorigenesis [209]. These observations demonstrate that inhibition of apoptotic pathways can potentiate Myc-induced tumorigenesis *in vivo*. Neither the mechanisms by which

Myc induces death in mammary epithelia nor the mechanisms by which the cells develop resistance to Myc-induced apoptosis is well understood.

In this study, we demonstrate that deregulation of Scribble promotes transformation of mammary epithelial cells *in vitro* and *in vivo* by disrupting morphogenesis, cell polarity and inhibiting cell death. We also report that deregulation of Scribble is selected-for during spontaneous mouse mammary tumorigenesis and in human breast cancer. These observations have led us to propose a model for investigating the mechanisms by which polarity proteins regulate initiation of carcinoma in mammals.

Materials and Methods

Antibodies

All antibodies used were purchased from commercial sources. They included β-actin (Sigma), β-catenin (BD Transduction Laboratories), Bim (Stressgen), cleaved caspase-3 (1:100 dilution, Cell Signaling), Scribble (1:500 dilution, C-20, Santa Cruz) and fluorophore conjugated secondary antibodies to mouse and rabbit IgG (Molecular Probes).

For immunofluorescence purposes, the Scrib antibody was conjugated to Alexa 647 using the Alexa-Fluor fluorophore labeling kit (Molecular Probes). Scribble antibody in 0.1% gelatin/PBS was concentrated to 100 μg/μL in 100 μL PBS using YM-30 columns (Microcon). Following manufacturers protocols I added 10 μL $NaHCO_3$ to the concentrated antibody and combined the reaction mixture with the fluorochrome for 60 min with mixing every 15 minutes. Fluorphore conjugated antibody was purified by centrifugation through a column loaded with the supplied resin. Antibody concentration and the degree of labeling were determined using a nanodrop (Thermo Scientific).

Cell Culture

MCF-10A cells were cultured as previously described [120]. Briefly, they were grown in growth media (DMEM/F12 media supplemented with 5% horse serum, 10 μg/mL insulin, 20 ng/mL epidermal growth factor, 0.5 μg/mL hydrocortisone, 100 ng/mL cholera toxin, 10 U/mL penicillin and 10 μg/mL streptomycin). Cells were split at a 1:4 dilution every 4 days. To trypsinize, cells were rinsed with PBS and incubated for 20 - 30 minutes with 1 mL 0.05% trypsin (Gibco). The cells were collected and resuspended in resuspension media (DMEM/F12 supplemented with 20% horse serum and 10 units/mL penicillin and 10 μg/mL streptomycin) to neutralize the trypsin. Cells were spun down and resuspended in growth media and re-plated at the appropriate concentrations.

For plating into 3D culture, 5000 cells were plated on a bed of 70 μL of Matrigel in assay media (2% horse serum, 10 μg/mL insulin, 5 ng/mL epidermal growth factor, 0.5 μg/mL hydrocortisone, 100 ng/mL cholera toxin, 10 U/mL penicillin, 10 μg/mL streptomycin and 2% matrigel in DMEM/F12). Media was changed every four days.

SUM-159 cells were grown in HAM's F12 media (Gibco) supplemented with 5% FBS, 25 μg/mL amphotericin B, 5 μg/mL Gentamycin (Gibco), 5 μg/mL Insulin and 1 μg/mL Hydrocortisone. MCF-7 and MDA-MB-231 cells were grown in DMEM supplemented with 10% FBS, 10 U/mL penicillin and 10 μg/mL streptomycin. All cells were split 1:3 every 4-5 days.

Retroviral packaging

Retrovirus preparation, infection and selection were performed as described previously [120]**.** Briefly, 239GPG [210,211] maintained in growth media (DMEM supplemented with 10.0% heat inactivated FBS, 2.0mM L-Glutamine, 1.0 μg/mL tetracycline, 2.0 μg/mL puromycin and 300 μg/mL neomycin) and split 1:10 every 4 days. 293GPG cells were transfected at approximately 80% confluence with 15 μg of retroviral plasmid DNA using Lipofectamine in Opti-MEM media. Viral supernatants were collected in collection media (DMEM supplemeted with 10.0% heat inactivated FBS, 2.0mM L-Glutamine). Supernatants collected from days 3 - 6 were combined and filtered through a 0.45 μM filter. Cells to be infected were plated at a density of $0.5X10^6$ cells/10cm plate and the following morning were infected with a total volume of 4mL of media containing 1 - 3 mL of viral supernatant (depending on the viral titer) + growth media (to 4 mL) and 8 μg/mL of polybrene. After a 6 hour incubation with the viral supernatant 6 mLs of fresh media was added.The following morning the media was changed. When the infected cells reached confluence they were split into the appropriate selection media (10 μg/μL puromycin, 10 μg/mL hygromycin or 300 μg/mL neomycin (G418).

RNAi constructs

Retroviral microRNA30 (miR30) based RNA interference (RNAi) vectors targeting expression of Scribble were designed as follows: a target sequence that recognizes the 3'UTR of Scribble (Scrib.RNAi-1) and one in the coding region of Scribble (Scrib.RNAi-2) (conserved between mouse and human) was identified using RNAi Codex (http://codex.cshl.edu). A 97-nucleotide oligonucleotide was synthesized that has a 5' miR30 flanking sequence - a sense strand Scribble target sequence - a common miR30 loop sequence - an antisense strand targeting Scribble - and a common 3'miR30 flanking sequence. The oligo sequences for the shRNA template strands used were as follows:

Scrib.RNAi-1 (5061):

5'- TGCTGTTGACAGTGAGCGAGGCGAGACTGTAACTAGTGATTAGTGAAGC CACAGATGTAATCACTAGTTACAGTCTCGCCGTGCCTACTGCCTCGGA -3'

Scrib.RNAi.2 (377):

5'- TGCTGTTGACAGTGAGCGAGCACGTGGAGTCGGTGGATAATAGTGAAGC CACAGATGTATTATCCACCGACTCCACGTGCCTGCCTACTGCCTCGGA -3'

The primers were amplified using polymerase chain reaction (PCR) primers with primers that recognize the miR30 flanking sequence and has Xho1 and EcoR1 restriction enzyme sites as follows:

5'miR30PCRxhoIF

5'- CAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCG -3'

3'miR30PCREcoRIF

5'- CTAAAGTAGCCCCTTGAATTCCGAGGCAGTAGGCA -3'

The PCR product was cloned into the pSM2c vector [212] was subsequently subcloned into MSCV-LTR-Puro-IRES-GFP (MLP) vector [213] (kindly provided by S. Lowe). MCF-10A, MCF-7, MDA-MB-231 and SUM-159 cells were retrovirally infected with the RNAi constructs and selected with puromycin.

Inducible Myc experiments in MCF-10A cells

The Myc.Estrogen receptor fusion (Myc.ER) in the pBabe retroviral backbone was a kind gift of G. Evan [214]. MCF-10A cells were retrovirally infected with the

Myc.ER construct. Myc activity was induced using 1 μM 4-hydroxytamoxifen (4-OHT) in growth media (2D) or assay media (3D).

Scribble-knockdown rescue experiments

pBluescript Scribble cDNA was generously provided by P. Humbert (Peter MacCallum Cancer Centre). The Scribble rescue vector was made by excising the 3' UTR of Scribble from the pBluescript Scribble cDNA construct using site directed mutagenesis with the following two primers:
5'-CCTGTGCCCTCCTAGGAATTCGTCGACGCGGCCGCCTCCGCGG-3';
5'-CCGCGGAGGCGGCCGCGTCGACGAATTCCTAGGAGGGCACAGG-3'.
This allowed me to insert a EcoRI and SalI site for ease of subcloning the Scribble cDNA to the LNCX2 retroviral vector. MCF-10A cells were retrovirally infected with the rescue or empty construct and selected for neomycin resistance.

Scribble mislocalization experiments

The Scribble P305L mutation was generated using site directed mutagenesis with the following primers:
5'-GCTGATGGCCCTGCTCCGCTCCCTGGG;
5'-CCCAGGGAGCGGAGCAGGGCCATCAGC.
Both wild type and mutant Scribble cDNA were subcloned into the LPCX retroviral vector (Clontech). LPC-Scribble wild type and LPC-Scribble P305L were retrovirally transduced into MCF-10A cells and stable cell lines were selected using puromycin.

Immunoblotting

Cells were trypsinized and rinsed with ice-cold PBS and lysed in radioimmunoprecipitation assay buffer [20 mM Tris (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% IGEPAL, 10mM NaF, 1 mM Na_3VO_4, 100 μg/mL aprotinin, 2.5 μg/mL leupeptin, and 1 μg/mL pepstatin]. Lysates were collected by scraping the plates and were centrifuged at 15,000 x g for 15 minutes at 4°C. Protein concentration

was normalized using the Biorad Protein assay. Lysates were run on poyacrylamide gels and transferred to PVDF membranes. Antigen detection was performed by incubating the membrane in blocking buffer (either 5% milk or 5% Bovine serum albumin (BSA) in TBS/T) for 1 hour at room temperature. Membranes were then incubated with antibody diluted in block buffer for 1 hour – overnight (4^{o}C). This was followed by 3 x 5 minute washes in TBS/T. Membranes were incubated for 1 hour with isotype specific secondary antibodies conjugated to horse radish peroxidase (HRP) diluted in blocking buffer. This was followed by 3x 5 minute washes in TBS/T. Finally, the membrane was treated with enhanced chemiluminesence (ECL) solution for 1 minute and exposed to autoradiography film.

Quantitative PCR

Total RNA was isolated from 32 breast tumors (generous gift of the Wigler Lab (CSHL)) and 4 normal breast tissue samples tumors (generous gift of the David Mu (CSHL)) (RNA isolation performed by Min Yu). The RNA was isolated using a Versagene RNA tissue kit (Gentra). 1 µg of RNA was reverse transcribed with the Taqman probe kit (Applied Biosystems) using random hexamer primers to generate cDNA. The resulting cDNA was used for quantitative PCR using SYBR Green Master Mix (Applied Biosystems) in 96 or 384 well plates in triplicate. Primers for Scribble were designed with the Primer Express software (version 1.5a; PE Applied Biosystems) so as to overlap exon-exon boundaries.

Scribble primers:

5’-CAGGCCAATAACCTGCTGAT-3’;

5’- GATGAATATGCCCTTCGTCGT - 3’**.**

GAPDH primers:
5’-AAATTCCATGGCACCGTCAA-3’,

5’-TCTCGCTCCTGGAAGATGGT-3’.

Q-PCR was performed on the ABI 7700 or ABI 7900 sequence detection system (Applied Biosystems). Sequence Detector Software (SDS version 1.9.1; Applied Biosystems) was used to extract the PCR data, which were then exported to

Excel (Microsoft, Redmond, WA) for further analysis. *Scribble* expression levels were normalized to *GAPDH* expression levels. The cycle difference between tumor sample and the average of the normal tissues (C_d) are ploted as $2_{|Cd|}$.

Immunofluorescence

Immunofloresece analysis were performed as previously described [120,215] For 2D immunofluoresence 25,000 cells were plated and grown on glass coverslips (in 12 well plate). Before fixation the coverslips were rinsed with PBS and fixed in 5% formalin or 4% fresh paraformaldehyde for 10 minutes at room temperature. Formalin/paraformaldehyde quenching was performed using PBS:Glycine (3x 5minutes). The fixed cells were permeablized with 0.2% Triton X-100/PBS for 10 minutes (4^{o}C) and were washed 3x 10 minutes in IF wash. Cells were blocked in blocking buffer (10% goat serum/IF wash) for 1 hour and then incubated with primary antibody diluted in blocking buffer overnight. This was followed by 3x 10 minute washes with IF wash. Coverslips were incubated with fluorophore conjugated antibodies (1:500) or directly conjugated antibodies (Scribble 1:500-1:1000) diluted in blocking buffer for 1 hour followed by 3x 10 minutes washes with IF wash. Coverslips were then incubated with 4',6-diamidino-2-phenylindole (DAPI) in PBS to stain nuclei. Coverslips were mounted on glass slides using Prolong antifade mounting media and sealed with nailpolish.

For 3D immunofluoresence the acinar structures were fixed, quenched, permebealized, washed and blocked similar to the protocol for 2D coverslips. A second blocking step was performed before adding primary antibody using F(ab)2 diluted 1:100 (1mg/mL stock) in blocking buffer for 30 minutes. Incubation with primary antibodies (but not secondary antibodies) was performed in F(ab)2-containing blocking buffer.

Microscopy

All microscopy/image acquisition was performed using an Axiovert 200M fluorescence microscope equipped with an apotome for optical sectioning and the Axiovision software package.

Tumor Immunofluoresence

PFA-fixed, Paraffin-embedded human samples were deparaffinized in xylene twice for 5 min. Antigen retrieval was performed using a pressure cooker to boil the sample in Trilogy for 15 min. Samples were blocked with 10% goat serum in 0.1% Triton:PBS for 1 hour. Staining with fluorophore conjugated Scribble in blocking buffer, was performed in a humidified chamber overnight at room temperature followed by PBS washes. Nuclei were stained with DAPI in PBS and coverslips were mounted using Prolong antifade mounting media.

Results

Loss of Scribble disrupts 3D acinar morphogenesis of mammary epithelial cells.

Although the expression of Scribble is frequently lost in breast cancer [89], the significance of this change is not known. To investigate the cellular effects of downregulating Scribble, we used two different RNA interference (RNAi) constructs in a miR30 based retroviral vector [213] to stably deplete Scribble in the immortalized but non-tumorigenic human mammary epithelial cells line MCF-10A. Cells were lysed and by immunoblot we observed efficient knockdown of Scribble expression with both shRNAs compared to cells infected with the Control shRNA targeting firefly luciferase **(Fig 2.1A).** Downregulation of Scribble expression did not induce gross changes in cell morphology in cells grown in high-density culture (**Fig 2.1B)** or changes in cell-cell junctions as monitored by the localization pattern of β-catenin compared to control cells (**Fig 2.1C**). These observations are consistent with previous studies using both MCF-10A cells and canine kidney epithelial cells grown as monolayers [101,216].

Numerous reports have demonstrated that growing cells on extracellular matrix can highlight features not seen in two-dimensional culture [118]. Wen MCF-10A cells are plated on a bed of extracellular matrix they a undergo morphogenetic program that starts with a highly proliferative phase (days 1-8) and than undergoes an apoptotic phase (days 6-9) resulting in the formation of proliferation-arrested 3D structures composed of polarized epithelial cells surrounding a central hollow lumen [117,126]. Overall these 3D acinar structures resemble breast acini *in vivo*. To determine the role Scribble plays during 3D morphogenesis, Control.RNAi and Scrib.RNAi cells were plated on 3D matrix. Scrib.RNAi acini did not show any evidence of hyperproliferation and appeared morphologically similar to Control.RNAi acini **(Fig. 2.2A).** To analyze changes in polarity, we monitored the orientation of the Golgi apparatus by staining acini for the *cis*-golgi protein, GM130 [55]. In Control.RNAi acini Golgi were always oriented towards the lumen, however, cells in Scrib.RNAi acini had a modest, but

reproducible, disruption of Golgi orientation demonstrating that Scribble plays a role during the establishment of apical-basal axis of polarity during acinar morphogenesis (**Fig. 2.2B**). In addition to the modest effect on apical-basal axis of polarity, there was a more dramatic effect on the organization of cells within an acinus. Whereas we rarely (< 6%) observe cells in the lumen of Control.RNAi acini, the majority of Scrib.RNAi acini (> 55%) retained cells in the luminal space, **(Fig. 2.2C)**.

Scribble Regulates Luminal Apoptosis in 3D acini

Since lumen formation in MCF-10A acini requires apoptosis of the centrally located cells [126,217] we investigated whether loss of Scribble inhibited apoptosis. We observed that only 20% of Scrib.RNAi acini showed significant evidence of luminal cell death as monitored by cleaved-caspase-3 (a marker of apoptosis) staining compared with almost 65% of control acini **(Fig. 2.3A,B).** This data demonstrates that the loss of Scribble expression blocks the centrally located cells from being cleared by apoptosis.

Scribble loss blocks Myc induced Apoptosis

c-Myc, an oncogene implicated in breast cancer, induces both proliferation and apoptosis in a number of cell types including mammary epithelia [218,219]. We investigated if Myc induces apoptosis in MCF-10A cells and if downregulation of Scribble affects Myc-induced apoptosis. To inducibly activate Myc, we used a Myc-estrogen receptor (ER) fusion protein that can be activated using the estrogen analog 4-hydroxytamoxifen (4-OHT) [214,220]. We note that 4-OHT does not have any non-specific effects on growth and morphogenesis of MCF-10A cells because the cells lack expression of the 4-OHT target, ERα [221].

To determine if Scribble loss blocks Myc-induced apoptosis, we activated Myc in Scrib.RNAi acini. Whereas activation of Myc in Control.RNAi acini induced apoptosis in more than 45% of day 6 structures, less than 15% of Scrib.RNAi acini showed evidence of cell death in response to Myc activation after 3 days of activation (**Fig. 2.4A,B**). It was previously shown that apoptosis of centrally

located cells requires upregulation of Bim, a BH3 domain only pro-apoptotic protein [217]. Consistently, activation of Myc induced an increase in the levels of Bim in developing (Day 8) 3D structures, and loss of Scribble suppressed the ability of Myc to induce Bim expression (**Fig. 2.4C**).

Rescue of Scribble.RNAi phenotype

To test whether we could rescue the Scribble dependant block in apoptosis we overexpressed a Scribble rescue construct in Control.RNAi.Myc.ER and Scribble.RNAi.Myc.ER cells. The Scribble shRNA1 targets the 3' UTR of Scribble. Thus we were able to construct a shRNA1 resistant Scribble cDNA construct that lacks the 3'-UTR target sequence complementary to the shRNA. Myc was stimulated in acini in 3D culture from day3 to day 6 and stained for cleaved-caspase-3. Only acini that co-expressed the shRNA (as determined by GFP expression) and the overexpressed Scribble rescue construct (intensely staining acini as determined by Scribble immunofluoresence) were quantified. We find that expression of the scribble rescue construct in Scribble.RNAi acini increased the number of Myc-induced cleaved-caspase-3 positive acini compared to Scribble.RNAi acini alone. (**Fig2.5A,B**) Furthermore, whereas Myc.ER.Scrib.RNAi acini failed to induce the BH3 only protein, Bim, we found that expression of the Scribble rescue construct resulted in increased levels of Bim in response to Myc stimulation **(Fig 2.5C)**. This demonstrates that the Scribble knockdown was specifically driving the block in apoptosis and was not the result of any off target effects.

Scribble is mislocaized in cancer cell lines and primary breast tumors

To determine the relevance of Scribble loss to human cancers, we investigated changes in expression of Scribble in human breast cancer cell lines. We did not observe a change in total protein levels as determined by immunoblot in the breast cancer cell lines MCF-7 or MDA-MB-231 compared to untransformed MCF-10A cells (**Fig 2.6A)**. However, whereas Scribble localization was membrane restricted in MCF-10A cells, breast cancer cell lines

(MCF-7, MDA-MB-231 and SUM-159) had varying degrees of Scribble mislocalized to the cytoplasm (**Fig 2.6B**). This observation raised the possibility that Scribble function may also be deregulated by loss of membrane localization. This possibility is consistent with previous findings in *Drosophila* mislocaling Scribble from cell-cell junctions as the result of a single amino acid point mutation in a LRR domain results in a fly phenotype identical to that observed for the *scrib* complete loss of function mutant [87]. In addition, a *scrib* allele that harbors a point mutation in an LRR domain resulted in the *rumz* mouse mutant and causs in a defective neuronal migration and keratinocyte migration [101,104]. Together these results demonstrate that mislocalization of Scribble from cell-cell junctions is sufficient to cause a loss of function in *Drosophila* and mammals.

Scribble mislocalizaion is sufficient to block normal morphogenesis and apoptosis

To directly test whether mislocalization of Scribble deregulates its function in mammary epithelial cells, we mutated a conserved Proline at position 305 to a Leucine (P305L), which is known to disrupt membrane binding of Scribble [89]. We retrovirally overexpressed the P305L and wild type Scribble (WT.Scrib) in MCF-10A cells The overexpression resulted in a modest (2-3 fold) increase in Scribble expression compared to endogenous Scribble levels in MCF-10A cells (**Fig 2.7A**). Immunofloresence analysis showed that the wild type protein localizes to cell-cell junctions whereas the P305L mutant localizes to the cytoplasm, demonstrating that Scribble uses the LRR domain to localize to cell-cell junctions in mammary epithelial cells (**Fig 2.7B**). To determine whether mislocalization of Scribble disrupts acinar morphogenesis, day 12 acini were immunostained with GM130 and DAPI (**Fig. 2.7C**). While the control and WT Scribble overexpressing cells had a well-developed lumen and polarized Golgi apparatus, P305L acini had no detectable lumen and a disrupted Golgi orientation. Consistently, the P305L expressing acini did not stain positive for cleaved Caspase-3 compared to control and wt Scribble acini (**Fig 2.7D**). These

phenotypes were similar to or more severe than those observed in Scrib.RNAi acini (**Fig. 2.2B**).

We generated MCF-10A and Myc.ER cell populations overexpressing either wild type (wt) Scribble or the P305L mutant. To determine whether mislocalization of Scribble blocks Myc-induced cell death, we monitored Myc-induced apoptosis in wt and P305L expressing cells. While Myc induced apoptosis in both control and wt acini, it failed to induce apoptosis in P305L acini (**Fig 2.8**), demonstrating that Scribble mislocalization is sufficient to inhibit Myc-induced cell death. These results suggest that the Scribble mislocalization observed in breast cancer cell lines represents a second mode of inactivating Scribble function during cell transformation or tumorigenesis.

Scribble is reduced and mislocalized in primary breast tumors

To determine if Scribble is deregulated in primary human breast cancers we analyzed both changes in levels of Scribble mRNA expression and changes in subcellular localization of Scribble protein. Total RNA was isolated from 32 human tumors and four normal breast samples. Seventeen tumors had more than a two-fold decrease in the level of Scribble expression compared to normal breast, as determined by Q-PCR analysis (**Fig. 2.9**) suggesting that Scribble levels are frequently downregulated during the evolution of breast tumors.

To determine if Scribble localization was deregulated in primary tumors both normal breast tissue and breast tumors were immunostained with an anti-Scribble antibody. Whereas Scribble was found only at the basolateral surfaces of epithelial cells within the TDLU structures of normal breast **(Fig. 2.10),** it was mislocalized in 10 out of 20 Ductal carcinoma in situ (DCIS) samples. However, all the tumors also had regions with normal Scribble localization demonstrating that tumors are mosaic for normal and mislocalized Scribble. The presence of a mosaic pattern rules out any immunostaining artifacts because the regions with proper cell-cell junction localization serve as an internal control for the immunostaining procedure. In addition, we collected optical sections and determined that Scribble was mislocalized in all optical sections of the tumor

tissue (data not shown). Thus we observed that Scribble was deregulated by both downregulation and by mislocalization in human breast cancer.

Taken together our results demonstrate that the polarity protein Scribble is a novel regulator of transformation in mammals. Furthermore, we demonstrate that Scribble can be deregulated in breast cancer by downregulation or mislocalization and that this deregulation disrupts polarity and morphogenesis, blocks apoptosis and cooperates with an oncogene to transform cells in culture.

Discussion

Understanding the mechanism through which normal and transformed cells achieve sensitivity or resistance to normal microenvironmental survival cues is essential for a deeper insight into cancer initiation and progression. In epithelial tissues, apical-basal polarity may establish the means by which signals from extracellular matrix can be properly interpreted by establishing the cellular context of the cells. Hence, an understanding of the molecular defects in apical-basal polarity such as of those observed histologically in the early hyperplastic stages of the neoplastic process could improve our understanding of disease progression. We report a role for Scribble, an apical-basal polarity regulator, in regulating the luminal apoptosis in a 3D model in both normal morphogenesis as well as oncogene-induced apoptosis.

During the development of polarized mammary epithelial ducts *in vivo*, apoptosis occurs in the developing terminal end bud creating a cell-free lumen [222,223]. Similarly MCF-10A cells grown in 3D matrix follow a morphogenetic program where centrally located cells undergo apoptosis to form a hollow lumen [126]. We report that Scribble loss-of-function results in defective luminal apoptosis during 3D morphogenesis in MCF-10A cells. Loss of Scribble expression or its mislocalization blocks the clearing of luminal cells by apoptosis. It is thought that luminal apoptosis is initiated as the result of detachment from the extracellular matrix (anoikis) [126,217]. However, loss of polarity by blocking β4 integrin signals results in enhanced sensitivity to apoptotic signals because of loss of matrix dependant survival cues [121]. This would suggest that loss of polarity regulators such as Scribble should result in more apoptosis. This is in contrast to what we observe. Hence an alternative explanation is that Scribble loss is a cell intrinsic survival factor and blocks apoptosis. This suggests that Scribble loss of expression may use a novel mechanism to regulate apoptosis.

A second aspect that we addressed is the cooperative effect mediated by Scribble loss-of-function with Myc-induced transformation of mammary epithelial cells. Previous reports have demonstrated that Myc-induced apoptosis occurs in a Bim dependant manner and will cooperate with molecules that block apoptosis

(Bcl2 overexpression or Bax loss) to enhance tumorigenesis in lymphoma and mammary gland models [208,209,213]. With this in mind the block of apoptosis observed in untransformed Scribble knockdown acini, suggested to us that Scribble might block oncogene-induced apoptosis and cooperate with Myc during transformation. Indeed we find that Scribble loss-of-function in Myc transformed acini blocks Myc-induced apoptosis suggesting a mechanism through which normal epithelial architecture (i.e., polarized epithelia) can keep Myc dependent transformation in check. Hence we demonstrate a role for Scribble in regulating the transformative potential of Myc.

This is an important question to address from a clinical perspective because not much is known about what molecular features modify Myc-overexpressing tumor prognosis in breast cancer. One report suggests that Myc amplification and overexpression correlated with modified patient response to anthracycline therapy and correlated with poor overall survival and distant metastisis [224]. In addition Scribble is located in a region commonly amplified in cancers (8q24.3) that is independent of the c-Myc amplicon (8q24.13) [225-227]. Studies looking at the cooperation betweens these two amplicons and particularly the role of Scribble expression could be very informative in understanding Myc overexpressing tumors.

In addition to loss of expression, mislocalization of Scribble results in loss-of-function. In *Drosophila* and mammalian models mislocalization, of Scribble results in Scribble loss-of-function [67,87,89]. We demonstrate that mislocalization phenocopies Scribble expression loss in mammary epithelial morphogenesis and transformation. Both loss of expression and mislocalization of Scribble in cancer have been documented. In lobular carcinomas *in situ* of the breast reduced Scribble expression has been observed in 81% of tumors [89]. In addition mislocalization has been observed in gastrointestinal cancers [107]. By RT-PCR I observed reduced Scribble expression in 17/32 primary breast tumors when compared to normal tissue. I also demonstrated that Scribble mislocalization is seen in breast cancer cell lines and in sporadically in DCIS of

the breast. This demonstrates that Scribble function can be regulated in diverse ways in disease states.

How Scribble is misregulated in cancer may be an interesting question to address. In a breast cancer cell line, MDA-MB-231, with cytoplasmic Scribble, re-expression of E-cadherin was sufficient to recruit Scribble to the membrane [89]. However, in lobular carcinoma *in situ* of the breast it was demonstrated that while 67% of LCIS had lower E-cadherin expression, 81% of tumors had reduced Scribble expression [89]. This 14% disparity suggests that an E-cadherin independent mechanism for Scribble mislocalization may exist. Hence, though we have some ideas as to the outcome of Scribble misregulation in cancer (i.e., defective apoptotic mechanisms) more work is necessary to identify the specific mechanisms of mislocalization and the regulation of its expression.

Experiments that may provide further insight into Scribble's role in regulating apoptosis could focus on Scribble's role in developmental apoptosis and Scribble misexpression/mislocalization in cancer related apoptosis. To address Scribble in development it would be insightful to determine what the expression pattern is within developing placodes and terminal end buds. Furthermore, knockout of Scribble expression very early in mammary gland development (i.e., in developing terminal end buds) may result in delayed or blocked luminal apoptosis similar to what has been observed in developing Bim-null mammary glands [222].

A second interesting line of study would be to determine whether Scribble loss-of-function confers drug resistance. Molecules that block apoptosis have been suggested to confer resistance to cytotoxic agents [228]. Does Scribble loss play a similar role in transformed mammary epithelia? This can by addressed by selecting cancer cell lines for clones that are resistant to apoptotic stimuli and determine whether Scribble expression is lost or mislocalized in the resistant clones. This type of approach could help us understand if Scribble misregulation can play a role in clinically relevant resistance pathways.

To summarize, our results demonstrate a unique role for Scribble in regulating apoptosis during acinar morphogenesis and neoplasia. These results

validate Scribble's role as a tumor suppressor gene in mammalian tissues through our demonstration that Scribble loss-of-function cooperates with oncogenes during oncogenic transformation. Thus epithelial polarity homeostasis may function as a tumor suppressor.

Figures

Figure 2.1

Scribble loss does not alter two-dimensional phase morphology or cell-cell junctions. (*Courtesy Lixing Zhan)*

(A) Immunoblot of Scribble expression demonstrates the efficient knockdown of Scribble in stable populations of MCF-10A cells expressing either of two independent Scribble shRNAs compared to cells expressing a control shRNA. Actin protein levels served as a loading control.

(B) Phase morphology of control and Scrib.RNAi cells grown on monolayer cultures show no differences (10x).

(C) Monolayer cultures were immunostained with β-catenin antibody to monitor presence of cell-cell junction. Nod differences are observed as the result of Scribble knockdown (10x). Scale bar = 50 μM

Figure 2.1

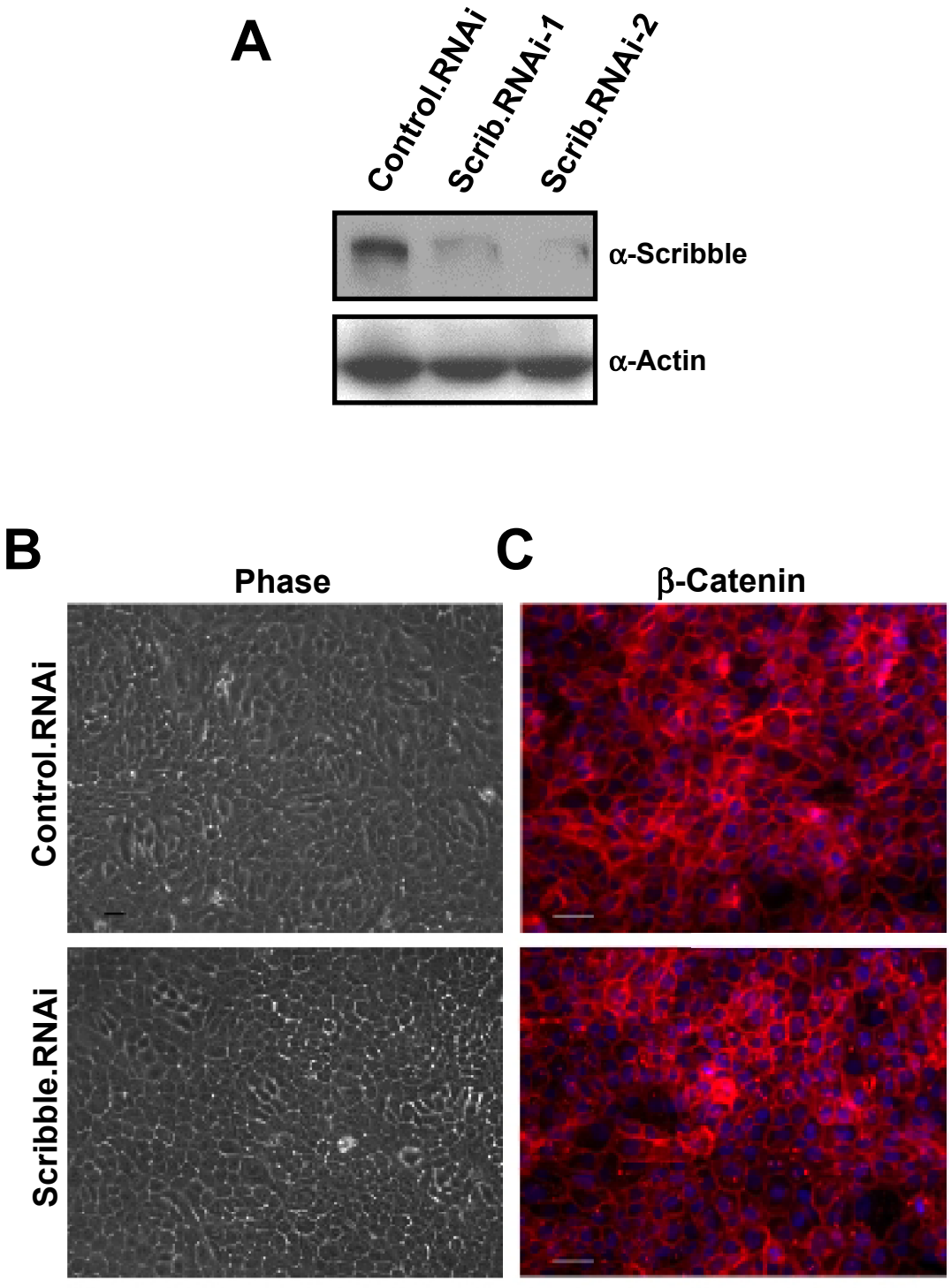
A
Control.RNAi
Scrib.RNAi-1
Scrib.RNAi-2
α-Scribble
α-Actin
B
Phase
C
β-Catenin
Control.RNAi
Scribble.RNAi

Figure 2.2

Scribble alters 3D organization of epithelial cells in acini (*Courtesy Lixing Zhan (A and C) and Ken Bergami(B))*

(A) Phase images of day 14 Control or Scrib.RNAi acini demonstrate no overt morphological differences (10x).

(B) Golgi orientation was visualized in day 16 acini stained with the *cis*-golgi marker, GM130 (Red) and DAPI (nuclei, Blue). The arrow points to a cell where the Golgi is oriented towards the basal surface of the cell, an event rarely seen in control acini (apotome, 63x).

(C) Control and Scribble.RNAi cells stained with DAPI demonstrate luminal filling of Scribble.RNAi acini compared to Control acini (apotome, 63x). Scale bar = 50 μM

Figure 2.2

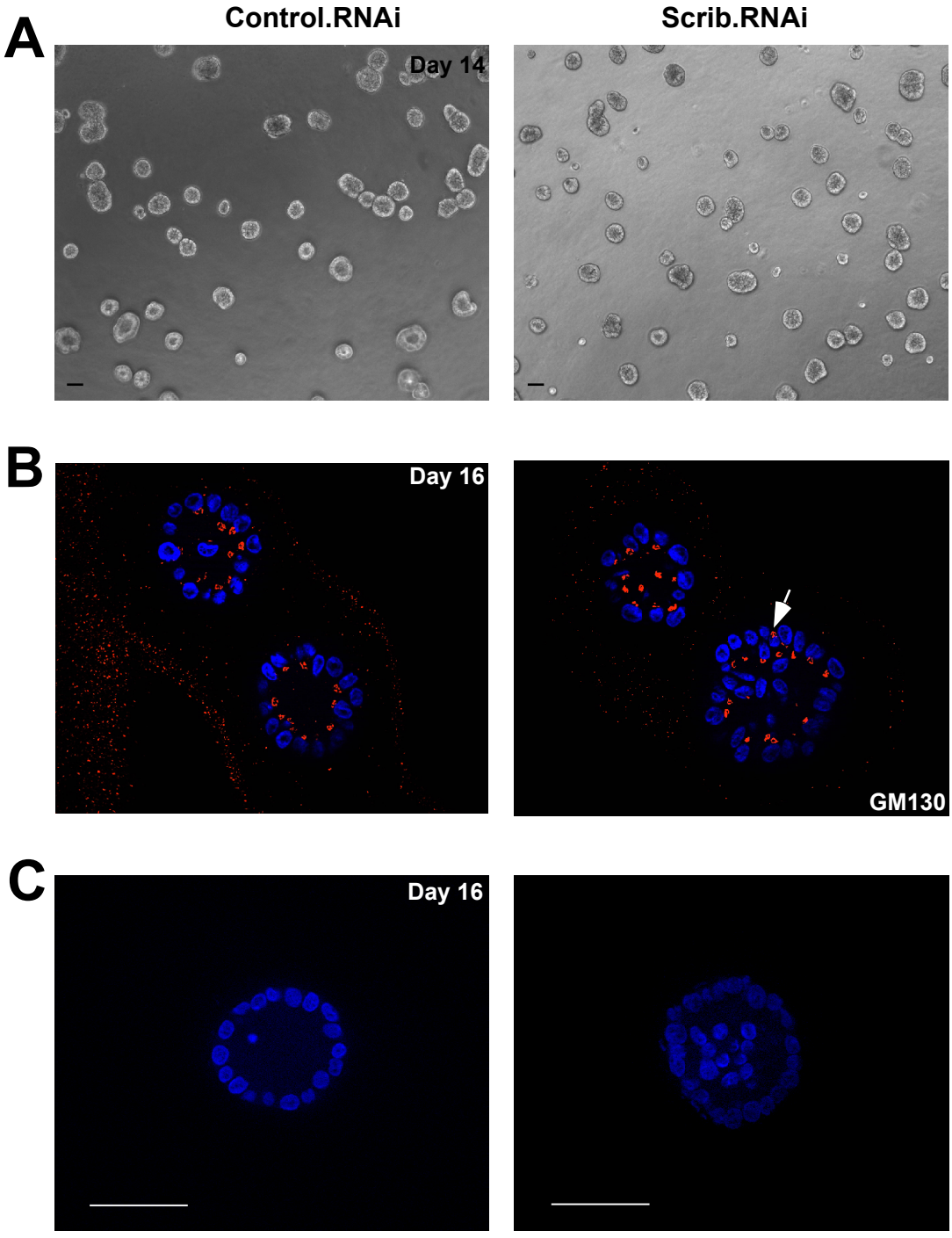

Figure 2.3

Scribble loss blocks luminal apoptosis in 3D acini (*Courtesy Lixing Zhan)*

(A) Control or Scrib.RNAi acini (day 8) immunostained for activated caspase-3 (Red, apoptosis marker), and DAPI (nuclei, Blue). Fewer activated-caspase-3 positive Scribble.RNAi acini compare demonstrates that luminal cells are not being cleared by apoptosis. Scale bar = 50μM.

(B) Day 8 Control and Scribble.RNAi acini positive for activated caspase-3 were counted and the percentage of the total acini are plotted. (n=3, mean ± SD, >150 acini/experiment).

Figure 2.3

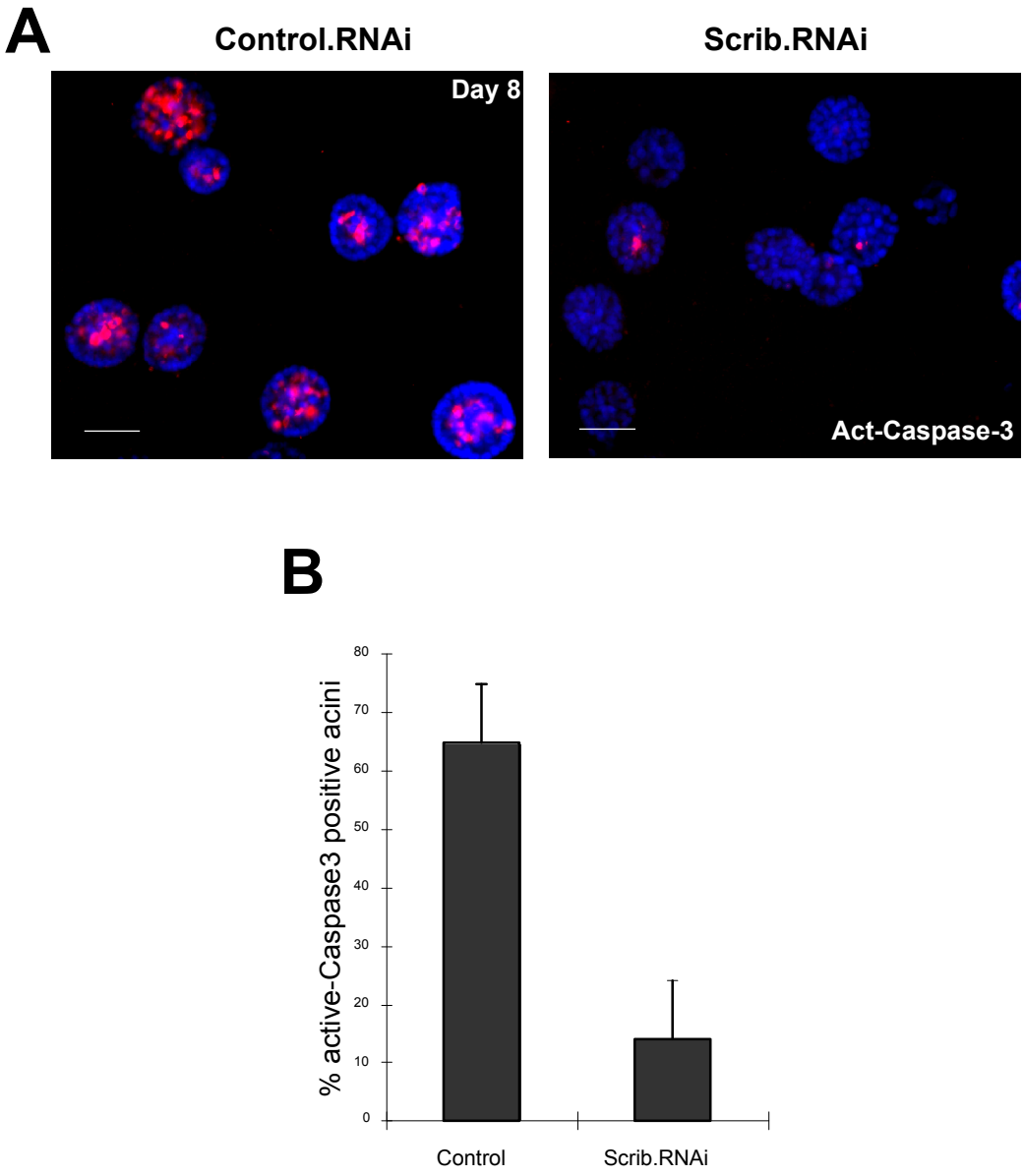
A
Control.RNAi
Scrib.RNAi
Day 8
Act-Caspase-3
B
% active-Caspase3 positive acini
80
70
60
50
40
30
20
10
0
Control
Scrib.RNAi

Figure 2.4

Scribble loss blocks Myc-induced apoptosis (*Courtesy Lixing Zhan)*

(A) Acini from MCF-10A cells with empty vector (control) or expressing Myc fused to estrogen receptor (Myc.ER), with and without Scrib.RNAi were stimulated (+/-) on day 2 with 4-OHT and immunostained on day 6 for activated caspase-3 (Red) and DNA (Blue). Scale bar, 50 μM

(B) Percentage of Control and Scribble.RNAi acini positive positive fo activated caspase-3 positive from (A) demonstrates a reduction in the number of Scribble.RNAi acini undergoing Myc-induced apoptosis (n=3, mean ± SD, >150 acini/experiment).

(C) Day 8 acini were stimulated with 4-OHT for 12 hours and lysates analyzed by immunoblot for Bim expression. Following myc induction, Bim expression is lower in Scribble.RNAi acini compared to Control acini suggesting a block in apoptosis. Actin protein levels served as a loading control.

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Figure 2.4

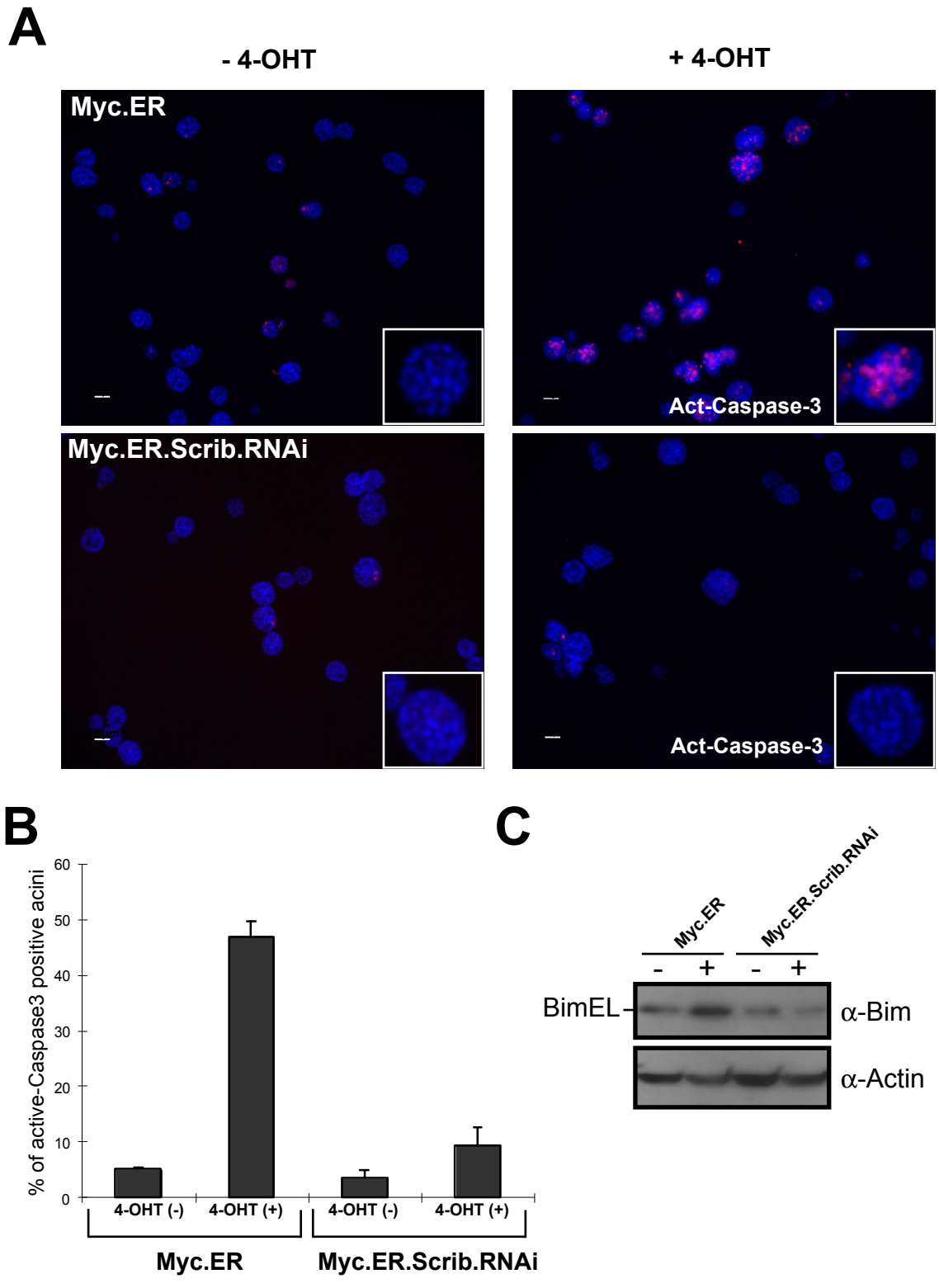

A
- 4-OHT
+ 4-OHT
Myc.ER
Act-Caspase-3
Myc.ER.Scrib.RNAi
Act-Caspase-3
B
% of active-Caspase3 positive acini
60
50
40
30
20
10
0
4-OHT (-)
4-OHT (+)
4-OHT (-)
4-OHT (+)
Myc.ER
Myc.ER.Scrib.RNAi
C
Myc.ER
Myc.ER.Scrib.RNAi
- + - +
BimEL
α-Bim
α-Actin

Figure 2.5

Rescue of Scribble function in Scrib.RNAi cells using an RNAi-resistant cDNA.

(A) Myc.ER.Scrib.RNAi cells stably expressing RNAi-resistant cDNA were stimulated with 4-OHT to activate Myc for 24 hours. Lysates were collected and analyzed for re-expression of Scribble (top panel) and a rescue off Myc-induced increases in expression of Bim (middle panel). We observe that Bim expression is induced with Scribble rescue construct. Actin protein levels served as a loading control.

(B) RNAi expressing acini were identified by the expression of green flurosecent protein (GFP) (upper panel). The corresponding changes in Scribble protein expression (lower panel) were determined by Scribble immunofluoresence (lower panel) (compare acini marked with *).

(C) Increased percentage of acini immunostaining for activated caspase-3 in GFP positive (shRNA expressing) acini that re-express Scribble demonstrates a rescue of Myc-induced increase in apoptosis. Together these results show that the expression of RNAi resistant cDNA rescues Scribble protein expression and rescues Myc-induced apoptosis (n=3, mean ± SD, >50 acini counted/experiment).

Figure 2.5

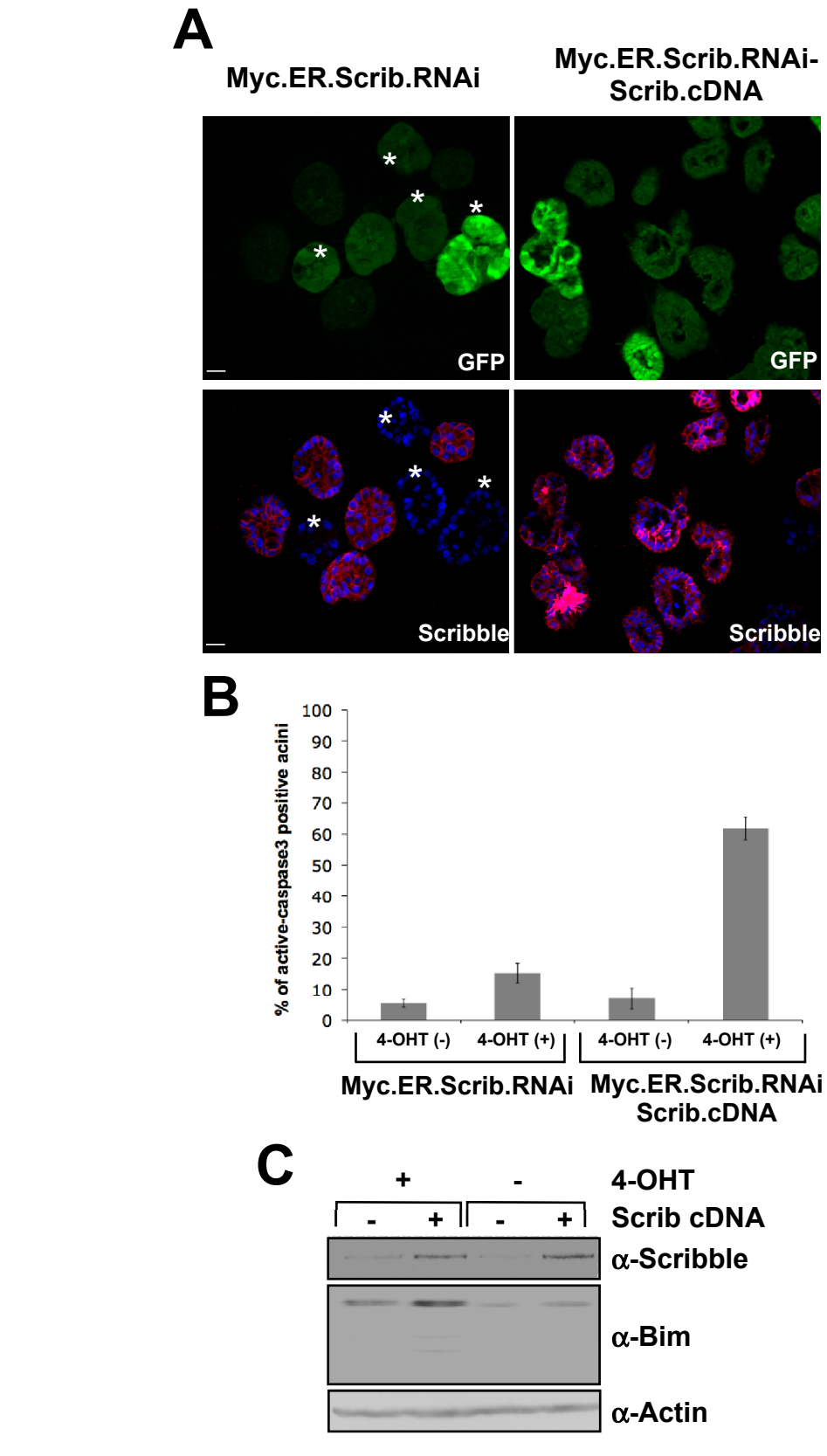

A
Myc.ER.Scrib.RNAi
Myc.ER.Scrib.RNAi-Scrib.cDNA
GFP
GFP
Scribble
Scribble
B
% of active-caspase3 positive acini
100
90
80
70
60
50
40
30
20
10
0
4-OHT (-)
4-OHT (+)
4-OHT (-)
4-OHT (+)
Myc.ER.Scrib.RNAi
Myc.ER.Scrib.RNAi Scrib.cDNA
C
+
-
4-OHT
-
+
-
+
Scrib cDNA
α-Scribble
α-Bim
α-Actin

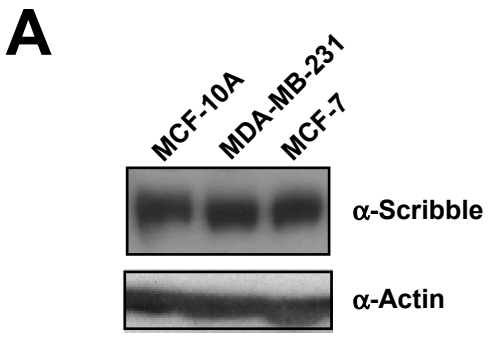
Figure 2.6

Mislocalization of Scribble is observed in breast cancer cell lines

(A) MCF-10A, MCF-7 and MDA-MB-231 cells were lysed and Scribble protein expression levels compared. No significant difference in expression levels was observed.Actin protein levels served as a loading control.

(**B**) MCF-10A, MCF-7, MDA-MB-231 and SUM-159 cells in monolayer cultures were immunostained with Scribble (Red) and DAPI (Blue, nuclei). Whereas in untransformed MCF-10A cells Scribble localizes to the membrane, in the three breast cancer cells lines varying degress and patterns of Scribble mislocalization were observed.

Figure 2.6


A
MCF-10A
MDA-MB-231
MCF-7
α-Scribble
α-Actin

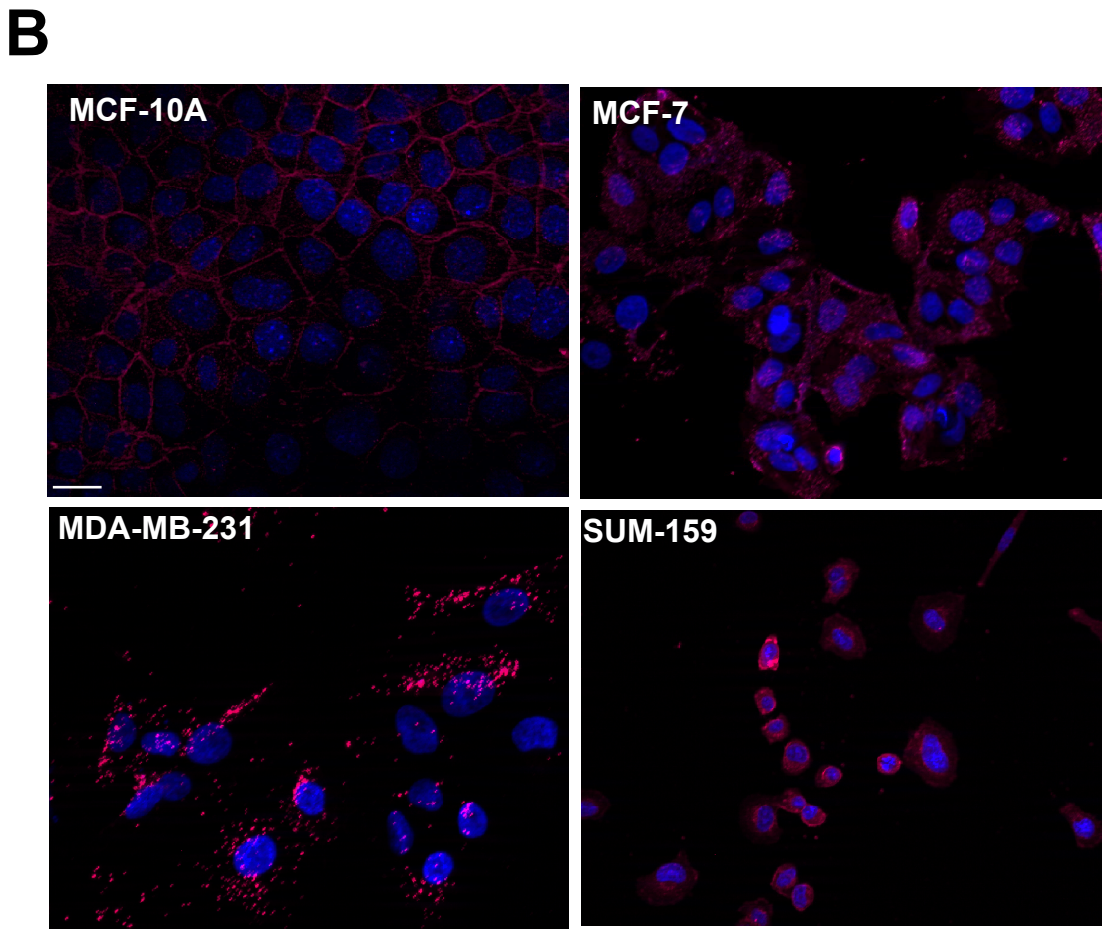

B
MCF-10A
MCF-7
MDA-MB-231
SUM-159

Figure 2.7

Mislocalization of Scribble phenocopies loss of scribble expression in MCF-10A cells.

(A) Overexpression of wild type (WT) and P305L mutant Scribble constructs in MCF-10A cells. Protein levels indicate approximately a 2-fold overexpression of both constructs. Actin protein levels served as a loading control.

(B) Immunofluoresence of day 8 acini demonstrates that while overexpression of WT scribble remains membrane bound, P305L Scribble is mislocalized (apotome, 63x). **(Courtesy Lixing Zhan)**

(C) Immunoflouresence of day 14 acini WT and P305L reveals a severe disruption of acinar morphogenesis with no luminal clearing in P305L mutants (DAPI, nuclei, Blue). In addition the *cis*-golgi marker, GM130 (Red), is arrayed in an apparently random pattern indicative of defective polarity (apotome, 63x).

(D) The lack of luminal clearing in (D) suggested defective apoptosis similar to or possibly even more severe than that observed in Scribble.RNAi acini. We quantified activated-caspase 3 positive acini in day 8 Control, WT and P350L acini. While WT Scribble had no effect on luminal apoptosis compared to Control acini, P305L mutant expression resulted in a dramatic reduction in cells undergoing luminal apoptosis. (n=3, mean ± SD, >75 acini/ experiment, n=3)

Figure 2.7

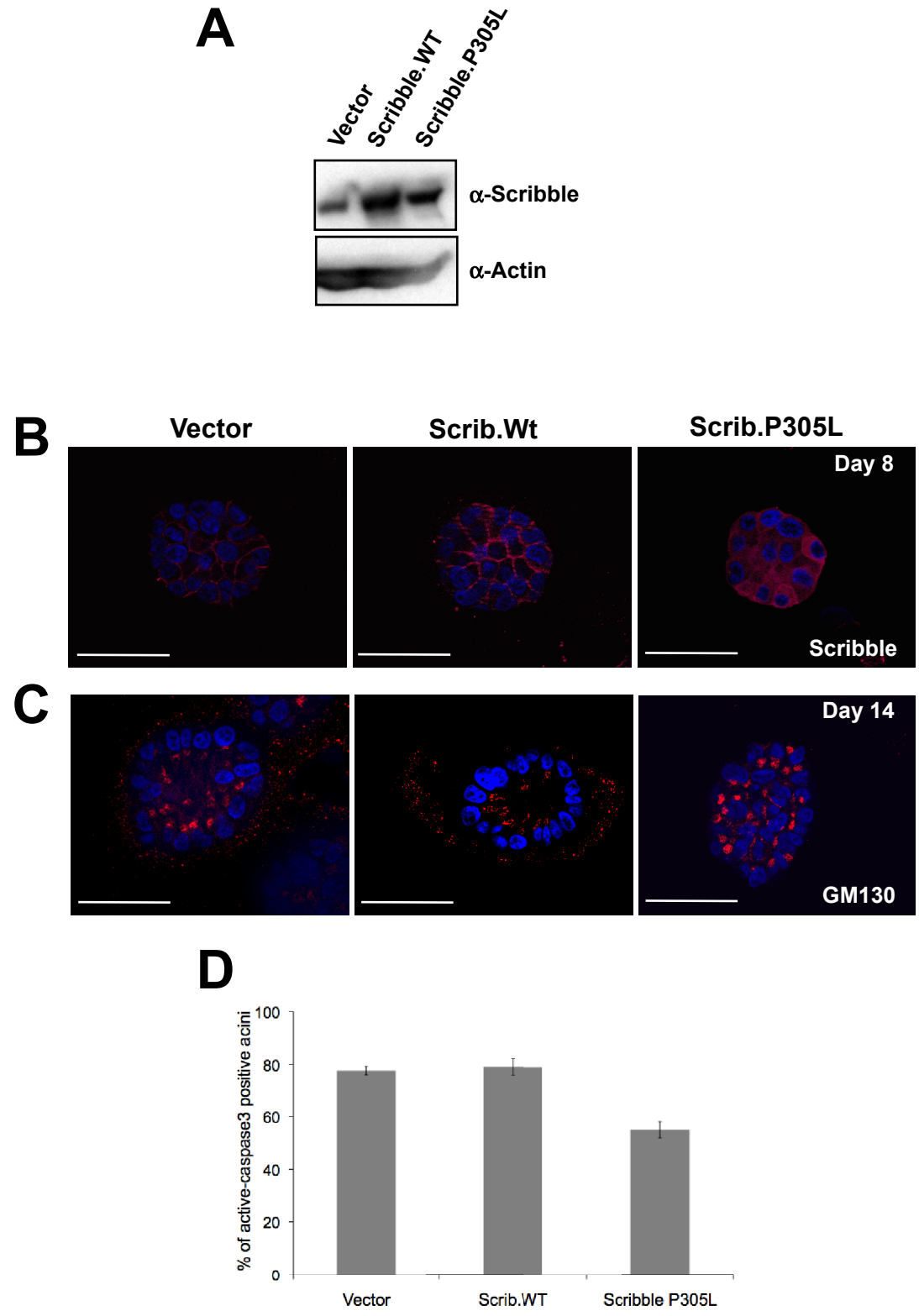

A
Vector
Scribble.WT
Scribble.P305L
α-Scribble
α-Actin
B
Vector
Scrib.Wt
Scrib.P305L
Day 8
Scribble
C
Day 14
GM130
D
% of active-caspase3 positive acini
100
80
60
40
20
0
Vector
Scrib.WT
Scribble P305L

Figure 2.8

Mislocalization of Scribble blocks Myc-induced apoptosis (*Courtesy of Lixing Zhan*)

Acini from MCF-10A Myc.ER cells infected with a Control vector, Wild type Scribble or Scribble P305L mutant were stimulated from day 3 to day 6. The percentage of acini that stains positive for activated caspase-3 were determined (n=3, mean ± SD, >150 acini/experiment). The statistically significant increase in the number of apoptosis-free acini demonstrates that P305L but not WT Scribble overexpression phenocopies effect of Scribble-loss on Myc-induced apoptosis.

Figure 2.8

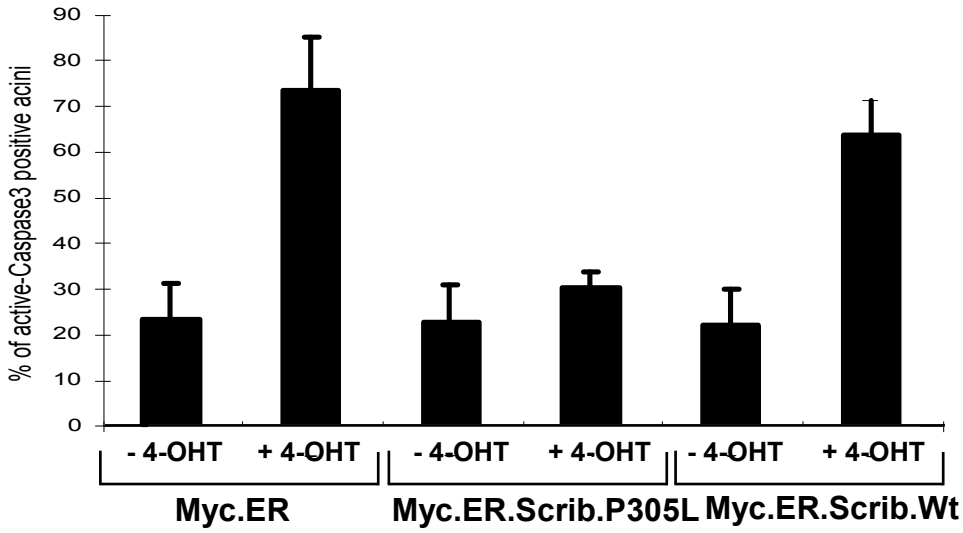

% of active-Caspase3 positive acini
90
80
70
60
50
40
30
20
10
0
- 4-OHT
+ 4-OHT
- 4-OHT
+ 4-OHT
- 4-OHT
+ 4-OHT
Myc.ER
Myc.ER.Scrib.P305L
Myc.ER.Scrib.Wt

Figure 2.9

Scribble mRNA expression is reduced in primary human breast tumors

RNA isolated from 32 primary human breast tumors and four normal breast tissues were analyzed for abundance of Scribble mRNA by quantitative RT-PCR. The graph represents fold change in Scribble expression over the average of abundance of Scribble mRNA in four normal breast samples. Scribble expression levels are reduced by more than two fold in 17/32 tumors.

Figure 2.9

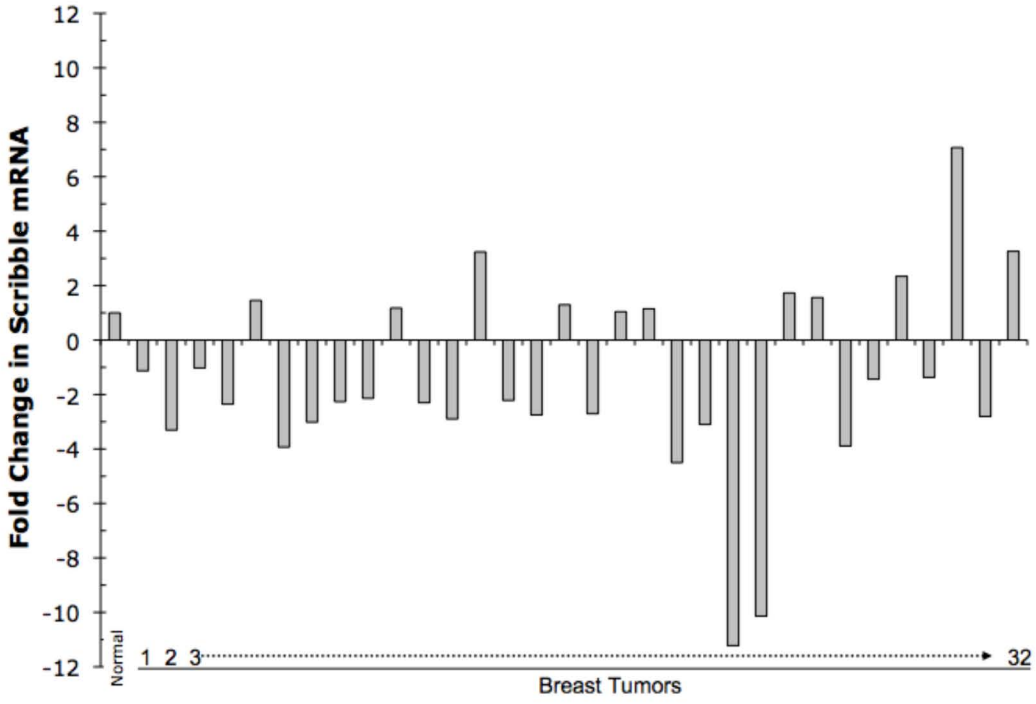
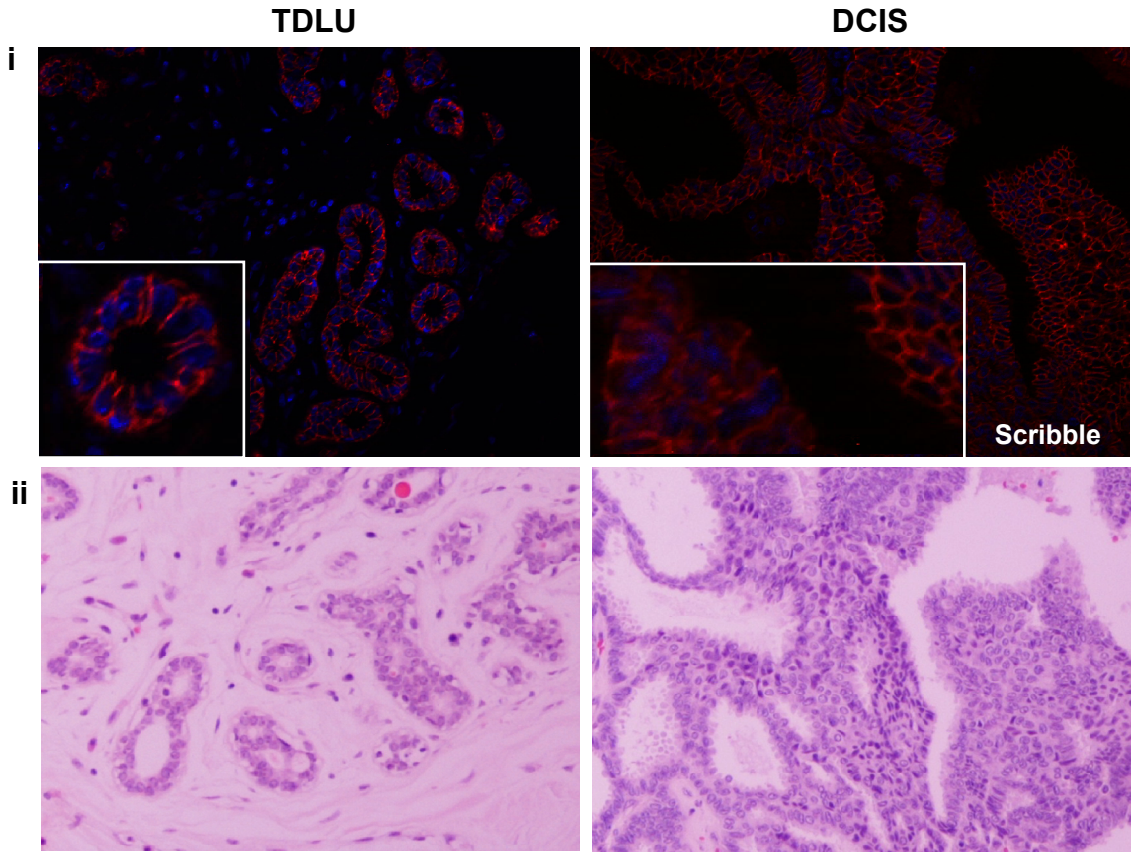

Fold Change in Scribble mRNA
12
10
8
6
4
2
0
-2
-4
-6
-8
-10
-12
Normal
1 2 3
32
Breast Tumors

Figure 2.10

Scribble is mislocalizaed primary human DCIS

Nine normal human breast and 20 DCIS tissue sections were immunostained using anti-Scribble antibodies (i) and a corresponding section were stained with H&E (ii). The inserts show higher magnification images to highlight regions with changes in Scribble localization compared to the basolateral expression pattern in normal terminal ductal lobular units (TDLU).

Figure 2.10



Chapter 3

Loss of the polarity regulator Scribble increases self-renewal of mammary progenitors, blocks differentiation and promotes tumorigenesis.

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[Disclosure of Contributions:
Elena Kabotyanski (Baylor) performed the *in vitro* differentiation assays
(Fig 3.11)
Heather LaMarca (Baylor) performed the Comma-1D Mammosphere assays
(Fig 3.7A)
Suling Liu (U. of Michigan) performed all the human primary mammary epithelial experiments
(Fig 3.7B,C,D)
Zhexun Wang (CSHL) began the Let7c flow sorting Experiments during his rotation.
(Fig 3.17 and 3.19B)
All other experiments were done by Avi Z Rosenberg]

Introduction

Cell polarity is an evolutionarily conserved property that generates cellular asymmetry, directionality, or both in cells and organs. Scribble, a scaffolding protein, is thought to regulate diverse aspects of cell polarity. Loss of Scribble in *Drosophila* and mammalian epithelia results in a partial to complete disruption of apical-basal polarity and organization of epithelial cells **(Fig 2.2B and 2.7B,C)** [67]. In addition to regulating apical-basal polarity in epithelia, Scribble also regulates other forms of polarity. For example, in migrating astrocytes, T cells, fibroblasts and epithelial cells, Scribble is required to establish front – back polarity to activate Rac signaling at the leading edge of migrating cells [101,102,229,230]. In the inner ear cochlea, Scribble is required for planar cell polarity that regulates the orientation of the hair cell stereociliary bundle [231]. In addition, Scribble regulates cell fate determination in mitotically dividing *Drosophila* neuroblasts (progenitor cells) by regulating spindle polarity and by controlling asymmetric distribution of proteins between the mother and the daughter cells [73]. However, it is not known what role, if any, Scribble plays during self-renewal and differentiation of progenitor cells in mammals.

Mammary gland morphogenesis provides a tractable platform to study cell polarization and differentiation because mammary progenitor cells can be induced to form differentiated structures that contain polarized epithelial cells both in culture and *in vivo* [126,129,232]. Studies over the past few decades have characterized the different stages of mammary gland development and differentiation. The adult virgin mammary gland consists of primary ducts with side branches that fill the entire mammary mesenchyme (fat pad). Pregnancy induces further differentiation resulting in increased branching complexity with extensive side branching and the subsequent development of milk-secreting lobuloalveolar structures. Both ducts and alveoli have a bilayered organization with an outer basal epithelial cell layer that is in direct contact with the mammary stroma and an inner luminal epithelial cell layer that lines the central lumen. These two cell layers can readily be distinguished based on the expression of cytoskeletal proteins, cell adhesion molecules and transcription factors. For

example, differentiated epithelial cells express cytokeratins 8 (CK8) and 18 (CK18) and E-cadherin, whereas the basal cells express CK5, CK14 and p63.

Transplantation of primary mouse mammary epithelial cells derived from the adult mammary gland into an epithelium-free syngeneic mammary fat pad can regenerate the cellular diversity and the complete mammary ductal network [129] demonstrating that adult primary mammary epithelial cell isolates have cells with stem cell activity. Recent studies have used a panel of cell surface markers to identify and characterize mammary stem cells [154]. For example, $CD45^-$ $CD31^-TER119^-$ (Lin^-) and $CD29^{hi}CD24^+$ or $CD24^{med}CD49f^{hi}$ cells have the capacity to generate an entire mammary ductal tree from a single mouse mammary epithelial cell in fat pad reconstitution assays [140,141]. In addition to these markers, human mammary epithelial cells expressing the enzyme aldehyde dehydrogenase 1 (ALDH1) are enriched in mammary gland repopulating units [156]. Cells that express these stem cell markers are enriched in cells expressing the basal markers CK14/p63 and rarely or never express luminal epithelial markers [139-141] suggesting that the basal layer of cells harbors the undifferentiated mammary progenitor population. However, the mechanisms by which these progenitors self-renew or differentiate into the various cell types found in mammary ducts are poorly understood.

We are, beginning to gain insights into the molecular mechanisms that regulate differentiation of mammary stem and progenitor cells. Several lines of evidence suggest that the Notch signaling pathway plays a critical role in this process. Notch4 was identified as a site of integration (*int3*) for the Mouse MMTV provirus. This results in constitutive production of the Notch intracellular domain (NICD) that translocates to the nucleus, activates transcription and induces mammary tumorigenesis [182,184,233-235]. In addition to promoting carcinomas, subsequent studies showed that expressing Notch4 ICD under the control of MMTV long terminal repeat or the WAP promoter blocks lobuloalveolar differentiation [182,236,237], demonstrating that activation of Notch blocks differentiation of progenitors cells. Consistent with these results, activation of Notch receptors enhances self-renewal and mammosphere-forming capacity of

human mammary progenitor cells in culture [159]. Curiously, conditional deletion or downregulation of a canonical binding partner for the NICD domain required for Notch induced transcription, C-promoter-binding factor 1/Recombination signal binding protein for immunoglobulin kappa J region (CBF-1/RBP-J), using MMTV-Cre or WAP-Cre, results in the expansion of luminal progenitor cells but blocks luminal differentiation [238,239]. This suggests that Notch activity is required for luminal differentiation. Interestingly, Notch receptors (1-4) and their ligands (Delta1, Delta3, Delta4 and Jagged2) are expressed in both luminal and basal cell lineages ligands in basal cells and receptors in luminal cells [239], suggesting that Notch plays a broader role in a cell-type and developmental-stage specific manner during differentiation of mammary stem cells.

Here I report that loss of the polarity regulator Scribble in mammary epithelial cells promotes Notch activity resulting in the expansion of cells that express basal markers and have increased progenicity. Loss of Scribble also blocks luminal differentiation, both during normal morphogenesis and pregnancy/hormone-induced differentiation. Furthermore, loss of Scribble promotes tumor initiation and results in tumors expressing basal markers. Thus, we demonstrate that the polarity protein Scribble is a critical regulator of self-renewal and differentiation pathways in the mammary gland during development and tumorigenesis.

Methods

Plasmids and Reagents

pBabe-NeuNT.GFP, was the generous gift of Carlos Arteaga (Vanderbilt University). pCMV-Keratin14 promoter-β-hemoglobin-intron-SC35YFP was a gift of Rajika Thakar and David Spector (CSHL). The retroviral pMARX-Let7c sensor construct used was previously described [155].

The following antibodies were purchased from commercial sources: β-actin (Sigma), p63 (H-137, Santa Cruz), Scrib (C-20, Santa Cruz), Cytokeratin 5, 6 and 14, (Covance) Cytokeratin 18 (Progen), E-cadherin, NuMA, phosphotyrosine and p-ERM (BD Biosciences), ErbB2 (Ab-15, Neomarkers), α-Tubulin (for loading controls) (Sigma) and pan-tubulin (for immunofluoresence)(Covance), fluorophore-conjugated secondary antibodies (Molecular Probes). β-Casein antibody was the generous gift of Dan Medina (BCM). The gamma secretase inhibitor, GSI XXI (Calbiochem), was dissolved in DMSO and used at a final concentration of 4 μM.

Comma-1D and Eph4 Scribble RNAi cells and recue experiment

Comma-D β-geo cells were kindly provided by Dan Medina (Baylor College of Medicine) and Joe Jerry (U Mass amherst) and were maintained as previously described [139]. They were cultured in DMEM\F12 w glutamine supplemented with epidermal growth factor, insulin and gentamicin. Cells were passaged every 4 days at a 1:4 dilution. Stable cell lines were generated by retroviral infection as previously described in Chapter 2. The Scribble RNAi and Scribble rescue constructs were described in Chapter 2.

Eph4 cells were culture in DMEM/F-12 supplemented with 2% FBS, 50 ug/mL gentamycin and 5 ug/mlL insulin. MCF10A, MCF-7 and SUM-159 cells were grown as described in Chapter 2.

Immunofluoresence

For immunostaining 25,000 cells were seeded and grown on glass coverslips were washed with PBS and fixed either with 4% PFA for 30 minuets at RT (p63, NuMA) or ice cold methanol for 10 min at -20^{O}C (cytokeratins). Subsequent staining steps were described in Chapter 2. For NuMA immunofluoresence, fluorophore-conjugated anti-mouse IgM antibodies were used.

Differentiation assay and β-casein qPCR

Control and Scribble shRNA expressing Comma-1D cells were plated in 10 cm plates pre-coated with Matrigel (BD Biosciences #354234) diluted 1:5 in DMEM/F12. Cells were seeded at a density of 1 × 10^6 cells/plate in complete growth medium until the cells were confluent [240]. Two days post-confluence, EGF was withdrawn and the cells were maintained in priming medium (DMEM/F12 with insulin (5.0 μg/ml) and 10% stripped horse serum) for 48 hrs. Then, hydrocortisone (1.0 μg/ml) and prolactin (3.0 μg/ml) were added to the medium to induce the expression of milk proteins. The cells were treated for various time points up to 24 hours. Total RNA was isolated from untreated and hormone-treated cells using TRIZOL reagent according to the manufacturer's protocol (InVitrogen). DNase I treated (15 min at 37^{0}C), total RNA (5.0 μg) was reverse-transcribed (Superscript, InVitrogen) and amplified by PCR using primers specific to exon VII of ß-casein gene and GAPDH primers to control for mRNA integrity. Primers for exon VII were as follows: exonVII-forward (5'-CATATGCTCAGGCTCAAACCATCTCT-3') and exonVII-reverse (5'-GTACTGCAGAAGGTCTTGGACAGAC-3'). Quantitative qPCR was performed on an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) using SYBR GreenPCR master mix (Molecular Probes, Eugene, OR).

Comma-1D mammosphere assay

Single cell suspensions of Control and Scribble shRNA expressing Comma-1D cells were plated in 6-well, ultra-low attachment plates (2.0 mL per well) at a density of 30,000 cells per well in DMEM/F12 medium supplemented

with 20 ng/ml EGF (Millipore, Billerica, MA) 20 ng/ml bFGF, B27, and 1X antibiotic-antimycotic (Invitrogen). Media was added after four days. The primary mammospheres were dissociated after 8 days as previously described [158] by manual manipulation. Single cells were re-plated at a density of 5000 cells/well and media added every 3-4 days. After 10 days the number of secondary mammospheres were counted in a minimum of 12 wells for each cell line.

Primary human MEC isolation and Mammosphere assay

Dissociation of primary mammary tissue

100-200 g of normal breast tissue from reduction mammoplasties were minced with scalpels in a sterile petri dish. The well-minced tissue was transferred to a tissue dissociation flask with 150-300 ml of 300U/ml of type 3 collagenase (Worthington Biochemical Corporation, Lakewood, NJ) and dissociated approximately 16 hours (overnight) on a rotary shaker at 37 °C. The dissociated tissue was centrifuged for 30 seconds at 40 x g in 50 ml centrifuged tubes and the pellets, which was highly enriched with epithelial organoids, was washed for several times with Hanks Balanced Salt Solution (HBSS) (GibcoBRL, Bethesda, MD) and centrifuged at 40 x g in 50 ml centrifuged tubes after each wash. 1 - 5 ml of pre-warmed trypsin-EDTA (Stemcell Technologies Inc, Vancouver, British Columbia, Canada) was added to the organoid pellet. The pellet was mechanically dissociated by pipetting with a P1000 for 3 minutes. Following dissociation 10 ml of cold HBSS with 2% Fetal Bovine Serum (FBS) (Atlanta Biologicals, Norcross, GA) was added and centrifuged at 100 x g for 5 min. After centrifugation, the supernatant was removed, and 2-4 ml of pre-warmed dispase (Stemcell Technologies Inc, Vancouver, British Columbia, Canada) and 200-400 ul of 1 mg/ml DNAse 1 (Stemcell Technologies Inc, Vancouver, British Columbia, Canada) was added and pipetted for 1 min. 10 ml of cold HBSS with 2% FBS was added and the cell suspension was filtered through a 40-μm cell strainer (Falcon) and then passed through a 22G pippetting needle with 90° blunt ends (Fisher Scientific) to obtain a single cell suspension. An aliquot of the single cell suspension was mixed with trypan blue stain

(GibcoBRL, Bethesda, MD) 50:50 and loaded in the hemocytometer. Doublets, triplets, and groups of higher numbers of cells were counted. The number of single cells was >99% in all experiments and over 85% of cells were viable.

Single cells from the epithelial organoids were plated in ultralow attachment plates (Corning) or 0.6% agarose-coated plates at a density of 100,000 viable cells/mL in primary culture and 5000 cells/mL during passaging. Cells were grown in a serum-free mammary epithelial basal medium (MEBM) (Cambrex Bio Science Walkersville, Inc, Walkerville, MD), supplemented with B27 (Invitrogen), 20 ng/mL EGF (BD Biosciences), antibiotic-antimycotic (100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate and 0.25 μg/ml amphotericin B) (GibcoBRL, Bethesda, MD), 20 μg/ml Gentamycin, 1 ng/ml Hydrocortisone, 5 ug/ml Insulin and 2-mercaptoethanol. Bovine pituitary extract was excluded. Mammospheres were collected by gentle centrifugation (1000 rpm) after 7–10 days and dissociated enzymatically (10 min in 0.05% trypsin, 0.53 mM EDTA; Invitrogen) and mechanically, using a pippetting needle with 90° blunt ends (Fisher Scientific). The cells obtained from dissociation were passed through a 40-μm sieve and analyzed microscopically for single-cell suspension. If groups of cells were present at a frequency >100 for 10,000 single cells, mechanical dissociation and filtering were repeated.

Fat pad transplantation and wholemount staining

Retrovirally engineered Comma-1D cells were transplanted to epithelium free mammary fat pads as previously described [139]. Briefly, endogenous epithelium was removed from 3-week-old Balb/C females. Cells (100- 10,000) were resuspended in 10 μL of RPMI media and injected into the epithelium free fat pad. Eight weeks following transplantation, glands were harvested and either stained as wholemounts with hematoxylin or fixed for immunhistochemistry in freshly prepared 4% paraformaldehyde. To determine ErbB2 driven tumor onset, weekly palpation was performed until the first palpable tumor formed. Subsequently tumor onset and growth was monitored every 3 days until tumors were 1.5 – 2.0 cm in diameter.

Immunhistochemistry

See Chapter 2 methods.

Xenografts

SUM-159 cells were injected subcutaneously into NOD/SCID mice in a 200 μL volume of 1:1 matrigel:PBS. Tumor onset was assessed weekly until the first tumor was detected. Subsequently tumor onset was monitored every 3 days.

Let7c sensor FACS analysis

Single cells suspensions of Let7c sensor expressing cells were resuspended at 1.0 x10^6/ mL in PBS and filtered with a cell strainer (BD Falcon). Let7c sensor positive (dsRed positive) cells were isolated by sterile sorting on a BD FACS Aria directly into the appropriate culture media. All subsequent sorts were performed on a BD LSRII cell analyzer. Data analysis was performed using the FACS DiVA software.

Notch Target QPCR and Rescue by Notch inhibition

Control and Scribble.RNAi Comma-1D cells were grown to confluence and RNA was Trizol extracted and reverse transcribed as described in Chapter 2. Notch target and control primer sets were as follows:

mHes1 5’-AAAGACGGCCTCTGAGCACA
5’-TCATGGCGTTGATCTGGGTCA;
mHey1 5’-TGGGGACCTAGACTACCAGC
5’-CCACCCTAAAGTCGCCAGTA;
mHey2 5’-TGAGAAGACTAGTGCCAACAGC
5’-TGGGCATCAAAGTAGCCTTTA
mGAPDH 5’-CCAATGTGTCCGTCGTGGATC
5’-GTTGAAGTCGCAGGAGACAAC

Microscopy and Image analysis

All fluorescence images were collected using an Axiovert 200M equipped with an Apotome imaging system. Images were analyzed using Axiovision and ImageJ software packages.

Results

Basal cells express low levels of Scribble in the normal and diseased human breast

Mouse and human breast ducts throughout the mammary gland have a bilayered organization with a layer of epithelial cells contacting the lumen and a layer of basal cells contacting the basement membrane. These two cell layers can be readily distinguished based on their differential expression of cytoskeletal proteins, cell adhesion molecules and transcription factors. For example, differentiated epithelial cells express CK8, CK18 and E-cadherin. In contrast, basal cells express CK5, CK14 and p63. Cells that exhibit stem cell properties have been reported to express basal markers [132,136,139,141,241]. In this study we use differences in expression of cytokeratins, E-cadherin and p63 to distinguish between the different mammary cell lineages *in vitro* and *in vivo*.

To understand the expression pattern of the polarity protein Scribble in normal human breast ducts, we co-immunostained breast tissue sections with Scribble and cell type specific markers **(Fig. 3.1)**. In the luminal epithelia Scribble expression was restricted to the basolateral membrane and co-stained with expression of the luminal marker E-cadherin. Intriguingly, Scribble expression was not observed in cells staining with the basal cell marker CK14 demonstrating that Scribble is differentially expressed in the cells making up the bilayered ducts in the normal breast.

Next we investigated if the differential expression of Scribble is conserved in premaligant breast lesions and ductal carcinoma *in situ* (DCIS). We co-immunostained a panel of 30 early hyperplastic columnar lesions and 25 DCIS samples for expression of Scribble and epithelial and basal cell markers. Both early lesions and DCIS were heterogenous for these cell types and had cells expressing epithelial and basal markers [242,243]. In columnar lesions and in DCIS lesions, Scribble expression was restricted to epithelial cell layers and was not observed in the basal cell (CK14 positive) layers **(Fig 3.2A,B)**. Thus the

inverse correlation between Scribble expression and basal cells observed in normal ducts is retained during disease progression.

Scribble knockdown results in increased numbers of basal cells

We next investigated if there is a causal relationship between low levels of Scribble expression and the basal cell type. To this end we used a pluripotent mouse mammary epithelial cells line, COMMA-1D-βgeo (CD), derived from a mid-pregnant Balb/C mouse [240]. This cell line is ideal for these studies because it maintains itself as a heterotypic mixture of differentiated epithelial, mesenchymal and undifferentiated basal/progenitor cell types in cell culture [139,240,244,245]. Furthermore, CD cells will regenerate an entire mammary ductal tree comprised of primary and secondary branches with differentiated alveolar structures when transplanted into epithelium-free mammary fat pads of syngeneic Balb/C hosts [139,246]. Thus the CD population is a good model to study the effect of a gene on mammary progenitor activity.

We investigated if downregulation of Scribble altered the representation of the different cell types in populations of CD cells. We downregulated expression of Scribble in CD cells using the shRNAs characterized in Chapter 2 **(Fig. 3.3A)**. While the cells expressing a control shRNA (Control.RNAi) did not differ from the parental cells and predominantly had a cuboidal/cobblestone epithelial morphology, downregulation of Scribble (Scrib.RNAi) resulted in cells with a more elongated morphology **(Fig. 3.3Bi, ii)**. Prompted by the morphology change we immunostained the cell populations with epithelial (E-cadherin, CK18), basal (CK14, p63) and myoepithelial (SMA) markers. Interestingly, downregulation of Scribble induced a 2-3 fold increase in the percentage of cells expressing basal markers (CK 14: 20.7%±9.5% versus 55.4%±8.9%; p63: 20.7%±7.3% versus 58.5%±13.0%) with a concomitant 2-3 fold decrease in percentage of cells expressing epithelial markers (CK18: 74.9%±16% vs 32.6%±11.8%) **(Fig 3.4)**. However, the representation of myoepithelial cells as determined by SMA staining was unchanged, with approximately 3% of the total population staining positively in both control and Scrib.RNAi cells (data not shown). To rule out any

off target effects of the RNA interference, we used the second Scribble shRNA and rescued Scribble expression by overexpressing an RNAi-resistant Scribble cDNA in Scrib.RNAi cells as in Chapter 2. We monitored changes in expression of CK6, a cytokeratin associated with proliferatin progenitor cells [136,142,247,248], and found that shRNA2 induced a similar increase in CK6 as shRNA1 **(Fig 3.5A)**. In addition, the percentage of CK6 positive CD cells expressing RNAi-resistant Scribble cDNA and shRNA1 did not differ significantly from control cells expressing the RNAi-resistant cDNA **(Fig 3.5B,C)**. Together these data rule out any off-target effects of expressing Scrib.RNAi. Our results demonstrate that downregulation of Scribble expression induces an increase in cells expressing basal cell markers.

Scribble knockdown does not induce an epithelial to basal cell type conversion

The increase in the percentage of basal cells observed in CD cells upon Scribble knockdown could be the result of the conversion of differentiated epithelial cells to basal cells [249,250] or the result of an expansion of basal cells. To distinguish between these two possibilities, we tested if the downregulation of Scribble expression would convert a cell line with homogenously, differentiated luminal epithelial characteristics to cell populations that express basal cell markers. The mouse mammary epithelial cell line Eph4 is a differentiated luminal epithelial cell line that exhibits a cobblestone morphology with well-formed adherens and tight junctions [196,251]. We downregulated expression of Scribble in Eph4 cells and investigated if there were changes in cell morphology and expression of the luminal epithelial markers E-cadherin or CK18 and the progenitor marker, CK6. Downregulation of Scribble did not induce a change in the cobblestone morphology **(Fig 3.6A,Bi)**, in the expression of epithelial markers **(Fig 3.6Bii,Biii)**, or in the percentage of cells expressing progenitor markers (CK6: 4.5%±2.4 vs 5.6%±3.1) **(Fig 3.6C)**. Thus, downregulation of Scribble does not induce the dedifferentiation of epithelial cells to basal/progenitor cells.

Scribble knockdown in CD and in primary human mammary epithelial cells increases cells with progenitor properties

Next we investigated if the increase in basal cells correlates with an increase in cells that have progenitor/stem cell properties and also whether Scribble downregulation induced changes can be observed in primary human mammary epithelial cells. In this regard, we have previously developed an *in vitro* assay where mouse and human mammary epithelial cells grown under non-adherent culture conditions form spheres (mammospheres) similar to those observed for neural progenitor cells [139,158,252]. The ability of cells to form mammospheres positively correlates with properties of mammary stem cells as determined by the ability of mammopheres to undergo self-renewal in secondary mammosphere cultures, differentiate into multiple cell lineages and to reconstitute a cleared mammary fat pad [158,175].

As has been previously reported [139], control CD cells were capable of forming mammospheres. Interestingly, CD Scrib.RNAi cells showed a 2 - 3 fold increase in the number mammosphere forming units **(Fig. 3.7A)** suggesting that the increase in the percentage of basal cells may relate to an increase in cells with progenitor properties. To test if downregulation of Scribble can induce an increase in progenitor cells in primary human breast cells, we repeated the mammosphere forming studies using cells derived from reduction mammoplasties. Three independent lentiviral shRNAs (one targeting the same region as that used in CD experiments and two independent sequences) were used to downregulate expression of Scribble in primary cell isolates derived from two independent reduction mammoplasties **(Fig. 3.7B)**. Downregulation of Scribble in primary human mammary epithelial cells induced changes in morphology in which the cells lost their epithelial morphology and acquired an elongated morphology **(Fig. 3.7C)**, similar to the morphological changes observed in CD cells **(Fig. 3.3B)**. Furthermore, downregulation of Scribble induced a two-fold increase in mammosphere forming capacity of primary human mammary epithelial cells **(Fig. 3.7D)**. Thus, we demonstrate that loss of Scribble

expression induces an increase in the percentage of cells with progenitor properties in both mouse and human mammary epithelia in culture.

Downregulation of Scribble induces an increase in mammary stem cells capable of reconstituting epithelium-free mammary pads in vivo

The most robust assay to determine mouse mammary stem cell activity is to test the ability of cells to engraft and undergo ductal morphogenesis in an epithelium-free mammary fat pad [154,253]. We tested if the Scribble.RNAi-induced increase in mammosphere forming capacity related to an increase in *in vivo* reconstitution ability. We transplanted control and Scribble.RNAi cells as a limiting dilution series to determine the repopulation efficiency and, in effect, the frequency of mammary gland repopulating units. Transplantation of 10,000 or more cells results in very efficient engraftment of both control and Scrib.RNAi cells resulting in mammary ductal outgrowths **(Fig. 3.8)**. However, at lower dilutions of cells (1000 cells or fewer), Scrib.RNAi cells showed a significant increase in reconstitution efficiency compared to control CD cells (P=0.004) Taken together, both the improved mammosphere-forming ability and the improved ability to reconstitute the mammary fat pad *in vivo* demonstrates that downregulation of Scribble induces an expansion of mammary progenitor cells.

Scribble is required for commitment of progenitor cells to luminal/alveolar lineage

Analysis of wholemounts of glands transplanted with Scrib.RNAi cells exhibited two obvious defects **(Fig 3.9A)**. First, the Scrib.RNAi outgrowths display mild hyperplasia **(Fig 3.9B).** This observation is consistent with our observation that downregulation of Scribble results in mammary tumors with low penetrance and increased latency (>48 weeks) [185]. Second, the Scrib.RNAi outgrowths were predominantly unbranched ducts with little or no tertiary branching **(Fig. 3.9B')**, whereas the control outgrowths had significant tertiary branching and lobuloalveolar differentiation.

To better understand the differences between the CD control and Scrib.RNAi outgrowths, we analyzed the histology of the ductal structures by

Hematoxylin and Eosin (H&E) staining and by immunohistochemistry. Control outgrowths had normal-looking bilayered ducts composed of columnar luminal epithelia surrounded by a layer of basal cells. In contrast, the ducts in Scrib.RNAi glands were dilated and consisted primarily of a monolayer of cells **(Fig 3.10A)**. Prompted by these differences, we performed an immunohistochemical analysis with cell type specific markers. As expected, the ducts derived from control CD cells had a layer of CK14 positive basal epithelia and an inner layer of differentiated luminal cells which expressed CK18, E-cadherin and Scribble **(Fig 3.10B,C)**. To our surprise, the ducts generated from Scrib.RNAi cells did not have an epithelial layer as they lacked cells that immunostained for epithelial markers such as E-cadherin or CK18 (**Fig. 3.10** and data not shown). The Scrib.RNAi ducts only had a layer of cells that stained positive for the basal marker CK14 (**Fig. 310B,C,D**).

Luminal epithelial cells have a characteristic apical-basal polarity as determined by the asymmetric distribution of membrane-restricted proteins. To rule out the possibility that the single layer of cells in Scrib.RNAi ducts have the characteristics of a polarized luminal epithelial cell layer, we immunostained ducts for the apical polarity marker phosphorylated-Ezrin/Radixin/Moesin (p-ERM). While the epithelial cells in control CD ducts showed a clear apical p-ERM signal (**Fig 3.10D**), the single layered ducts in Scrib.RNAi glands did not show significant p-ERM signal in the apical surface. These results demonstrate that Scribble expression is required for progenitors to differentiate into bilayered ductal structures *in vivo* that have a basal cell layer and a polarized luminal epithelial layer.

Scribble is required for pregnancy-induced differentiation of luminal epithelia.

Pregnancy hormones induce differentiation of mammary epithelia to generate lobuloalveolar structures that secrete milk proteins. To rule out the possibility that Scrib.RNAi cells are arrested in a luminal progenitor state but can nevertheless be forced to undergo luminal differentiation, we investigated if the transplanted glands can respond to lactogenic hormones and functionally

differentiate to form secretory alveolar structures. We mated mice that had been transplanted with Scrib.RNAi cells in one gland and control CD cells in the contralateral gland and analyzed them at day 16 of pregnancy. In control glands we observed extensive accumulation of fat lipid droplets by H&E staining, indicative of milk lipid synthesis (**Fig 3.11A**). In contrast, we did not observe any fat lipid droplets in the Scrib.RNAi glands (**Fig 3.11A**). Furthermore, immunohistochemical analysis for the milk protein β-casein showed significant expression levels of β-casein in the lumens of alveoli from control glands but undetectable levels in the Scrib.RNAi glands (**Fig 3.11B**).

To determine if hormone-induced expression of β-casein mRNA expression was also inhibited, CD cell cultures on Matrigel were treated with lactogenic horones, insulin, hydrocortisone and prolactin, and changes in β-casein mRNA expression levels were determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR) [125,240,254]. Downregulation of Scribble significantly blocked the ability of CD cells to induce β-casein gene expression in response to lactogenic hormones (**Fig 3.12**). The detectable levels of β-casein expression in the Scrib.RNAi cells in culture, but lack of β-casein in the alveoli *in vivo* may be due to the difference in sensitivity between immunohistochemistry and qRT-PCR methods or the clonality of an individual alveolus *in vivo*. Thus, both *in vitro* and *in vivo* observations demonstrate that downregulation of Scribble blocks lactogenic hormone-induced functional differentiation of mammary epithelial cells.

Scribble is required for development of mammary tumors with luminal characteristics

Several recent studies have demonstrated that breast tumor subtypes are frequently associated with specific oncogenic or tumor suppressor events. For example, BRCA1 loss is associated with basal type tumors whereas ErbB2 is associated with tumors that express luminal epithelial markers such as E-cadherin and CK18 [243,255,256]. Consistent with this relationship, transgenic

mouse mammary tumors induced by expression of ErbB2 under the control of MMTV promoter also express luminal markers suggesting that expression of ErbB2 may play a causal role towards development of luminal tumors [257-260]. We reasoned that transformation of CD cells with ErbB2 should induce tumors with luminal characteristics and should provide another context to determine if downregulation of Scribble alters the generation of cells with luminal characteristics.

Control and Scrib.RNAi CD cells were retrovirally engineered to express activated ErbB2 (**Fig. 3.13A**) and their tumor initiating capacity was analyzed by transplanting the cells into epithelium-free mammary fat pads of syngeneic hosts. Downregulation of Scribble significantly decreased the latency of tumor onset from 105 days to 45 days (**Fig 3.13B**), possibly as a result of more progenitor cells that could be targets for ErbB2-induced tumorigenesis in the CD Scrib.RNAi cells. The control and Scrib.RNAi tumors were histologically distinct (**Fig. 3.14A**). When we analyzed the tumors for expression of luminal or basal markers we observed that the tumors derived from control CD.ErbB2 cells express luminal markers such as CK18 (**Fig 3.14Bi**) and E-cadherin (data not shown). However, tumors derived from Scrib.RNAi ErbB2 cells express only markers associated with basal epithelia (CK5 and CK14) (**Fig 3.14Bi, ii**) but not epithelial markers such as E-cadherin and CK18. This demonstrates that lack of Scribble blocks the ability of ErbB2 to induce tumors with luminal characteristics. This observation may provide insight into the clinical observations of a subset of very aggressive ErbB2 positive primary human breast tumors which possess a basal/basoluminal phenotype [260,261] and suggest that either loss Scribble expression or other genetic events that phenocopy Scribble loss play critical roles during the genesis of this type of tumor.

Taken together these data demonstrate that the genetic make-up of the cell that is being transformed by an oncogene can dictate the subtype of tumor that ensues and compliments the notion that tumor subtypes are dictated by the type of oncogenic events [142].

Loss of Scribble promotes symmetric division and expansion of progenitor cells

To begin to understand the mechanism by which loss of Scribble induces an increase in mammary stem cells, we performed colonogenic assays by plating CD cells at a single cell density. Previous studies have shown that CD cells have a population of bi-potent progenitors which when plated at single cell density give rise to populations that have both CK5 (basal) and CK18 (luminal) positive cells. This suggests that the progenitor cells undergo asymmetric division where one cell remains a basal cell and the daughter cell differentiates into a CK18-positive luminal cell [139]. Control and Scrib.RNAi cells were plated at single cell density and the resulting colonies were co-stained for CK5 (basal) and CK18 (luminal). We segregated the colonies into those that were mono-potent for CK5 positive cells or bi-clonal for CK5 and CK18 positive cells (**Fig. 3.15A**). Interestingly, the loss of Scribble resulted in an increase in the number of clones that were basal only (CK5 positive) and a decrease in the number of bi-potent colonies (CK5 positive/CK18 positive) (K5+/K18- = 27.5% vs 51.8%; K5+/K18+ = 6.1% vs 2.3%) (**Fig 3.15B**). These results suggest that knockdown of Scribble expression increases the ability of CK5 positive cells to divide symmetrically and maintain the basal state and conversely, decreases the ability of these cells to divide asymmetrically and differentiate into luminal cells.

Next, we directly tested if the cells show a decrease in asymmetric cell divisions using markers such as Nuclear Matrix Apparatus protein (NuMA) and Partitioning defective 3 (Par3), which have been used to identify asymmetrically dividing cells in populations of *Drosophila* neuroblasts, mammalian hematopoetic progenitors and basal cells of skin [262-265]. Loss of the Drosophila homolog of NuMA, Mud, is required for positioning of the spindle in neuroblasts [263,266]. In addition to spindle polarity, asymmetrically dividing progenitor cells also establish cellular polarity by segregating proteins to the cell cortex that is asymmetrically distributed the daughter cells, we refer to this property as Mitotic Cell Polarity (MCP) (see Discussion for more details). Actively dividing CD cells were immunostained with tubulin and NuMA (**Fig 3.16A**) or Par3 (data not shown) to monitor changes in MCP. The distribution of NuMA and Par3 was monitored in

mitotic cells in metaphase that were identified by the presence of a metaphase plate and a mitotic spindle. About 28% of mitotic control cells exhibit MCP as defined by asymmetric distribution of NuMA and Par3, whereas only about 10% of Scribble.RNAi cells exhibit MCP (**Fig 3.16B**). These observations demonstrate that loss of Scribble results in a loss in MCP, which can contribute to the symmetric expansion of the mammary stem cells observed in response to downregulation of Scribble expression.

Let7c microRNA sensor as an independent reporter to identify basal/progenitor cells

We have recently demonstrated that expression of the Let7c microRNA (miRNA) is repressed in a subpopulation of CD cells that are associated with progenitor status, $ALDH^{bright}/Sca^{high}$ [155]. A miRNA sensor was developed to dynamically identify live cells with repressed Let7c microRNA [155]. The design of the sensor has been previously reported. Briefly, the let7c miRNA target sequence was engineered into the 3' untranslated region (UTR) of the cDNA encoding for the dsRed fluorescent protein. Expression of this construct in cells that express the Let7c miRNA results in an RNAi pathway-dependant degradation of dsRed mRNA and thus no red fluorescence. In contrast, in cells with repressed Let7c miRNA, the dsRed mRNA will not be degraded and results in expression of the fluorescent dsRed protein. Thus, progenitor cells with repressed Let7c miRNA can be readily detected as RFP-positive cells using florescence microscopy or by flow cytometry.

We infected CD and an untransformed human mammary epithelial cell line, MCF-10A, with the Let7c sensor and sensor positive and negative cells were isolated from the infected population by flow cytometry. We monitored this enriched population for changes in the percentage of dsRed positive cells at every passage. Following several rounds of cell divisions, dsRed positive cells give rise to cell populations that had both dsRed positive and dsRed negative cells. The percentage of dsRed positive cells decreased with every passage and after approximately eight passages (32 days) achieved steady state frequencies

(0.5±0.07% and 2.5±0.9%, CD and MCF-10A respectively) (**Fig. 3.17**). Importantly, we never observed dsRed negative cells converting into dsRed positive cells over the course of the experiment. These observations demonstrate that the Let7c sensor positive cells have progenitor properties because they are capable of generating sensor-negative cells and not vice versa.

To understand the relationship between dsRed positive state and CK14 expression, we transfected dsRed positive cells with a reporter expressing yellow fluorescent protein (YFP) under the control of the CK14 promoter. More than 46% of the CK14 positive population were Let7c sensor positive and 40% of the sensor positive population were CK14 positive (data not shown), suggesting that dsRed marks a significant fraction of the basal cells in the population. Since presence of the Let7c sensor is associated with expression of progenitor/basal cell markers such ALDH, Sca1 [155] and CK14, the dsRed sensor serves as an independent tool to monitor changes in the percentage of progenitor cells in a cell population.

Loss of Scribble promotes symmetric division and maintenance of a progenitor state as monitored by Let7c sensor

Next we determined if loss of Scribble induces an increase in the Let7c sensor positive population. Knockdown of Scribble induced a more than two fold increase in the number of dsRed positive cells (CD.Scrib.RNAi: 0.5%vs1.45% and MCF-10A.Scrib.RNAi: 2.5%vs6.7%) (**Fig 3.17**). These data add further strength to our conclusion that loss of Scribble induces an increase in mammary progenitor cells.

We reasoned that the Let7c sensor should function as a live-cell reporter to monitor differentiation of progenitor cells by following the presence or loss of dsRed signal. MCF-10A cells were flow sorted to enrich for dsRed positive cells and plated at low dilution to follow a very small population of cells in real time. Several fields with dsRed positive cells were followed over a period of 72 hours and images were collected every hou. Both control and Scrib.RNAi cells proliferated to populate the field of view at the same rate. In addition, we did not

observe any difference in proliferation rates over six passages (split once every four days).

We tracked Let7c sensor positive cells over 72 hours to differentiate between asymmetric versus symmetric cell division of sensor positive cells. In both control and Scrib.RNAi populations sensor positive cells divided every 12 - 17 hours. We followed a sensor positive cell in control and Scrib.RNAi population over five divisions. In the control population, a single Let7c sensor positive cell underwent two symmetric and three asymmetric divisions to result in four Let7c sensor positive cells out of the 16 progeny cells (**Fig 3.18**). Interestingly the frequency of asymmetric divisions correlated with the number of asymmetric divisions identified using MCP markers such as NuMA and Par3 (**Fig 3.16**). We did not observe any pattern of choice between symmetric and asymmetric divisions. Thus, it is likely that there are cell intrinsic and extrinsic factors that regulate the choice of the type of division.

In Scribble.RNAi populations, the Let7c sensor positive cells we observed underwent only symmetric divisions and showed no evidence of asymmetric divisions. However, when the sorted population reaches steady state, there are only about 2.5-fold more sensor positive cells in the Scrib.RNAi population than in the control population, suggesting that not all Scribble knockdown cells undergo symmetric division every time. A more thorough analysis will be required to understand what factors regulate the choice between symmetric and asymmetric divisions. Nevertheless, the results provide direct evidence for the ability of Scrib.RNAi cells to undergo symmetric cell divisions at the expense of asymmetric divisions.

Scribble knockdown expands the tumor initiating population in cancer-derived cell lines

It is likely that blocking differentiation and increasing the number of progenitor cells has implications for breast cancer initiation, maintenance and progression. The data presented in Fig 3.13 shows that loss of Scribble cooperates with ErbB2 during mammary tumorigenesis *in vivo* demonstrating

that Scribble loss can promote initiation of tumorigenesis. To test whether loss of Scribble increases tumorgenicity of highly tumorigenic cell lines, we downregulated expression of Scribble in SUM-159, a basal-type breast cancer derived cell line and MCF-7 cells a luminal-type breast cancer cell line which contains a large population of tumor initiating cells (i.e., “tumor stem cells”)[267]. To monitor changes in percentage of cells with progenitor properties, we expressed the Let7c sensor in these breast cancer cell lines. Parental SUM-159 cells achieved a steady state frequency of 5.2% of Let7c sensor positive cells and MCF-7 cells achieved a steady state level of 3.7%. Scribble loss resulted in a more than two-fold increase (MCF-7: 8.2% and SUM-159: 11.6%) in the percentage of cells expressing the Let7c sensor (**Fig 3.19A,B**). In addition to Let7c sensor expression, Scribble downregulation in SUM-159 cells also induced a two-fold increase in the number of ALDH1 positive cells, an independent marker of tumor initiating cells (**Fig. 3.20**)**.** To rule out the possibility that this is restricted to SUM-159 cells, we downregulated Scribble expression in another breast cancer derived cell line, MCF-7. This data demonstrates that loss of Scribble in breast cancer cell lines increases the percentage of cells with progenitor properties.

To determine if Scribble loss in SUM-159 cells altered tumor initiation potential *in vivo*, we injected 10,000 control and Scrib.RNAi cells sub-cutaneously into NOD/SCID mice. While, both control and Scrib.RNAi cells gave tumors at the same frequency, Scrib.RNAi cells resulted in tumors with significantly shorter latency than the parental SUM-159 cells (median latency 35 vs 49 days p= 0.008) (**Fig. 3.21**) demonstrating that loss of Scribble increases the tumor initiation capability of breast cancer derived cell lines.

Loss of Scribble blocks differentiation by activating Notch signaling pathway

Studies in *Drosophila* neuroblasts have demonstrated that dysregulation of a member of the Scribble polarity complex, Lgl, promotes neural progenitor cell expansion by blocking asymmetric division and activating the Notch signaling pathway [80]. Notch has been described as a key regulator of asymmetric cell

division as well as self-renewal of *Drosophila* and mammalian stem and progenitor cells [159,264,268].

We investigated if loss of Scribble expression resulted in changes in the Notch pathway by monitoring changes in expression of Notch target genes such as Hes1, Hey1 and Hey2. RNA was isolated from control or Scrib.RNAi cells and changes in levels were recorded as fold change in Scrib.RNAi cells compared to levels present in control cells. Interestingly, all three target genes were upregulated 2- to 5-fold in Scrib.RNAi cells compared to the levels observed in control cells (**Fig. 3.22**). To confirm that the changes in mRNA levels are specifically due to activation of Notch, we treated the cells with the γ-secretase inhibitor XXI (GSI) that blocks generation of Notch ICD, a required step for nuclear translocation and activation of gene expression [179,269]. Treatment of the cells with GSI returned the levels of Notch target genes to within one-fold of the expression observed in control cells (**Fig 3.22**). These results demonstrate that downregulation of Scribble activates the Notch signaling pathway.

Next we determined if activation of Notch signaling is required for the loss of the Scribble-induced increase in the number of basal cells in CD. Control and Scrib.RNAi cells were grown in the presence of GSI for two passages and cells were immunostained for expression of epithelial (CK18) and basal (CK14) markers (**Fig 3.23A**)**.** Treatment of control cells with GSI did not have any significant effect on the percentage of CK18 and CK14 positive cells (**Fig 3.23B**). However, treatment of Scrib.RNAi cells with the Notch inhibitor resulted in a four-fold increase in the number of CK18 positive cells and a 1.5- fold decrease in CK14 positive cells (**Fig. 3.23B**) demonstrating that increased Notch signaling is required for the loss of Scribble-induced increase in basal cells. Taken together, these data demonstrate that Scribble is a critical regulator of mammary progenitor cell self-renewal and differentiation and that loss of Scribble activates the Notch signaling pathway to maintain an undifferentiated state.

Thus, we identify the polarity gene Scribble as a critical regulator of epithelial differentiation in culture and *in vivo* and report that Scribble can contribute to tumor heterogeneity by regulating cell differentiation pathways.

Discussion

Here we demonstrate that the polarity protein Scribble regulates asymmetric cell division and Notch pathway activation to control mammary epithelial progenitor cell self-renewal and differentiation.

Scribble is known to regulate different types of cell polarity. Loss of Scribble in *Drosophila* follicular and imaginal disc epithelia induces a defect in apical – basal polarity and a loss of tissue structure [67]. Consistent with this observation, loss of Scribble in mammalian epithelia interferes with establishment of apical-basal polarity as demonstrated in Chapter 2 of this thesis and front-back polarity of migrating epithelial cells and astrocytes [101,216,229]. Although the precise mechanisms by which Scribble regulates these process is yet to be understood, it is thought to involve either activation of Rac/Cdc42 signaling or disruption of E-cadherin function depending on the cell type [89,101,216,229]. In addition, we have also demonstrated that Scribble is required for cell death and lumen formation during three-dimensional morphogenesis of mammary epithelial cells in a Rac activation dependent manner [185]. Thus, in differentiated epithelial cells Scribble is known to regulate multiple cellular process related to cell architecture and morphogenesis.

In the *Drosophila* nervous system, loss of the Scribble/Dlg/Lgl pathway blocks differentiation by inducing symmetric cell division and expansion of progenitor cells at the expense of differentiated neurons in a Notch-pathway dependent manner [73,80,270,271]. We demonstrate that loss of Scribble in mammary progenitor cells blocks asymmetric cell division and induces self-renewal ofprogenitors at the expense of epithelial cell differentiation in part by activating Notch signaling. These effects of Scribble are likely to be cell type specific because inactivation of Scribble in progenitor cells activates Notch signaling, whereas inactivation of the Scribble/Lgl pathway in *Drosophila* follicular and imaginal disc epithelial cells does not activate Notch signaling [241,272]. Taken together, the results demonstrate that Scribble functions in a highly cell-context dependent manner both in *Drosophila* and in mammals.

Self-renewal of stem cells has been associated with asymmetric cell division. For example, during *Drosophila* neurogenesis, neuroblasts (NB) divide asymmetrically to self-renew [71]. The size of the daughter cell and their position within the tissue are controlled by proteins that regulate size and axis of the mitotic spindle. The NBs are larger in size and are retained in the neuroepithelial layer, whereas the differentiating ganglion mother cell (GMC) is smaller in size and leaves the neuroepithelial layer because the mitotic spindle is oriented perpendicular to the plane of neuroepithelium [273]. Cell polarity proteins such as Dlg are required for this process, and mutation of Dlg polarity proteins results in a loss of size asymmetry and the randomization of spindle axis [73,78]. This form of polarity is usually referred to as spindle polarity.

In addition to spindle size and axis, cellular proteins are also distributed to the cell cortex to be asymmetrically distributed between the daughter cells in dividing NBs [71,72]. Proteins that belong to the Partitioning defective polarity complex are segregated to the daughter cell that maintains its NB progenitor state. However, the daughter cell that receives Numb (a negative regulator of Notch signaling) differentiates into a GMC likely due to the ability of Numb to inhibit Notch signaling [271]. Interestingly loss of function mutation in mud (*Drosophila* homolog of NuMA) uncouples cortical polarity from spindle polarity in mitotic neuroblasts demonstrating that control spindle polarity and pathways that regulate asymmetric segregation of proteins are separable processes [78,263,266]. Interestingly, the asymmetric distribution of proteins in mitotically dividing progenitor cells is highly conserved during evolution. Mammalian neuroepithelium and keratinocyte progenitor cells are also known to asymmetrically distribute proteins during mitosis [265,274]. In this manuscript, we report asymmetric distribution of NuMA and Par3 proteins in mammary epithelial progenitor cells. Thus, the ability of cells to asymmetrically distribute proteins during mitosis is likely to play critical roles during differentiation of multiple organs. To distinguish asymmetric distribution of proteins from the process of spindle polarity, we refer to the former as Mitotic Cell Polarity (MCP). MCP is the ability of a cell to asymmetrically distribute proteins to the cell cortex during

mitosis. It is likely that progenitor cells use both spindle polarity and MCP to self renew. Although our results are interpreted in the context of progenitor cells, we cannot rule out the possibility that MCP occurs in non-progenitor cells. Further analysis into mechanisms that regulate MCP is likely to provide critical insights into self-renewal pathways in stem and progenitor cells.

Our results also identify a mechanism by which mammary progenitor cells differentiate. We demonstrate that Scribble is required for the differentiation of progenitor cells into epithelial cells. Loss of Scribble activates Notch, which in turn is required for expansion of the undifferentiated, basal cell population. Our results are consistent with previous results where overexpression of Notch ICD in the mammary gland induces poorly differentiated mammary tumors [236] and increased Notch activity increases self-renewal and mammosphere forming capacity of primary human mammary epithelial cells [159].

However, our results may not be consistent with previous studies where inhibition of the Notch pathway by conditional knockout of the Notch ICD binding partner Cbf-1/RBP-J in MMTV-Cre positive cells results in an increased proliferation of basal cells [238]. In addition, recent studies where inhibition of Notch signaling was accomplished by knock down of Cbf-1 in $CD29^{hi}CD24^{+}$ mammary stem cells, or by knock down of Notch3 in bipotent mammary progenitor cells inhibited luminal differentiation, suggest that Notch activity is required for luminal differentiation [239]. Unlike the above studies where the Notch pathway was inactivated in cells that are either MMTV-positive or pre-sorted for CD surface makers, our experiments were performed in a mixed cell population. It is likely that Scribble knockdown in unsorted cells of a mixed population activates Notch in all cell types including cells that have the mammary stem cell properties and could be responsible for our results. Thus, it is highly likely that Notch activity may both promote expansion of stem cells and promote restriction of cells to luminal lineage depending on cell context. These inconsistencies strongly argue in favor of a need for more detailed analysis of the role played by the Notch pathway in the mammary gland.

In addition to Notch, there are likely to be other regulators of mammary differentiation. Recent studies from the Werb and Visvader laboratories has shown than a zinc-finger domain containing transcription factor, GATA-3, is expressed in luminal epithelial cells and is required for luminal differentiation. Loss of GATA-3 during early mammary gland development results in severe disruption of mammary gland morphogenesis. Acute loss of GATA-3 in adult mammary gland results in accumulation of undifferentiated luminal cells that do not express luminal markers such as E-cadherin, demonstrating that GATA-3 is critical regulator of luminal differentiation [275,276].

The ability of Scribble expression to distinguish between luminal and basal cell types in DCIS raises the possibility that Scribble may contribute to generation of different cell types in cancer. Interestingly, we show that while expression of ErbB2 in parental CD cells induced tumors that express epithelial markers, expression of ErbB2 in Scrib.RNAi cells induced tumors that exclusively express basal markers, suggesting that the genetic make-up of the target cells can dictate the subtype of the ensuing tumor. Pathways that control normal differentiation of mammary progenitors may also be of interest in the context of tumor heterogeneity [242]. It is known that more than 60% of DCIS of the breast have intra-tumor heterogeneity, where there are multiple intrinsic subtypes and the tumor cells differ in nuclear grade, estrogen receptor expresion, and ErbB2 and p53 status within an individual tumor. It is possible that during tumor evolution the intrinsic subtypes compete with each other and the poorly differentiated, aggressive subtype dominates the tumor. Very little, if any, is known about the mechanisms that regulate intra-tumor heterogeneity. It is possible that somatic and stochastic inactivation of proteins that regulate self-renewal and differentiation pathways, such as Scribble, can play important roles during generation of tumor heterogeneity and evolution. Further studies will be necessary to test this hypothesis.

Thus, we identify a novel function for the polarity gene Scribble as a regulator of asymmetric cell division during mammary progenitor cell self-renewal and differentiation. In addition, our results identify an unexpected role for

Scribble in determining the differentiation status of tumors, demonstrating that changes in polarity pathways play important roles not only during normal differentiation but also during tumor progression.

Figures

Figure 3.1

Scribble is differentially expressed in the normal human breast

Tissue sections of normal human mammary ducts were immunostained for Scribble, E-cadherin, cytokeratin 14 and DAPI. In the normal breast, basal cells are cytokeratin 14 positive and express less Scribble (see higher magnification in in ii). Scale bars=20 μM

Figure 3.1

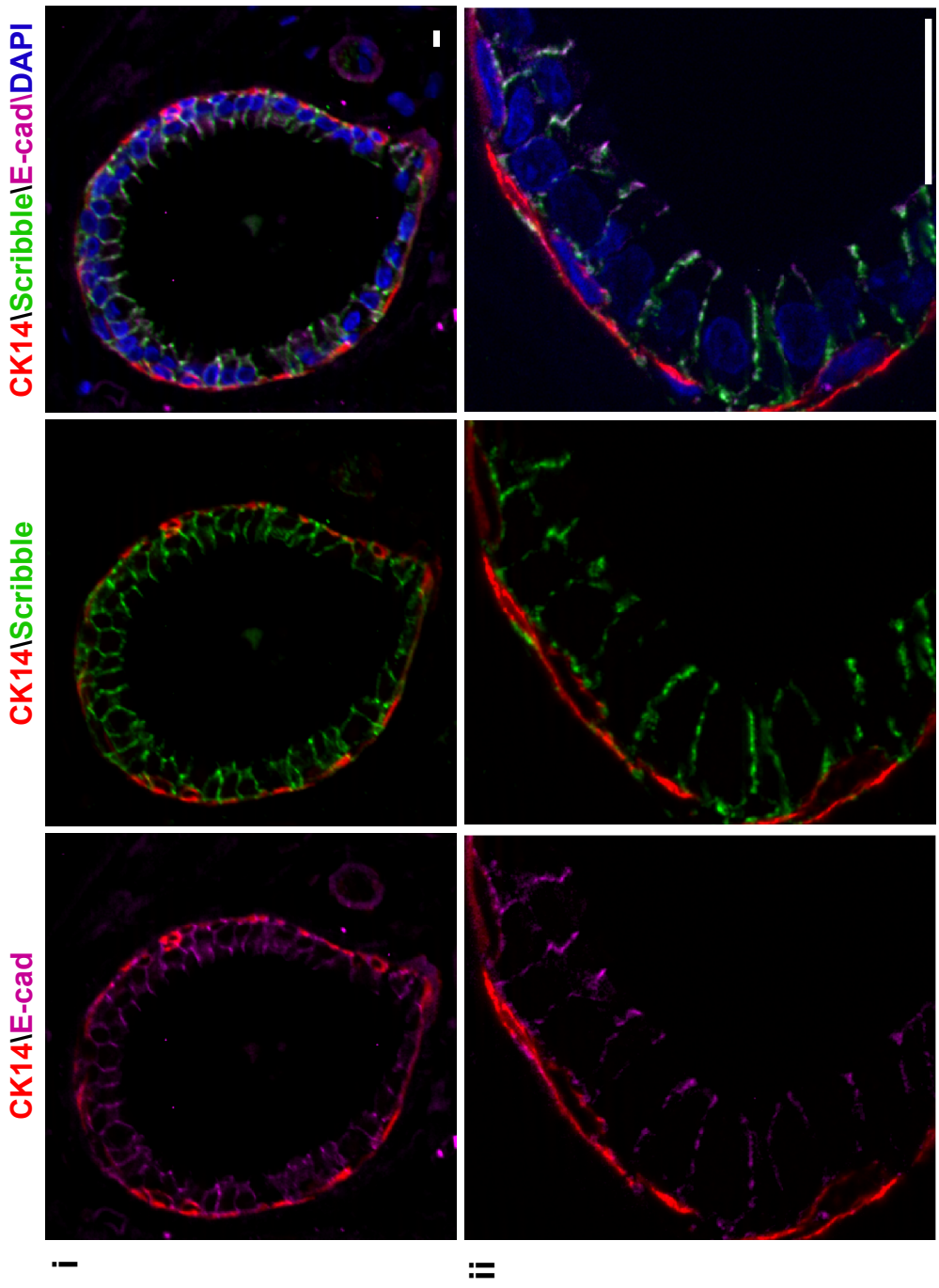


Figure 3.2

Scribble is differentially expressed in early breast lesions

Tissue sections from columnar hyperplasia **(A)** and ductal carcinoma in situ **(B)** were immunostained for Scribble, cytokeratin 14 and DAPI. In both lesions cytokeratin 14 positive cells are present that express less Scribble. Scale bars=20 μM

Figure 3.2

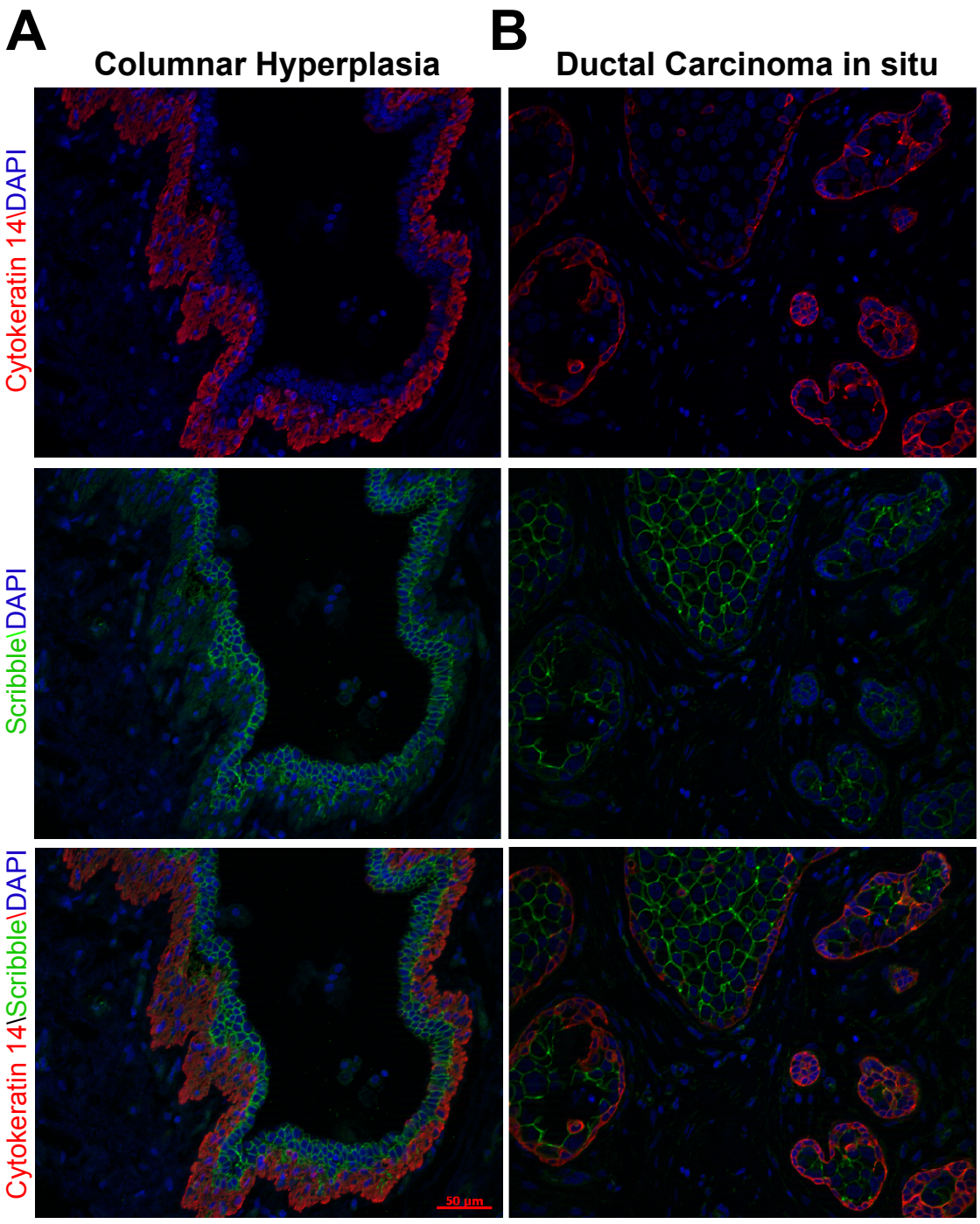

A
Columnar Hyperplasia
B
Ductal Carcinoma in situ
Cytokeratin 14\DAPI
Scribble\DAPI
Cytokeratin 14\Scribble\DAPI
50 µm

Figure 3.3

Scribble knockdown in Comma-1D cells results in altered phase morphology

(A) Immunoblot for Scribble expression in Comma-1D cells stably expressing two different Scribble shRNAs. Actin protein levels served as a loading control. **(B)** Phase morphology of control and Scrib.RNAi cells at low (i) and high (ii) magnification. Scale bars=20 μM

Figure 3.3

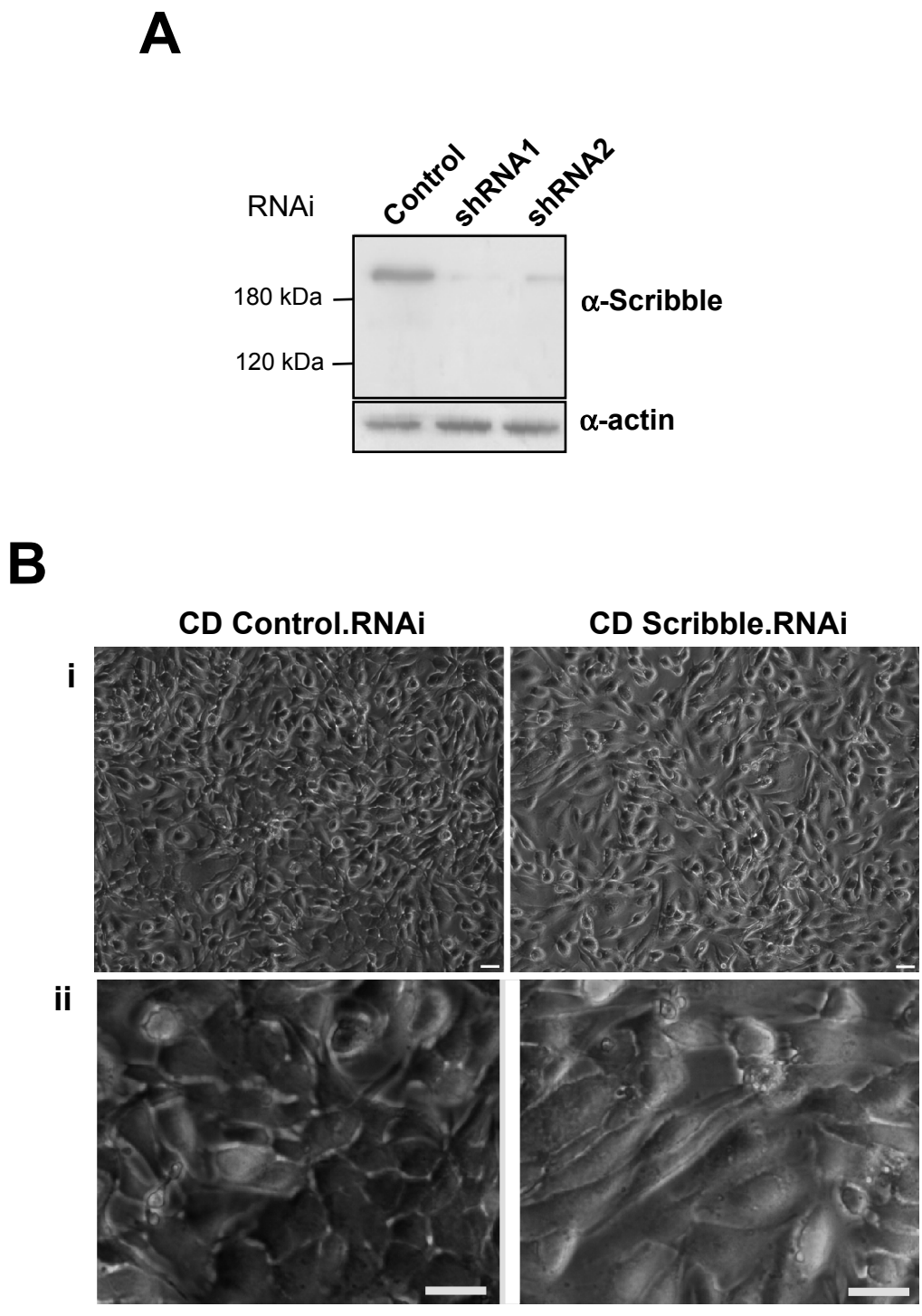

A
RNAi
Control
shRNA1
shRNA2
180 kDa
120 kDa
α-Scribble
α-actin
B
CD Control.RNAi
CD Scribble.RNAi
i
ii

Figure 3.4

Scribble knockdown expands the population of cells expressing basal markers

CD cells expressing Control and Scribble shRNA were immunostained for E-cadherin(red)/CK14(green)/DAPI(blue) **(A)** or p63(green)/CK18 (red)/DAPI(blue) **(B)**. **(C)** Percentage of CK14, p63 and CK18 positive cells were determined. The data represents an average of three experiments (>1500 cells were quantified/experiment, n=3, **p<0.01, *p<0.05). Scale bars=20 μM

Figure 3.4

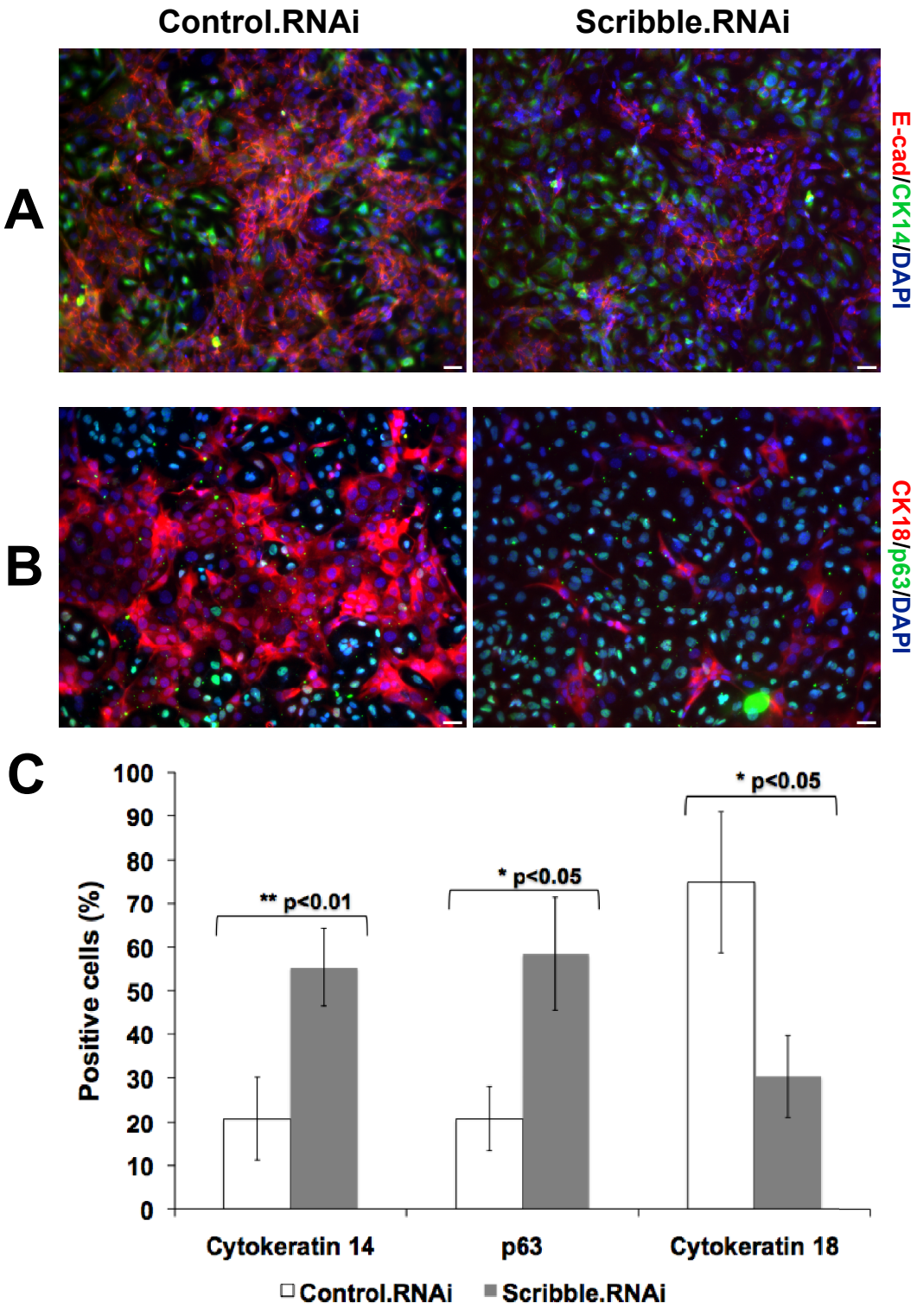

Control.RNAi
Scribble.RNAi
A
E-cad/CK14/DAPI
B
CK18/p63/DAPI
C
Positive cells (%)
100
90
80
70
60
50
40
30
20
10
0
** p<0.01
* p<0.05
* p<0.05
Cytokeratin 14
p63
Cytokeratin 18
Control.RNAi
Scribble.RNAi

Figure 3.5

Scribble knockdown phenotype is rescued by overexpression of Scribble cDNA

(A) Quantitation of CK6, a marker of proliferating progenitor cells, in CD.Control as well as CD.Scrib.RNAi cells expressing two independent shRNAs. (n=3, Scribble.RNAi1 **p<0.01; Scribble.RNAi2 *p<0.05) **(B**) Immunoblot for control and Scrib.RNAi cells expressing an RNAi-resistant Scribble cDNA. Tublin levels were used as a loading control. **(C)** RNAi-resistant cDNA expressing control and Scrib.RNAi cells were analyzed for changes in CK6 expression. Graph represents percentage of CK6 positive cells. (n=3, no significant difference).

Figure 3.5

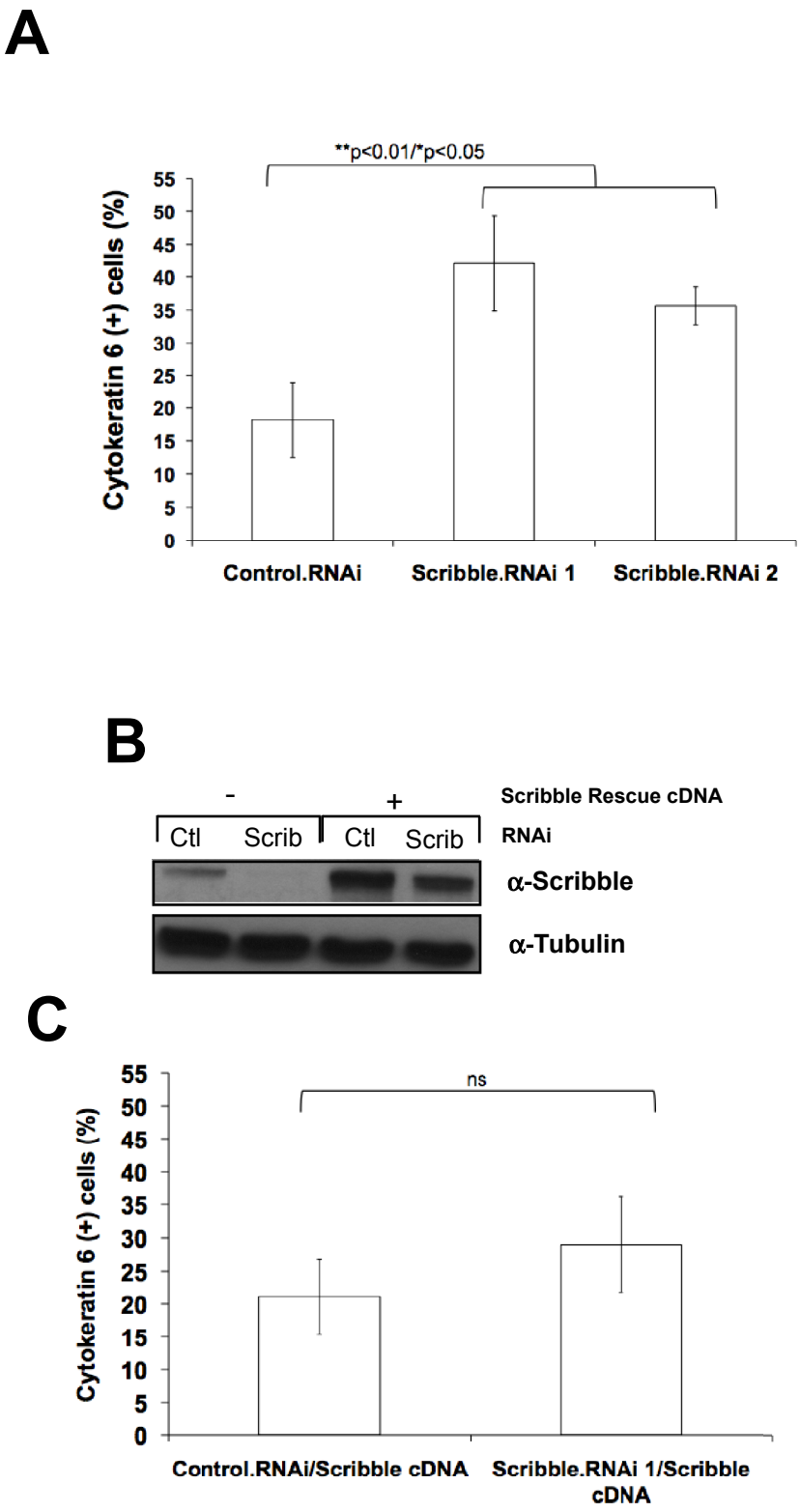

A
**p<0.01/*p<0.05
Cytokeratin 6 (+) cells (%)
55
50
45
40
35
30
25
20
15
10
5
0
Control.RNAi
Scribble.RNAi 1
Scribble.RNAi 2
B
-
+
Scribble Rescue cDNA
Ctl
Scrib
Ctl
Scrib
RNAi
α-Scribble
α-Tubulin
C
ns
Cytokeratin 6 (+) cells (%)
55
50
45
40
35
30
25
20
15
10
5
0
Control.RNAi/Scribble cDNA
Scribble.RNAi 1/Scribble cDNA

Figure 3.6

Scribble knockdown does not result in a morphological or cell fate change in Eph4 cells, a differentiated epithelial cell line

(A) Immunoblot demonstrating Scribble knockdown in EpH4 cells. **(B)** Phase morphology of Eph4 cells expressing control or Scribble shRNA. **(C)** Immunostain for CK6 (green)/CK18(red)/DAPI(blue) and **(D)** E-cadherin(red)**. (E)** Quantitation of CK6 positive cells in control and Scrib.RNAi expressing Eph4 cells. The data represents an average of three experiments (n=3, >150 cells/experiment, no significant difference). Scale bars=20 μM

Figure 3.6

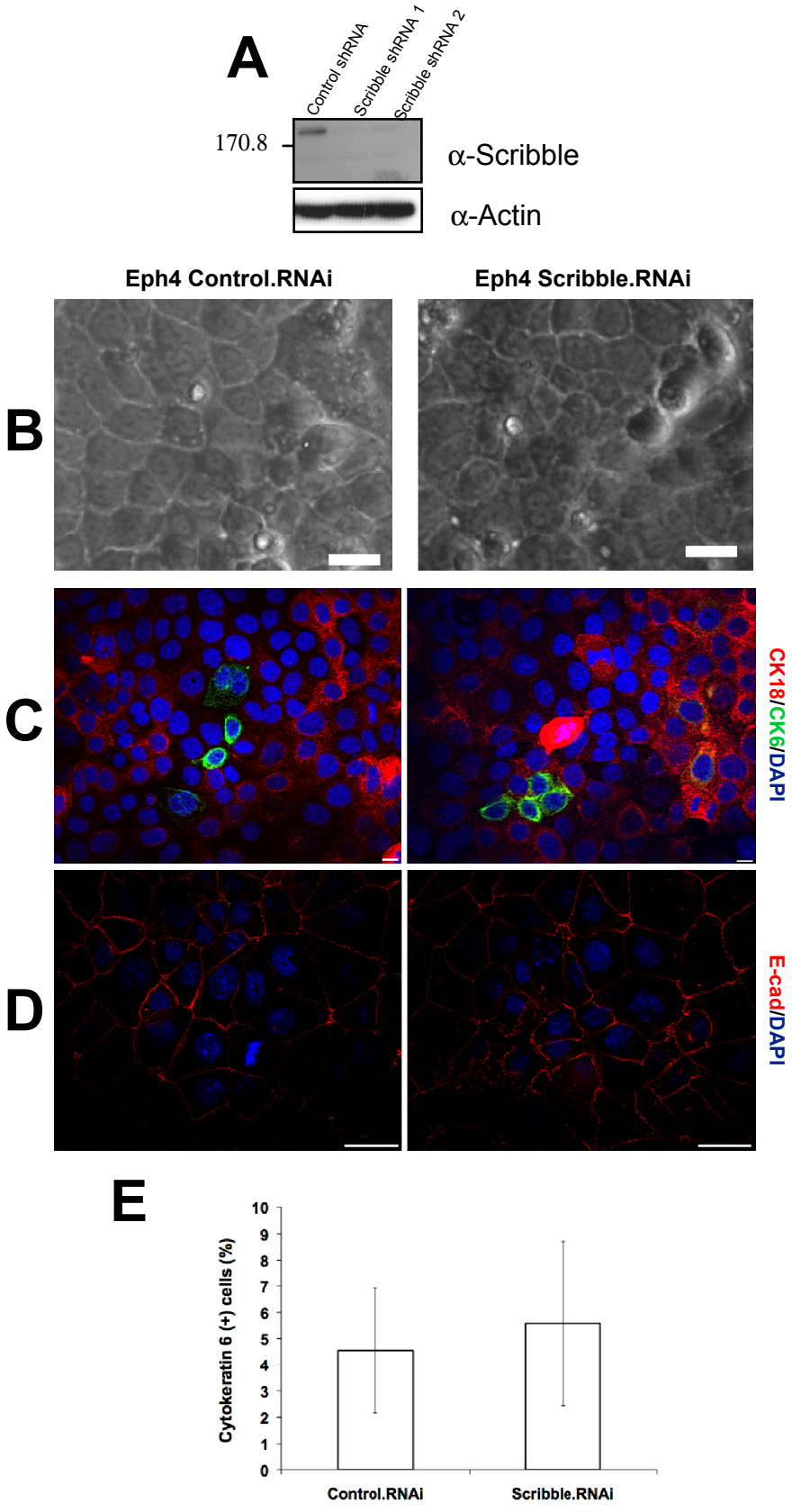

A
Control shRNA
Scribble shRNA 1
Scribble shRNA 2
170.8
α-Scribble
α-Actin
Eph4 Control.RNAi
Eph4 Scribble.RNAi
B
C
CK18/CK6/DAPI
D
E-cad/DAPI
E
Cytokeratin 6 (+) cells (%)
10
9
8
7
6
5
4
3
2
1
0
Control.RNAi
Scribble.RNAi

Figure 3.7

Loss of Scribble expression enhances secondary mammosphere formation
(A) Control and Scrib.RNAi CD cells were subjected to the mammosphere-forming assay. The data represents the percentage of secondary mammospheres formed (n=4, ***p<0.005). **(B)** Immunoblot demonstrating knockdown of scribble in primary human mammary epithelial cells derived from reduction mammosplasties infected with three independent Scribble shRNAs **(C)** Phase morphology of primary human mammary epithelial cells expressing control or Scrib.RNAi**. (D)** Control and Scrib.RNAi infected human mammary epithelial cells were subjected to the mammosphere-forming assay. The data represents the percentage of secondary mammospheres formed (n=2).

Figure 3.7

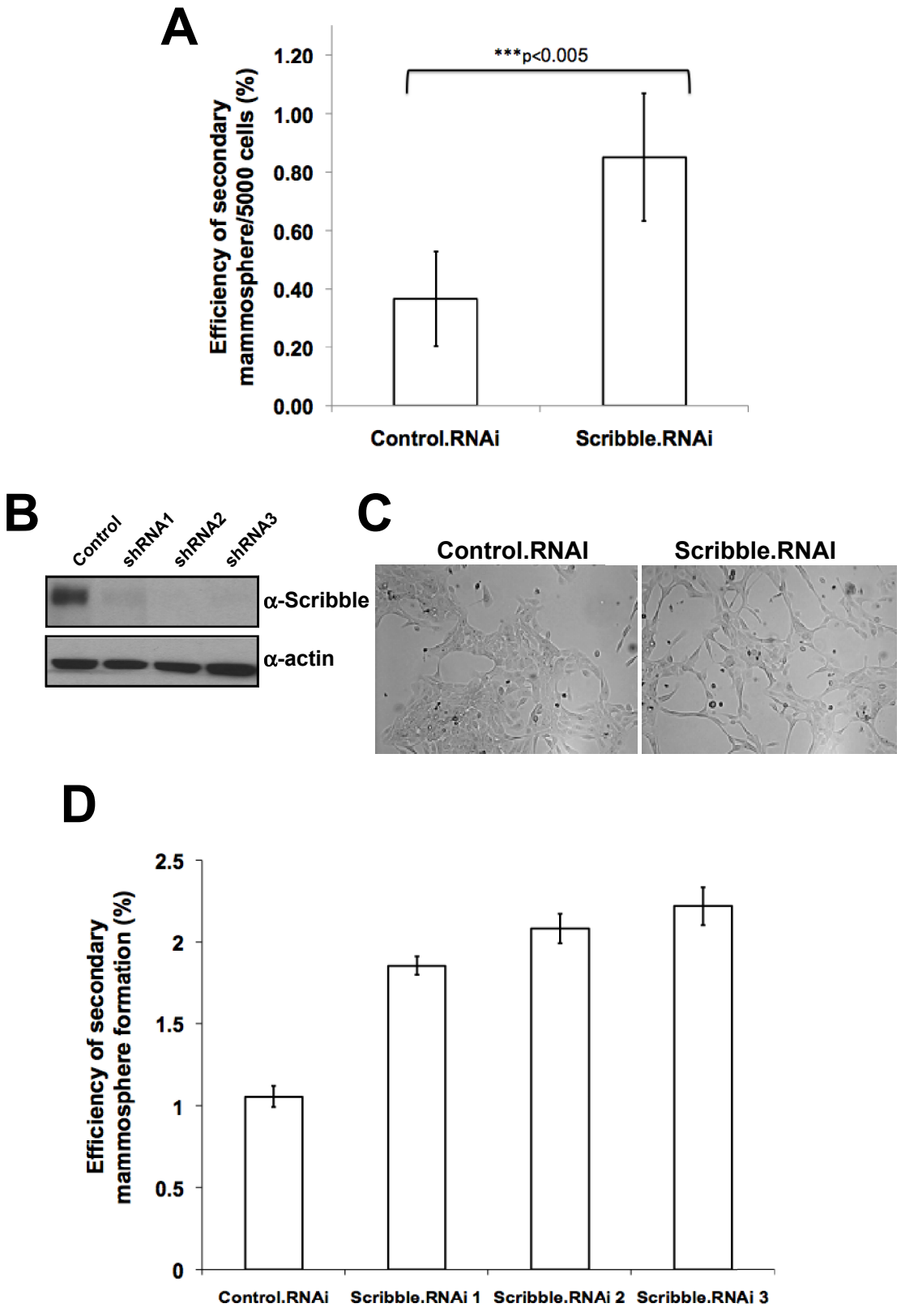

A
***p<0.005
Efficiency of secondary mammosphere/5000 cells (%)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Control.RNAi
Scribble.RNAi
B
Control
shRNA1
shRNA2
shRNA3
α-Scribble
α-actin
C
Control.RNAI
Scribble.RNAI
D
Efficiency of secondary mammosphere formation (%)
2.5
2
1.5
1
0.5
0
Control.RNAi
Scribble.RNAi 1
Scribble.RNAi 2
Scribble.RNAi 3

Figure 3.8

Scribble knockdown results in improved outgrowth potential from CD cells

Limiting dilutions of Comma-1D control and Scribble.RNAi cells were transplanted to the cleared mammary fat pads. Mammary whole mounts were analyzed eight weeks after transplantation to detect the presence of ductal outgrowth. Table represents number of glands with successful ductal outgrowths (P=0.004).

Figure 3.8

Dilution / shRNA	100	500	1000	10,000
Control.RNAi	2/15	5/24	6/22	15/17
Scribble.RNAi	5/15	11/24	11/22	14/17

Figure 3.9

Scribble.RNAi mammary outgrowths display regions with nodular hyperplasias and reduced tertiary branching

(A) Wholemounts of representative virgin glands transplanted with Control and Scrib.RNAi cells (Eight weeks post-transplantation). **(B)** Higher magnification of wholemounts demonstrates dense, hyperplastic regions in Scribble.RNAi glands. **(B')** Higher magnification of Scribble.RNAi wholemount demonstrates reduced tertiary branching patterns.

Figure 3.9

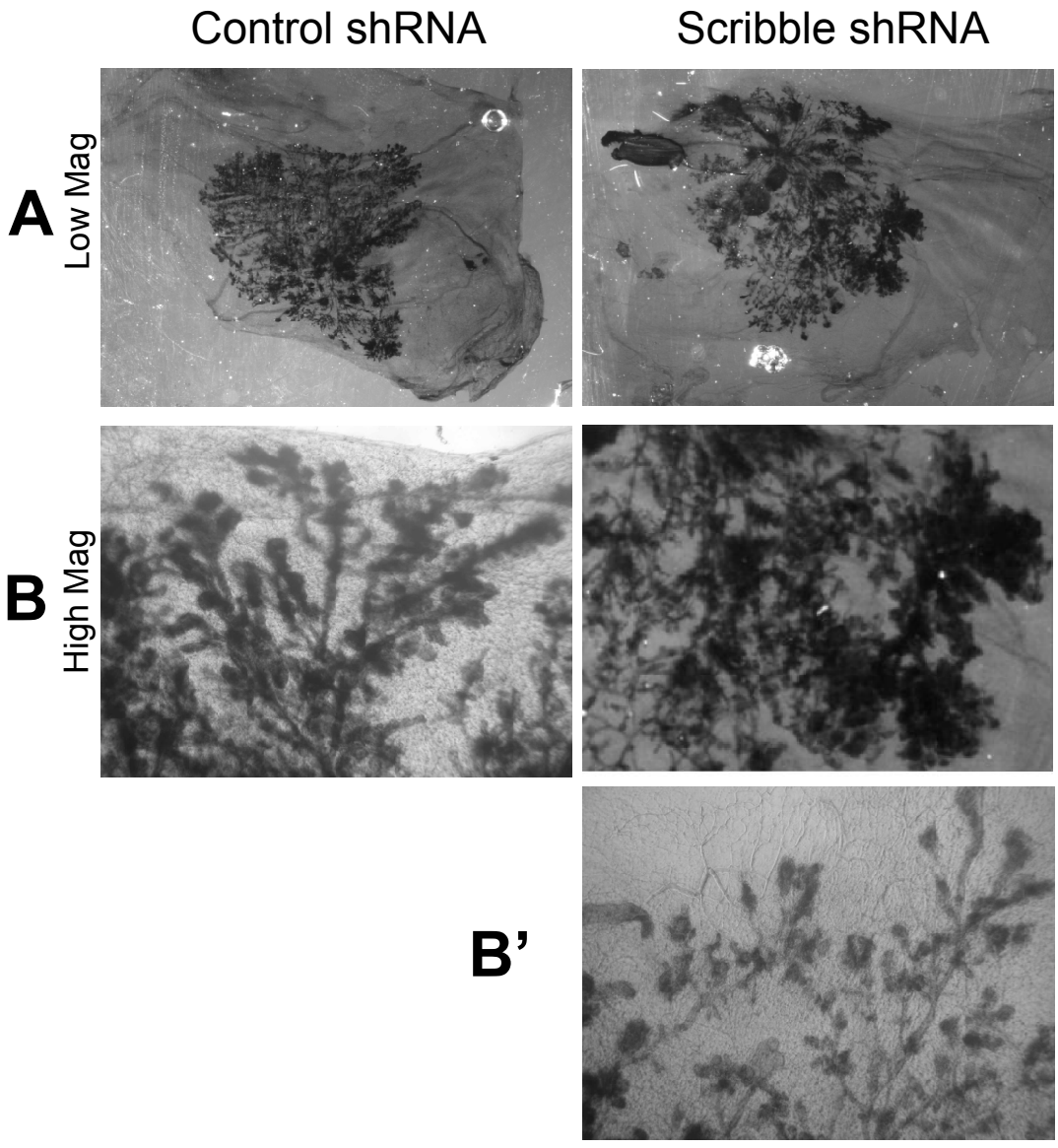


Figure 3.10

Ductal structures in Scribble.RNAi outgrowths are monolayers and do not exhibit luminal epithelial features

(A) H&E staining of tissue section from Control and Scrib.RNAi outgrowths in virgin glands. **(B,C,D)** Tissue sections from control and Scribble.RNAi glands were immunostained with indicated markers. Scale bars=20 μM

Figure 3.10

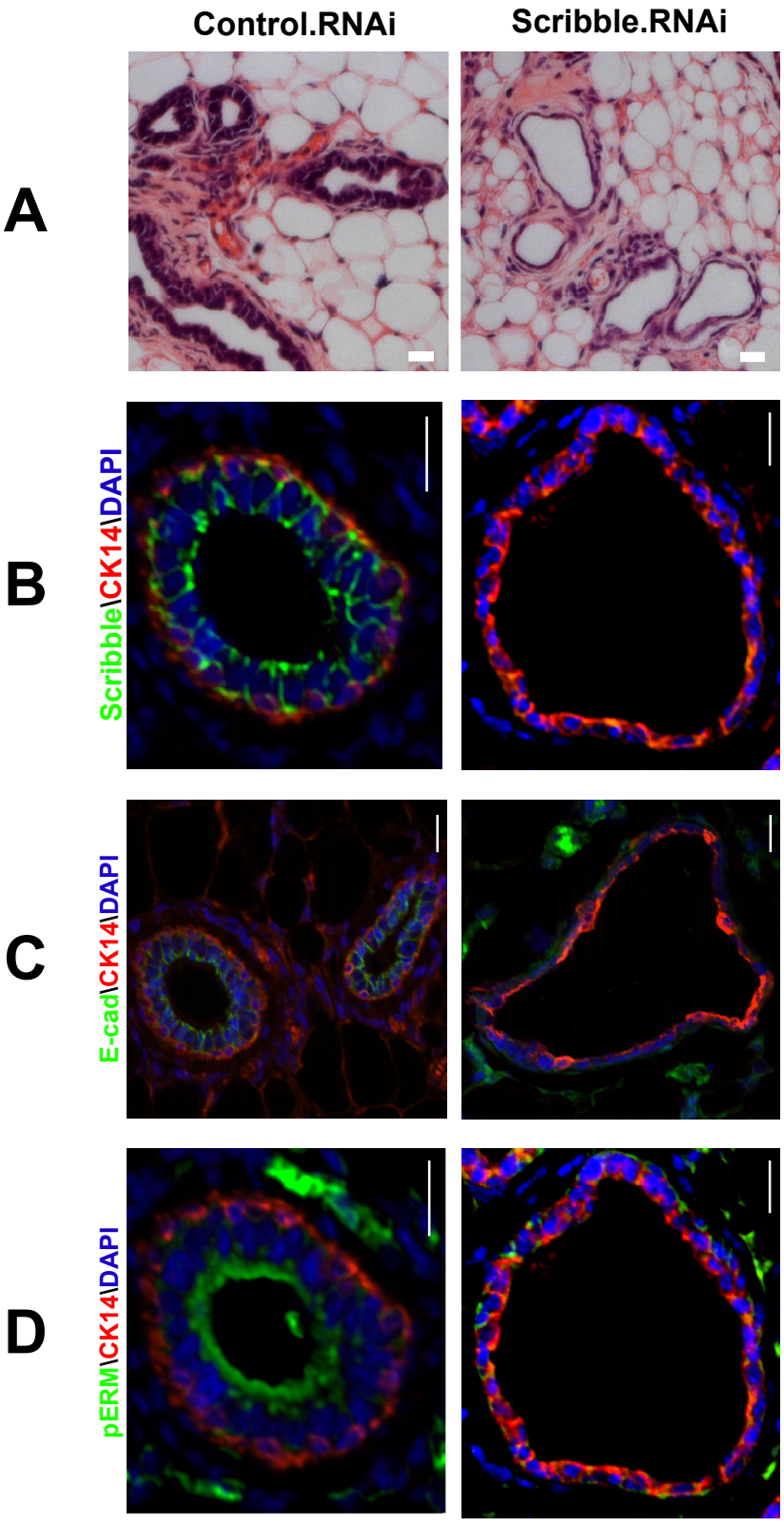

Control.RNAi
Scribble.RNAi
A
B
Scribble\CK14\DAPI
C
E-cad\CK14\DAPI
D
pERM\CK14\DAPI

Figure 3.11

Scribble knockdown glands fail to undergo pregnancy-induced differentiation

(A) H&E stained tissue sections of Scrib.RNAi or control glands from mice at day16 of pregnancy. **(B)** Tissue sections from **A** were immunostained with β-casein antibodies. Scale bars=20 μM

Figure 3.11

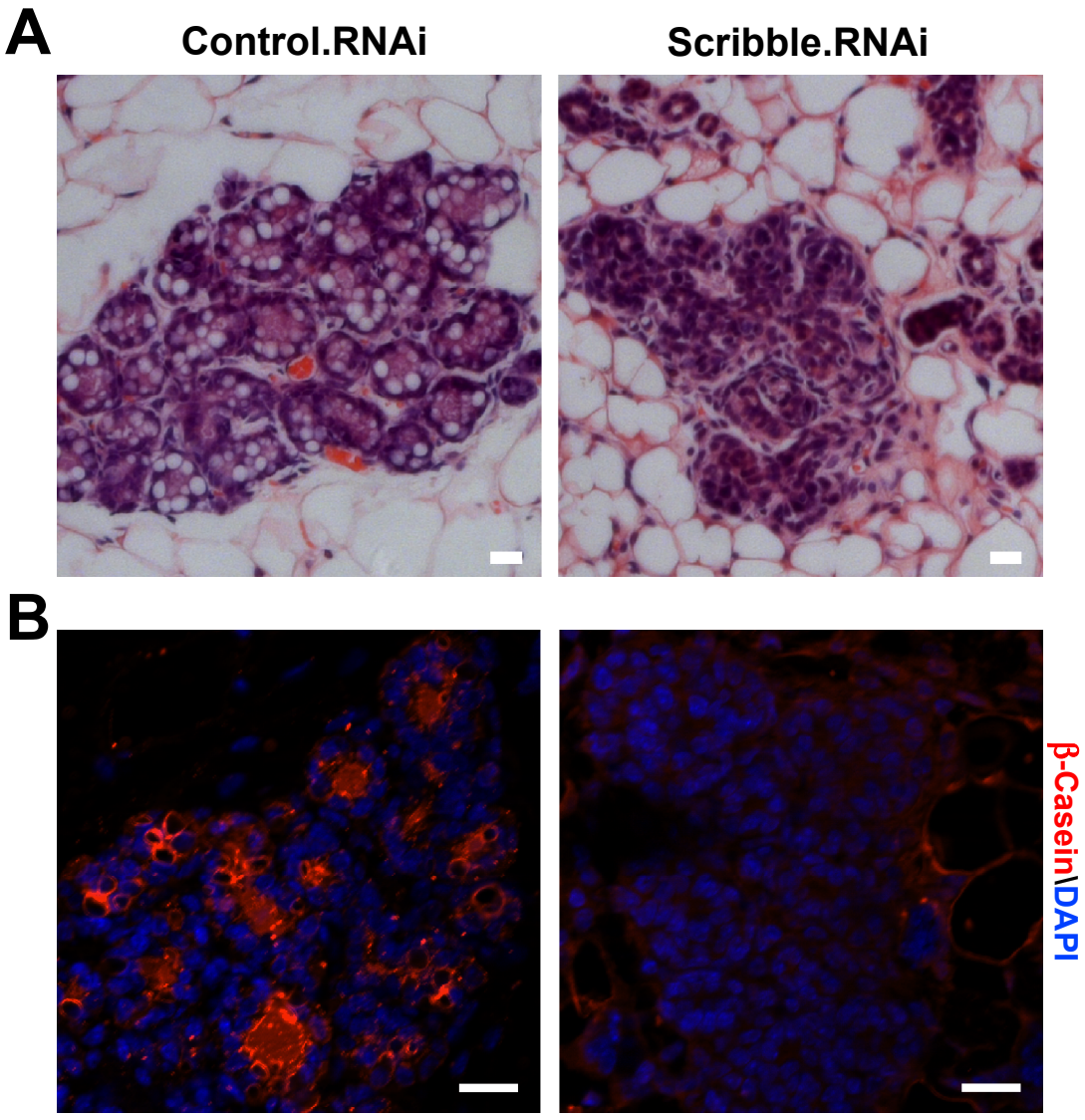


Figure 3.12

Scribble knockdown blocks lactational differentiation *in vitro*

Comma-1D cells in culture were treated with prolactin, insulin and hydrocortisone and mRNA was collected at various time-points up to 12 hours and analyzed for changes in β-casein mRNA by qRT-PCR.

Figure 3.12

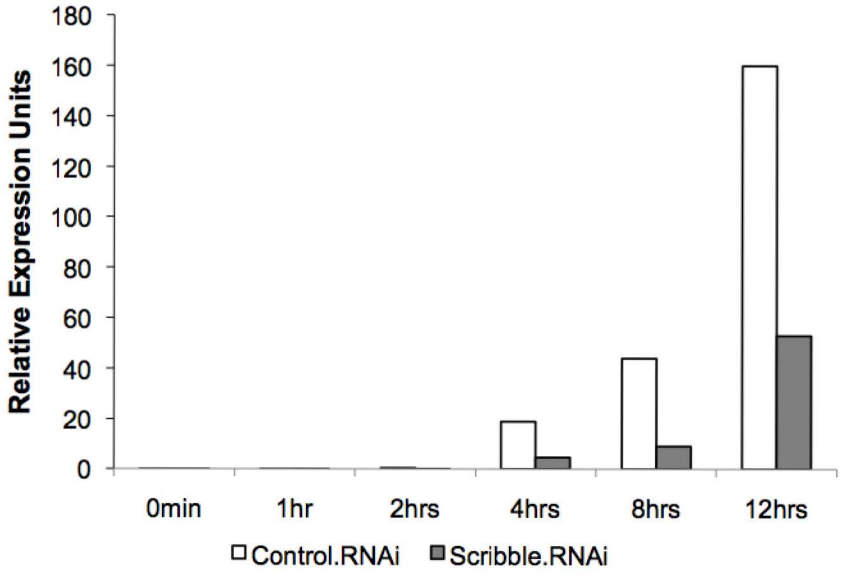

Relative Expression Units
180
160
140
120
100
80
60
40
20
0
0min
1hr
2hrs
4hrs
8hrs
12hrs
Control.RNAi
Scribble.RNAi

Figure 3.13

Loss of Scribble expression promotes ErbB2 driven tumorigenesis

(A) Immunoblot of control and Scrib.RNAi CD cells expressing activated ErbB2. Actin served as loading control. **(B)** Kaplan-Meyer graph representation of tumor onset of ErbB2.control.RNAi (Control, black line) vs ErbB2.Scribble.RNAi (Scribble, red line) cells transplanted into Balb/C mammary fat pads (median onset 104 days vs 45 days, n=12).

Figure 3.13

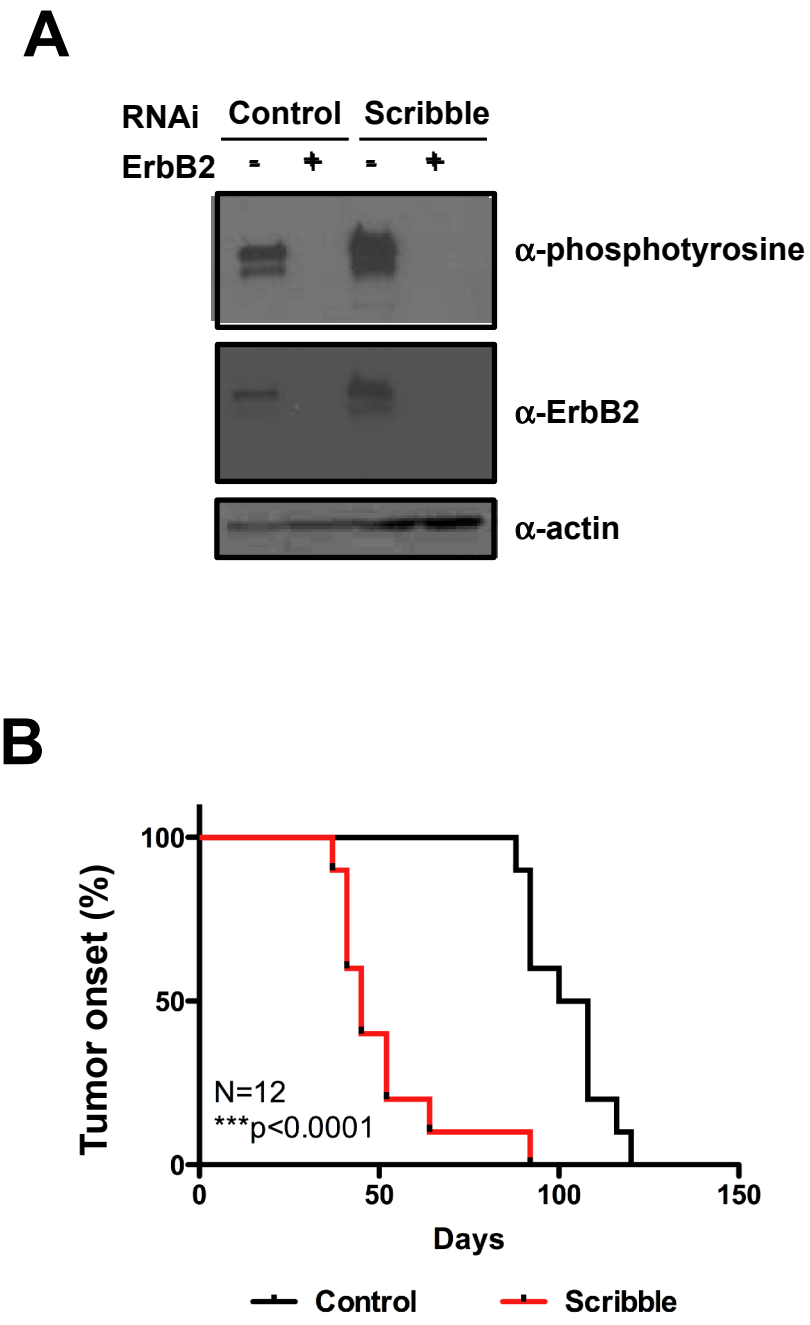

A
RNAi
Control
Scribble
ErbB2
-
+
-
+
α-phosphotyrosine
α-ErbB2
α-actin
B
Tumor onset (%)
100
50
0
N=12
***p<0.0001
0
50
100
150
Days
Control
Scribble

Figure 3.14

Loss of Scribble expression promotes ErbB2 driven basal tumors

(A) H&E stained tissue section of tumors derived from of ErbB2.control.RNAi and ErbB2.Scribble.RNAi cells. **(B)** Tumor sections immunostained with indicated markers **(i, ii)**. Scale bars=20 μM

Figure 3.14

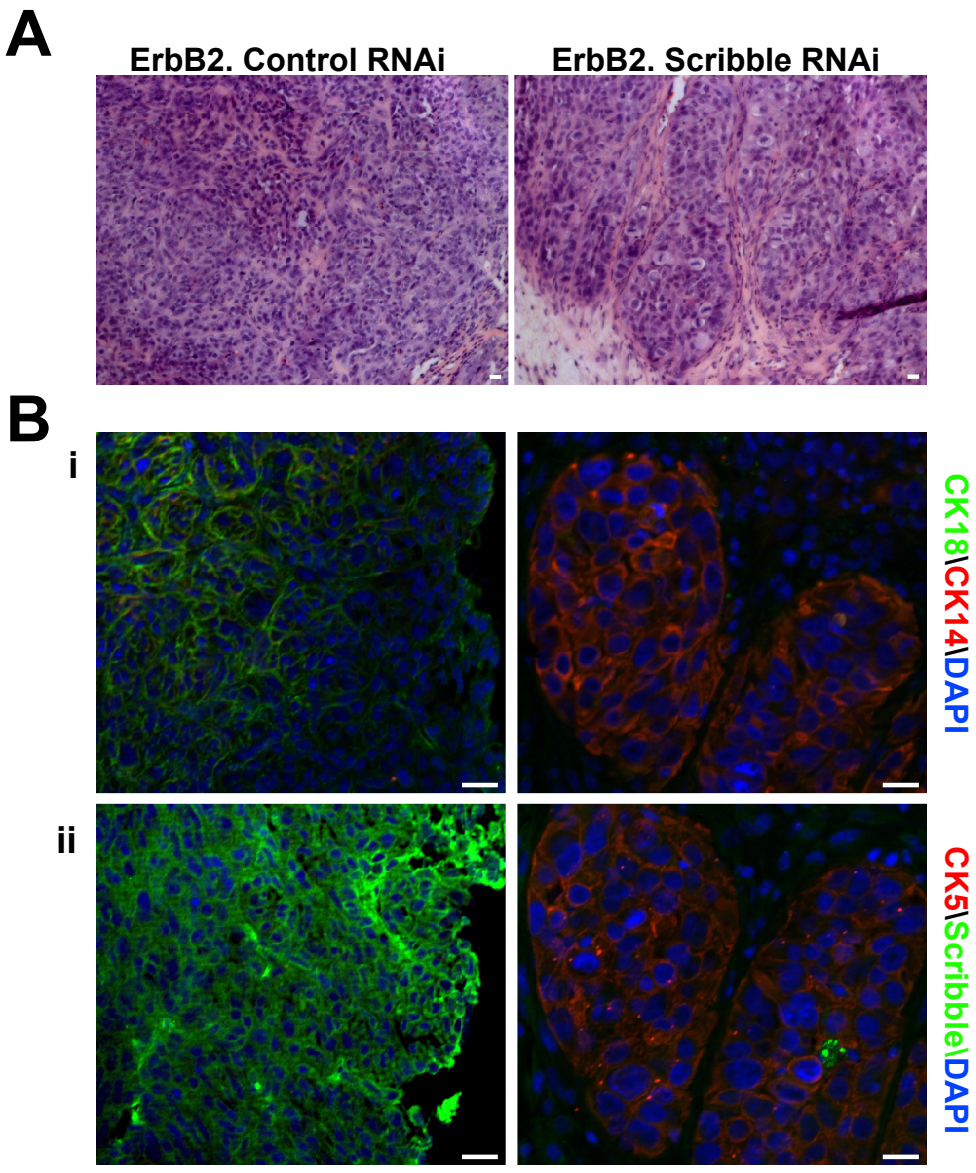

A
ErbB2. Control RNAi
ErbB2. Scribble RNAi
B
i
ii
CK18\CK14\DAPI
CK5\Scribble\DAPI

Figure 3.15

Scribble loss enhances basal colony formation and decreases bipotent colony formation

(A) CD cells were plated at colonogenic density and the resulting colonies were immunostained for basal (CK5) and luminal (CK18) cytokeratins. **(B)** Percentage of colonies that express only basal maker (CK5) and those that express both basal (CK5) and epithelial (CK18) were determined. Data represent the average of two experiments.

Figure 3.15

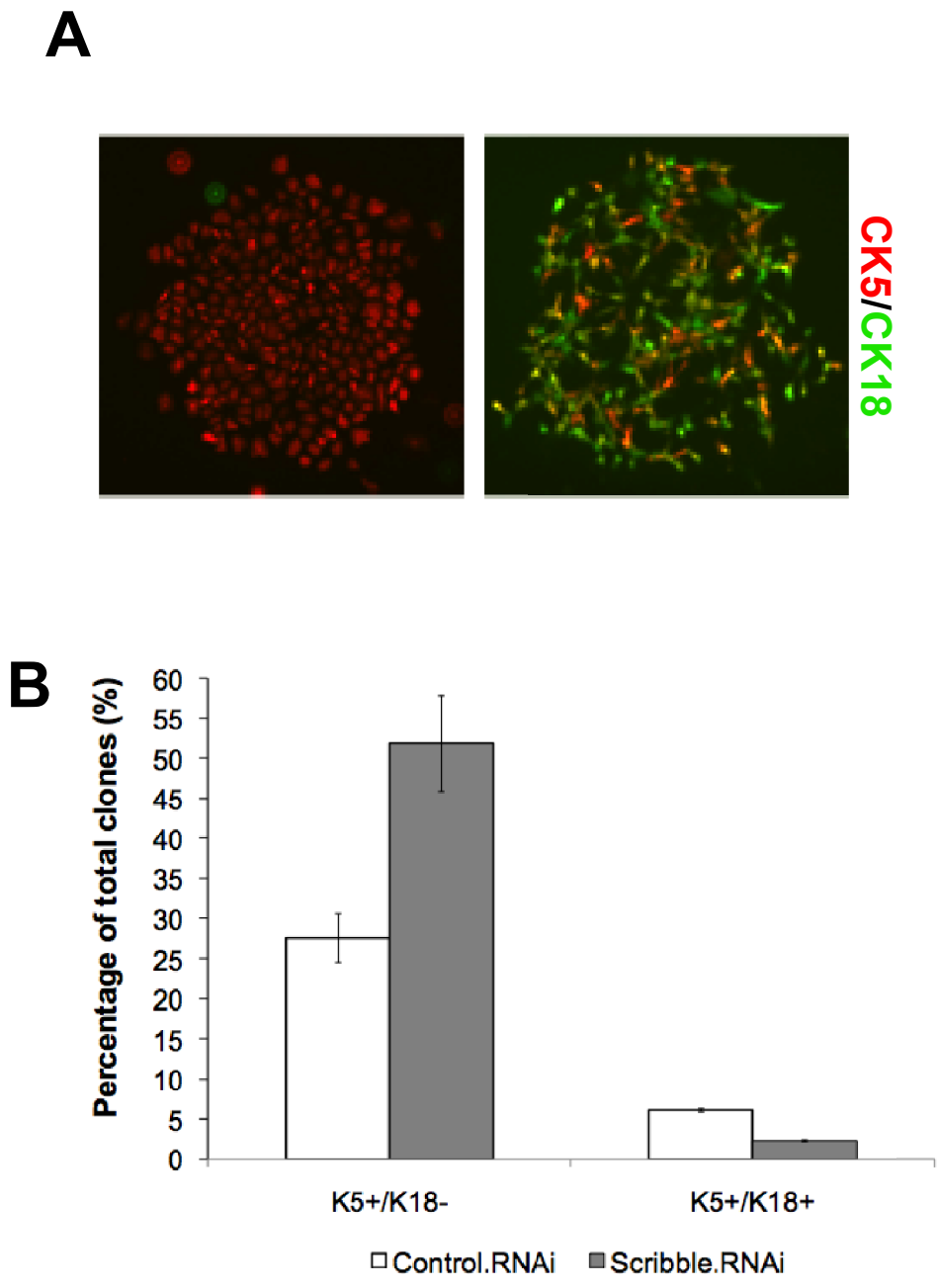

A
CK5/CK18
B
Percentage of total clones (%)
60
55
50
45
40
35
30
25
20
15
10
5
0
K5+/K18-
K5+/K18+
Control.RNAi
Scribble.RNAi

Figure 3.16

Scribble knockdown results in fewer asymmetric divisions in CD cells

(**A)** Comma-1D cells stained with NuMA and tubulin. A representative image of an asymmetrically dividing cell shown in the top panel and an example of a symmetrically dividing cell is shown in the bottom. **(B)** Percentage of mitotic cells that display asymmetric or symmetric division (n=2, >30 mitotic cells/experiment)

Figure 3.16

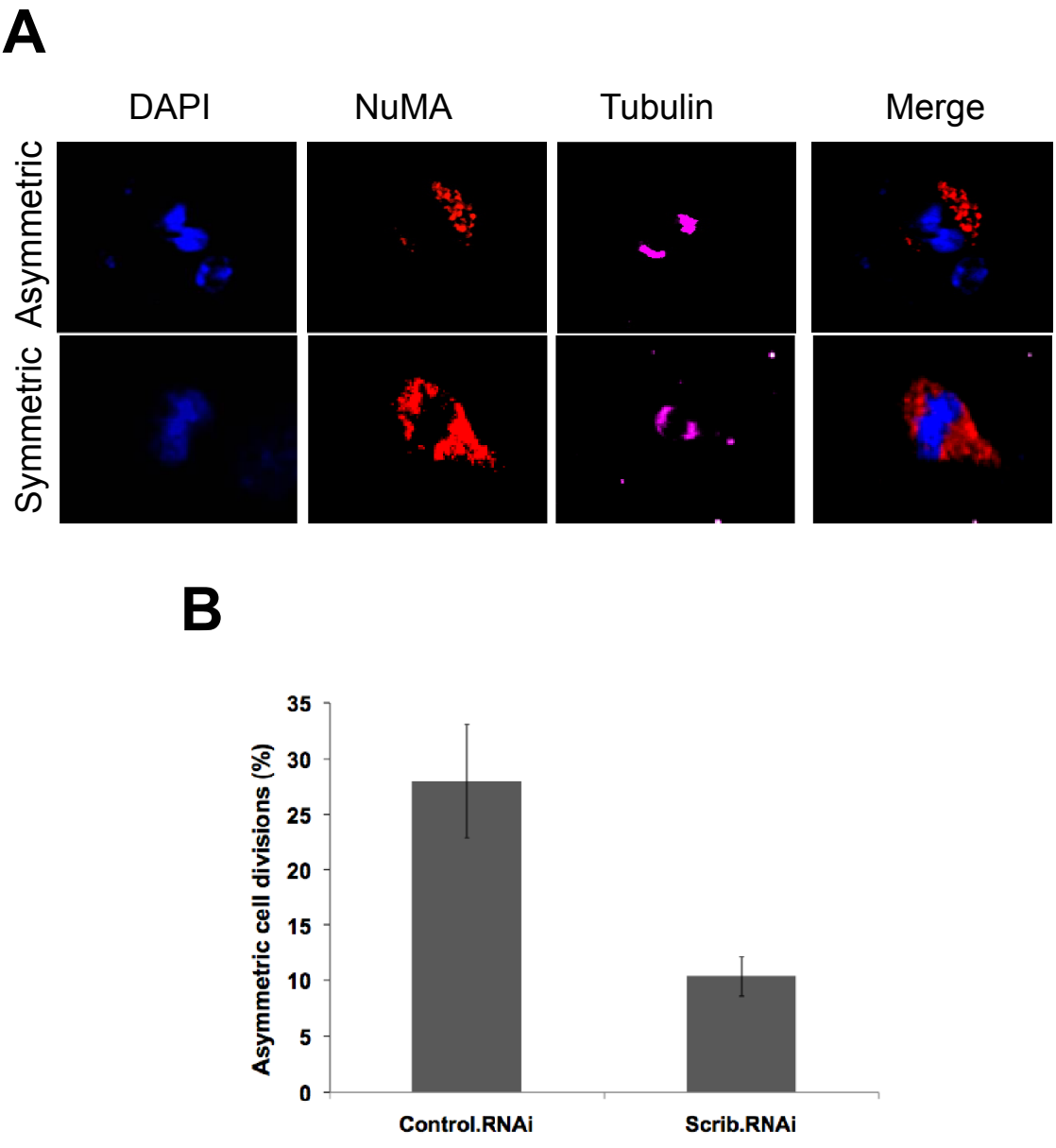

A
DAPI
NuMA
Tubulin
Merge
Asymmetric
Symmetric
B
Asymmetric cell divisions (%)
35
30
25
20
15
10
5
0
Control.RNAi
Scrib.RNAi

Figure 3.17

Comma-1D and MCF-10A cells with Scribble knockdown have more Let7c sensor positive cells

CD and MCF-10A cells expressing Let7c sensor were analyzed by Flow cytometry to detect presence of dsRed positive cells. The graph represents percentage of cells positive for dsRed (n=3, 10^6 cells sorted/experiment,***p<0.005)

Figure 3.17

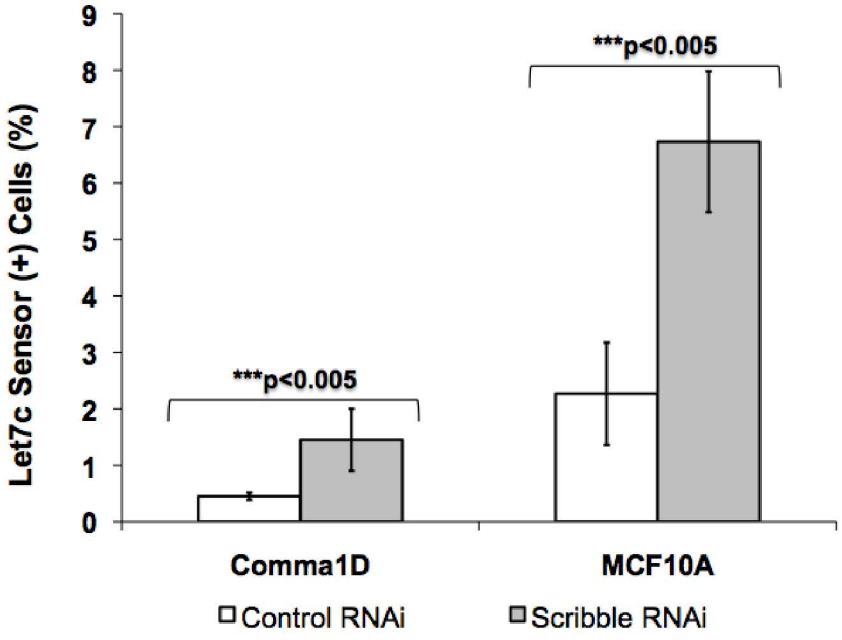

Let7c Sensor (+) Cells (%)
9
8
7
6
5
4
3
2
1
0
***p<0.005
***p<0.005
Comma1D
MCF10A
Control RNAi
Scribble RNAi

Figure 3.18

Scribble knockdown blocks asymmetric division of Let7c sensor positive cells

Sensor positive (red) cells were plated at a low dilution and fields with 4 - 5 red cells were followed over 72 hours. Both phase and RFP images were collected every hour. RFP and RFP-Phase merged views of representative fields at representative time points are shown. (See movies in Supplemental figure 3 for the entire series).

Figure 3.18

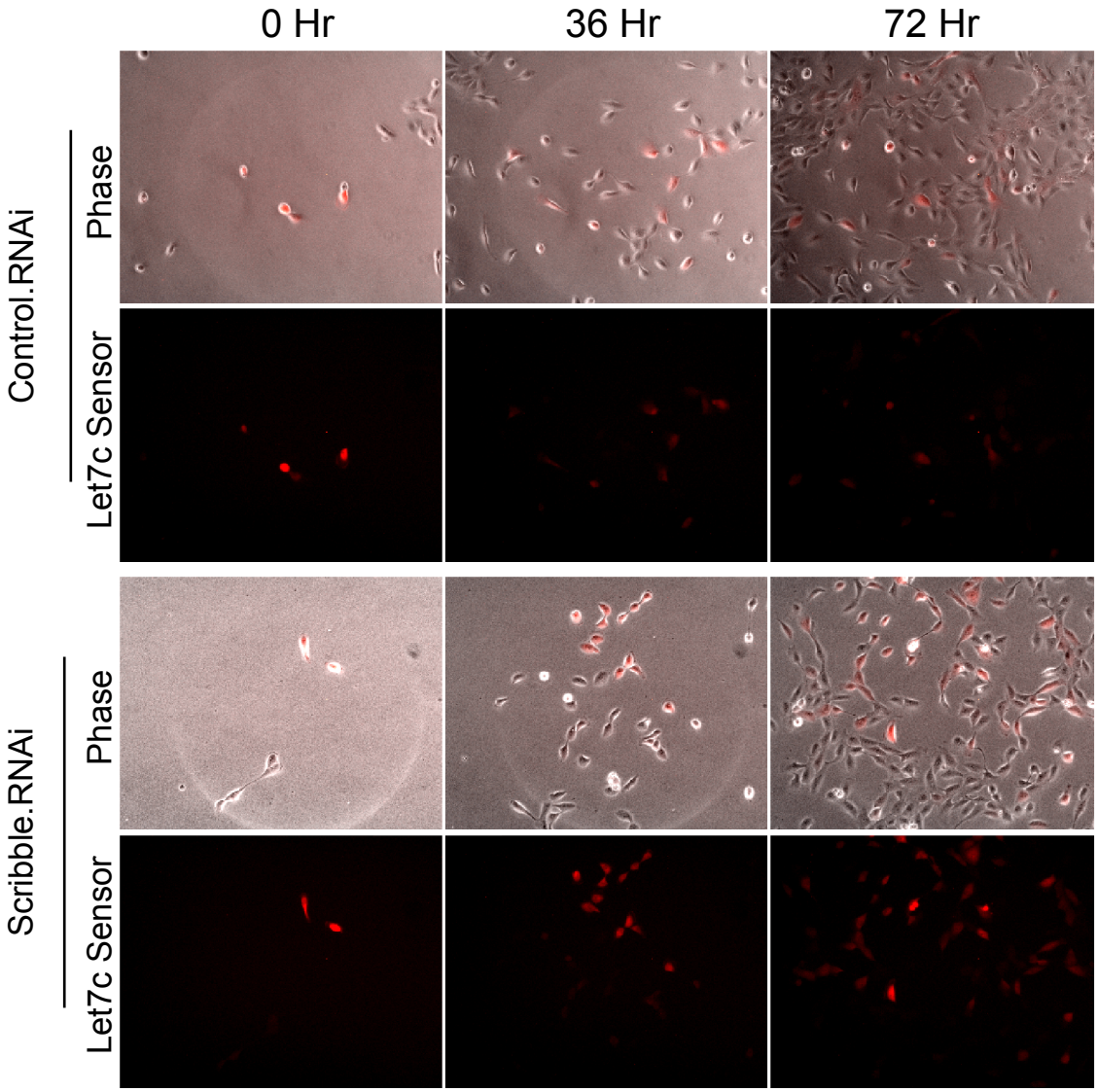
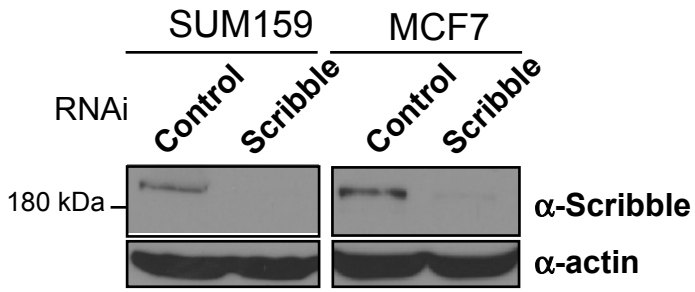

0 Hr
36 Hr
72 Hr
Control.RNAi
Phase
Let7c Sensor
Scribble.RNAi
Phase
Let7c Sensor

Figure 3.19

Scribble loss increases Let7c sensor positive cell population in breast cancer cell lines

(A) Immunoblot demonstrating Scribble knockdown in the SUM-159 and MCF-7 breast cancer cell lines. Actin protein levels served as a loading control. **(B)** Cells from **A** expressing engineered to express the Let7c sensor were analyzed by flow cytometry. Graph represents percentage of Let7c sensor positive cells in control and Scrib.RNAI population (n=3, 10^6 cells sorted/experiment,***p<0.005, **p<0.01).

Figure 3.19


SUM159
MCF7
RNAi
Control
Scribble
Control
Scribble
180 kDa
α-Scribble
α-actin

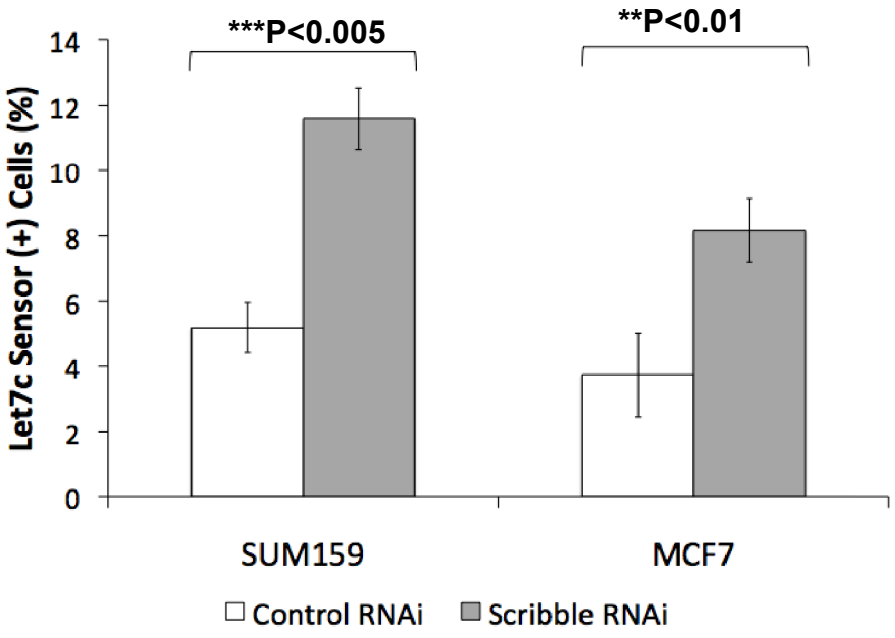

***P<0.005
**P<0.01
Let7c Sensor (+) Cells (%)
14
12
10
8
6
4
2
0
SUM159
MCF7
Control RNAi
Scribble RNAi

Figure 3.20

Loss of Scribble increases percentage of ALDH positive cells in SUM-159 cells

SUM-159 cells expressing control.RNAi or Scrib.RNAi were immunostained for Aldehyde dehydorgenase (n=2, >750 cells counted/expt).

Figure 3.20

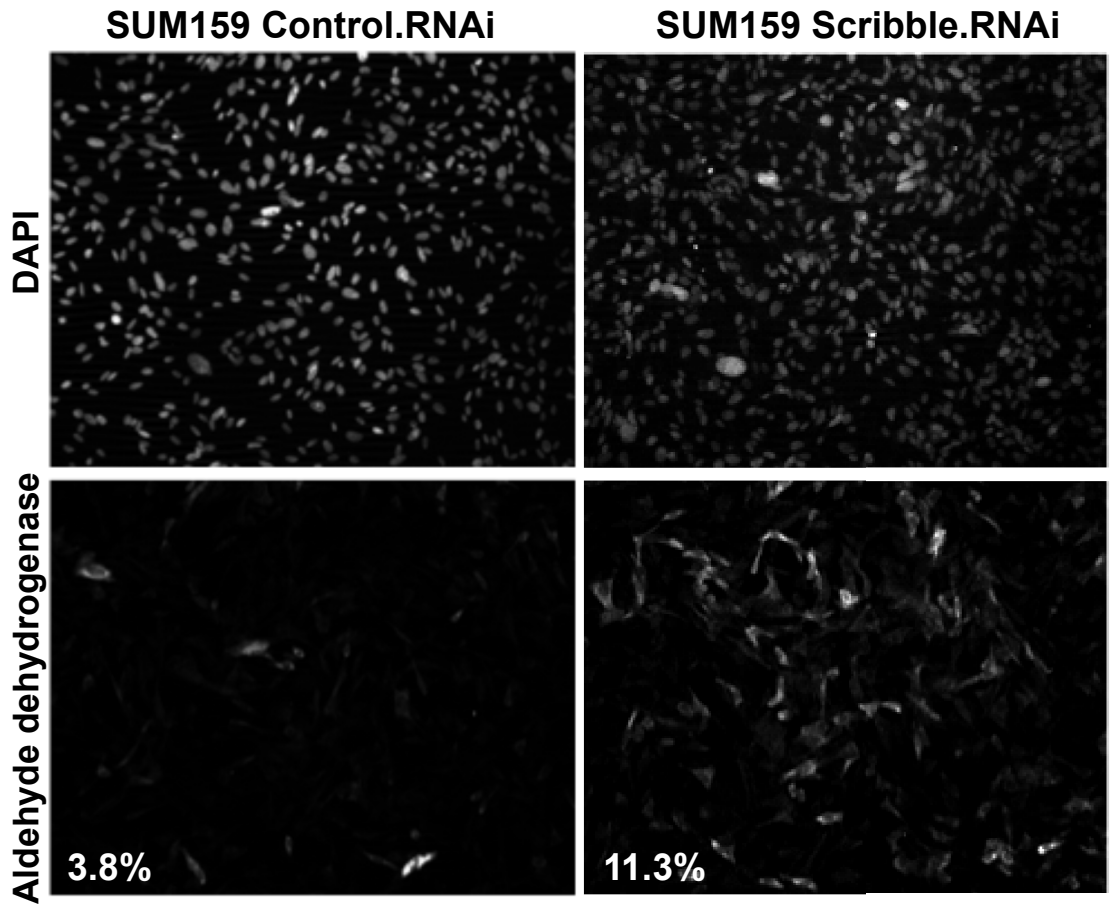

SUM159 Control.RNAi
SUM159 Scribble.RNAi
DAPI
Aldehyde dehydrogenase
3.8%
11.3%

Figure 3.21

Scribble loss enhances the tumorgensicity of SUM-159 cells

10,000 cells were injected subcutaneously into NOD/SCID mice and the Kaplan-Myer plot represents time to tumor onset.

Figure 3.21

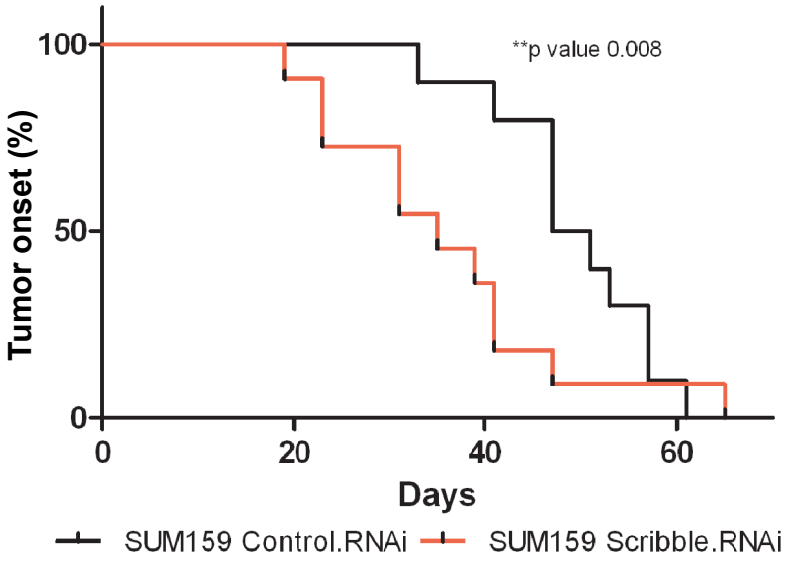

100
50
0
Tumor onset (%)
**p value 0.008
0
20
40
60
Days
SUM159 Control.RNAi
SUM159 Scribble.RNAi

Figure 3.22

Notch signaling activity is increased upon Scribble knockdown in CD cells

RNA isolated from control.RNAi (white), Scribble.RNAi (black) and Scribble.RNAi cells grown in the presence of (Gamma secretase inhibitor XXI (GSI) (4 μM)) (grey) were analyzed for expression of Notch target genes Hey1, Hey2, and Hes1 by qRT-PCR. The data represent the fold change in expression in Scribble.RNAi cells over the control.RNAi cells. (n=3, ***p<0.005).

Figure 3.22

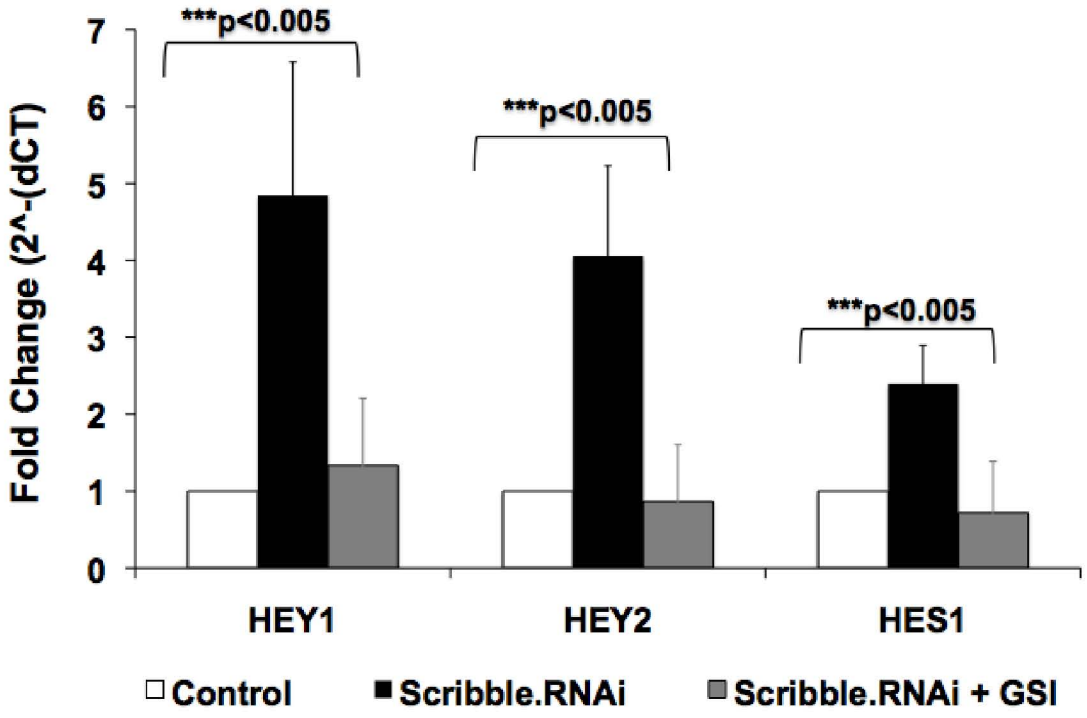
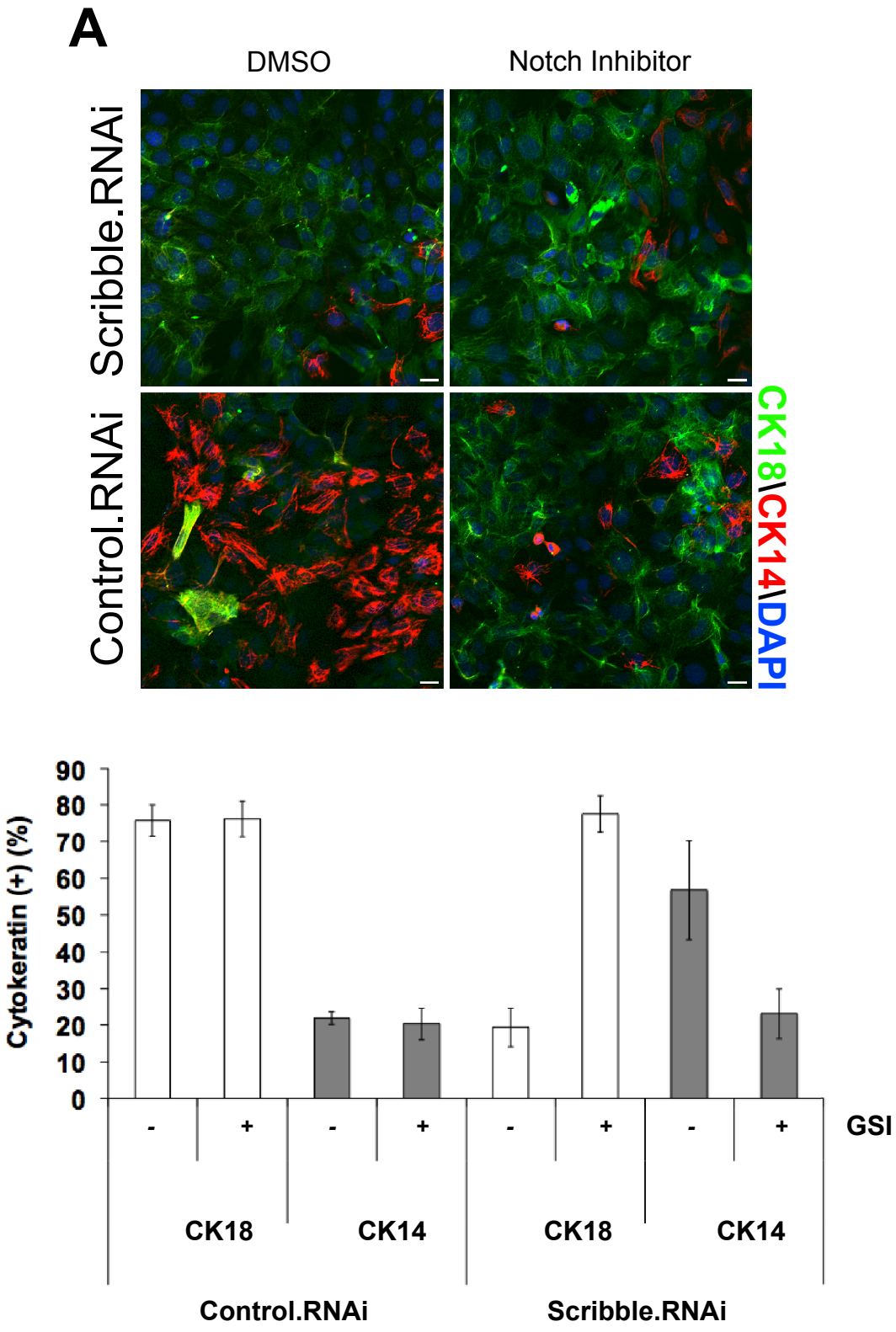

***p<0.005
***p<0.005
***p<0.005
Fold Change (2^-(dCT))
7
6
5
4
3
2
1
0
HEY1
HEY2
HES1
Control
Scribble.RNAi
Scribble.RNAi + GSI

Figure 3.23

Notch inhibition blocks Scribble-loss dependant increase in cells expressing basal cytokeratins and promotes luminal differentiation

(A) Control and Scribble.RNAi cells treated with GSI (4 μM) for two passages and cells were stained with CK14 (red) and CK18 (green). **(B)** Graph represent percentage of CK positive cells (n=3, >2500cells/expt). Scale bars=20 μM

Figure 3.23


A
DMSO
Notch Inhibitor
Scribble.RNAi
Control.RNAi
CK18\CK14\DAPI
Cytokeratin (+) (%)
90
80
70
60
50
40
30
20
10
0
-
+
-
+
-
+
-
+
GSI
CK18
CK14
CK18
CK14
Control.RNAi
Scribble.RNAi

Chapter 4

Conclusions and Future Directions

Following the identification of the polarity regulator Scribble in *Drosophila* it was quickly demonstrated to function as a tumor suppressor gene in *Drosophila* [67-69]. However, Scribble's function as a polarity regulator and tumor suppressor in mammalian model systems had not been fully addressed. In addition, though some correlations between Scribble expression and cancers have been made [89,106], Scribble was not demonstrated to play a causal role in disease initiation and progression. In my thesis I brought demonstrate two aspects of Scribble's function that are relevant to mammary gland biology and neoplasia. In chapter 2, I demonstrated that Scribble is required for normal mammary acinar morphogenesis and oncogene-induced apoptosis. In Chapter 3, I demonstrated that Scribble plays an important role in mammary gland development and neoplasia by regulating mitotic cell polarity and thereby cell fate decision of mammary gland progenitor cells. Thus establishing a role for Scribble during mammary morphogenesis and tumorigenesis.

Scribble was initially ascribed a role in neoplastic processes because its loss-of-function alone resulted in the disruption of tissue architecture and mild overgrowth [277]. Strong oncogenic signals such as Ras or Notch cooperated to promote massive overgrowth and invasion of the transformed tissue [68,69]. Likewise, our results demonstrate that Scribble loss alone only results in a mild tumor initiation phenotype as demonstrated by the mild polarity defects, block in luminal apoptosis, mild hyperplasia and low penetrance mammary tumors. Furthermore, with the addition of an oncogenic stimulus, c-myc, mammary epithelial cells underwent a neoplastic transformation. Scribble's abrogation of myc-induced apoptosis is similar to the effect reported for other regulators of apoptosis in myc-dependent oncogenesis. Previous reports in lymphoma and mammary gland models have demonstrated that myc-induced apoptosis occurs in a Bim-dependant manner and that myc overexpression cooperates with

molecules that block apoptosis (Bcl2 overexpression or Bax loss) to enhance tumorigenesis [208,209,213].

To understand the mechanism underlying the regulation of apoptosis, Dr. Lixing Zhan, has elaborated a Scribble-dependent Rac1 activity that initiates a Jnk-mediated apoptosis cascade to mediate luminal and oncogene dependant apoptosis [185]. This has provided us with a detailed mechanistic insight into how Scribble modifies oncogene dependent apoptotic stimuli as well as morphogenetic apoptotic stimuli.

In contrast to the block of apoptosis observed in differentiated mammary epithelial cells, an entirely different aspect of Scribble's biology can be observed when expression is lost in undifferentiated cells. I demonstrated that Scribble expression is required in mammary epithelial progenitor cells to maintain the mitotic cell polarity necessary for differentiation of epithelial cells. Intriguingly in Drosophila neuroblasts a similar role has been reported for Scribble, where scribble loss results in the expansion of progenitor populations [88]. This increase is dependant on the asymmetric distribution of proteins required for exiting the neuroblast state [278]. Though this aspect of Scribble biology is restricted to undifferentiated cells, its impact is relevant to all of the subsequent differentiation steps of the breast progenitors including ductal morphogenesis and lactation. Furthermore, Scribble loss increases the number of with markers of cancer stem cells. Thus Scribble loss in undifferentiated cells plays a role in subsequent normal and diseased stages in the breast.

Open questions

These results open up a plethora of exciting questions integral to understanding Scribble in development and diseased states. In particular a deeper understanding of the biochemical means by which Scribble regulates MCP. My analysis has demonstrated that Scribble-dependent suppression of Notch activity is critical to MCP. Studies in *Drosophila* neuroblasts suggest that control over Notch activity by the negative regulator Numb is vital for the differentiation of GMCs following asymmetric mitotic division [80]. Similarly,

mammalian hematopoietic stem cells have more Notch activity in cells expressing low Numb following mitotic division [264]. To address whether a similar mechanism functions in mammary epithelial progenitors it will be necessary to investigate the biochemical link between Scribble and suppression of Notch activity.

This regulation of Notch activity by Scribble may be relevant to diseased states as well. Recent findings suggest that Notch activity is of prognostic importance in breast cancer. A Notch 1 signature was identified in a microarray and meta-analysis study and was significantly associated with basal tumors and poor survival [278]. In general basal like breast tumors are associated with poor prognosis [243,260], However, what signaling pathways define these poorly differentiated tumors is still unclear. Our results suggest that scribble expression may lie upstream of the activated Notch signature in basal –like tumors. If indeed the aberrant Notch activity regulates tumor differentiation status, it may suggest a novel therapeutic approach to treat aggressive basal-like breast tumors. For example, Notch inhibitors could possibly be used as a differentiation therapy to differentiate these tumors therby generating a more clinically responsive tumor. Thus the insight gained into Scribble's role in self-renewal processes resulting in basal cell expansion may prove useful in thinking about novel therapeutic approaches.

Another issue to address is whether Scribble expression functions in a temporal way in determining disease outcome (i.e., apoptosis versus cell-fate decisions). We have already generated an Scribble RNAi mouse model in collaboration with Prem Premisrut (Lowe Lab, CSHL). Using the Scribble shRNA sequence (RNAi1) used extensively in the experiments presented in this thesis. Briefly, FLP mediated recombination was used to generate ES cells with a tetracycline inducible Scribble shRNA in the mouse collagen A1 locus [279]. Transgenic mice carrying the shRNA can then be mated with tissue specific Tet transactivators to spatially control Scribble expression. Furthermore, the mice can be crossed to different oncogene expressing mouse lines to determine the *in*

vivo dependence of oncogene mediated transformation on temporally controlled Scribble expression. Future investigations directly addressing the role of Scribble loss prior to oncogene-induced transformation versus following oncogene-initiated transformation has already begun. Based on the results presented in this thesis I expect that use of this model will allow us to comprehensively study the evolution of different subtypes of breast cancer. Furthermore, we can study what the effect of re-initiating Scribble mediated apoptosis regulation and polarity will have on tumor progression/regression.

Finally a finer understanding of the expression pattern of Scribble in the normal breast as well as diseased breast would provide us with a better idea of where and how Scribble expression is relevant in development and disease. A better understanding of Scribble expression patterns in the normal breast will be necessary to determine precisely which cells express Scribble and to what extent. Then to determine if Scribble expression modifies clinical outcome it will be necessary to survey a broad but comprehensive spectrum of human breast cancers and to determine if Scribble misregulation correlates with clinical outcome. In this survey both Scribble expression as well as localization must be taken into account. For example: Does loss of Scribble expression correlate with development of drug resistance? Does Scribble misexpression correlate with more aggressive basal-like tumors of the breast? Do changes in Scribble correlate with a particular oncogenic event and does it modify the tumors response? Does Scribble affect long-term outcome? For example, data suggests that Myc amplification and overexpression correlated with modified patients response to anthracycline therapy and correlated with poor overall survival but did not correlate with tumor subtype [224]. Using Scribble expression can this cohort be broken down further to help us identify the refractory subset of patients?

Concluding remarks

I have thus demonstrated that regulation of Scribble is critical for the differentiation programs during mammary gland development. These data present the first evidence of a role for polarity regulators in the differentiation of the mammary gland. Furthermore, we elaborated two means through which Scribble mediates a tumor suppressive effect: by regulating apoptosis in differentiated cells and via differentiation of progenitor cells. Hence the data presented herein will lay the foundation for future work in pursuit of a deeper understanding of the regulatory roles played by polarity in development and neoplasia. My hope is that the understanding my work provided with regards to Scribble expression and tumor initiation can impact patient care and suggest new methods for changing the course of disease progression.

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