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Mammary Epithelial Cell Fate & EGFR-Rasgrp1-Ras Effector Signals

by

Alexandr Joseph Samocha

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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by
Alexandr J. Samocha

DEDICATION AND ACKNOWLEDGEMENTS

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Mammary Epithelial Cell Fate & EGFR-Rasgrp1-Ras Effector Signals

Alexandr J. Samocha

Abstract

Mammary stem cells (MaSC) are a heterogeneous population that give rise to progenitor cells subsequently developing into differentiated cells. The exact mammary epithelial cell (MEC) hierarchy and particularly the signals that govern this hierarchy are largely unknown. Several studies have implied a role for EGFR signals in the mammary epithelium but the exact role that of EGFR (Epidermal Growth Factor Receptor) has remained rather elusive. Insights from a 3-D *ex vivo* model derived from human breast tissue and studies using a hypomorphic EGFR allele have put forth a model that the strength of EGFR signals may impact cell fate decisions in the mammary gland. We obtained recent evidence indicating that the Ras guanine nucleotide exchange factor (RasGEF) Rasgrp1 can dampen EGFR signals in epithelial cells.

We demonstrate that RasGEF Rasgrp1 is expressed in mammary epithelium and use cleared fat pad approaches to reveal a previously unrecognized functional role for RasGRP1 in the mammary gland. These approaches involved the transplantation of mammary epithelial cells (MECs) from *Rasgrp1*-deficient animals and from mice with a single point mutation in *Rasgrp1*, the *Rasgrp1*^{Anaef} model. Biochemically, loss of Rasgrp1 as well as the *Rasgrp1*^{Anaef} point mutation resulted in increased EGF-induced Ras-effector kinase signaling. MECs showed increase activation of the ERK-, Akt-, and mTORC1-S6- kinase pathways upon EGF stimulation when the MECs were isolated from

Rasgrp1$^{-/-}$ or the *Rasgrp1*^{Anaef} females. Using a model cell line, Eph4, we demonstrated that Rasgrp1's suppressive action depends on its catalytic activity.

Functionally, *Rasgrp1* perturbation led to shortened mammary ductal trees in mammary whole mounts, increases in numbers of proliferative terminal end buds (TEBs), and sustained proliferation of mammary ductal cells (with high phospho-Akt) in areas where proliferation has normally halted. Organoid assays to reveal that *Rasgrp1* perturbation led to a gain-of- function EGF-driven phenotype. With WT MECs, EGF drove the formation of spheres in organoid assays. By contrast, EGF induced robust branching of *Rasgrp*$^{1-/-}$ and *Rasgrp1*^{Anaef} MECs that could be prevented when EGFR inhibitors are added. Thus, Rasgrp1 critically controls the mammary morphogenetic program as indicated by elevated branching morphogenesis upon EGF-EGFR signaling in the absence of Rasgrp1 function.

MEC colony assays using *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef} cells revealed that progenitors form more and larger colonies than WT counterparts and with increased proliferation (Ki67) in response to EGF and strong staining for pAkt, all of which were reduced with EGFR inhibitors in the assays. Lastly, capitalizing on FACS profiling of mammary epithelial cells we established that Rasgrp1 perturbation led to alterations in cell fate of stem- and progenitor-cells in the mammary gland. *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef} mammary glands revealed decreased total cellularity of CD31-/CD45-/Ter119- lineage-negative cells but increased luminal CD49f^{medium}/EpCAM^{high} and basal CD49f^{high}/EpCAM^{medium} cell numbers that encompasses the MaSCs.

In sum, we provide mechanistic insights on the biochemical signals that impact MACSs and progenitor cells. We show that Rasgrp1 is required to balance MEC EGFR signals and regulate mammary stem and progenitor cell fate to dampen proliferation so that cells can exit mitosis and maturate and differentiate.

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Chapter 1

Mammary gland structure, development, and epithelial cell hierarchy

ABSTRACT

The mammary gland is a dynamic organ comprised of a network of bi-layered ductal epithelial structures that develops predominantly during puberty. In the ductal phase, during puberty, robust proliferation and branching morphogenesis occurs leading to development of the mature “ductal tree” from its rudimentary tree laid down in embryogenesis. Bulbous terminal end buds (TEBs) form at the tips of ducts, and cell proliferation and migration drive invasion of TEBs into the fat pad, and branching occurs through TEB bifurcation as well as secondary side-branch sprouting. TEB formation, branching elongation, and invasion into the stroma are controlled by a wide variety of signals or cues. Pubertal development can be studied in different *ex vivo* models, including but not limited to, organoids and Matrigel-embedded colony forming assays. Organogenesis is fueled by specific progenitor and mammary stem (MaSC) cell populations. Interestingly, mammary progenitor cell populations are highly heterogeneous, although this complicates understanding cell fates, developmental transitions, and the cellular hierarchy. A theoretical mammary epithelial hierarchy has been constructed based upon gene and extracellular receptor expression profiles, which serves as a working model. In Chapter One, I cover the structure of the mammary gland, organogenesis during puberty, models of mammary development, MaSCs, and insights into the epithelial developmental hierarchy.

INTRODUCTION

The mammary gland is complex and multifaceted organ, whose intricate properties make it poised for developmental studies. Unlike most other organs, the bulk of mammary gland development occurs late, during puberty. Development is cyclical – the primitive epithelial tree is created during embryogenesis but extensively remodeled and expanded during puberty, pregnancy, and lactation [1,2,3]. Stromal and epithelial factors coordinate to build the functional mammary epithelial cell (MEC) tree. The onset of pubertal hormones, combined with growth factor receptor signals, shapes mammary tubulogenesis [4]. Generation of the mature epithelial ducts, important for milk production in lactation, involves the coordination of branching, distinct progenitor cell populations, and receptor signaling. Novel three-dimensional *ex vivo* culture methods have been developed to more deeply interrogate organogenesis under controllable conditions [5,6].

In order to more fully understand mammary gland development, we need to understand epithelial progenitor and stem (MaSC) cells. For reasons still unknown, delineating the exact character of the MaSC population is difficult. MaSCs have a high degree of heterogeneity as well as distinct lineage-priming/specification as a function of unique cues. A developmental hierarchy has been constructed based upon extracellular receptor and transcriptome profiles [7]. This working model remains open for revision, constantly being adapted by ongoing work on characterizing MaSCs. In this chapter, I cover some of the historic work as well as more recent insights on MaSCs and the developmental hierarchy.

Mammary gland structure, function, and pre-pubertal development

The mammary gland is comprised of many different interacting cell types. Epithelial cells form a primate structure early during embryonic development, which later form the nipple and fill out the fat pad. Mature epithelial cells can later differentiate into alveolar cells and subsequently produce milk proteins. The epithelial ducts are surrounded by fat cells, which becomes innervated with a variety of stromal cells. Stromal cells include, but are not limited to, endothelial cells, immune cells (T cells, macrophages), and fibroblasts [8]. Maturation of the gland is regulated at first by mesenchymal interactions, and later by hormone and growth factor receptor signaling during puberty and pregnancy. The cyclical and delayed maturation of the gland makes the breast relatively unique among mammalian organs.

Mammary epithelial cells (MECs) can be broadly separated into two distinct types: luminal and basal. Luminal cells form the bulk of the mammary ducts. Luminal progenitor cells, which will be covered in greater detail later, can go on to form ductal or alveolar cells. Luminal populations are often subclassified based upon hormone and growth factor receptor status. The basal epithelium can also be roughly broken down into two major subtypes: myoepithelial cells and mammary stem cells (MaSCs). Myoepithelial cells form the outside of the ductal epithelial tree and assist in the motility of milk protein along the lumen. MaSCs are a small, heterogeneous, and to date poorly defined population that drive development and repair. Together, the mammary epithelial subtypes interact to

carry out the organ's functions.

Although this review will be focused predominantly on pubertal development of the mammary gland, it is important to understand the morphogenic processes that occur during embryonic development. Formation of the gland begins at E10 with the appearance of two lines of multilayered ectoderm. At E12, these ectodermal lines form five placodes that consist of multiple layers of columnar cells [9,10]. By E13 signals exist with the mesenchyme that are able to drive specific differentiation of luminal and basal cells [11]. Initial mammary cell specification is believed to be controlled by *wnt* genes. *Wnt10b*, *Wnt3*, and *Wnt6* are expressed adjacent to the mammary placode and *Wnt5a* and *Wnt11* are expressed in the underlying layer of mesenchyme [12,13]. The mammary placode subsequently matures, largely through input from the GLI3 / FGF10 signaling cascade. Perturbations to either the transcription factor *Gli3* or growth factor *Fgf10* results in impaired progression from primitive ectodermal structures into a fully formed mammary placode [14,15]. The maturation following placode development is rapid. At E14 placodes expand into a larger sphere of cells, and at E16 epithelial cells begin branching thereby forming a rudimentary duct with ten to twenty sprouts [16, 17]. Mammary epithelial cells assemble into their normal morphology between E16-E18, forming a bilayered duct with an inner lumen [18]. Ultimately, this rudimentary duct serves as a platform for the spectacular metamorphosis that occur during pubertal development.

Pubertal Development of the Mammary Gland at a Glance

Puberty is the most dynamic and striking period during the development of the mammary gland. Early pubertal morphogenesis is driven by the elevated expression of ovarian hormones, like estrogen and progesterone. The rudimentary duct undergoes significant expansion, resulting in the formation of bulbous multilayered structures called terminal end buds (TEBs) [19]. TEBs are the proliferative centers that drive elongation, bifurcation, and branching until the entirety of the mammary fat pad is filled, thereby creating the mature epithelial tree [20]. The branching aspect of tree formation occurs as a result of TEB bifurcation. The TEB contains cap cells, a rapidly growing and dividing progenitor cell population that later goes on to form the tree's outer myoepithelial layer [21]. Cyclical surges of hormones result in secondary and tertiary branches. In wild type C57BL6 mice, onset of pubertal development occurs at approximately 5 weeks of age, reaches its midpoint around 7 weeks, and concludes around 9 weeks (**Figure 1.1**). Pubertal morphogenesis is a coordinated process that involves branching, invasion, and a host of extracellular signals that promote growth and differentiation.

Epithelial Migration and Invasion During Pubertal Morphogenesis

Growth factor-induced proliferation is at its peak just prior to ductal initiation [6] Mammary epithelial cells need to coordinate migration and express genes important for motility and invasion in order to spread branches through the dense stromal adipocytes. Coordination is achieved through controlled proliferation. Prior to migrating, MECs exist in a quiescent bilayered form. Following hormone- and growth factor-mediated stimulation, MECs reorganize to form a pre-invasive, highly proliferative, incompletely polarized,

multilayered epithelium [22]. This rearrangement is reversible and follows along with the cyclical stimulation of migration. Cells found within the newly formed ducts reorganize dynamically as the duct elongates, while maintaining E-cadherin and ß-catenin-mediated cell-to-cell contacts [23]. The multilayered cap cells lack complete polarization, while cells more distal to the TEB can maintain luminal and basal identities. Cell polarity and these cell adhesion proteins are critical regulators of mammary development [24,25]. Interestingly, no evidence exists for a chemotactic gradient in mammary epithelial branching [26, 27].

Steroid hormones induce the expression of ligands that can activate receptor tyrosine kinases (RTKs), which in turn drive ductal elongation of epithelium into the adipocyte-rich stroma. The leading mammary epithelial surface (basal cells) is smooth, with no actin-based protrusions into the extracellular matrix [28]. This branching resembles what has been observed in the mouse salivary gland and kidney [29,30]. The genetic requirements for branching tubulogenesis are increasingly well-understood, but only recently have advances been made toward better understanding the mechanism of action [31]. In 2016, Huebner et al. discovered that phosphorylated ERK, a downstream effector in the MAP kinase pathway, is spatially enriched in cells found at the front of growth ducts [32]. Inhibition of MAPK intermediates acutely attenuated migration and duct elongation in *ex vivo* culture models. It was further observed that MECs shift into and out of the high motility elongating edge, and that proliferation is not required for ductal protrusion [32]. 3D tracking found cells moving between front and interior positions, although most cells remain within their original compartment in the organoid model. Further, individual GFP

labelling of migrating cells revealed anisotropic protrusion of branch cells and isotropic protrusion of organoid body cells [32]. Protrusions do not extend into the ECM, however. In sum, pubertal duct branches preferentially extend in the direction of elongation, cell migration is potentially sufficient for branching morphogenesis, and specific RTK downstream effectors mediate the expansion.

Signaling Control During Ductal Morphogenesis; hormones, FGF, EGF, MMP, and TGFß

Ductal morphogenesis is a somewhat stochastic process, regulated by the combinatorial input of diverse signals. The stochastic aspect is perhaps best exemplified by the fact that there are substantial differences between mammary glands of mouse littermates. This has led the field to conclude that predetermined genetic control of pubertal development is not a possibility, unlike in the development of other epithelial tissues [33]. Development is dictated by mechanical factors and molecular signals from the surrounding stroma [34]. As a result, maturation of the breast is context-dependent.

The pubertal developmental process is initiated in large part by the expression of ovarian and pituitary hormones [35]. These signals cooperate to facilitate growth and communication between epithelial and stroma cells. Genetic knockout of estrogen receptor alpha (ERα) leads to incompletely developed mammary ductal trees, and exogenous administration of estrogen in mice lacking ovaries rescues ductal morphogenesis [36,37]. Estrogen facilitates stromal cell expression of hepatocyte growth

factor (HGF) and epithelial expression of amphiregulin (AREG) [38,39]. AREG can communicate with the epidermal growth factor receptor (EGFR), whose expression is essential on mammary stromal cells [40]. Estrogen's collective functions serve to regulate local cell growth during pubertal development.

Estrogen is not the only steroid hormone important for pubertal mammary gland development, however. In fact, estrogen alone is insufficient to restore ductal morphogenesis when other input (like from the pituitary gland) is missing. Exogenous administration of growth hormone, normally produced by the pituitary gland, can restore the impaired branching phenotype of pituitary gland-deficient mice [41]. Growth hormone induces stromal insulin-like growth factor 1 (IGF1) expression, which binds to IGFR on epithelial cells [42]. Together growth factor and IGF1 act as global regulators of ductal morphogenesis.

Several growth factor receptors and receptor tyrosine kinases (RTKs) are involved in the integrated signaling environment that directs mammary morphogenesis. Fibroblast growth factors (FGF) and their receptors (FGFRs) are critically important for growth and branching. FGF2 and FGFR2 in particular shape the profile of ductal outgrowth and MEC proliferation. Genetic deletion of FGFR2 disrupts ectodermal and placode formation during embryonic mammary organogenesis [43]. Mosaic inactivation of *fgfr2* generated FGFR2-null MECs. Mammary epithelial cells lacking FGFR2 have a proliferative disadvantage when compared to their wild-type and FGFR2-heterozygous counterparts, and are depleted within TEBs [44]. Inducible *fgfr2*$^{-/-}$ mice reveal a similar phenotype;

proliferation is significantly attenuated and TEBs are completely absent from the glands [45]. In sum, FGF2 is a key regulator of luminal epithelial cells and it plays a specific role in the TEBs of elongating ducts.

The EGFR family of signaling proteins plays a pivotal role in directing pubertal mammary gland development in conjunction with FGF and FGF2. For a more detailed review on their functions, see **Chapter 3**. In brief, perturbations to each of the four EGFR family members results in developmental defects. In *ex vivo* culture, FGF2 addition can rescue growth and branching in EGFR-null organoids [46]. Mice with dominant negative EGFR display reduced proliferation and inhibited duct maturation [47]. Deletion of ErbB2 shunts ductal outgrowth [48,49]. ErbB2 also controls TEB formation through its regulation of cellular compartmentalization. ErbB3 deficiency results in small TEBs and increased branch density. Decreased TEB size occurs as a result in an increase in apoptosis, controlled via observed changes in the PI3K signaling pathway. Anti-apoptotic transcription factor Bcl-2 is reduced in ErbB3-/- mice [50]. Mice deficient for ErbB4 have defect occurring later in breast development, specifically during the formation of milk-producing luminal cells [51].

Lastly, in addition to hormones and growth factor signals, matrix metalloproteases (MMPs) and the morphogen TGFß have been identified as important local factors in pubertal development. MMPs are expressed in distinct spatial patterns within the mammary gland while TGFß exists within varying concentration gradients throughout the stroma. MMP14 is highly expressed in TEBs, MMP3 is largely stromally expressed, and

MMP2 is expressed throughout the tissue but at lower levels in secondary and tertiary branches [52]. Defects in MMPs result in developmental impairments. MMP2-null mice have delayed onset of branching early in puberty and increased tertiary branches at the end of pubertal, due to its role in promoting cell survival [53]. MMP3 loss diminishes lateral branching through its interaction with extracellular matrix proteins collagen IV and laminin 111 [53]. Changes to TGFß elicit similar dramatic effects. Upregulation of TGFß leads to hypoplastic ductal outgrowth and TGFß-deficient mice have increased MEC proliferation and side branching [54,55]. These factors contribute to the physical aspect of how epithelial ducts invade into the adipocyte-rich stroma.

3D Models to Study Breast Development

Novel three-dimensional culture methods have allowed for *ex vivo* mammary development studies with a focus on the mechanistic role of stimulatory and inhibitory conditions and the roles of distinct cell populations. Mammary epithelial cells can be cultured as organoids, which allow for the analysis of the factors that contribute to branching morphogenesis. Isolated murine MECs are placed in Matrigel and expand to recapitulate normal ductal morphology [5,6]. Depending on the stimulating conditions, the MEC clusters form either branching organoids or hollow cysts (**Figure 1.2A**).

Isolated single mammary epithelial cells can be plated in Matrigel pellets in a colony forming assay. Single MECs are able to seed into the surrounding matrix and clonally expand. The number of colonies that are able to form, as well as the size of the colonies,

provides a representation of the proliferative capacity of progenitor cells within the total MEC population (**Figure 1.2B**). Similarly, single MECs can also be cultured short-term in ultra-low adherence plates to generate mammospheres. Mammosphere assays interrogate the function and clonogenic capacity of MaSCs. Only MaSCs with self-renewal potential are able to survive non-adherent conditions. MaSC activity can be quantified by the number of mammospheres that are able to form. Use of these three powerful tools has allowed for new insights into the mechanisms by which ductal morphogenesis is directed.

Luminal cells and luminal-specific progenitors

Epithelial cells in the mammary gland can be broadly separated into two types: luminal and basal. As discussed earlier, those two types can then be broken down further into subgroups. Each group contains mature and progenitor cell populations that are distinguished by gene and extracellular ligand profiles. Several classifications of luminal cells exist within the mammary gland. Luminal cells are segregated based upon functional, morphological, and expression profiling evidence. Luminal cells can be broadly separated into two lineages: ductal and alveolar. Mature ductal cells can be either estrogen receptor (ER) positive or negative, and feature the following ligand expression profile: $CD49f^{lo}CD29^{lo}CD24^{+}CD14^{-}EpCAM^{hi}c\text{-}kit^{-}Sca1^{+}CD61^{-}CD49b^{-}$ [7]. Mature alveolar cells are always ER- and are $CD49f^{lo}CD29^{lo}CD24^{+}CD14^{-}EpCAM^{hi}Sca1^{lo}CD61^{-}$. Alveolar cells can be distinguished by their secretory morphology and histologically by the accumulation of milk proteins within.

Transcription factors drive luminal cell fate commitment throughout development. *Elf5* transcription factor is one of the major drivers of luminal cell specification and differentiation. Elf5 is able to directly repress *Slug* transcription, thereby blocking basal cell determination and promoting luminal cell fate [56,57]. RANKL acts as a downstream paracrine effector molecule for *Elf5* activity [58]. Transcription factor *Gata-3* is another key regulator of luminal cell identity. *Gata-3* expression is important for the maturation of luminal progenitor cells into mature ductal and alveolar cells [59]. *Stat5a* drives the expansion of luminal progenitors and subsequent differentiation into alveolar cells [60]. Though these three factors are well-studied, work is ongoing to determine the full network of signals that regulate luminal cell fate decisions.

Luminal progenitor populations can be distinguished using many of the above extracellular markers. For example, ductal progenitors are $CD61^{+}CD49b^{+}CD14^{+}c\text{-}kit^{+}$ whereas their mature counterparts are negative for the surface expression of these proteins [61,62,63]. Sca-1 expression can be used to separate ductal progenitors from alveolar progenitors since alveolar progenitor cells lack membrane expression of the protein. Expression of these extracellular receptors is not ubiquitous between mouse model lines, either. Neither c-kit nor CD61 are expressed in mammary epithelial cells derived from C57BL6 mice, while they are both found in MECs from FVBN mice [64].

The fate and biology of these luminal progenitor cell populations was further examined by lineage tracing experiments. K8-creER targeted cells exclusively differentiate into luminal

cells when observed *in vitro* [65]. MECs marked with this label are maintained in small numbers following several rounds of growth and differentiation. *Elf5*, a well-studied luminal progenitor cell gene, was utilized in a separate study to trace this restricted progenitor population throughout pubertal development [66]. *Elf5*-expressing cells exclusively produced mammary epithelial cells with a luminal identity and were a major driver of branching morphogenesis [67]. In 2014, Rios et al. confirmed that these *Elf5*-expressing cells are short-lived and restricted progenitors, as the positive cells could not be detected in the mammary glands of lineage tracer mice after 20 weeks. Combined, these findings show that there may exist both short- and long-lived progenitor cells that exclusively mature into luminal subtypes.

Basal cells and progenitors

Unlike luminal cells, the factors that control and label different basal populations are less clear. Both stem cells and bipotent progenitor cells have been identified within the broader basal cell lineage. The use of extracellular markers to distinguish these discrete populations, however, has not yet yielded clear profiles. Mature myoepithelial basal cells are typically $CD29^{hi}CD49f^{hi}CD24^{+}EpCAM^{lo/med}$ [7]. The undifferentiated population that is enriched for MaSCs is believed to be $CD49f^{hi}CD29^{hi}CD24^{hi}EpCAM^{lo/med}Sca1^{-}$. Neither markers specific for a restricted basal cell progenitor nor for a restricted myoepithelial cell progenitor have been identified. Luminal progenitors, discussed above, are thought to arise from an early basal cell population.

Several epithelial-mesenchymal transition (EMT) factors have been identified as molecular regulators of the basal/MaSC population. Transcription factor *Slug* is a key for the basal/MaSC lineage as it suppresses luminal cell fate. *Slug*-deficient mice have delayed ductal morphogenesis and aberrantly express luminal signature genes on basal cell populations [68,69]. Changes to *Sox9* results in similar basal defects. Tumor protein TP63 loss blocks the formation of the primitive epithelial duct [70]. Perhaps one of the strongest drivers of basal cell identity, *p63* overexpression in luminal cells led to an identity shift to a basal phenotype [71]. Notch1/3 signaling has been observed to downregulate TP63 expression as basal progenitors restrict to a luminal cell fate [72]. The transcription factor p53, on the other hand, restricts basal / MaSC renewal and drives differentiation [73]. Work is ongoing to better define basal-specific regulatory factors.

Restricted and Bipotent Progenitor Cells

A tremendous amount of work has been performed in order to establish the identity of restricted luminal / basal cells and bipotent progenitors. *In vitro* experimentation confirmed that, given specific culture conditions, the restricted differentiation potential of both luminal and myoepithelial cells can be maintained [74]. Ultra-low adherent mammosphere culture created MEC colonies with both restricted unipotent differentiation potential and a bipotent stem-like phenotype [75]. Mammary transplantation studies, in which the rudimentary duct is surgically cleared and MECs are injected into the fat pad and allowed to grow, have further confirmed the identity of these two separate progenitor cell populations. A single MEC is also able to fully reconstitute a mature epithelial ductal tree

[76]. This study and others assayed the rare, multipotent MaSC populations.

Lineage tracing studies were performed in order to hone in on the exact nature of mammary progenitor populations. In 2011, van Keymeulen et al. established that all mammary epithelial lineages are derived from cytokeratin 14 (K14) expressing embryonic progenitors [65]. Following this population throughout puberty revealed that K14+ MaSCs promote basal expansion and maintenance. MaSCs expressing K8 promoted luminal cell expansion and maintenance throughout puberty. When cultured *in vitro* these restricted progenitors maintain their identity, while only basal cells display multipotency in transplant / reconstitution assays [65]. Rios et al. used lineage tracing experiments to identify a cytokeratin 5 (K5) expressing bipotent progenitor population [67]. K5 cells labelled at the onset of puberty contributed to the formation of both mature luminal and myoepithelial cells. The factors that separate these bipotent progenitors, which are primed to differentiate from MaSCs that have high self-renewal, is still an active field of research.

Mammary Stem Cells: More Questions Than Answers

While there is a general consensus about some of the factors that mark and or regulate the MaSC population, the field has been left with more questions than answers when trying to specifically define MaSCs [77]. For example, the estimates for stem cell frequency, derived from calculating the number of mammary repopulating units, varies dramatically. Estimates show MaSC frequency between 1 in every 100 total cells, to 1 in every 4,900 [78]. Extracellular markers used to sort MaSCs have proven insufficient to

exclusively identify MaSC populations, but rather, only identify populations within which MaSCs are enriched [79,80,81]. Embyronic stem cell marker Oct4 has been found to label human mammary stem cells, but is insufficient to specifically separate MaSCs from other progenitor cells [82,83].

Some progress has been made towards separating MaSCs from mature myoepithelial and basal progenitors by using transgenic mouse models. MaSCs residing in the terminal end bud (TEB) cap cells exclusively express the phosphatase s-Ship [84]. MaSCs during pubertal development are not just found in cap cells, however. Lgr5, given its well-known role in intestinal epithelial populations, became of interest as a potential marker of the MaSC population [85]. Again, the Lgr5 story is unclear. Whether Lgr5+ cells have increased repopulating activity and whether they trend towards quiescence or self-renewal has not been clearly delineated. LGR5+ cells are only present near the nipple, and not at all TEBs. The exact nature of Lgr5+ and Lgr5- negative MaSC populations is an ongoing field of research [61,86,87]. The Lgr5 question is emblematic of the larger picture – that MaSCs are a highly heterogeneous population. Isolating a basal population enriched for MaSCs from fetal mice reveals a distinct stem cell identity. Fetal-derived MaSCs display augmented clonogenic potency and repopulating efficiency when compared to their adult stem cell counterparts [88]. Further, these fetal MaSCs express a unique extracellular receptor pattern, one that is intermediate between basal and luminal cells, which are also different from pubertal MaSCs [89]. The heterogeneity is further compounded when looking at adult mammary glands. Adults MaSCs have been found to display “lineage priming,” in which restricted differentiation programs exist within

seemingly pluripotent and self-renewal competent stem cells [90, 91]. Recent RNAseq studies have began to further subdivide the MaSC pool. In 2017, Pal et. al found an early progenitor subset marked by CD55 that exists between basal and luminal cells [92]. In sum, the spatio-, temporal, and functional heterogeneity of MaSCs has made their study complicated and bolstered the importance of finding new regulatory factors. The earlier discussed stochastic aspects of mammary gland development in each individual further complicates matters.

Concluding Remarks & a Model of Mammary Epithelial Cell Developmental Hierarchy: A Model

The mammary gland is a complex and highly dynamic organ. Decades of work have gone into profiling the various developmental states, epithelial subtypes, and molecular regulators of morphogenesis and cell fate decisions. This work has culminated in the construction of a model of the mammary epithelial cell hierarchy. Questions still remain regarding the identity of stem and progenitor cell populations, however.

The above *in vitro* and *in vivo* findings suggest that breast epithelial cells can be arranged into developmental hierarchy (**Figure 1.3**). In this hierarchy, mature luminal and myoepithelial cells are replaced by restricted progenitors, which is replenished by the MaSC population [93]. Bipotent progenitors exist within this hierarchy, but the exact placement is unclear and has therefore been excluded. Extracellular ligand expression and transcription factor regulation has served as the basis for the construction of this

model. The extraordinary complexity of the mammary stem cell population has complicated a clear understanding of the drivers for epithelial cell fate decisions. Additional insight can possibly be gained from the study of effector molecules downstream of extracellular receptor activation, which I will discuss in Chapter Two.

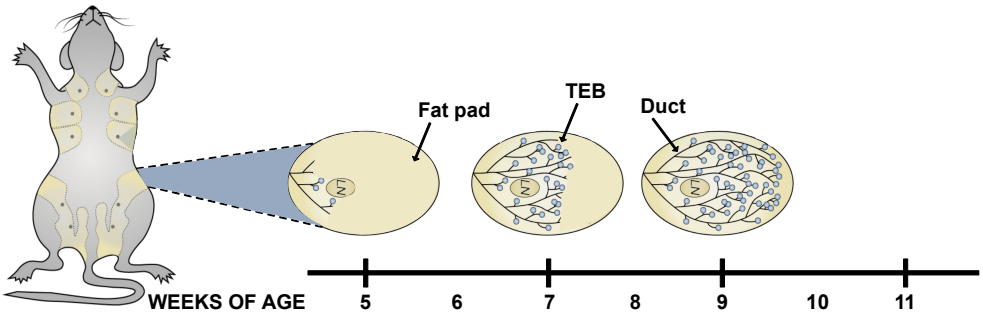


Figure 1.1. Pubertal Mammary Gland Development. Cartoon depicting the stages of pubertal development in the mammary gland of C57BL6 mice. The rudimentary duct can be found within the mammary fat pad prior to 5 weeks-of-age. Onset of puberty occurs at 5 weeks, in which terminal end buds (TEBs) begin to appear. Pubertal development is characterized by TEB bifurcation and ductal elongation. By approximately 9 weeks, the epithelial ducts fill the fat pad and pubertal developed ceases.

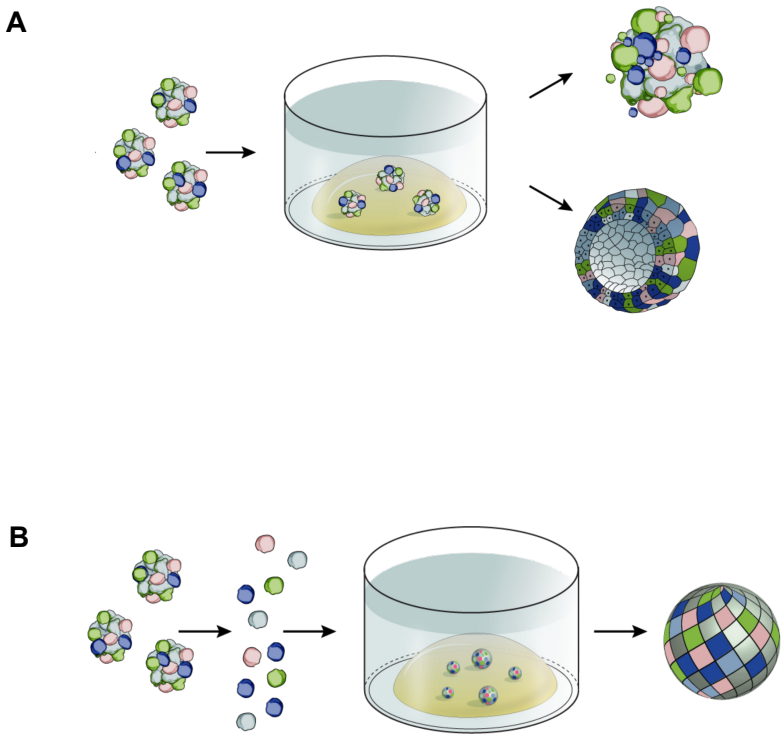


Figure 1.2. 3D *Ex Vivo* Models to Study Ductal Morphogenesis (A) Cartoon representation of organoid generation. Mammary epithelial cells (MECs) are isolated from mice and left in small multicellular clusters. These MECs clusters are then placed in Matrigel and allowed to grow in three-dimensional culture. Depending on the culture conditions, MECs form either multicellular hollow cysts or branched bilayered organoids. (B) Cartoon representation of the MEC colony forming assays. Isolated MECs are separated into single cells and then placed in matrigel. A proportion of these MECs are able to form colonies. The size and number of MEC colonies acts as a stand in for progenitor cell activity.

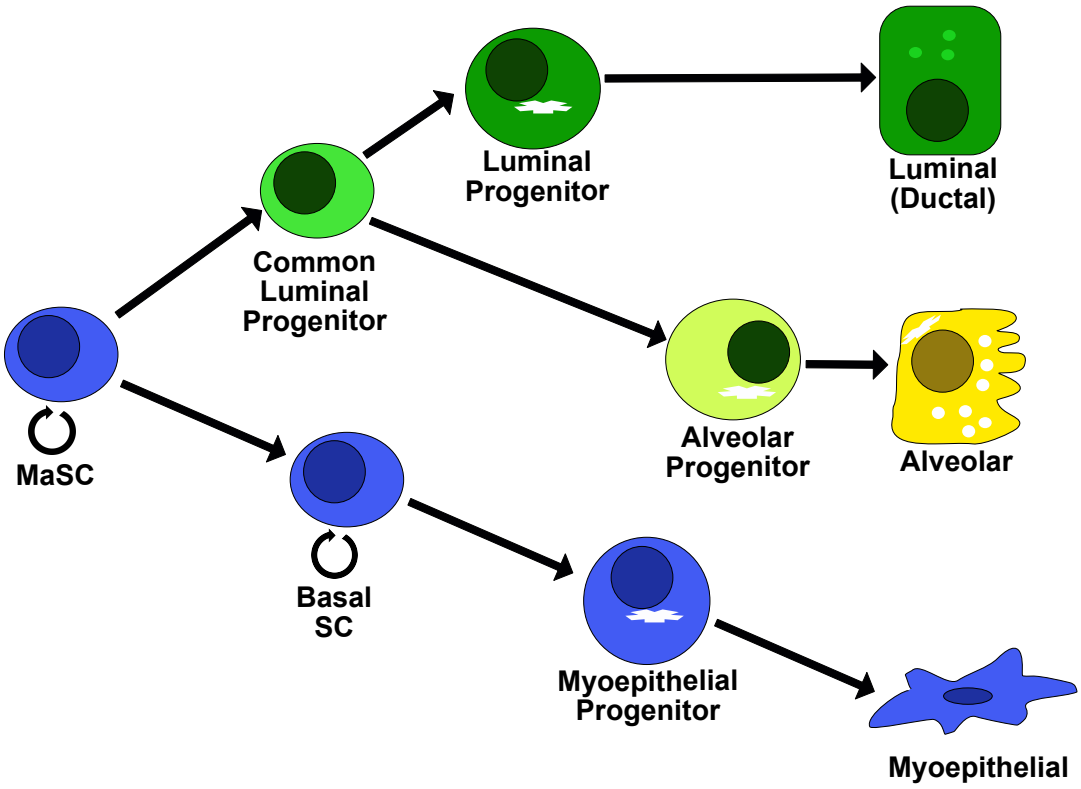


Figure 1.3. The Mammary Epithelial Developmental Hierarchy. A cartoon representation of the mammary developmental hierarchy model. Progression through this hierarchy moves from left to right. The mammary stem cell (MaSC) population contains both MaSCs primed for self-renewal and bipotent progenitors primed to become restricted basal / luminal progenitors. Luminal cells differentiate from a common restricted progenitor into mature ductal or alveolar cells. Restricted myoepithelial progenitors go on to create mature myoepithelial cells.

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Chapter 2

Ras Signals, Effector Pathways, and Tissue Homeostasis

INTRODUCTION

In **Chapter 1**, I discussed the various factors that control maturation of the mammary gland. Pubertal progression is regulated by the proliferation and cell fate decisions of mammary stem (MaSC) and mammary epithelial cell (MEC) populations. Despite numerous studies that have worked to detail the mammary hierarchy, the factors and effector molecules that regulate its assembly are not well known. In the Roose lab we had several lines of evidence that suggested that Ras signaling could play a key role in balancing growth with differentiation in the mammary gland. In this chapter, I will describe receptor tyrosine kinases (RTKs) upstream of Ras, Ras activation by three families of guanine nucleotide exchange factors (GEFs), Ras downstream effector pathways, and how perturbations to RasGEFs can result in surprising homeostatic changes in intestinal epithelial cells.

Defining Ras

Ras is a small membrane-bound GTPase protein that requires GTP-associated activation in order to exert its wide-ranging cell biological effects [1]. Ras is activated downstream of diverse extracellular signals, generally regulating cell proliferation, apoptosis, and differentiation. Ras cycles between two tightly bound GTP-binding states, Ras-GTP (active) and Ras-GDP (inactive) [2,3]. Following receptor stimulation, a small amount of the cell's total Ras is loaded with GTP. RasGEFs are required to dissociate GDP from Ras and subsequently release nucleotide-free Ras. Ras will preferentially associate with

GTP given the higher cytoplasmic concentration of GTP [4]. GTP-hydrolysis to GDP is required to inactivate Ras. While Ras features a low level of intrinsic hydrolytic activity, Ras' ability to convert GTP to GDP is enhanced by Ras GTPase activating proteins (RasGAPs) [5].

Ras activity has been recognized to play an important physiological role during development and in disease. Studies of Ras-activating mutations found in human tumors revealed dramatic changes to proliferative potential and cell fate decisions [6]. Ras signals to a large network of downstream signals, which will be detailed later in this chapter. Over three decades of study have elucidated Ras' central role in regulating cellular and tissue homeostasis.

Ras Activation Occurs by Three Families of RasGEFs

Normal activation of Ras occurs through the interaction with RasGEFs. Three families of RasGEF proteins have been characterized. Dominant negative Ras (Ras^{S17N}) was found to exert its blocking function by usurping RasGEF binding [18, 19]. These studies, as well as many others, highlight the important role for RasGEFs in cell and tissue biology. The structure, functional domains, catalytic activity, and expression pattern varies between the three families of RasGEFs. Distinct functional defects in lymphocytes occur in mice lacking unique RasGEFs [20,21,22]. This argues for specialized functions for each RasGEF.

Ras guanine nucleotide releasing factors (RasGRFs) include RasGRF-1 and RasGRF-2 (reviewed in 23). RasGRFs have multiple domains, including a REM-CDC25 core catalytic domain. Unique to RasGRFs are its two PH domains, which promote membrane localization of RasGRFs in response to stimulation. RasGRFs feature a coiled-coil domain for oligomerization and an ilimaquinone domain for calmodulin binding. A summary of RasGRF functional domains can be found in **Figure 2.1A**. RasGRFs have tissue-specific expression [24]. RasGRF-1 and RasGRF-2 are found mainly in the central nervous system [25]. RasGRF-2, but not RasGRF-1, is expressed in T cells [26]. RasGRF-2 may only play a modest role in T cells, as *Rasgrf2* deficient mice display normal T cell maturation [26].

Son of sevenless (SOS) RasGEFs are ubiquitously expressed [24]. There are two members of the SOS family, SOS1 and SOS2. SOS RasGEFs have six distinct functional domains: including a Ras exchange motif (REM) and proline-rich (PR) domain. SOS' functional domains can be found in **Figure 2.1A.** SOS features two distinct Ras binding sites in its catalytic core, which is unique among RasGEFs. There is one site for GDP to GTP exchange, thereby activating the Ras protein, and another site for allosteric regulation of SOS by Ras [27,28]. Ras binding in the allosteric pocket enhances SOS' GEF activity and creates a site for membrane anchorage. Allosterically-activated SOS contributes to the creation a positive feedback loop for SOS activity [33]. This unique Ras interaction is shown as a structure cartoon in **Figure 2.1B.**

The PR motif can bind SH domain-containing proteins including Grb2, a critical protein for SOS recruitment and activity [29,30]. The PR motif is also important for feedback

control for the SOS protein [31]. SOS1 function is tightly regulated, found in an autoinhibited state in the absence of specific protein and lipid membrane signals [32].

The final family of RasGEFs, the Ras guanine nucleotide releasing proteins (Rasgrps) is perhaps the most unique. Much less is known about the Rasgrp family. Rasgrp's are structurally quite different than SOS or RasGRF [34,35,36]. There are four members of the Rasgrp family, Rasgrp1-4, each with specific expression patterns and nuanced biological functions [5]. Rasgrp2 is a GEF for Rap, while the other three are GEFs for Ras. Rasgrps contain the standard CDC25-REM domain for Ras binding, with the unique EF hand motif for binding calcium [37]. Heterogeneity exists between the specific molecular structure of the EF hand, CDC25-REM, and C1 domains between Rasgrp family members, however, indicating their distinct biochemical regulation [38]. The structural domain organization of Rasgrp can be found in **Figure 2.1A**. Structurally, Rasgrp1 and Rasgrp3 are the most similar Rasgrps. Rasgrp proteins are recruited to the plasma membrane following extracellular stimuli and bind to DAG (**Figure 2.1C**). Rasgrp proteins all have specific expression patterns. Rasgrp1, for example, is expressed in T cells, in the brain, in keratinoctyes, in intestinal epithelial cells, and in mammary epithelial cells [20,39,40,17].

Depletion or perturbation to RasGEF proteins has been demonstrated to cause significant changes to the balance of proliferation with differentiation. Developmental changes to Rasgrp proteins impact hematopoiesis. Rasgrp1 is important to T lymphocyte development, as *Rasgrp1*$^{-/-}$ mice have impaired single positive selection [20]. *Rasgrp1*$^{-/-}$

mice have increased proliferation of epithelial cells in the small intestine and colon [17]. Perturbations to *Rasgrp1* and *Rasgrp3* results in splenomegaly, *Rasgrp3* is required for splenic B cell proliferation, and Rasgrp-mediated Ras/ERK signals sensitize immature B cells to negative selection [11,41]. Elevated levels of Rasgrp1 expression, alongside the classical mutant *KRas*, initiates oncogenic Ras signaling in T cell acute lymphoblastic leukemia [42]. *Rasgrp1* overexpression contributes to tumor progression in K5 skin cancer mouse models [43]. *Rasgrp1*$^{-/-}$ exacerbates *KRas* mutant and APC^{min} colorectral cancer progression and worsens survival [17]. Finally, RasGRP3 drives MAPK activation in uveal melanoma [53].

In sum, the three families of RasGEFs each feature distinct structure and function and changes to RasGEF expression promotes dramatic changes during development and in disease progression.

Receptors Upstream of Ras Signaling

Work from the Roose lab and others has confirmed several receptors that act upstream of Ras activation. Ras proteins have been extensively studied in the T and B lymphocytes. T-cell receptor (TCR) stimulation recruits Zap-70 to the ITAM domain, which in turn contributes to the assembly of the active LAT signalsome [7]. The LAT complex is then able to induce generation of diacylglycerol (DAG) to the plasma membrane, which is required for the binding of certain RasGEFs [8,9]. Ras then becomes GTP-bound and able to initiate context-dependent downstream effector pathways.

Ras activity can also be found following the activation of the B-cell receptor protein (BCR). Antigen-mediated stimulation of the BCR results in the phosphorylation of Syk, the binding of PLCγ, and subsequent recruitment of the RasGEF SOS [10, 11]. This too can lead to generation of DAG, and activation of other RasGEFs. Ras downstream of BCR activation plays a critical role in B cell fate decisions during hematopoiesis.

Other receptors have been implicated in Ras activation in immune cells. Cytokine receptor IL7R signals to Ras through the RasGEF Ras guanine nucleotide-releasing protein 1 (Rasgrp1) [12]. T cells with a deficiency for RasGEFs *Rasgrp1* or *SOS1* show a defect in ß-selection that results from dysregulated signals through the TCR and ILR7 [13,14]. Recent work has shown that Ras and its associated GEFs can be activated through weak, tonic signals too. It was observed that tonic LAT-HDAC signals are able to sustain the expression of Nur77 and Irf4 to regulate naïve CD4 T cells [15]. Myers et al. (*in preparation*) found that perturbations to the RasGEF *Rasgrp1* results in significant changes to Ras-signaling mediated tuning of T cells [16].

Outside of the immune compartment, Ras activity has been found downstream of many other receptors. For example, Rasgrp3 was found to be activated downstream of GPCR activity in GNAQ mutant uveal melanoma. Constitutive signals from Gαq promote PKC release and subsequent DAG-mediated membrane recruitment of Rasgrp3. This, in turn, activates the MAPK pathway [53].

Growth factor receptors and receptor tyrosine kinases often activate Ras-Ras effector pathways following binding to an agonist. SOS1- and Rasgrp1-mediated activation of Ras occurs downstream of epidermal growth factor receptor (EGFR) [17]. Following agonist binding, EGFR will either homo- and heterodimerize with EGFR-family proteins, leading to downstream phosphorylation events and RasGDP for GTP exchange. A more thorough review of EGFR and its relationship with Ras and RasGEFs can be found in **Chapter 3**.

Ras Signaling Through Downstream Effector Pathways

Activated RasGTP signals through a large number of downstream effector pathways. A cartoon summary of the three major effector pathways can be found in **Figure 2.2**. Perhaps the best characterized of these is the RAF-MEK-ERK MAP kinase pathway. Ras activity cascades through the phosphorylation and subsequent activation of RAF-MEK-ERK [3,44,45]. Phosphorylated ERK then induces changes the activation of transcription factors like MYY, ELK-1 and MSK1 to promote proliferation, differentiation, and cell survival. P-ERK has also been postulated to play a role in the feedback regulation of the RasGEF SOS [46].

Class I phosphoinositide 3-kinases (PI3Ks) also play an important role downstream of Ras activation. PI3Ks get recruited to upstream receptors and to Ras itself [54,55,56]. Activation of these PI3K proteins occurs in both Ras-dependent and Ras-independent mechanisms [57,58]. Ras, compared to other small GTPases like Rho, binds to specific isoforms of phosphoinositide kinases [59]. For example, the binding of Ras to p110alpha

phosphoinositide kinase, is involved in Ras-driven tumorigenesis [60]. Activated PI3K results in the phosphorylation of PDK1 and subsequent activation of AKT. One of the important products of this phosphorylation cascade is phosphatidylinositol-3,4,5 triphosphate (PIP_3) [47,48]. PH domain-containing proteins that bind PIP_3 results in the downstream activation of numerous effectors including, but not limited to: eIL4E, Bcl-2, and p53. The PI3K-AKT pathway is involved with the regulation of protein synthesis, proliferation, cell survival, and apoptosis (**Figure 2.3**).

Both the MEK-ERK and PI3K-AKT pathways induce phosphorylation and activation of the mTORC1-S6 pathway. mTORC1 phosphorylates S6 kinase, which in turn produces the ribosomal protein S6. Research into the full breadth of S6 functions is ongoing. S6 has been confirmed to play a key role in mRNA translation and in the regulation of proliferation, cell size, and glucose homeostasis [49].

Changes to RasGEFs alter tissue and cellular homeostasis in the gut

Many pathways that act downstream of Ras activation work to control the delicate balance of cellular growth and differentiation. Changes to these pathways, elicited via perturbations to RasGEFs, can dramatically alter tissue and cellular homeostasis. The RasGEF Rasgrp1 in particular has been implicated to play a key role in maintaining this delicate balance. Work from the Roose lab has demonstrated that T cells that lack *Rasgrp1* have impaired maturation. *Rasgrp1* deficient T cells are unable to properly undergo single positive selection. When *Rasgrp1* is point-mutated (R519G), T

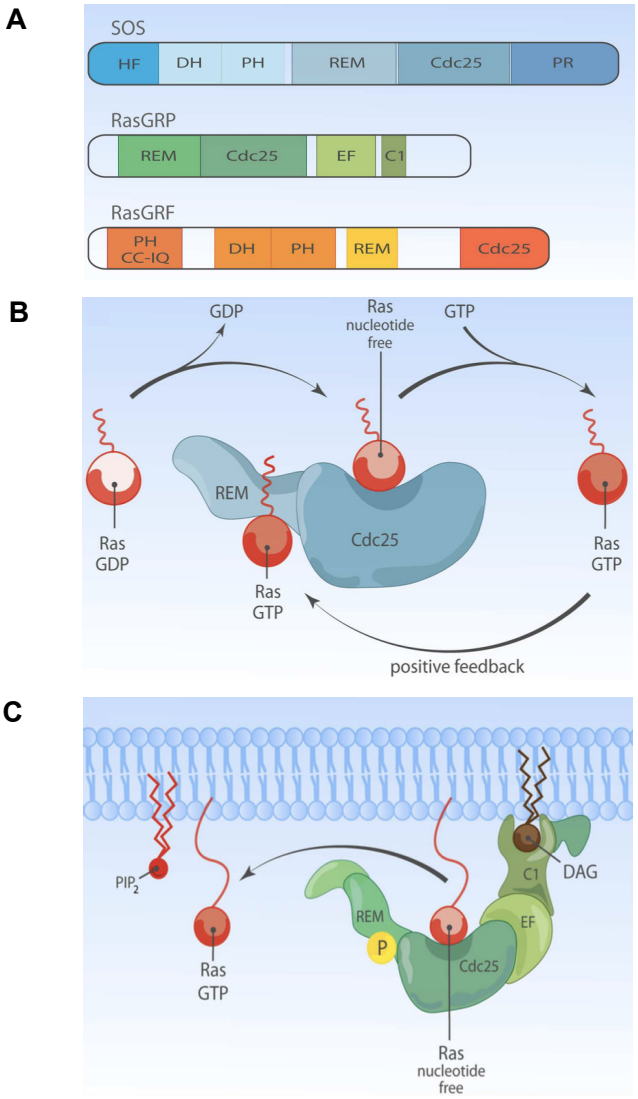
lymphocyte maturation proceeds normally, but favor Th2 differentiation [16]. Our recent work in intestinal epithelial cells (IECs) has demonstrated an unexpected role for SOS1 and Rasgrp1 in gut homeostasis as well [17].

The intestine is a dynamic organ featuring continuous renewal of epithelial cells along the villi. Stem and progenitor cells in the villus crypts expand as they move into the transit amplifying zone, and then differentiate into different IEC lineages as they move along the villi. The signals that balance proliferation with differentiation in this progenitor cell pool are poorly understood. The generation of progenitor cells is controlled in part by Wnt signaling [50]. The epidermal growth factor receptor (EGFR) and downstream Rasgrp1/SOS1-Ras signals are also involved. Paradoxically, Rasgrp1 opposes SOS1-Ras signals in normal intestinal epithelial cells.

In 2015, Depeille et al. showed that two distinct RasGEFs, SOS1 and Rasgrp1, act downstream of EGFR but are functionally opposite in the context of both normal intestinal homeostasis and during colorectal cancer [17]. Rasgrp1 creates negative feedback that acts to limit EGFR-SOS1-Ras signals. *Rasgrp1*$^{-/-}$ mice feature enhanced progenitor cell proliferation, increased migration up the villi, and higher apoptosis. Cell fate decisions are altered, too. *Rasgrp1*$^{-/-}$ mice produce more goblet cells compared to their wild type counterparts (**Figure 2.4**) [51]. In two separate colorectal cancer models, KRasG12D and Apc^{min}, loss of *Rasgrp1* exacerbates the serrated hyperplasia and adenoma formation phenotypes, respectively [52]. The incidence and disease progression of these *Rasgrp*$^{-/-}$ mutant models is enhanced.

Concluding Remarks

In sum, perturbations to the RasGEF *Rasgrp1* can severely unbalance epithelial cell proliferation and differentiation. Although labelled a lymphoid specific RasGEF at first, it has become clear that Rasgrp1 executes essential functions in other tissues. These findings further support the notion of non-redundant funtions for RasGEFs and emphasizes the importance of more than just downstream effectors and receptor ligands in modulating Ras activity.



Adapted from Jun et al. *Frontiers in Immunol. 2013*

Figure 2.1. Functional domains and structure of RasGRF, SOS, and Rasgrp. (A) Cartoon representation of functional domains of the three RasGEF family members. (B) Cartoon depicting Ras binding to the SOS protein. Ras is able to bind both in the CDC25 catalytic pocket and become loaded with GTP. That RasGTP can then activate downstream effector pathways, or bind the allosteric regulatory site in the REM motif. Binding to the allosteric pocket creates a positive feedback loop. (C) Cartoon depicting Ras' interaction with Rasgrp. Rasgrp's C1 domain anchors the protein to the plasma membrane through binding DAG. Nucleotide-free Ras can bind to the catalytic domain, resulting in active Ras.

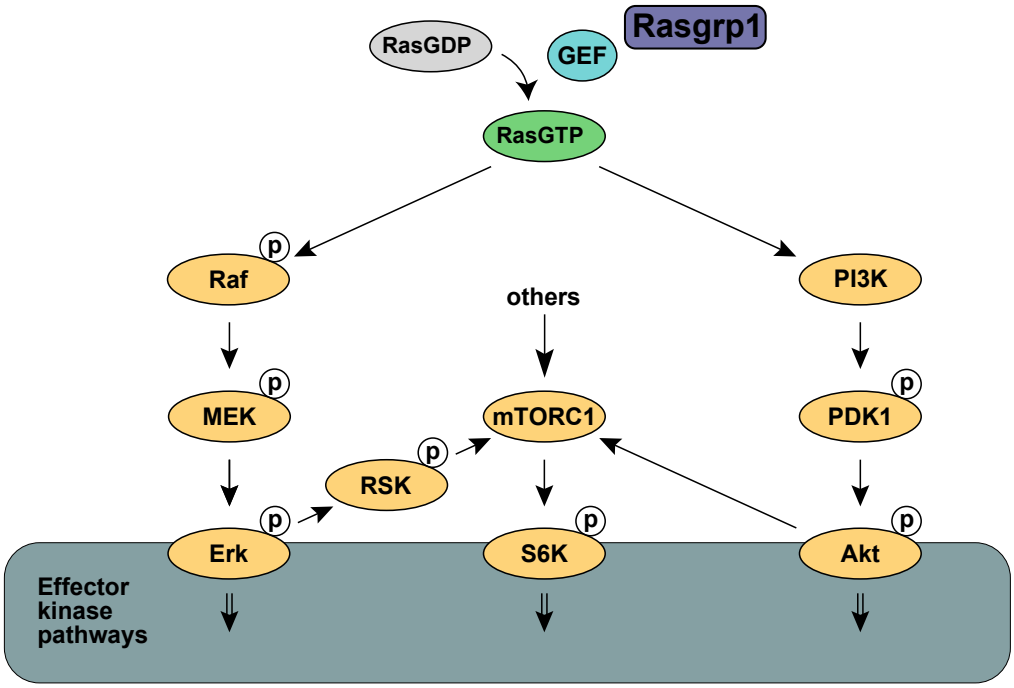


Figure 2.2. RasGTP predominantly signals to the MAPK and PI3K pathways. Cartoon depicting the predominant signaling targets for RasGTP. Upon activation by RasGEFs, like Rasgrp1, RasGTP induces a phosphorylation cascade. The MAPK pathway signals to pERK, and the PI3K to pAKT. Both pathways can activate the mTORC1 complex and generate pS6 kinase.

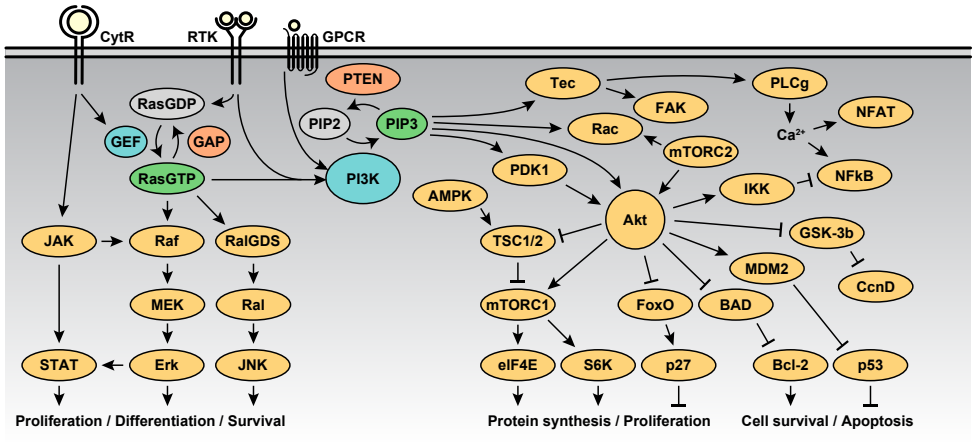
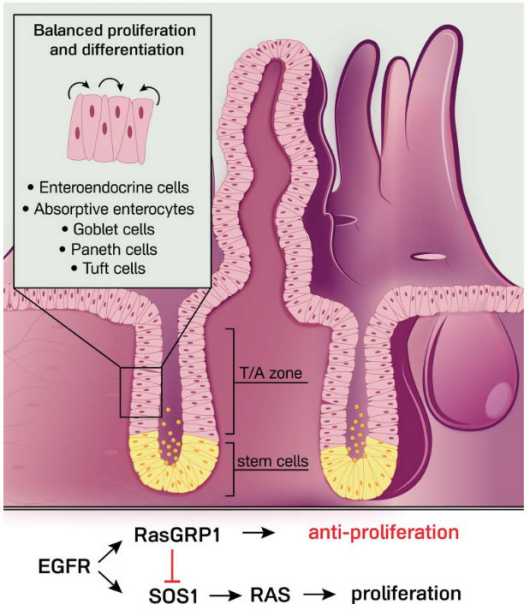


Figure 2.3. Ras Signaling Network. Cartoon representation of Ras upstream and downstream factors. Cytokine receptors (CytR) and receptor tyrosine kinases (RTK) can activate Ras. Ras and G-protein coupled receptors (GPCR) can activate the PI3K pathway. The JAK/STAT, MAPK, and RalGDP pathways regulate proliferation and survival. mTORC1 and FoxO pathways regulate protein synthesis. Bcl-2 and p53 regulate cell survival.



Adapted from Depeille et al. *Cell Cycle. 2015*

Figure 2.4. Rasgrp1 can limit EGFR-SOS1-Ras signals in the intestine. Cartoon depiction of intestinal morphology. The transit amplifying zone (T/A zone) features balanced proliferation and differentiation. Intestinal epithelial cells can differentiate into enteroendocrine, goblet, paneth, and tuft cells. EGFR-Rasgrp1-Ras signals are anti-proliferation.

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Chapter 3

EGFR Signaling, Downstream Effectors, and Epithelial Cell Fate

INTRODUCTION

In **Chapter 1** I described mammary morphogenesis from fate to function. I touched on the importance of regulatory factors in the balance of proliferation with differentiation, and how the mammary epithelia cell (MEC) hierarchy is shaped by the activity of mammary stem (MaSC) and progenitor cell populations. In **Chapter 2**, I discussed Ras signaling. Ras is activated downstream of cytokine receptors and receptor tyrosine kinases. Ras becomes activated by RasGEFs, like Ras guanine nucleotide releasing protein 1 (Rasgrp1), and signals to downstream effector pathways. Receptor-RasGEF-Ras signaling events are responsible for regulating a variety of cellular functions including, but not limited to, proliferation, survival, and cell fate decisions. Here, I will focus on epidermal growth factor receptor (EGFR). This chapter will discuss the EGFR family proteins found within the mammary gland, how EGFR proteins signal, the effects of perturbing EGFR proteins, and how EGFR strength of signal may play a role in MEC fate decisions.

EGFR Family and Ligands

The EGFR family consists of EGFR (ErbB1/Her1), ErbB2/Her2, ErbB3/Her3, and ErbB4/Her4. EGFR signaling involves a complex network with a ligand-receptor input, an intermediate processing core, and output of transcriptional regulator and cell biological responses [1]. Every ErbB receptor therefore consists of an extracellular ligand binding domain (except ErB2), a transmembrane motif and the cytoplasmic domain with kinase activity. ErbB receptors can both homo- and heterodimerize, with 10 possible

combinations—six heterodimers and four homodimers. ErbB family member and their ligands are bivalent. Each receptor is able to bind to ligands and each ligand can bind two receptors [2]. Historic studies have shown that ligand bound ErbB complexes have a one to one receptor to ligand ratio [3]. Despite clear structural and functional overlap, each of the four EGFR family members plays a unique role in cell biology.

There is a high level of homology between kinase domains of the four EGFRs (~60-80%) with divergence occurring predominantly in the C terminus (shared identity is only ~10-25%). ErbB proteins have tissue specific expression, though they are commonly expressed together. As the name implies, EGFRs are found in several epithelial cell types such as the lung, intestine, and the breast. ErbB2 has no known ligands, but frequently dimerizes with the other three EGFR members because of its unique and extended interaction loop [4]. ErbB3 is kinase dead, its cytoplasmic domain unable to initiate phosphorylation cascades. ErbB3 is able to transphosphorylate its own intracellular domain to assist heterodimerization, as well as allosterically activate other EGFRs, however [5]. EGFR is the most commonly found member within activated ErbB complexes. Interestingly, the EGFR family has also been found to interact with other receptor tyrosine kinases like IGF-R and c-MET [6].

Up to 13 different ligands have been found to bind EGFR family proteins: EGF, HB-EGF, transforming growth factor (TGF), amphiregulin (AREG), epiregulin (EREG), epigen (EPG), betacellulin (BTC), and neuregulins 1-6 (NRG) [7,8,9]. Ligand binding induces phosphorylation at tyrosine residues on the cytoplasmic domain [10]. As noted

previously, each EGFR family member with the exclusion of ErbB2 bind a select group of ligands. EGF and TGF are the key ligands for EGFR and ErbB3/B4 preferentially bind neuregulins. The EGFR family members and their preferred ligands can be found in **Figure 3.1**. Evidence suggests that EGFR phosphorylation and the duration and amplitude of signaling events are influenced by the binding of different ligands. This results in divergent cellular responses. For example, AREG is more potent in stimulating ductal elongation compared to EGF [11]. These ligand-specific nuances are important during development and cancer.

EGFR and Downstream Effector Pathways

Multiple phospho-tyrosine sites on the EGFR cytoplasmic tail enable the binding and activation of downstream effector molecules. The phospho-tyrosine sites in the EGFR allow for recruitment of distal signaling proteins with *src* homology (SH) and phosphotyrosine binding domains [13]. Biochemical binding assays has revealed that each EGFR has specific effector binding partners, which creates a high degree of flexibility and differential responses depending on microenvironmental and external stimuli [12]. EGFR's ability to activate Ras can result in the downstream activation of a number of pathways, reviewed in **Chapter 2**. In short, EGFR largely signals through the PLCγ, MAPK-ERK, JNK, and PI3K pathways.

SH2 and SH3 motifs are responsible for the growth factor receptor binding protein-2 (Grb2) mediated SOS association and Ras activation [14]. PLCγ binds SH2 domains and

activates protein kinase C (PKC) kinases, which contribute to increased Raf/ERK activation [20]. PLCγ generates diacyl glycerol (DAG) which acts as a membrane anchor for Rasgrp1 recruitment. Rasgrp1 activation in response to EGFR dimerization may perhaps play a different role in the activation of downstream effector molecules (**Figure 3.2**) [15]. p21 Ras isoforms are able to bind and stimulate Raf, an early member of the MAPK pathway [16,17]. The ERK signal transduction cascade can result in the activation of p38 and the ribosomal subunit kinase (RSK) family of proteins [18].

JNK lies downstream of MKK4/7 activation, produced by Ras/Raf. JNK signals contribute to the generation of cellular stress, differentiation, and cell growth [52]. PI3K is an essential downstream effector of EGFR pathway activation. EGFR-dimerization recruits PI3K subunit P85 [53]. The phophotidylinositol products generated by activated PI3K can result in the membrane localization and activation of AKT and PDK1 [19]. In addition to the more commonly activated pathways, Stat5 has also been identified as a common binding partner for EGFR and ErB4 [12].

EGFR-Effector Signals and Transcriptional Responses

The end result of these signaling cascades is the modulation of transcription via transcription factor binding. Both the ERK and PI3K pathways result in the upregulation and enhanced activity of many transcription factors like p50, p53, c-Rel, Rel-B, and NFkB [21, 22]. The Ras pathway in particular leads to the expression and subsequent nuclear localization of the transcription factors FOS and MYC. Transcription factors c-Jun and

ATF-2 act downstream of JNK activation [52]. cAMP and FOXO transcription factors are also commonly activated downstream of EGFR-mediated PI3K-AKT stimulation [23]. Anti-apoptotic transcription factor Bcl2 also lies downstream of PI3K-AKT [41]. Ultimately, these factors execute the cell biological functions associated with EGFR family members: regulation of apoptosis, cell cycle progression, survival, and protein synthesis.

EGFR During Mammary Gland Development

As I discussed in **Chapter 1**, the mammary gland is a highly dynamic organ comprised of bi-layered ductal epithelial structures that matures at the onset of puberty, and then again later during pregnancy and lactation. A large number of MEC intrinsic and extrinsic factors coordinate to promote mammary branching morphogenesis. Epithelial differentiation is modulated at all developmental stages by signals from the surrounding stroma. The EGFR family of proteins plays a key role in this control, both in the epithelial cells themselves as well as from the stroma (**Figure 3.3**).

ErbB receptor signals contribute the most during puberty, and then later during pregnancy [4]. Mammary morphogenesis requires the epithelial expression of EGFR-ligand AREG [11]. AREG release from MECs stimulates EGFR in the stroma, which then reciprocally induces growth signals back to the epithelial cells.

The vast majority of what is understood about the EGFR family's role in mammary development comes through the use of genetic mouse models. *Egfr*-deficient mice perish

just after birth, which complicated deciphering the exact nature of EGFR during mammary organogenesis [24,25]. Luetteke et al. generated *Waved-2* mice to circumvent this problem. The *Waved-2* allele has a point mutation near EGFR's cytoplasmic kinase domain that reduces activity; it's hypomorphic [26]. *Waved-2* mice have defective mammary development with diminished branching and a reduction in ductal invasion [27,28]. Use of a dominant negative EGFR protein using the mammary-specific MMTV promoter confirmed its role during pubertal development. Mice with the dominant negative EGFR display reduced proliferation and inhibited duct maturation [29]. *Egfr's* importance in the stroma was confirmed via the generation of mixed tissue recombinants from transplanting neonatal epithelial cells from wild type or *egfr*-deficient mice [30,31].

Perturbations in other EGFR family genes also results in dramatic mammary developmental phenotypes. Specifically, deficiency in the *ErbB2-4* results in impaired ductal outgrowth during puberty. Deletion of *ErbB2* shunts ductal outgrowth [32,33]. ErbB2 also controls terminal end bud (TEB) formation through its regulation of cellular compartmentalization. TEBs are the proliferative structures that drive ductal elongation. *ErbB3* deficiency results in small terminal end buds (TEBs) and increased branch density. The decreased TEB size occurs as a result in an increase in apoptosis as a result of decreased activity through the pro-survival PI3K signaling pathway. Anti-apoptotic transcription factor Bcl-2 is reduced in *ErbB3*$^{-/-}$ mice [34]. While the effects of *ErbB4* deletion are minimal during puberty, loss of *ErbB4* impairs lobuloalveolar development and lactation that are accompanied by perturbed Stat5a signals [35].

EGFR activity is also involved in changes to the mammary gland during pregnancy and lactation. Here I focused primarily on the role of EGFR signals during mammary development; for its role during pregnancy and lactation I refer you to a review by Eccles et al. [4]. Briefly, PI3K signals in response to ErbB3 activation regulate the formation of alveolar buds and expansion of the mammary ducts during pregnancy. ErbB2 and ErbB4 are also required for alveolar development when bound to NRG1. ErbB2/3/4 heterodimers are important for lactation. Cleavage of ErbB4 and generation of intracellular fragments maintains milk protein production by promoting differentiation in luminal progenitor cells [36].

In sum, despite many studies into the role of EGFR proteins in the mammary gland, the exact nature of each member has not been fully elucidated. Stromal and epithelial expression of the EGFR family is critically important at all stages of mammary development. A better understanding of EGFR and its downstream effectors is needed to create a clearer picture of the signals and processes that regulate the complex process of mammary organogenesis.

EGFR Strength of Signaling and Mammary Epithelial Cell Fate

As reviewed in **Chapter 1**, mammary epithelial cells are organized into a developmental hierarchy based upon extracellular ligand and gene expression patterns [reviewed in 37]. The exact nature of these populations, and the factors that balance their proliferation with

differentiation, are not well understood. Recent evidence has emerged, however, that EGFR signaling in MECs may be a key player in better defining this hierarchy.

In 2011, a report by Pasic et al. began to decipher EGFR's potential role in controlling MEC fate decisions during development. An *ex vivo* organoid model was utilized using cells taken from normal human breast tissue. They observed that different EGFR ligands can elicit discrete cell fate decisions. EGF stimulation of human breast organoids initiated a significant expansion of the basal (myoepithelial) population. Conversely, AREG stimulation drove organoids towards a luminal (ductal) cell fate. Interrogation of the downstream effector pathways that I discussed earlier revealed that this deviance in cell fate decisions was due in part to the strength of downstream MEK-ERK signals – in which stronger activation EGFR-Ras-MEK-ERK selectively expanded the basal cell population and weaker activation drives luminal expansion [38].

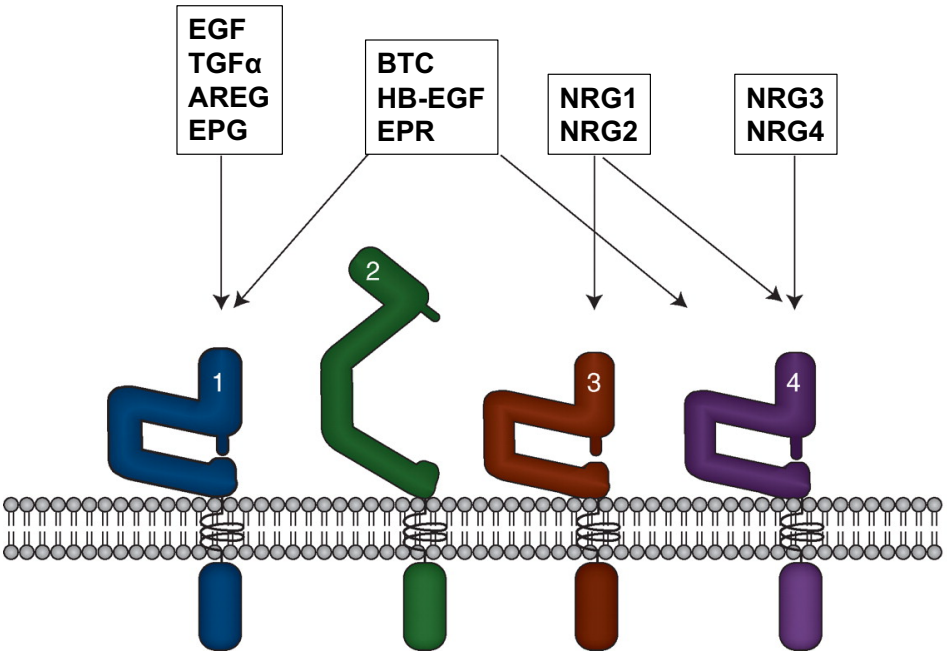
Mukhopadhyay et al. expanded our insights into this initial model in 2013. Using an hTERT-immortalized human stem/progenitor cell pool, they observed similar cell fate decision changes that were dependent on the strength and duration of EGFR signals. Once more, it was observed that stimulation with the weak agonist AREG promoted luminal cell fate and a strong agonist (TGFα) drove cells towards a basal cell identity. In contrast to the data presented in Pasic et al., however, Mukhopadhyay et al. found that EGF stimulation did not drive MaSCs down a specific lineage. The addition of U0126, an inhibitor against the MAPK pathway, significantly reduced differentiation into

CD49f^{lo}EpCAM^{hi} and EpCAM^{lo} cells [39]. Taken together, it appears that the duration and amplitude of EGFR signals affects MEC fate choices (**Figure 3.4**).

Closing Remarks

Since many of the signaling effectors triggered by the EGFR lay downstream of Ras, it is of interest to consider the strength and duration of Ras activation as the cell fate determination factor. A famous study in the mid 90's reported that nuances in receptor-Ras signaling can affect cell fate in a PC-12 cell line system [40]. Stimulation of rat adrenal carcinoma cells (PC-12) with different EGFR ligands produced altered cell fate. In the PC-12 system, EGF is a weaker agonist compared to the strong nerve growth factor (NGF). EGF stimulation led to a short window of Ras activation and cell proliferation, while NGF stimulation elicited prolonged Ras activation, exit from the cell cycle and differentiation [41]. Since that report in 1995, very little work has followed up on this, perhaps because it is challenging to couple quantitative biochemical measurements to cell fate decisions, especially in *in vivo* studies. The Roose lab uncovered that the balance between differentiation and proliferation also hinges on the amplitude and duration of EGFR-Ras signals in the intestinal epithelium. Aberrantly sustained activation of EGFR-Ras effectors in a genetic mouse model drives increased proliferation of the progenitor cell pool in the intestine and also impacts specific cell fate choices [15]. We postulate that the specific pattern of Ras activation and Ras effector pathway signals mediated by specific RasGEFs, like Rasgrp1 and SOS1, strongly influence these cell fate decisions [38].

Growth factor receptor signaling, EGFR in particular, is a complex and fascinating area of study. Decades of work have created a multifaceted picture of how and why EGFR signals initiate and propagate, but the field is still left with many intriguing questions. Until the turn of the century, EGFR's role in mammary gland development was thought to be exclusively from the stroma. When *egfr* is specifically removed from mammary epithelial cells from an adolescent mouse before the onset of puberty, ductal elongation can occur normally [29]. As researchers demonstrated, though, this is not the full story. Dominant negative EGFR and changes to any other the other ErbB proteins within MECs all result in profound developmental deficiencies. How exactly EGFR-Ras signals control cell fate requires future study.



Adapted from Nancy E. Hynes, and Christine J. Watson.
Cold Spring Harb Perspect Biol. 2010

Figure 3.1. The EGFR Family and Ligands. Cartoon depiction of the EGFR (ErbB) family of receptor proteins. EGFR (1), ErbB2 (2), ErbB3 (3) and ErbB4 (4). Common ligands for each receptor shown. ErbB2 has no known ligands. ErbB3 lacks catalytic activity on its cytoplasmic domain. The EGFR peptides can homo- and heterodimerize to form 10 unique complexes (not shown).

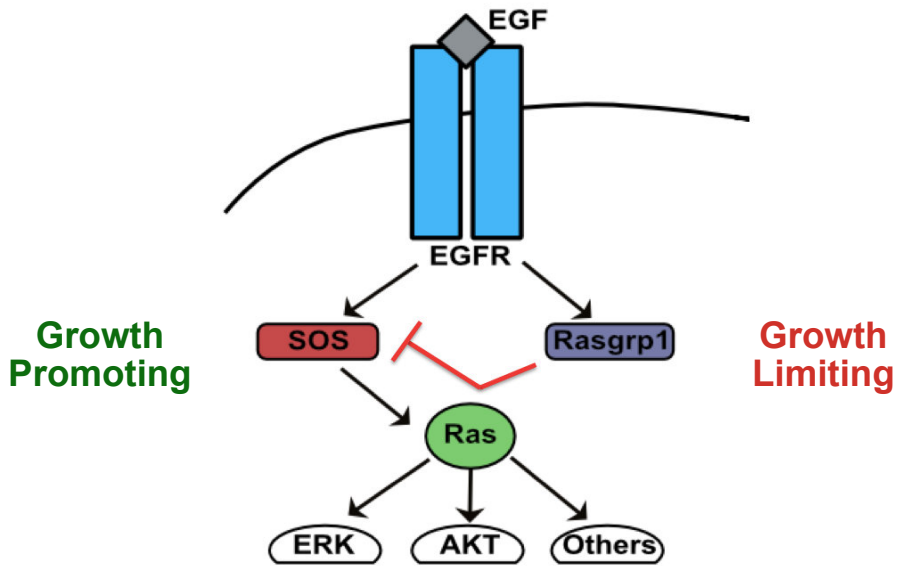
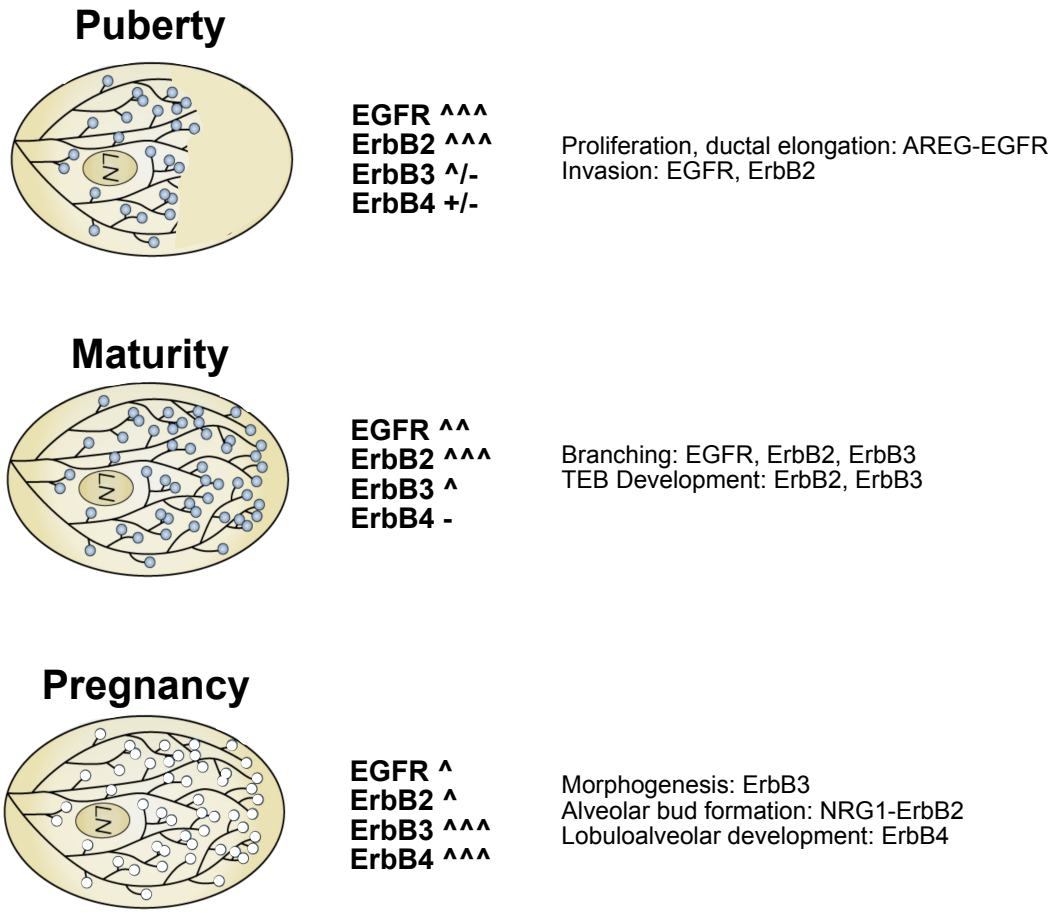


Figure 3.2. SOS1 and Rasgrp1 Downstream of EGFR in Intestinal Epithelial Cells. Signaling cartoon of EGFR-RasGEF-Ras signals in intestinal epithelial cells (IECs). In *Rasgrp1*$^{-/-}$ mice, IECs have increased EGFR-SOS1-Ras activation and an increase in downstream effector pathway activation. Rasgrp1 is growth limiting in IEC stem and progenitor cell populations. SOS1 is growth promoting.



Adapted from Eccles SA. *Int. J. Dev. Biol.* 2011.

Figure 3.3. The EGFR Family During Mammary Gland Development. Cartoon depicting three stages of mammary gland development: puberty, maturity and pregnancy. Strong levels of signaling is indicated by the (^) symbol, low levels by the (-) symbol. Below each stage is a brief summary of EGFR functions.

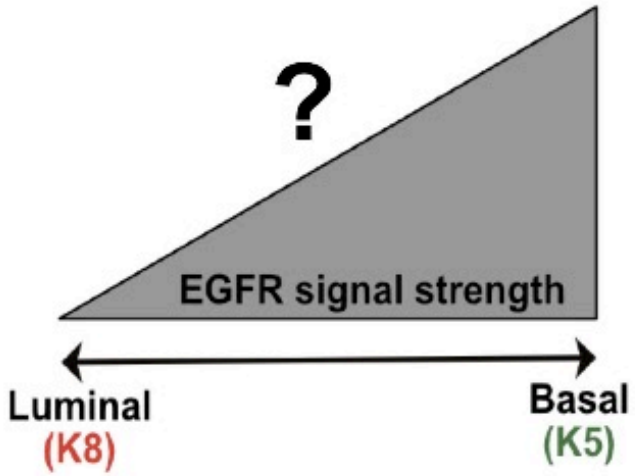


Figure 3.4. Strength of EGFR Signaling in Mammary Epithelial Cell Fate. Cartoon depiction of the hypothetical model for strength of EGFR signaling's effect on mammary stem and progenitor cell fate decisions. Weak and transient activation of EGFR-Ras pathways has been observed to promote a luminal (ductal) cell fate. Luminal cells can be detected by cytokeratin 8 (K8) expression. Strong and sustained activation of EGFR-Ras pathways drives a basal (myoepithelial) cell fate. Basal cells can be detected by cytokeratin 5 (K5) expression. This model has not been rigorously confirmed.

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Chapter 4

Aims of my Ph.D. Studies

Ras signaling has been implicated to play a key role in in regulating the balance between proliferation and differentiation across a wide range of tissues. Links between EGFR, Ras signaling, and mammary epithelial cells suggest a potential and important role of Ras activating proteins during pubertal mammary gland development. My working hypothesis has been that specific activation patterns of Ras effectors, mediated by specific Ras GEFs, are required to elicit cell fate changes and balance proliferation and differentiation in the pubertal mammary gland. In our studies, we utilized mouse models in which perturbations in *Rasgrp1*, either knockout or point mutation, led to an EGF induced gain-of-function growth response in mammary epithelial cells. In Chapters 5-7, I present how Rasgrp1 in mammary epithelial cells acts as a critical factor in the balance of proliferation with differentiation and how it regulates pubertal development, progenitor cell populations, and potential implications in human breast carcinomas.

In Chapter 5, I present a manuscript currently under review that encompasses the predominant focus of my thesis work. Within the manuscript we identified and previously unknown role for *Rasgrp1* expression within the mammary epithelial cell progenitor population. We demonstrate that two Ras exchange factors create balanced EGFR signals in mammary epithelial cells. We show that perturbation of the RasGEF Rasgrp1 leads to delayed pubertal development and an intrinsic increase in proliferative capacity of mammary epithelial cells. Our analyses revealed gain-of-function EGFR-Ras-kinase signals that lead to altered mammary organoid cell biology. Studies in the stem and progenitor cell compartments showed that the increase in EGFR-Ras-kinase effector signals in *Rasgrp1* perturbed models drives augmented proliferation and significant

alterations cell maturation. Our findings provide important insight into the characterization of the signals and processes that control the mammary stem and progenitor cell populations.

In Chapter 6, I detail our future plans to explore Rasgrp1's role in the mammary stem cell population. Following my results detailed in Chapter 5, we observed Rasgrp1's ability to promote differentiation and, when lost, we see an associated increase in the progenitor and stem cell pools. Here, I detail four approaches I am taking now to tease out Rasgrp1's specific contribution. We utilize FACS sorts with additional extracellular markers, organoids and mammospheres generated from sorted basal cells treated with stem cell driving factors like Wnt and Rspondin, RNAseq experiments on sorted MECs, and CyTOF analysis on the entire mammary epithelial cell population.

In Chapter 7, I present our preliminary evidence that Rasgrp1 may play a role in patient outcome in breast carcinoma. I discuss the potential connection between Rasgrp1 expression, changes in mammary epithelial subtypes, and differences in breast cancer biology.

Chapter 5

Restrained EGFR Signals Balance Mammary Epithelial Stem Cell Fate

ABSTRACT

Mammary stem cells (MaSC) are a heterogeneous population that give rise to progenitor cells subsequently developing into basal, myoepithelial, luminal, and ductal cells. The exact mammary epithelial cell (MEC) hierarchy or signals that govern this hierarchy are largely unknown. Recent evidence implies that the RasGEF Rasgrp1 can dampen EGFR signals in epithelial cells. Here we establish that Rasgrp1 supports proper mammary ductal tree development and suppresses proliferation of MECs in maturing ducts. Rasgrp1-/- or point-mutated Rasgrp1Anaef MECs demonstrate increased activation of ERK-, AKT-, and S6- kinase pathways upon EGF stimulation. Loss of Rasgrp1 function leads to gain of branching capacity and progenitor cell proliferation in organoid and colony formation assays. Whereas overall epithelial outgrowth is reduced in Rasgrp1$^{-/-}$ or Rasgrp1^{Anaef} mammary glands, basal/MaSC as well as luminal progenitor cell populations are increased. In sum, Rasgrp1 is required to balance MEC EGFR signals and regulate mammary stem and progenitor cell fate.

INTRODUCTION

The mammary gland is a dynamic organ comprised of a network of bi-layered ductal epithelial structures that develops predominantly during puberty. In the ductal phase, during puberty, robust proliferation and branching morphogenesis occurs leading to development of the mature "ductal tree" from its rudimentary tree laid down in embryogenesis. In this ductal phase, bulbous terminal end buds (TEBs) form at the tips of ducts, cell proliferation and migration drives invasion of TEBs into the fat pad, and branching occurs through TEB bifurcation or secondary side-branch sprouting (**Figure 1A**) (for reviews see (Hinck and Silberstein, 2005; Huebner and Ewald, 2014; Sternlicht and Sunnarborg, 2008; Wiseman and Werb, 2002)). TEBs consist of a mass of inner body cells surrounded by cap cells; polarized mammary ducts are comprised of a central luminal epithelial cell layer and a myoepithelial layer located at the basal position adjacent to the basement membrane (Huebner and Ewald, 2014; Wiseman and Werb, 2002).

Mammary stem cells (MaSCs) are heterogeneous (Visvader and Stingl, 2014), giving rise to progenitor cells that split into two lineages; (i) basal or myoepithelial progenitors that produce myoepithelial cells and (ii) luminal progenitors that generate luminal epithelial cells of the ducts and alveoli (Asselin-Labat et al., 2008). In support of bi-potent MaSCs, flow cytometry-aided studies revealed that single cells can reconstitute the mammary epithelium when transplanted into a cleared fat pad (Shackleton et al., 2006; Stingl et al., 2006). Flow cytometry further aided the resolution of subpopulations in the mammary gland and has helped identify a CD49f^{hi}CD29^{hi}CD24$^{+/med}$Sca1^{low} population (Shackleton et al., 2006; Shehata et al., 2012; Sleeman et al., 2007; Stingl et al., 2006), which defines

an extensive basal cell compartment, some of which are MaSCs. In sum, the mammary gland is continuously dynamic and contains a diverse array of cell populations that includes a stem cell component.

The EGFR family consists of EGFR (ErbB1/Her1), ErbB2/Her2, ErbB3/Her3, and ErbB4/Her4 with ligands that include EGF, transforming growth factor-alpha (TGFα), and amphiregulin (AREG) (Hynes and Watson, 2010). *Egfr*-deficient mice die shortly after birth (Miettinen et al., 1995; Threadgill et al., 1995), which prevents direct analyses of the mammary glands in these animals. *Waved-2* mice carry a point mutation near the kinase domain of the EGFR, which reduces kinase activity (Luetteke et al., 1994). *Waved-2* mice display impaired mammary development with reduced branching and decreased ductal invasion into the fat pad during puberty (Fowler et al., 1995; Sebastian et al., 1998). Mixed tissue recombinants and transplant of neonatal ductal epithelium and fat pad from wild type or *egfr*-deficient mice revealed an important role for EGFR in the stroma (Sebastian et al., 1998; Sternlicht et al., 2005; Wiesen et al., 1999).

The exact role that the EGFR may play on the epithelial cells has remained rather elusive. Several studies imply a role for EGFR signals in the mammary epithelium; deletion of *ErbB2* results in impaired ductal outgrowth (Andrechek et al., 2005; Jackson-Fisher et al., 2004), *ErbB3* deficiency leads to mammary outgrowth defects with smaller TEBs and increased branch density (Jackson-Fisher et al., 2008), and *ErbB4* deletion impacts the mammary gland during lactation but not during puberty (Tidcombe et al., 2003). Furthermore, expression of a dominant negative form of the EGFR from the MMTV

promoter inhibits pubertal mammary duct development with reduced proliferation in the ducts (Xie et al., 1997).

Mechanistic understanding of the role of EGFR signaling in mammary gland epithelial lineages is largely lacking. For example, it is not known if EGFR signals impact the previously mentioned (MaSC) fate decisions in the mammary gland. Capitalizing on a 3-D *ex vivo* model derived from human breast tissue and combinatorial use of different ligands, Pasic *et al*. reported that EGFR signaling is critical for duct development and that sustained EGFR signaling plays a role in myoepithelial lineage specification (Pasic et al., 2011). This study and the *waved-2* mouse studies (Fowler et al., 1995; Sebastian et al., 1998) imply that the strength of EGFR signals impacts cell fate decisions in the mammary gland.

The strength and duration of receptor-Ras signaling can affect cell fate; EGF stimulation of PC-12 cells (rat adrenal pheochromocytoma) leads to brief Ras activation and cell proliferation, while NGF (Nerve Growth Factor) stimulation results in sustained Ras activation, exit from mitosis, and differentiation (Marshall, 1995). Growth factor receptors, like the EGFR, signal through Ras guanine nucleotide exchange factors (RasGEFs) to activate the small GTPase Ras and kinase pathways that lie downstream of activated Ras (Vigil et al., 2010). We established that the RasGEFs RasGRP1 (Ras guanine nucleotide releasing protein-1) and SOS1 (Son of Sevenless-1) lie downstream of the EGFR but play opposing roles in the intestinal epithelium (Depeille et al., 2015). RasGRP1 suppresses proliferation whereas SOS1 stimulates growth (Depeille et al.,

2015). These studies were inspired by the fact that SOS1 and RasGRP1 are structurally very different (Iwig et al., 2013; Margarit et al., 2003; Vercoulen et al., 2017) and generate distinct Ras-kinase signals in lymphocytes (Daley et al., 2013; Das et al., 2009; Jun et al., 2013a; Jun et al., 2013b; Kortum et al., 2013b; Ksionda et al., 2013; Roose et al., 2007).

Here we show that deletion of *Rasgrp1* or a single amino acid substitution, *Rasgrp1*^{Anaef}, which impairs receptor-Rasgrp1 signals (Daley et al., 2013), leads to gain-of-function EGFR signals in mammary epithelium cells and altered morphogenesis with increased basal/MaSC as well as luminal progenitor cell populations.

HYPOTHESIS AND OBJECTIVES

Hypothesis

We postulate that specific activation patterns of Ras effectors, mediated by specific Ras GEFs, are required to elicit cell fate changes and balance proliferation and differentiation in the pubertal mammary gland.

Objectives

- Understand how Rasgrp1 shapes the character of EGFR-Ras-Ras effector signals and impacts mammary epithelial cell lineages during development.

- Characterize the expression of Rasgrp1 in mammary epithelial cells

- Determine how Rasgrp1 impacts pubertal development

- Elucidate the role of EGFR-Rasgrp1 signaling in the cellular fate of MEC lineages

RESULTS

Expression profiling and cleared mammary fat pad transplantation reveal a functional role for Rasgrp1 in the mammary gland epithelium

Our studies on the strength of EGFR signaling in intestinal epithelium (**Figure 1B**) (Depeille et al., 2015) motivated us to examine expression of RasGRP1 in other epithelia. The topology and make-up of mammary ducts and TEBs can be recognized by immunohistochemistry on cryosections of mammary tissue (**Figure 1A).** Dual staining for Cytokeratin-5 and Cytokeratin-8, which are markers of basal and luminal cells, respectively, labels mammary epithelial cells (**Figure 1C**). Immunohistochemistry for Rasgrp1 revealed expression in both ducts and TEBs (**Figure 1D**). Western blot analysis of isolated mammary epithelial cells (MECs) from wild type, point mutated *Rasgrp1*, or *Rasgrp1*-deficient mice as control, confirmed expression of Rasgrp1 in both wild type and *Rasgrp1*^{Anaef} MECs, whereas the Rasgrp1 protein was not present in the *Rasgrp1*$^{-/-}$ MECs as expected (**Figure 1E**). As we have observed previously, *Rasgrp1*^{Anaef} cells have a slight reduction in total Rasgrp1 protein (Daley et al., 2013). When we isolated MECs from mice and ran Taqman analyses for *Rasgrp1* mRNA levels we demonstrated that *Rasgrp1* is roughly equally expressed in MECs from wild-type and *Rasgrp1*^{Anaef}, and *Rasgrp1* is absent in *Rasgrp1*$^{-/-}$ MECs as expected (**Figure 1F**).

As a first assessment of whether Rasgrp1 may play a functional role in mammary gland epithelial cells and development we employed the *Rasgrp1*-deficient mouse model, with a T cell developmental defect (Dower et al., 2000), as well as the *Rasgrp1*^{Anaef} model with

an Arginine to Glycine mutation at position 519 (Arg 519 Gly) in *Rasgrp1*. T cell development is largely intact in *Rasgrp1*^{Anaef} mice, though mice beyond 12 weeks show autoimmune features (Daley et al., 2013). The *Rasgrp1*^{Anaef} mutation has a mild Rasgrp1 autoinhibitory defect in resting cells but causes severely hypomorphic, receptor-induced Rasgrp1-Ras-kinase signals in stimulated cells (Daley et al., 2013). In the current study we used *Rasgrp1*^{Anaef} mice at 11 weeks or younger. In addition, all mice were on a C57BL/6 background, which have somewhat slower pubertal development than FVB mice (**Supplemental Figure S1A**). We isolated mammary glands from 7-week-old mice and transplanted isolated MECs from these into a cleared mammary fat pad of 4-week-old wild type recipient mice with the mammary gland at the contralateral side as an internal control (Kessenbrock et al., 2013; Lawson et al., 2015b) (**Figure S1B**). Transplantation of wild type MECs into wild type recipients resulted in the typical formation of a ductal tree in development when analyzed 5 weeks post-transplant. Transplantation of *Rasgrp1*$^{-/-}$ or *Rasgrp1*^{Anaef} cells resulted in substantially accelerated branching morphogenesis, suggesting a gain-of-function phenotype when *Rasgrp1* is deleted or point-mutated (**Figures 2A and 2B**).

We previously reported that deletion of *Rasgrp1* results in increased Ras signaling in the intestinal epithelium as a consequence of increased EGF-induced EGFR-Sos-Ras signals (Depeille et al., 2015). Since strength of ligand to EGFR signaling has been proposed to influence mammary gland development and lineages choices in this organ (**Figure 2C**) (Fowler et al., 1995; Pasic et al., 2011; Sebastian et al., 1998), we investigated the impact of *Rasgrp1* perturbations on EGF-induced kinase pathways that

lie downstream of Ras (**Figure 2D**). EGF stimulation of *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef} MECs resulted in enhanced ERK phosphorylation (P-ERK), a downstream effector of the MAP kinase pathway, compared to EGF stimulated wild type MECs (**Figure 2E**). As downstream effector of phosphatidylinositol-3 kinase (PI3K) signaling, Akt promotes protein synthesis, cell proliferation, cell metabolism and cell survival (Wickenden and Watson, 2010). We observed significantly elevated Akt phosphorylation (P-Akt) in EGF stimulated *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef} MECs (**Figure 2F**). mTORC1 integrates signals from multiple pathways including ERK-RSK and AKT and mTORC1 signals to the ribosomal S6 signaling pathway and other targets that tie in with translation and metabolism. EGF stimulation of *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef} MECs resulted in robustly increased levels of pS6 compared to wild type (**Figure 2G**). In sum, perturbations in Rasgrp1 result in increased signals through ERK-, AKT-, and S6-effector kinase pathways in total MECs stimulated with EGF.

Rasgrp1 perturbation leads to developmental delay in the ductal phase

To assess the impact of *Rasgrp1* perturbation on the ductal phase during puberty, we systematically analyzed 5-, 7-, 9-, and 11- week-old C57BL/6 mice to capture pubertal development stages (**Figure S1A**). *Rasgrp1*$^{-/-}$ or *Rasgrp1*^{Anaef} females revealed an increase in TEB numbers at 7, 9, and 11 weeks (**Figures 3A and 3B**). The increased TEB numbers throughout puberty was accompanied by reduced lengthening of the ductal tree in *Rasgrp1*$^{-/-}$ or *Rasgrp1*^{Anaef} females (**Figures 3C and 3D**). These results indicated a delayed maturation of the ductal tree. Concomitantly, MECs did not exit mitosis but

continued to proliferate in 9-weeks old *Rasgrp1*$^{-/-}$ or *Rasgrp1*^{Anaef} mice, evidenced by the high number of Ki67-positive, proliferative MECs. By contrast, proliferation had decreased at that age in wild type females (**Figure 3E and 3F**). In *Rasgrp1*$^{-/-}$ or *Rasgrp1*^{Anaef} animals, we observed high levels of proliferation in already established mammary ducts, distant from the TEB. This proliferation is atypical as the proliferative drive during pubertal development is normally limited to the TEBs. In agreement with our *in vitro* EGF-induced effector kinase pathway analyses, immunohistochemistry for P-Akt revealed increased levels in ducts from *Rasgrp1*$^{-/-}$ or *Rasgrp1*^{Anaef} females, assessed by immunohistochemistry (**Figure 3G**). In sum, Rasgrp1 perturbation delays the outgrowth of the ductal tree; *Rasgrp1*$^{-/-}$ or *Rasgrp1*^{Anaef} leads to increased Ras effector levels and increased proliferation. Cell division is incompatible with simultaneous cell differentiation and we hypothesize that differentiation is perturbed in *Rasgrp1*$^{-/-}$ or *Rasgrp1*^{Anaef} mammary glands. We will come back to this point.

Rasgrp1 perturbation elicits gain-of-function EGF responses in organoids and colony forming assays

First, we wanted to assess the organ-forming and proliferative potential of MECs through organoid assays. Digestion of the mammary gland with digestive enzymes results in the production of clusters of MECs that can be plated in Matrigel supplemented with distinct growth factors (**Figure 4A**). FGF2 potently promotes branching of MECs in organoids (Ewald et al., 2008; Mroue and Bissell, 2013), whereas EGF typically induces sphere formation and outgrowth in the absence of branching (Pasic et al., 2011). As expected,

FGF2 stimulated robust branching in 60 to 80% of the organoids from 5-, 7-, and 9-week-old mice of all three genotypes. Patterns were overall similar with a slight increase in branching for *Rasgrp1*^{Anaef} at 5 weeks (**Figures 4B and 4C**). Surprisingly, EGF also induced efficient branching when the MEC clusters were of a *Rasgrp1*$^{-/-}$ or *Rasgrp1*^{Anaef} genotype (40-60%), pointing to a gain-of-function phenotype when *Rasgrp1* is perturbed (**Figures 4D and 4E**). This gain-of-function branching effect was accompanied with increased proliferation measured by Ki67 (data not shown). Combined addition of two distinct EGFR inhibitors, Erlotinib and Gefitinib (EGFRi), in the Matrigel assay blocked the EGF-induced branching (**Figure 4F**). Thus, Rasgrp1 critically controls the mammary morphogenetic program as indicated by elevated branching morphogenesis upon EGF-EGFR signaling in the absence of Rasgrp1 function.

MEC clusters can be further digested into single cells to probe their proliferative capacity in sphere-forming assays (**Figure 5A**). Plating out single cells resulted in increased colony formation for *Rasgrp1*$^{-/-}$ or *Rasgrp1*^{Anaef} MECs. The increased colony formation was negated by the inclusion of the Erlotinib/Gefitinib combination in the Matrigel, demonstrating that this effect was dependent on EGFR kinase signaling (**Figure 5B**). Immunohistochemistry on samples from these assays revealed that the increased numbers of *Rasgrp1*$^{-/-}$ or *Rasgrp1*^{Anaef} colonies were marked by high levels of P-Akt (**Figures 5C and 5D**). Akt phosphorylation was abrogated by the inclusion of the EGFR inhibitors in the Matrigel (**Figure 5E**). The increased numbers of colonies that formed from the *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef} MECs also displayed high levels of the proliferation marker Ki67 (**Figure 5F**), whereas levels of the apoptosis marker cleaved caspase-3 were

similar in colonies of WT, *Rasgrp1*$^{-/-}$, or *Rasgrp1*^{Anaef} MECs (**Figure 5G**). Taken together, our findings indicate that *Rasgrp1* perturbation in MECs leads to a strong gain-of-function branching phenotype with increased proliferation of progenitor cells in response to EGF.

Rasgrp1 catalytic activity suppresses EGFR-induced colony growth

The organoid and colony formation assays pointed to a suppressive function for Rasgrp1 in mammary epithelial cells. Suppression is lost when Rasgrp1 is deleted but also when Rasgrp1 is mutated to the *Rasgrp1*^{Anaef} version. We next utilized the Eph4 mammary epithelial cell line to gain further molecular insights in suppression by Rasgrp1. Eph4 cells are devoid of Rasgrp1 expression (**Figure 6A**), providing an "empty vessel/add-back" system. Stimulation of unmanipulated Eph4 cells with EGF resulted in robust outgrowth of colonies. Addition of EGFRi or omission of EGF (No GF) to the MEC colonies resulted in reduced outgrowth of Eph4 cells in these assays, visualized by Hoechst 33342 fluorescent nuclear staining (**Figures 6B and 6C**). With this Eph4 platform we next demonstrated that retroviral expression of Rasgrp1 suppresses sphere formation compared to empty vector control (**Figure 6D**). Expression of a catalytically inactive form of Rasgrp1 with an Arg_{271} to Glu substitution (R271E) did not suppress growth (**Figures 6E and 6F**), and all growth was inhibited by exposure to the Erlotinib/Gefitinib combination (EGFRi) (**Figure 6G**). Thus, Rasgrp1 requires its catalytic activity to suppress EGF-EGFR signals, which is line with the signaling model cartoon presented in **Figure 1B**.

Rasgrp1 and luminal and basal cell fate

We next used FACS (Fluorescence-activated Cell Sorting) profiling of mammary epithelial cells (Inman et al., 2015) to define cell subpopulations. Historically $CD29^{hi}CD24^{+/med}Sca1^{low}$ profiles have been used but the field now generally accepted that a $CD49f^{hi}$ $EpCAM^{low}$ defines basal cells (Shackleton et al., 2006; Shehata et al., 2012; Sleeman et al., 2007; Stingl et al., 2006). Based on limiting dilution-transplantation assays, roughly 1 in 40 to 1 in 800 basal cells can be defined as a bi-potent MaSC (mammary stem cell) (Inman et al., 2015; Visvader and Stingl, 2014). MaSCs are thought to give rise to progenitor cells before splitting off into the two branches of the luminal progenitors and the basal, myoepithelial progenitors, although the exact mammary epithelial hierarchy is unknown (reviewed in (Inman et al., 2015; Visvader and Stingl, 2014)).

To assess a potential role of Rasgrp1 in this hierarchy, we first determined the total cellularity of $CD31^{-}CD45^{-}Ter119^{-}$ lineage-negative cells that are also $CD49f^{+}EpCAM^{+}$ in the mammary gland. Eight-week-old *Rasgrp1*$^{-/-}$ or *Rasgrp1*^{Anaef} females demonstrated a significant reduction in $CD31^{-}CD45^{-}Ter119^{-}$ lineage-negative cells measured by quantitative FACS analysis with co-analyzed counting beads (**Figure 7A**). Capitalizing on staining and expression analysis of additional extracellular markers we further analyzed subsets via analysis of EpCAM and CD49f (Kessenbrock et al., 2017) (**Figure 7B**) to focus on basal and luminal populations (**Figure 7C**). We established that, in addition to the decreased cellularity, *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef} females have increased

percentages of both luminal CD49f^{medium}/EpCAM^{high} and basal CD49f^{high}/EpCAM^{medium} cells (**Figure 7C**). We observed an approximate 3.5x increase in luminal cells and 5x increase in basal cells in *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef} through the analysis of percentages of cell subsets. This increase in the proportion of luminal and basal cells in *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef} mammary gland was observed in 4 pairs of two females (**Figure 7D**). We also observed a concomitant decrease in the stromal population in the lineage-negative, live, CD49f and EpCAM gate (**Figures 7C and 7D**). Finally, we determined that the absolute number of luminal and basal cells, after correction for total singlets and normalized to WT, was notably higher in *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef} mice (**Figure 7E**). Our results here support the model that that stronger and more sustained EGFR signals drives progenitor cells more strongly towards a basal cell fate (**Figure 2C**) (Fowler et al., 1995; Pasic et al., 2011; Sebastian et al., 1998) and that perturbations to *Rasgrp1* results in enhanced MEC proliferation.

DISCUSSION

Here we demonstrated that perturbations in Rasgrp1, either via deficiency in *Rasgrp1* or *Rasgrp1*^{Anaef}, alter mammary gland morphogenesis and impact MASCs- and progenitor-cell biology. Biochemically, we found that catalytic activity of Rasgrp1 is required to dampen EGFR-Ras-effector kinase signals, particularly Akt and mTORC1-S6 kinase signals. Biologically, Rasgrp1 perturbation results in delayed mammary gland development and sustained epithelial cell proliferation outside the TEB region. Lastly, capitalizing on FACS profiling of mammary epithelial cells we establish that Rasgrp1 perturbation leads to decreased total cellularity of CD31^{-}CD45^{-}/Ter119^{-} lineage-negative cells but increased luminal CD49f^{medium}EpCAM^{high} and basal CD49f^{high}EpCAM^{medium} cell numbers that encompasses the MaSCs.

The RasGEFs SOS1 and RasGRP1 are structurally very distinct (Iwig et al., 2013; Margarit et al., 2003; Vercoulen et al., 2017). Utilization of SOS1 and RasGRP1 versus solely RasGRP1 generates different Ras-kinase signals in lymphocytes (Daley et al., 2013; Das et al., 2009; Jun et al., 2013a; Jun et al., 2013b; Kortum et al., 2013b; Ksionda et al., 2013; Roose et al., 2007). In concordance with their roles in distinct Ras-kinase signals, T lymphocytes deficient for *Sos1* (Guittard et al., 2015; Kortum et al., 2013a) demonstrate functional alterations that are distinct from those caused by deficiency in *Rasgrp1* (Dower et al., 2000; Kortum et al., 2013b; Priatel et al., 2002) or a by the *Rasgrp1*^{Anaef} single amino acid variant of *Rasgrp1* (Daley et al., 2013). The concept that two distinct RasGEFs downstream of the same receptor in the same cell can drive distinct

cell fate outcome has now become more general by our work presented here, and from past studies in the intestinal epithelium (Depeille et al., 2015). We previously demonstrated that loss of one or two alleles of *Rasgrp1* results in increased Ras-ERK signals in intestinal epithelial cell proliferation (Depeille et al., 2015). The increased phosphorylation of S6 that we observed in EGF-stimulated MECs was most striking. S6 receives input from mTORC1 as well as the ERK and Akt pathways (See **Figure 2D**). mTOR signaling is known to drive alterations in cell metabolism, including the increase in cell growth, cap-dependent translation and elongation, ribosome biogenesis, and switches in metabolic programs (Laplante and Sabatini, 2012; Zoncu et al., 2011). While speculative for now, it is possible that the interplay between SOS1 and RasGRP1 RasGEFs also functions to create controlled metabolic balance in mammary epithelial cells.

Our results here support the model that strength or duration of EGFR signals impact the fate of progenitor cells but also bring nuances that it is not a simple choice between luminal and basal cell fates (**Figure 2C**) (Fowler et al., 1995; Pasic et al., 2011; Sebastian et al., 1998). We observed increased proportions and absolute numbers of basal CD49f^{high}/EpCAM^{medium} cells in *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef} females, a population that overlaps with the MaSC identity but also increases in the luminal CD49f^{medium}/EpCAM^{high} population. The ability of a single transplanted cell to reconstitute an entire ductal tree with both luminal and basal, myoepithelial cells may suggest the existence of a clear, bi-potent MaSC and differentiation hierarchy. In reality, this is not straightforward and is very actively debated (reviewed in (Inman et al., 2015; Visvader and Stingl, 2014)). The

CD49f^{hi}CD29^{hi}CD24$^{+/med}$Sca1^{low} FACS profile as well as the CD31^{-}/CD45^{-}/Ter119^{-} CD49f^{high}/EpCAM^{medium} profile identify a basal cell population with MaSCs. Perhaps most indicative of a heterogeneous MaCS compartment are studies using *Lgr5* reporter mice. Lgr5 is a Wnt signaling target gene and an R-spondin receptor in intestinal stem cells (Barker et al., 2013). Reconstitution experiments with the *Lgr5* reporter have yielded contrasting results of Lgr5^{+} cells as having enriched repopulating activity compared to Lgr5^{-} cells (Plaks et al., 2013), both Lgr5^{-} and Lgr5^{+} cells having the same repopulating activity (de Visser et al., 2012; Rios et al., 2014), and Lgr5^{-} cells having enhanced activity (Wang et al., 2015). It should be noted though that these cleared fat pad transplantation assays can have a lot of variability; furthermore, this is perhaps more of an injury model than a pure stem cell model. Systematic use of better organoid systems for the mammary gland that include Wnt and R-Spondin ligands (Jamieson et al., 2017) may provide more comprehensive insights. Other avenues include recent single cell gene expression analyses of the mammary gland (Lawson et al., 2015a; Nguyen QH, in press; Pal et al., 2017) that help delineate and characterize rare cell subpopulations. Our work here sheds light on the intricacies of the biochemical signals that impact the mammary stem and progenitor cell fate choices.

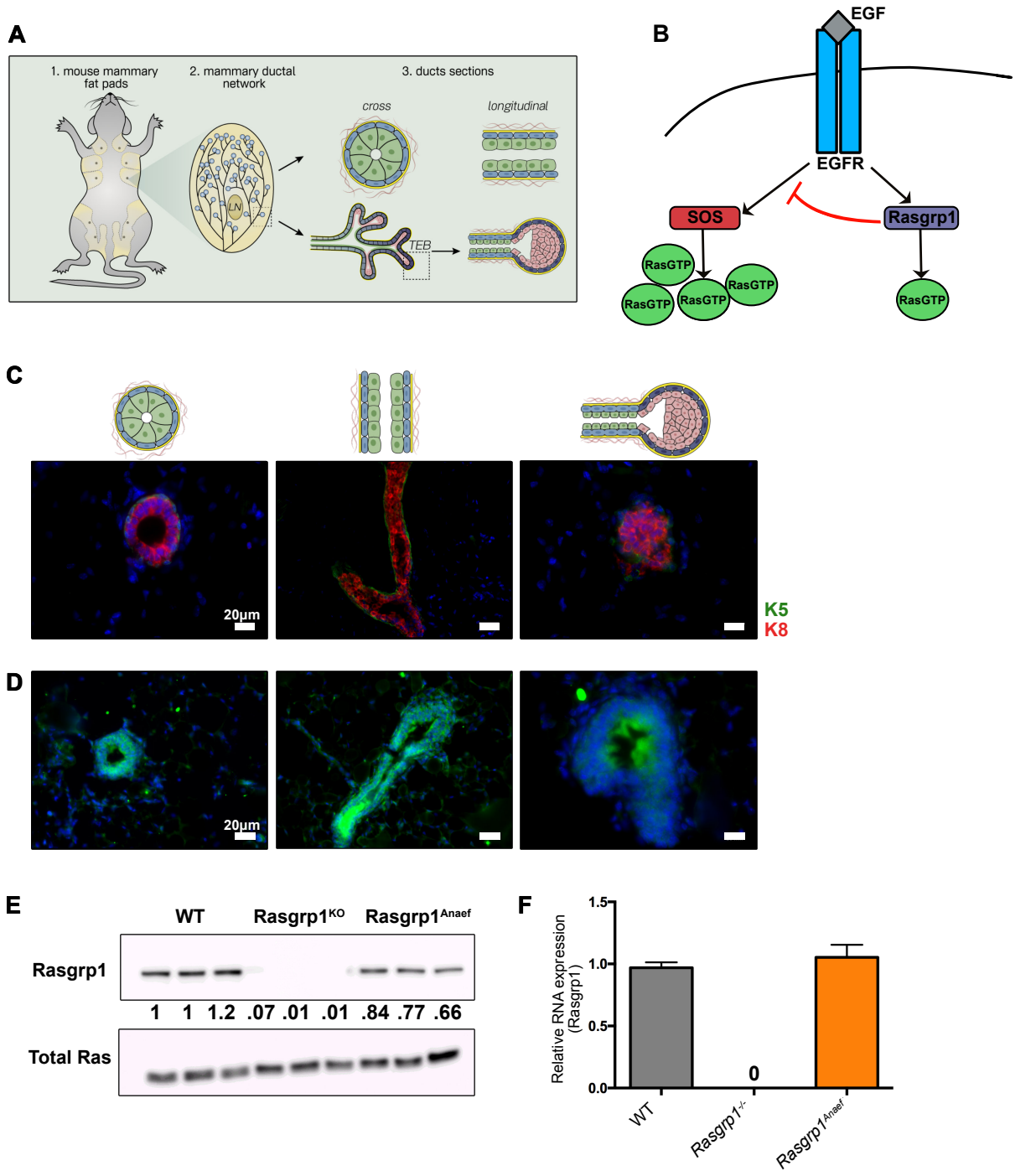


Figure 5.1. Rasgrp1 is expressed in the mammary epithelium during pubertal development. (A) Cartoon depicting the architecture of the mammary ductal network and duct and TEB (terminal end bud) make-up. (B) Cartoon of SOS and RasGRP1 Ras activation downstream of the EGFR. SOS is a potent Ras activator. RasGRP1 opposes EGR-SOS signals. (C, D) Immunofluorescence (IF) staining for mammary epithelial cell (MEC) markers in a wild-type (WT) C57BL/6 mammary gland. Cytokeratin-5 (K5, green) marks basal cells and cytokeratin-8 (K8, red) marks luminal cells. Cartoons depict

positions in epithelial ductal tree and possible sections. (D) IF for Rasgrp1 expression (green) in luminal and basal MECs in 8-week-old WT mammary glands. DAPI (blue) counterstain marks cell nuclei. Representative examples are shown. Scale bar, 20μm. (E) Western blot analysis of Rasgrp1 protein expression in murine mammary epithelial cells, normalized for cell number at extraction. WT, *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef} replicate samples produced from the same mouse samples. Blotting for total Ras serves as loading control. Ratiometric densitometry quantification below the blot with Rasgrp1 levels arbitrarily set at 1.0 in the first WT control. (F) *Rasgrp1* mRNA expression in MECs. RNA from isolated MECs was subjected to Taqman RT-PCR for *Rasgrp1* transcripts. Expression was normalized to βActin RNA levels.

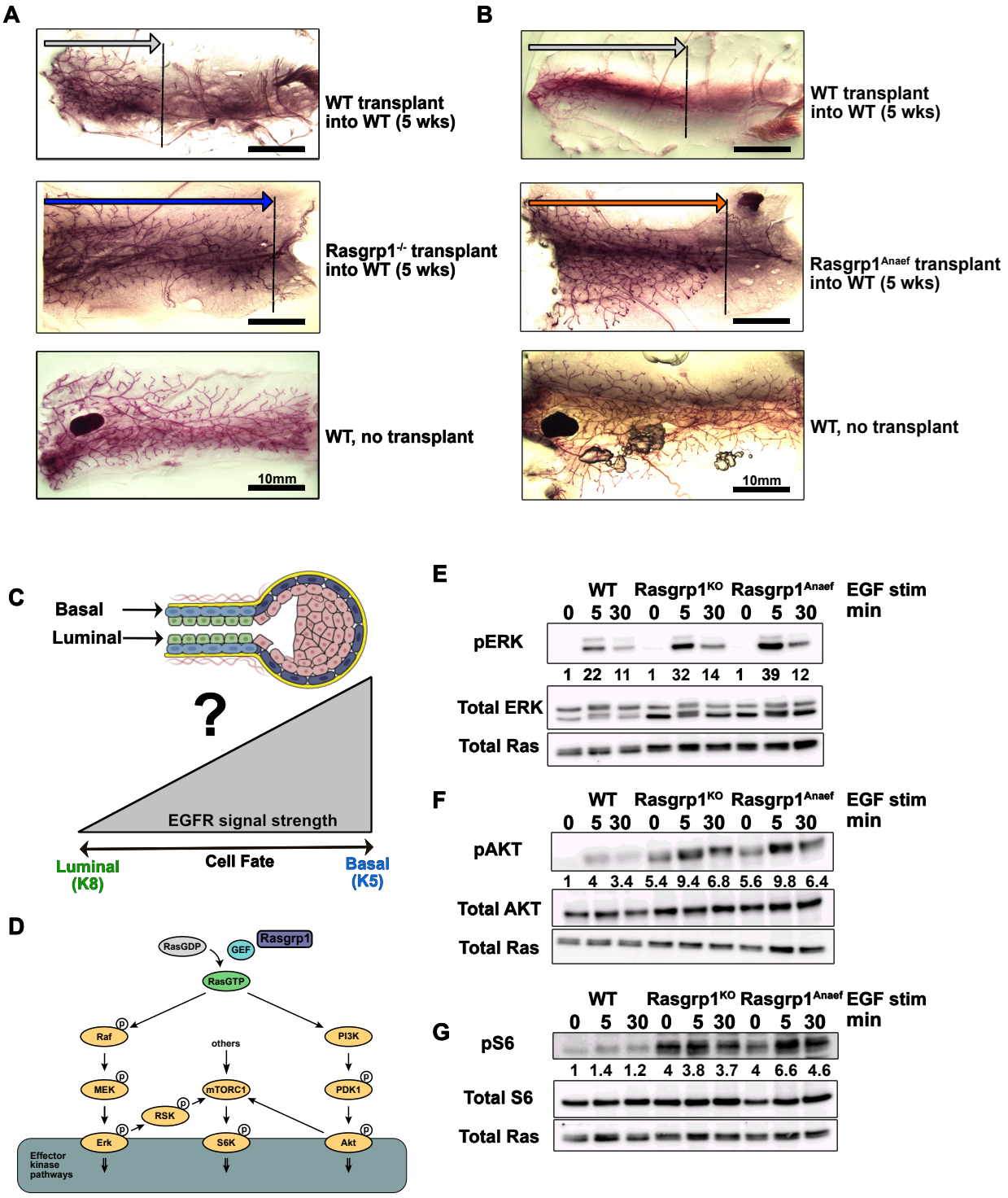


Figure 5.2. Rasgrp1 suppresses ductal tree formation following MEC transplantation and dampens EGFR-signals to ERK-, AKT-, and S6- effector kinase pathways. (A) Wild type and *Rasgrp1*$^{-/-}$ mammary epithelial cells (MECs) transplanted into recipient cleared 5-week-old WT mammary fat pad. Ductal elongation was allowed for 6 weeks before images were acquired. Stereo dissection scope-capture of whole mounted and carmine stained mammary gland. Scale bar, 10mm. (B) As in 2A but now

comparing wild type to *Rasgrp1*^{Anaef} MEC transplants. Scale bar, 10mm. n=3, total mice transplanted. (C) Cartoon depicting the hypothesis that duration and amplitude of EGFR signals may play a role in mammary epithelial cell fate decisions. (D) Cartoon of effector kinase pathways downstream of RasGTP, the product of RasGEFs like SOS1 and RasGRP1. (E -G) *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef} MECs reveal stronger EGF-induced ERK, AKT, and S6 kinase signals. Western blot detection of phosphorylated ERK-, AKT-, and S6- proteins after 0, 5, and 30 minutes of EGF stimulation. Total ERK, AKT and S6 is consistent between samples. Total Ras serves as additional loading control. Ratiometric densitometry quantification of P-ERK/ERK, P-AKT/AKT, and P-S6/S6 is indicated below the blots with WT 0 stimulation arbitrarily set at 1.0. Blots in 1E-1G are representative examples of three independent experiments.

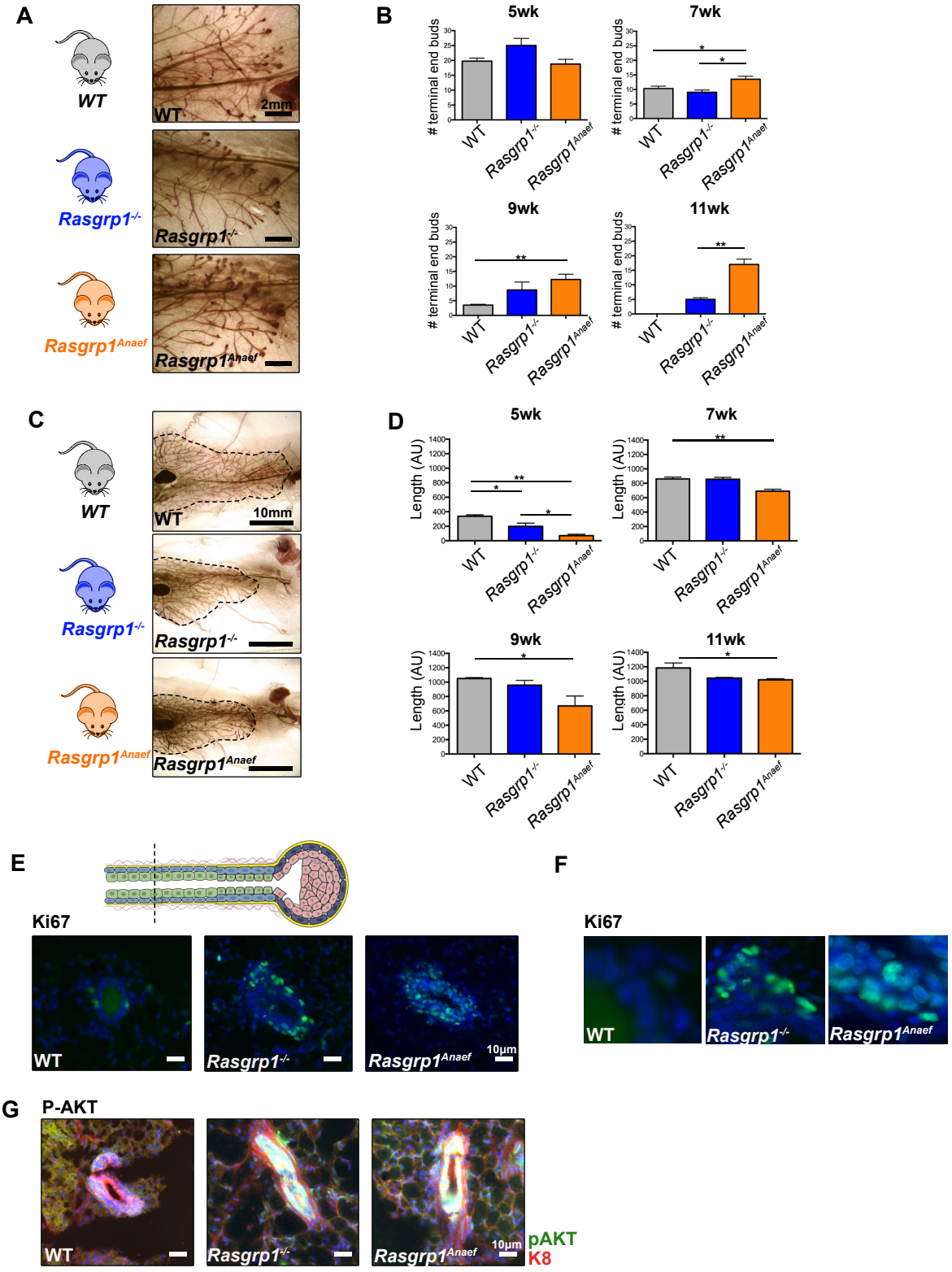


Figure 5.3. *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef} mice display altered ductal tree maturation accompanied by increased AKT signaling and proliferation in MECs. (A, B) Mice with Rasgrp1 perturbations show elevated number of the proliferative epithelial structures, terminal end buds (TEBs). Representative images from 7-week-old, carmine-stained

whole mounts. Scale bar, 2 mm. (B) Quantification of TEBs. *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef} mice show increased numbers of TEBs compared to their WT counterparts (*p < 0.05, **p < 0.005; n=4). (C, D) Rasgrp1 perturbations result in decreased ductal elongation. 7-week-old mammary whole mounts from WT, *Rasgrp1*$^{-/-}$, and *Rasgrp1*^{Anaef} were carmine stained and imaged on a stereo dissection scope. Scale bar, 10mm. (D) Quantification of ductal elongation of 5-, 7-, 9-, and 11-week old mice. Length is measured from the nipple-proximal end of the lymph node to the furthest epithelial branch. At early pubertal development, *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef} have significantly shorter ductal elongation (*p < 0.05, **p < 0.005; n= 4). This impairment persists through 7, 9, and 11 weeks of age. (E, F) Ki67 immunofluorescence (green) on cross-sections of terminus proximal mammary ducts from 9-week-old WT, *Rasgrp1*$^{-/-}$, and *Rasgrp1*^{Anaef} mice. DAPI (blue) counterstain marks cell nuclei. Representative examples are shown. Scale bar, 10 μm. (F) Higher magnification of Figure 3E. (G) AKT phosphorylation (green) by IHC on cross-sections of terminus proximal mammary ducts from 9-week-old WT, *Rasgrp1*$^{-/-}$, and *Rasgrp1*^{Anaef} mice. K8 (luminal) stained in red. DAPI (blue) counterstain marks cell nuclei. Exposure was equal in all three images and set to highlight that there is some P-AKT in WT. Representative examples are shown. Scale bar, 10 μm.

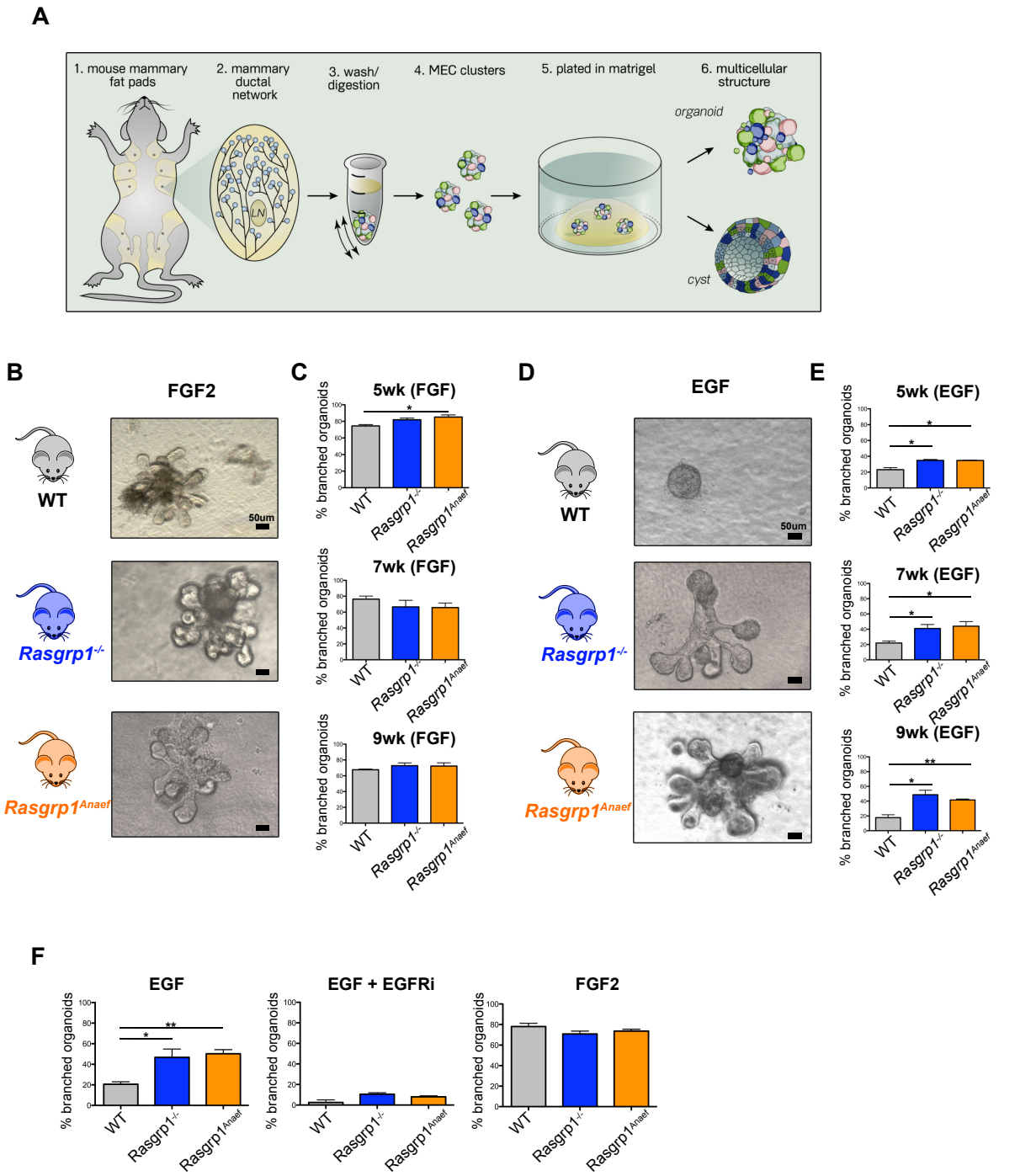


Figure 5.4. Perturbations to Rasgrp1 results in a gain-of-function EGF-induced branching in mammary organoids. (A) Cartoon representation of the organoid assay. Isolated mammary fat pads are enzymatically digested. Differential centrifugation separates mammary epithelial cell clusters, which will become organoids when placed in Matrigel pellets. (B, C) Organoids derived from WT, *Rasgrp1*$^{-/-}$, *Rasgrp1*^{Anaef} mice all respond robustly to the branching agonist FGF2. Representative images are shown for FGF2-stimulated branched organoids derived from 7-week-old mice. At 5 weeks, *Rasgrp1*^{Anaef} organoids respond more strongly to FGF2 stimulation (*$p < 0.05$), n=3.

Differences between all other groups are not significant (ns). Scale bar, 50 μm. (D, E) *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef}-derived organoids have augmented branching in response to EGF stimulation (*p < 0.05, ** p < 0.005, n=4). Representative organoid images are shown for each group, derived from 7-week-old mice. Scale bar, 50 μm. (F) Combination treatment with Erlotinib and Gefitinib EGFR inhibitors (EGFRi) reverses the gain-of-function organoid branching phenotype that occurs in EGF stimulated organoids from 9-week old *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef} mice. *p < 0.05, **p < 0.05, n=4. As positive control, FGF2 stimulation results in strong branching in organoids across all three groups.

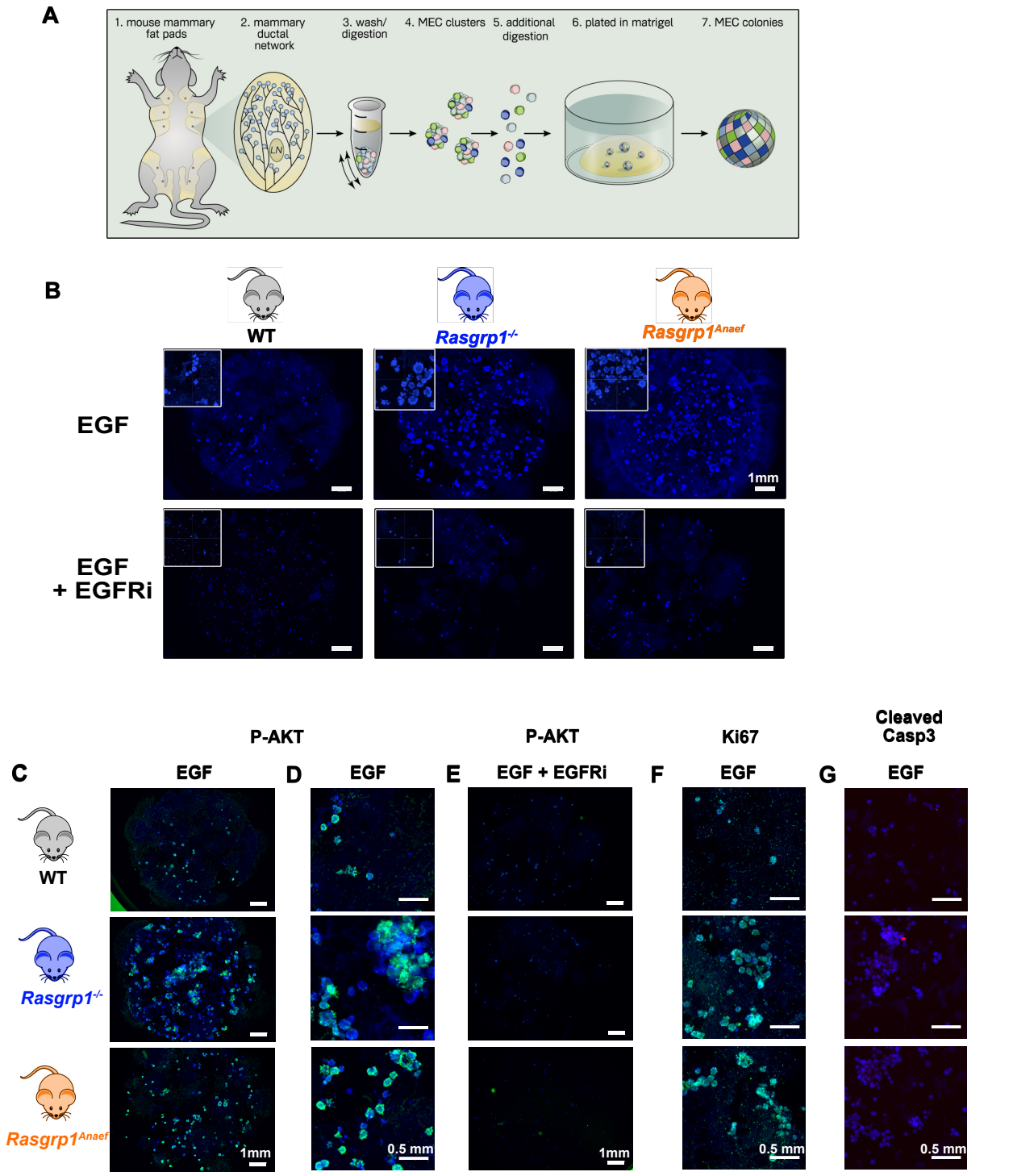


Figure 5.5. Mammary epithelial progenitor cell proliferation is increased in *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef}. (A) Cartoon representation of mammary epithelial cell colony forming assay. Fat pads are isolated from WT, *Rasgrp1*$^{-/-}$, and *Rasgrp1*^{Anaef} mice and enzymatically digested. Differential centrifugation separates mammary epithelial cell clusters. Grouped cells underwent modest typsinization and filtering to obtain single MECs. Single cells are placed into Matrigel pellet and followed for 10 days to assay colony

number and size. (B) *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef} single MECs form more numerous and larger clusters in response to EGF compared to WT. In each Matrigel pellet, 2500 cells were initially loaded. Addition of EGFR inhibitors reverses the gain-of-function phenotype. Hoechst 33342 nuclear counterstain. Scale bar, 1 mm. Zoom in images (top left). (C, D) AKT phosphorylation of colony formation assay on the three indicated, single MEC populations plated without EGFRi. Scale bar, 1 mm. (D) Higher magnification of images displayed in Figure 5C. Scale bar, 0.5 mm. (E) AKT phosphorylation of colony formation assay on the three indicated, single MECs populations plated with EGFRi. Scale bar, 1 mm. (F) Ki67 staining of colony formation assay on the three single MEC populations plated to assess cell proliferation. Scale bar, 0.5 mm. (G) Cleaved caspase-3 staining of colony formation assay on the three single MEC populations plated to assess apoptosis. Scale bar 0.5 mm.

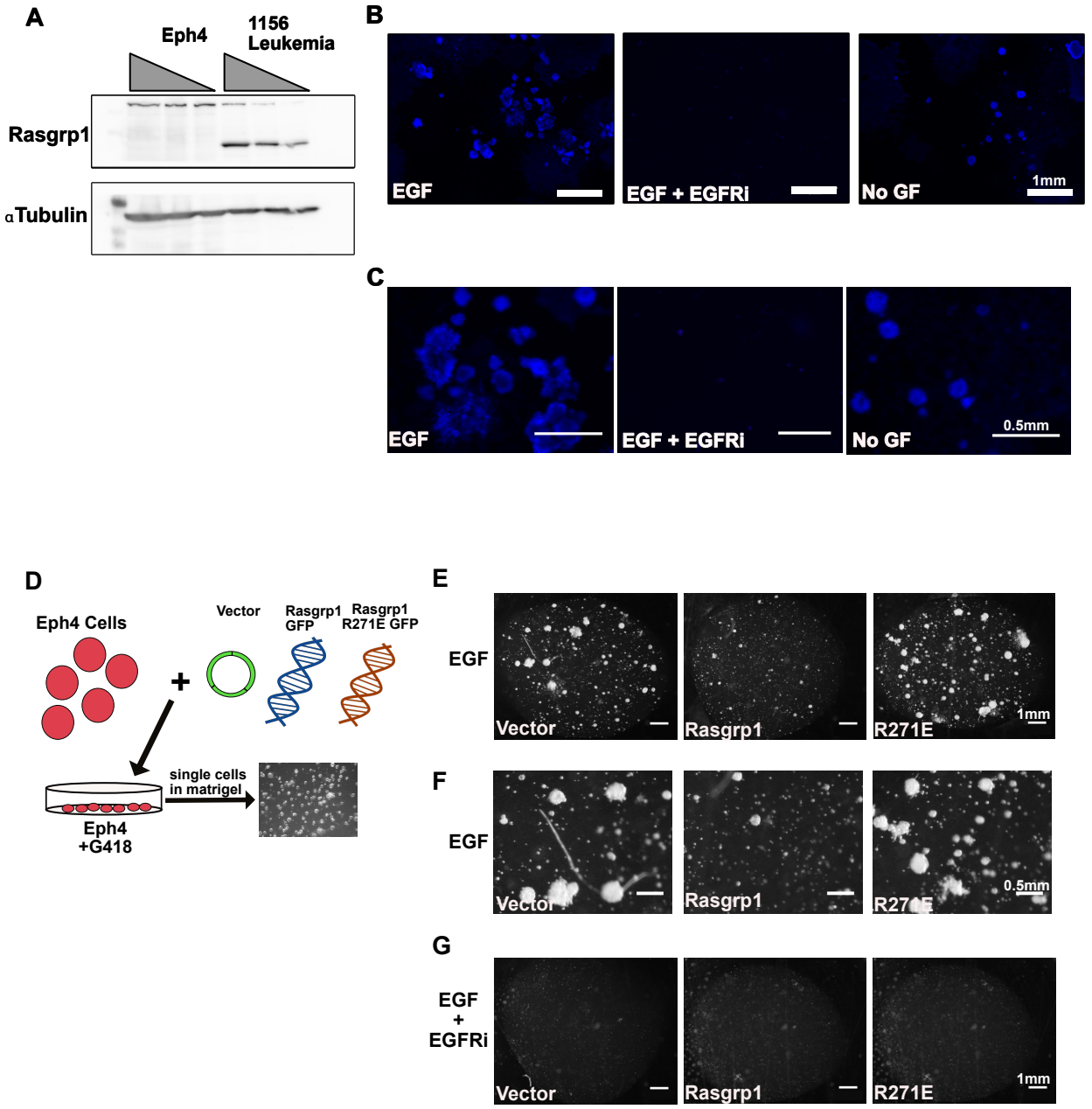


Figure 5.6. Catalytic activity of Rasgrp1 is required to suppress growth. (A) Western blot analysis for Rasgrp1 expression in the Eph4 MEC cell line and 1156 T cell leukemia cells that served as positive control. α-tubulin serves as loading control. Representative blot of 3 independent assays. (B) EGF stimulates robust growth of Eph4 cells in Matrigel-embedded colony forming cultures. Hoechst nuclear counterstaining was used on Eph4 colonies in matrigel. Omitting EGF (no GF) or inclusion or EGFRi reduces colony growth. Scale bar, 1 mm. (C) Zoom in images from Figure 6B. Scale bar, 0.5 mm. (D) Eph4 cells were used as a model to introduce GFP-labelled empty vector, Rasgrp1, or a catalytically inactive version of Rasgrp1 (R271E). (E) Catalytically active Rasgrp1 is required to abrogate enhanced colony formation in response to EGF. Stereo dissection scope images. Scale bar, 1 mm. (F) Zoom in images from Figure 6E. Scale bar, 0.5 mm.
(G) EGFRi reduces colony growth of transfected Eph4 cells. Scale bar, 1 mm.

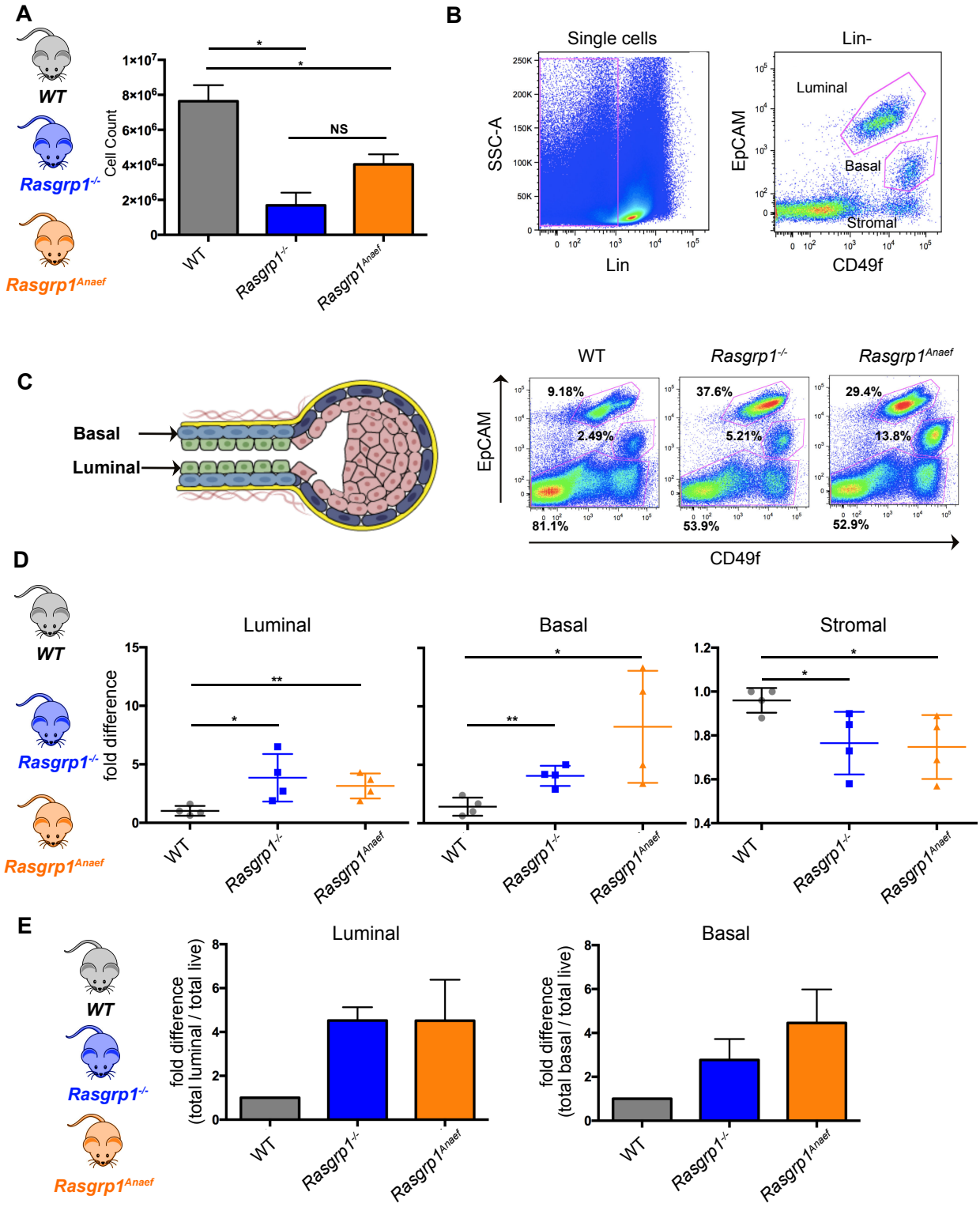
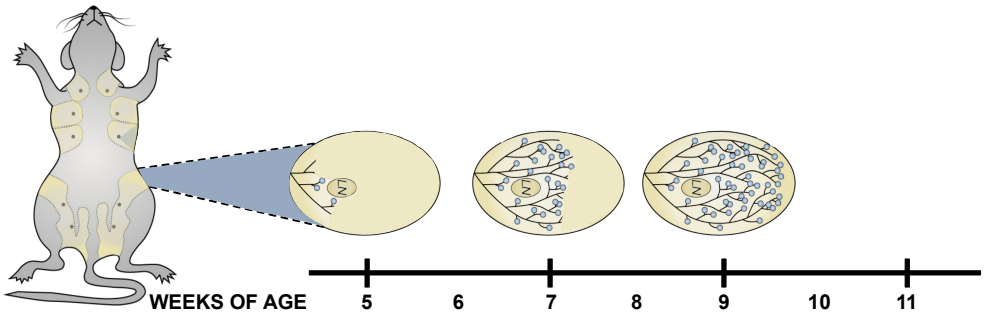


Figure 5.7. Perturbations to Rasgrp1 alters mammary epithelial cell fate during pubertal development. (A) Quantitative FACS analysis was employed to count the total number of CD49f-EpCAM positive mammary epithelial cells in the indicated mouse models at 8 weeks of age. *p < 0.05, n= 4. (B) Example of gating strategy to analyze luminal and basal cell types. (C) Depiction of cell types in duct and TEB. Representative

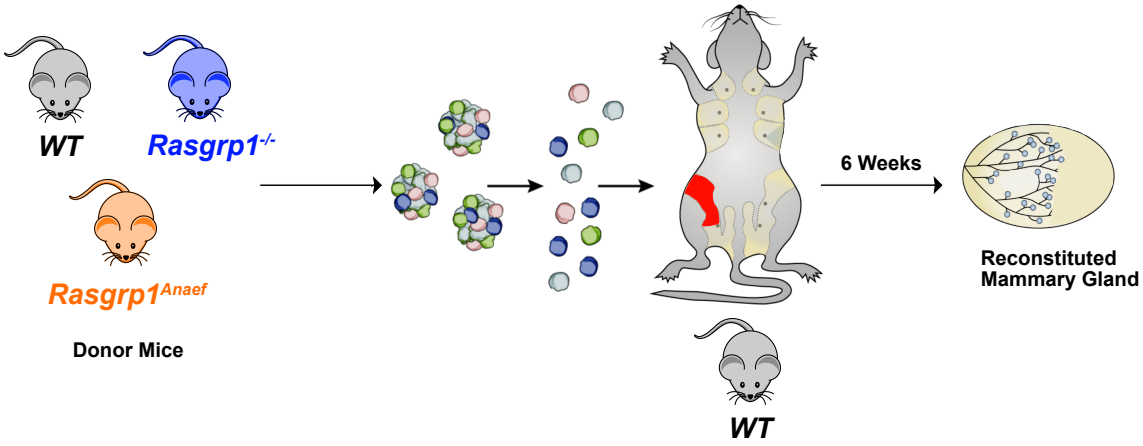
EpCAM and CD49f dot plots of mammary epithelial cells gated on lineage negative live singlets. Each mouse model is a pool of two age-matched littermates. (D) Quantification of fold difference in luminal, basal and stromal cell populations defined by the staining in figure 7C in the three mouse models. Each data point is from a single, two-female pool that was experimentally determined. Percentage values were taken from events in each gate in 7C and Supplemental Figure S2. One WT female was arbitrarily set at 1.0 and other ratios were calculated relative to this 1.0 value. *$p < 0.05$, ** $p < 0.005$, n= 8. (E) Ratiometric analysis of absolute number of luminal and basal cells divided by the total number. Values determined from quantitative FACS experiments, utilizing actual cell count. WT luminal and basal cells set to 1.0. Fold change determined after normalizing for total singlets obtained in each experiment. *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef} mice show increased luminal and basal cells. n= 8.

SUPPLEMENTAL FIGURES

S1A

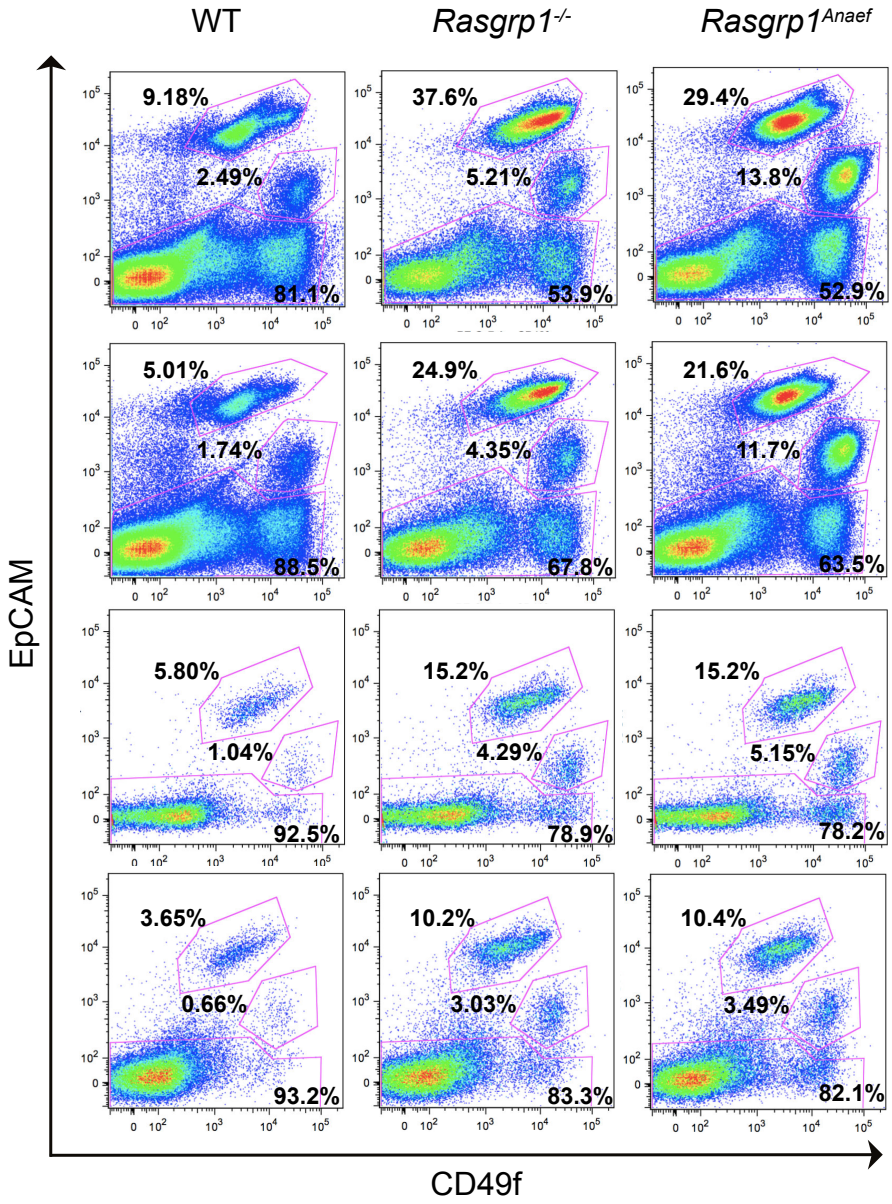


S1B



Supplemental Figure 5.1. Mouse experimental design. (A) Cartoon depicting pubertal mammary gland development in C57BL/6 mice. Shown is the #4 inguinal fat pad. Onset of pubertal development from the primitive duct occurs at 5 weeks, reaches an approximate midpoint at 7 weeks, and concludes at 9 weeks of age. Terminal end buds (TEBs), the driver of ductal bifurcation and elongation, subside following pubertal development. These time-points were selected for whole gland and mammary epithelial cell (MECs) isolation for experiments performed in Figure 3 and Figure 5. (B) Cartoon depicting mammary fat pad clearing and transplantation experimental design. MECs were isolated from wild type, *Rasgrp1*$^{-/-}$, *Rasgrp1*^{Anaef} mice at 7 weeks of age. MEC clusters are digested into single cells. These cells are then injected into the fat pad of a 5-week old wild type in which the endogenous primitive duct has been surgically cleared. The contralateral fat pad is left untouched, serving as a control. Transplanted mice are monitored for 6 weeks and then sacked to assess ductal outgrowth in the reconstituted mammary gland.

S2A



Supplemental Figure 5.2. Panel of Basal and Luminal MEC FACS. (A) Dot plots of mammary epithelial cells gated on lineage negative live singlets. Each mouse model, in each dot plot, is a pool of two age-matched littermates. The ratio of luminal and basal cells between wild type, *Rasgrp1*$^{-/-}$, and *Rasgrp1*^{Anaef} are consistent across experiments, despite changes in total cellularity across FACS experiments. The quantitation of the dot plots is found in Figure 7D.

METHODS

Mice

Mice were handled according to the Institutional Animal Care and Use Committee regulations, described in the Roose laboratory University of California, San Francisco (UCSF) mouse protocol AN84051 'Ras Signal Transduction in Lymphocytes and Cancer.' Rasgrp1 knockout (*Rasgrp1*$^{-/-}$) mice were provided by J. Stone as described in Depeille et al. (*Nat Cell Biol*. 2015). The R519G point-mutated Rasgrp1 Anaef (*Rasgrp1*^{Aanef}) mouse strain was established through ethylnitrosourea (ENU)-mediated mutagenesis of BL6 mice as previously described (Randall et al., 2009). Wild type (WT) C57BL/6 mice were bred and used as controls.

Antibodies

Primary antibodies were obtained from the following sources and used at the indicated concentrations: P-MEK 1/2 (1:250; 2338S), P-p44/42 (1:250; 9102S), p44/42 MAPK (1:250; 9102), P-AKT S473 (1:250; 4058L), AKT (1:250; 9272), P-S6 ribosomal protein S235/236 (1:300; 2211L), S6 ribosomal protein (1:300; 2317), Cleaved caspase-3 (1:300; 9661S) from Cell Signaling; Ki-67 (1:500; ab15580), Rasgrp1 (1:100; ab37927), Cytokeratin 5 (1:100; ab52635) from Abcam; CD49f-PE-Cy7 (1:100; Invitrogen 25-0495-82); EpCAM-APC (1:63; Invitrogen 17-5791-82); CD31-450 (1:170; Invitrogen 48-0311-82); CD45-450 (1:170; Invitrogen 48-0451-82); Ter119-450 (1:170; Invitrogen 48-5921-82) from eBioscience; α-tubulin (1:2000; T6074) from Sigma-Aldrich; Troma-1 cyokertain 8 (1:100; AB_531826) from Developmental Studies Hybridoma Bank; murine Rasgrp1 m199 from Depeille et al., 2015.

Secondary antibodies were obtained from the following sources and used at the indicated concentrations: Alexa Fluor 448 (1:500 in Matrigel IF, 1:250 slide IF; A21206), Alexa Fluor 555 (1:500 in Matrigel IF, 1:250 slide IF; A21434), Alexa Fuor 568 (1:500 in Matrigel IF, 1:250 slide IF; A21069).

Cell lines and reagents

Cell lines were cultured at 37°C in 5% CO_2. Unmodified Eph4 cell lines were obtained courtesy of Zena Werb. Eph4 is a nontumorigeneic cell line derived from spontaneously immortalized mouse mammary epithelial cells. Eph4 cells were grown in DMEM / F21 media + 10% FBS with pen/strep. Aliquot vials of Eph4 were frozen down in growth media containing 10% DMSO. For lipofection experiments, Eph4 cells with *Rasgrp1 GFP* and *Rasgrp1 R271E GFP* plasmids were grown under selection (G418 sulfate solution, Axenia Biology). Human recombinant epidermal growth factor (hEGF) protein was purchased from Life Technologies and dissolved in PBS. Human recombinant fibroblast growth factor 2 (hFGF2) protein was purchased from Global Stem (GSR-2001) and dissolve in PBS. Insulin, transferrin, sodium selenite (ITS; 25-800-CR) was purchased from Corning. Gefitinib (S1025) and erlotinib (S1023) were purchased from Selleck Chemicals and dissolved in DMSO.

Mammary organoid prep

4- to 9-week-old mice were selected for our experiments. CO_2 euthanized mice were placed chest up and sprayed with 70% ethanol. We made a medial cut distal down the abdominal skin, which was peeled away from the peritoneum of the abdominal cavity.

Inguinal (#4) fat pads were removed and cut with a scalpel until loosened. Mammary tissue and mammary epithelial cells (MECs) are transferred into 0.45 μM filtered collagenase solution; DMEM/F12 (UCSF CCF, CCFAA010-167201), 5% FBS, 50mg/mL gentamicin (Gibco, 83-50721M), 5 μg/mL insulin (Sigma, I5508), 2 mg/mL trypsin (Gibco, 27250-018) and type IV collagenase (Gibco,17104-019). Glands were shaken at 37°C for 35 min. The tissue then underwent differential centrifugation and treatment with 2 U/μl DNAse (Sigma D4263-1VL) to isolate MEC clusters. Organoid density was determined before placing MEC clusters into growth factor-reduced Matrigel (BD 354230) at 2 organoids/μL. Organoids in Matrigel pellet are placed on a glass bottom chamber slide (Lab-Tek®, 177379) in the 37°C incubator for 20 min to solidify before DMEM/F12 media is added. Growth factor media used: 2.5 nM FGF, 2.5 nM EGF, 1 μM gefitinib erlotinib, in 1x pen/strep, 1x ITS DMEM/F12. Growth and branching of organoids are followed for up to 10 days.

Single mammary epithelial cell preparation for FACS

Single cell isolation for FACS adopted from a protocol provided by Kessenbrock K. and Lawson D (University of California, Irvine). #1- #5 fat pads were removed from 4- to 9-week old mice and placed in a dry 10-cm dish. The tissue was chopped with a razor until slurry-like in consistency. Mammary tissue was shaken in collagenase medium (2 mg /mL collagenase type IV, Sigma C5138, in DMEM / F12, Corning 10-090) at 37°C for 1 hour. Digested tissue is spun down at 1500 rpm for 5 minutes and then washed with PBS. MECs are freed using 0.05% Trypsin/EDTA (Corning 25-0520), and excess DNA is

removed using DNAse (Worthington LS002139). Single MECs are counted and placed into FACS tubes.

Basal and luminal FACS stain on primary mouse MECs

We used flow cytometry to assess luminal (CD49f/EpCAM^{hi}) and basal/mammary stem cell-containing CD49f^{hi}/EpCAM populations (Shackleton et al., 2006; Stingl et al., 2006). Single MECs were isolated from WT, Rasgrp1$^{-/-}$, and Rasgrp1^{Anaef} mice and placed into 500 ul DMEM / F12 FACS tubes (Falcon, 352235). Single color controls were made for CD49f, EpCAM, and Lin- (CD31, CD45, Ter119) in addition to a no stain control. FACS tubes are placed at 4°C in the dark for 20 minutes. Cells are spun down at 1500 rpm for 5 minutes and aliquoted into new FACS tubes through the cap filter. FACS tubes are kept on ice. FACS stains were performed on an LSRII and an FACS Aria II machine. Sytox blue (ThermoFisher Scientific, S3457) is added to Lin- and ALL tubes just prior to their run, in order to differentiate lin-/live (MECs) from lin+/dead (stroma).

Mammary whole mount and carmine stain

Protocol was adapted from Kouros-Mehr H. et al. (Cell. 2006 Dec 1; 127(5): 1041–1055.) Inguinal #4 mammary fat pads were removed carefully from experimental mice. Glands were spread on glass slides (Fisherbrand, 12-550-15) and fixed overnight in 3:1 ethanol to glacial acetic acid. Slides were transferred to 70% EtOH then 50% ethanol for 10 minutes. Slides were washed with tap water slowly to remove ethanol. Slides were placed in new slide holder containing Carmine Red (carmine, Sigma C1022; aluminum potassium sulfate, Sigma A7167). Mammary tissue was then transferred to 70% then

95% ethanol for 15 minutes, and 100% ethanol for 15 x 3 minutes. Tissue was then transferred to Histo-Clear II (National Diagnostics, HS-202) for 2 hours, and then placed into fresh Histo-Clear. Slides were imaged using a dissection scope. Number and location of terminal end buds was assays. Image analysis software (ImageJ) was used to determine length of mammary duct branching.

Cleared mammary fat pad transplantation assay for mammary epithelial organogenesis

We followed the protocol detailed in from Lawson, D.A. et al (Cold Spring Harb Protoc.; 2015(12): pdb. prot078071.). In short, mouse mammary tissue was dissociated for the isolation of 10^3-10^6 MECs from WT, Rasgrp1$^{-/-}$and Rasgrp1^{Anaef} mice. MECs were resuspended in 20 μL Matrigel-GFR: primary growth medium 1:1 and kept on ice. Recipient mice aged 21-35 days were surgically cleared of their rudimental mammary epithelial duct in the #4 inguinal fat pad, located in the region of the mammary fat pad between the nipple and the proximal lymph node. MECs suspended in Matrigel mixture were then injected into the cleared mammary fat pad. Mice were followed up daily for the next week to assay for health. Epithelial organogenesis took place over 6 weeks, after which the transplanted inguinal fats pads were isolated and stained with carmine.

Immunofluorescence of mammary whole mount sections

Mammary fat pads were dissected, placed on glass slides, fixed in 4% PFA for 1 hour, and place overnight in 30% sucrose overnight. Mammary tissue was placed in 1:1 OCT (Tissue-Tek)/30% sucrose for 1 hour. Tissue embedded in OCT. Sections (15 μm-thick,

Leica CM 1950 Cryostat) were obtained, setting the cooling block to maximum cold. Sections were dried at room temperature for 1 hour, then incubated in 0.3% Triton X100 (Sigma-Aldrich) in PBS. Slides were rinsed with PBS and blocked for 1 hour (10% normal donkey serum, 3% bovine serum albumin, 0.1% Triton X100 in PBS). Primary antibodies were diluted in blocking buffer, added to the tissue, and incubated overnight at 4°C overnight. Slides were washed with PBS and incubated in secondary antibodies (diluted in 5% normal donkey serum, 0.1% Triton X100) at room temperature in the dark for 2 hours. After additional PBS rinses, DAPI (Sigma-Aldrich) counterstain was added. Images were obtained using a motorized, upright fluorescence microscope (Zeiss Axio Imager M2, Carl Zeiss). Images were collected using Imager M2 camera.

3D mammary epithelial colony forming cell assay

MEC clusters were first obtained following the protocol described in this manuscript. MEC clusters were then suspended in in 2mL of 0.05% trypsin/EDTA and placed in the 37°C incubator for 6 minutes. Cells were taken out every 2 minutes and pipetted up and down several times to assist in the separate of clusters into individual cells. 5 ml of HBSS + 2% fetal bovine serum was added to stop trypsinization. Now single cells were repelleted and filtered over a 100 μM strainer. MECs were counted for the colony forming assay or frozen down in DMEM / F12 + 50% FBS + 10% DMSO. For Eph4 cells, 0.25% trypsin/EDTA was used to detach cells from the plate and inspected for single cellularity. 20-μL Matrigel platforms were created in a 48-well plate and let sit for 20 minutes at 37°C to solidify. Isolated MECs are resuspended in growth factor reduced matrigel to give 2500 cells / 20μl. 20 μl of the Matrigel-cell suspension was added on top of the Matrigel platform in

each well and placed in the 37°C incubator for 20 minutes to solidify. Growth factor and inhibitor medium were then added. Growth factor media used: 2.5 nM FGF, 2.5 nM EGF, 1 μM gefitinib erlotinib, in 1x pen/strep, 1x insulin transferrin, sodium selenite DMEM/F12. Size and number of colonies of organoids are followed for up to 10 days.

Nuclear counterstain of organoids and colony-forming assay

Nuclear counterstaining for visualization was done using Hoechst® 33342 (ThermoFisher Scientific, H1399) and following the provided protocol. In short, Hoechst® dye is diluted 1:2000 in PBS and added to MECs for 10 minutes, protected from the light. Staining solution was removed and cells were washed 3x with PBS. Images were collected using an inverted fluorescence phase contrast microscope (Keyence BZ-X710, BZX Viewing Software).

Lipofection of Eph4 MECs

Plasmids for wild-type *Rasgrp1 GFP* and catalytically dead *Rasgrp1 R271E GFP* were generated at the UCSF virus core. Eph4 cells were lipofected with *Rasgrp1 GFP* and *Rasgrp1 R271E GFP* following the Lipofectamine® Reagent (Invitrogen, 18324-012) transfection protocol. In short, 70-90% confluent Eph4 cells are incubated in media containing Lipofectamine® and DNA in Opti-MEM (Gibco, 31985-062). Eph4 cells were followed for 1-3 days and then analyzed.

Immunofluorescence on 3D cultured mammary colonies and organoids

Protocol adapted from Huebner RJ et al. (Huebner et al., 2016). Mammary epithelial cell colonies and organoids were grown in a 50:50 growth factor-reduced Matrigel: DMEM /F12 suspension. Growth factor media was removed and cells were fixed in warm 4% PFA (32% solution; 15174-3; Electron Microscope Sciences) for 25 minutes at 37°C with gentle rotation. The plate was then washed 3x in PBS at room temperature (RT) for 10 minutes. 0.5% Triton X-100 in PBS was added to wells for 30 minutes at RT. Cells were blocked for 3 hours in 10% FBS in PBS at RT. Primary antibodies were diluted in blocking buffer, placed in wells, and let sit overnight at 4°C. Wells were washed 10% FBS in PBS at RT and then blocked for 40 minutes. Secondary antibodies were diluted in blocking buffer, placed into wells, and let sit for 2 hours at RT. Wells were washed in PBS. Nuclear counterstain was added following the protocol described in this manuscript. Wells were washed with PBS and then imaged on the Keyence BZ-X710 inverted fluorescence phase contrast microscope.

Western Blot

Cells were placed in 6-well plates and starved for 2 hours at 37°C in PBS. After resting, cells were stimulated for 0, 2, 5 and 30 minutes in EGF. Cells were lysed with ice-cold NP40 with added protease inhibitors (10 mM sodium fluoride, 2 mM sodium orthovanadate, 0.5 mM EDTA, 2 mM phenylmethylsulphonyl fluoride, 1 mM sodium molybdate, aprotinin (10 mg/mL), leupeptin (10 mg/mL), pepstatin (1 mg/mL)). After lysing for 30 minutes, lysate was centrifuged at 4°C and resuspended in 2X sample buffer. Lysates were run on pre-cast NuPAGE™ 4-12% Bis-Tris gel (NP0335BOX, Invitrogen) and transferred to PVDF membranes. Protein was incubated with primary antibodies

overnight. Western blots were visualized with enhanced chemo-luminescence substrate (Thermo Scientific, 32106) and imaged on a Fuji LAS 4000 image station (GE Healthcare). Bands were quantified with Multi Gauge software, and densitometry was determined within linear range of the exposure. Values were normalized to loading controls. Intensity was reported as fold difference from control sample.

RNA Extraction and real-time PCR

Total RNA was isolated from the Eph4 cell line using RNeasy Kit (Qiagen). RNA underwent reverse-transcription with random primers (Invitrogen) and reverse transcriptase. RNA quantity and quality was assayed via NanoDrop spectrophotometer (Thermo Fisher) 260/60/280. Eph4 samples were made in triplicate and real-time PCR was performed using the RealPlex2 (Eppendorf). Expression was normalized to ß-Actin (Mm02619580_g1, Applied Bio Systems) and quantified using the CT comparison method. This method was detailed by Eppendorf. Rasgrp1 primer (Mm00448564_m1) was obtained at Applied Bio Systems.

Quantification and Statistical analysis

For Western Blot analyses, densitometric intensity values were compared using paired *t*-test.

Mammary ductal tree branch lengths were determined utilizing ImageJ analysis of captured pictures. Pixel values were transformed into metric values using provided scale information. Terminal end buds were quantified by manual counting and statistical significance was determined using paired *t*-tests. Percent branched organoids were

determined by inspecting 50 randomly selected in-Matrigel organoids for each mouse and averaged between the total *n* of each condition. Significance was determined by paired *t*-tests. Hoechst labelled MEC colonies were counted using captured photographs of the Matrigel pellet. A Muse^{TM} cell analyzer was used to provided quantitative cell counts. Analysis of graphs were doing using Graphpad Prism 5 software. FlowJo (v 8.8.6) was used for all FACS analyses and generation of plots.

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Chapter 6

A Potential Role for Rasgrp1 in Mammary Stem Cells

INTRODUCTION

As we demonstrated in **Chapter 5**, *Rasgrp1* plays an important role in balancing growth with differentiation in mammary epithelial cell (MEC) populations. We determined that *Rasgrp1* is expressed in pubertal MECs, perturbations to *Rasgrp1* result in substantial changes to the proliferative capacity of MECs. Colony-forming assay experiments showed that progenitor cell populations obtained from *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef} mice have increased capacity for seeding and expansion. Flow cytometry-based experiments revealed an expansion of both luminal and basal cells compared to WT; with an approximate 3x expansion in the luminal compartment and 4x expansion in the basal compartment. In sum, our findings suggest that *Rasgrp1* supports proper maintenance of mammary progenitor cell populations.

Many questions remain outstanding as to the specific nature of *Rasgrp1*'s role in stem and progenitor cell maintenance. Despite strong effects on MEC progenitor cell populations, *Rasgrp1* expression is detected in both mature and early-stage cells. Early progenitor and mammary stem cell (MaSC) populations are thought to reside in the basal cell compartment, with the ability to fully reconstitute the mammary gland in transplantation experiments [1,2,3]. The most widely used markers to distinguish MaSCs within the basal cell population are CD24, CD29, CD49f, CD14, CD61, and Sca-1 [4,5]. The experiments we presented in **Chapter 5** demonstrate changes to the MaSC population, but *Rasgrp1*'s expression in both luminal and basal groups complicates our interpretation. Additionally, despite increases in the MaSC population's ability to

proliferate when *Rasgrp1* is perturbed, we see expansions both luminal and basal populations. We do not observe a restricted expansion of MaSC-marker positive basal cells, either *in vitro* or *in vivo*.

The identity and function of MaSCs is one of the most important outstanding questions in the mammary biology field. Our knowledge deficit calls into questions the fundamental developmental hierarchy of mammary epithelial cells. Without drilling down into the specific nature of MaSCs, the MEC hierarchy as we understand it remains unclear. Therefore, we propose further interrogation into MaSCs and *Rasgrp1*'s potential role in their regulation. I believe that our Rasgrp1 mouse models may provide an opportunity to create clarity in regards to both the MEC hierarchy and MaSCs [6,7]. We propose the following set of experiments to address the open question of Rasgrp1 and to build a new understanding of mammary stem and progenitor cells.

EXPERIMENTATIAL APPROACH

Differentiating stem and progenitor cell markers through use of additional extracellular receptors

For the purpose of our work in **Chapter 5**, we sought to separate isolated MECs at the most basic level, luminal from basal. To do so, we utilized fluorescence-activated cell sorting (FACS) for extracellular markers. We followed the antibody design used in historic studies, CD49f and EpCAM, to distinguish bulk luminal and basal cells [8]. For our follow

up experiments, we would like to use additional, MaSC-enriching, markers to isolate in which cellular compartment exactly *Rasgrp1* perturbation is enacting its greatest effect.

We first will combine out panel of antibodies that distinguish MECs (CD49f and EpCAM) and stromal cells (CD31, CD45, Ter119) with additional extracellular markers. CD14 expression is found on all luminal progenitor cells and could serve as a good marker to subdivide the luminal population we previously identified [9,10]. CD49b could be used in additional to or instead of CD14, as it is another receptor expressed by all luminal progenitor cells. Sca-1 identifies a luminal cell subpopulation that express a substantial level of differentiation proteins, like estrogen receptor and cytokeratins K8 and K18 [10]. It is important to note that, while useful in other studies, CD61 and c-Kit may be less useful in identifying progenitor cell populations, as previous work has confirmed their expression in FVB/N mice but not C57BL/6 mice [11,12].

Inclusion of CD55 as an extracellular marker is also of great interest to us for future FACS experiments. CD55 has been well studied in the hematopoietic system, in which its expression at one of the earliest developmental branch points has aided the construction of a cellular hierarchy [13]. RNAseq experiments from the Visvader group has recently revealed an intriguing role for CD55 in distinct mammary progenitor populations during pubertal development [14]. During pre-puberty, CD55 is expressed predominantly in basal-like cells. Later in development, the expression of CD55 shifted to luminal cells. When combined with CD14, the authors found that CD55 expression exists within a potent progenitor cell that later restricts to a luminal cell fate [14]. Given these findings,

we seek to combine our existing panel of markers with CD14 and CD55. Our strategy for highlighting these new progenitor cell populations is depicted in **Figure 6.1**. We hypothesize that we will see an expansion of the $CD55^{+}CD14^{+}$ population in *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef} MECs. Given what is understood about intensity of EGFR signaling and MEC fate described in **Chapter 3**, we may also observe a modest decrease in the number of committed luminal progenitors ($CD55^{+}$ MECs during mid-late puberty).

Additionally, we will utilize a separate panel of extracellular markers to isolate a basal population enriched for MaSCs. The separate panel will allow for identification of MaSCs that is commonly found in historic studies. Following the precedent established by Shackleton et al. and Stingl et al. 2006, we will use the following panel of antibodies on freshly isolated murine mammary epithelial cells: CD49f, CD29, CD24, and Sca-1 [2,3]. The $CD49f^{hi}CD29^{hi}CD24^{+/med}Sca\text{-}1^{low}$ population is enriched for MaSCs. Given our observed changes in the total basal cell pool as well as in our colony-forming assay experiments, we hypothesize that in mice in which *Rasgrp1* has been perturbed there will be an expansion of the MaSC-enriched population.

Utilizing these combinatorial FACS panels will address the exact nature of *Rasgrp1* expression during pubertal development. Pinpointing the specific MEC compartment in which *Rasgrp1* is most highly expressed will give insight into the specific mechanism by which *Rasgrp1* regulates MEC progenitor and stem cell populations. We will also take a parallel approach with CyTOF, which I will describe later in this chapter.

Revealing changes to MaSC activity utilizing mammary mammosphere, colony formation, and organoid culture assays

In **Chapter 5** we detailed a strong EGF-induced gain-of-function branching response in *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef} organoids (**Figure 5.4**). Branching responses are driven by the proliferating terminal end bud-like cap cells, containing the MaSC population. We further reinforced the idea that *Rasgrp1* influences MaSC maintenance via our matrigel-embedded colony forming assays (**Figure 5.5**). The seeding capacity and size of the MEC colonies acts as a stand in for progenitor cell proliferation. In sum, we have shown that several *ex vivo* assays can be used to assay phenotypic effects of *Rasgrp1* perturbation.

Our experimentation focused on the use of two growth factors, FGF2 and EGF, to assay changes to the organ-forming and proliferative potential of *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef} MECs. FGF2 is a potent agonist of branching in mammary organoids, and can serve as a positive control for morphogenic potential [11,12]. EGF is a weak branching activator in organoid culture, usually inducing the formation of a hollow cyst [13]. EGF is critical to our studies, however, as we observed the connection of EGFR-Rasgrp1-Ras effector signals. EGF-EGFR signaling is known to be critically important in basal-like and progenitor cells, and overexpression of EGFR family proteins has been associated with increased in ALDH-1 positive MaSCs [15].

To hone in on the specific effects of *Rasgrp1* perturbation on the MaSC pool, we will include additional growth factors to our *ex vivo* culture experiments. One of the most

critical factors to assay is Wnt. The Wnt/ß-catenin pathway is a major contributor to MaSC maintenance. Wnt3A stimulation of MaSC-enriched pools leads to a clonal expansion of that population [16]. Wnt4 acts as a paracrine effector of signaling to progenitor cells, eliciting mitogenic effects in MaSC populations upon estrogen and progesterone binding [17]. Wnt10b is not only essential for embryonic development and placode formation, it also drives stem cell formation and mammary line specification [18]. Wnt6, Wnt5A, and Wnt5b are basal signature genes. We believe therefore that the inclusion of Wnt proteins into our functional assays could provide further insight into MaSC activity. Wnt3A is of particular interest given its role later in pubertal development and in stimulating self-renewal in MaSCs.

R-spondin proteins are ligands that induce Wnt signals,and are therefore another potential avenue of study within the context of *Rasgrp1* perturbation. R-spondin mediated Wnt activation drives fate determination in luminal cells and has been shown to induce ductal budding and branching [19,20]. Genetic knockout studies have found that R-spondin1 is required for normal mammary epithelial morphogenesis during pubertal development [21]. Whole mount carmine stains and transplant assays confirmed substantial defects in branching. These changes are indicative of R-spondin1's potential ability to regulate the survival and formation of MaSCs. *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef} MECs may therefore respond more strongly to the addition of R-spondin1 than their wild type counterparts.

In addition to the organoid and Matrigel-embedded colony formation assays we performed in **Chapter 5**, we will be performing a mammosphere assay. The organoid assay provides an understanding of organogenesis and branching, and the colony-forming assay gives insight into the activity of MEC progenitors. Mammosphere assays address the function of MaSCs [2,3]. Single MECs isolated through FACS are placed into ultra-low adherence plates. Only the MECs with high clonogenic (stem) potential are able to survive the non-adherent conditions. The capacity for MECs from our different mouse models to generate mammospheres will contribute better understanding *Rasgrp1*'s role in MaSCs specifically.

Finally, we plan to perform all the above functional assays (organoid, colony formation, and mammospheres) with the growth factors alone and in combination (FGF2, EGF, Wnt3a, R-spondin1) using sorted basal cells in a limiting dilution. It is generally accepted that MaSCs reside in the basal pool, so we want to use exclusively those cells in our functional assays. We hypothesize that the increased proliferative phenotype we have observed in progenitor populations from *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef} will drive organoid, colony, and sphere formation at less dense concentrations than their wild type counterparts. We suspect that *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef} MECs will also respond more strongly to activators of the MaSC population Wnt3a and R-spond1, given what we have previously observed. The proposed layout for all experiments is depicted in **Figure 6.2**.

The proposed experiments will provide likely key functional insights into the role for *Rasgrp1* expression in MaSCs. Furthermore, we hope that these studies will aid in the

functional characterization of the MaSC population and will open up future experimentation into the factors that regulate them.

Profiling the effects of Rasgrp1 perturbation on MEC populations utilizing single-cell RNAseq

The difficulty in deciphering the exact extracellular receptor profile of MaSCs has driven the field to explore new ways to address the identification of distinct mammary progenitor populations. RNAseq, especially when performed on single cells, has proven a fruitful avenue of exploration. The global analysis of single cell transcriptomes has shed light onto the lineage, cell-to-cell relationships, biomarkers, and rare subsets that exist within complex tissues [14]. RNAseq and subsequent expression principal component analysis has created entirely new networks of interconnectivity and highlighted the vast heterogeneity of organs. Comparative RNAseq between single cells derived from genetically modified mouse models is an informative way to interrogate both wide- and small-scale changes in lineage relationships. We propose that single cell RNAseq performed on sorted basal cells from wild type, *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef} will reveal a specific MaSC population that is strongly regulated by *Rasgrp1* expression.

Single cell RNA profiling experiments performed in Pal et al. have greatly assisted the construction of the mammary developmental hierarchy [14]. Their analysis of 3308 adult total mammary epithelial cells confirmed the expected luminal, basal, and progenitor RNA clusters, based upon signature genes [22]. It also revealed an unexpected intermediate

cluster of MECs, with an expression profile that exists somewhere between the basal and luminal profile. Comparing pubertal and adult single MECs generated seven separate expression clusters. Importantly, one of the seven clusters appear to be the MaSCs, with enhanced expression of Aldh1a3, CD55, Sparc, Tgfbr3. In sum, Pal et al. observed a large-scale shift in gene expression from relatively homogenous and basal to distinct lineage-restricted programs during pubertal development [14].

Mammary stem cells have also been observed in disease contexts through the use of single-cell analysis. Early-stage metastatic breast carcinoma cells possess a distinct stem cell-like expression signature when analyzed via single cell RNAseq [23]. Further profiling revealed differences between high- and low-metastatic tumor burden when looking at expression clusters for EMT, survival, and dormancy. Much like in development, single cell RNAseq on metastatic cells allowed for construction of a hierarchical relationship between distinct clusters.

We are interested in how expression pattern shifts, and hierarchical clustering, change in the context of *Rasgrp1* perturbation. We hypothesize that *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef} MECs will have a delayed shift to restricted lineage expression profiles, or perhaps a prolonged maintenance of the basal and stem cell profiles. We propose using principle component analysis followed by heat map, t-SNE plot, and ternary plot generation to represent changes induced by *Rasgrp1* perturbation. We anticipate that our results will generally align with those in Pal et. al (adapted for **Figure 6.3**), with significant changes occurring in the basal and progenitor cell clusters. We believe that large-scale profiling of

MECs from wild type, *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef} will provide substantial advancements in our understanding of *Rasgrp1*'s role during ductal morphogenesis.

Building a new mammary epithelial hierarchy through CyTOF interrogation

Flow cytometry has been instrumental in the deconvolution of the functional complexity of biological systems. The use of both extracellular and intracellular markers has led to the creation of developmental hierarchies based upon clustering expression profiles together. Historically, flow cytometry has made use of fluorescently tagged antibodies. Different emission spectra for fluorophores allows for combinatorial analysis. Fluorescence-based systems have their limitations, however. Up to twelve different colors are used in routine flow cytometry, and up to 17 total colors have been discovered. Spectral overlap is quite common between frequently used antibodies, complicating the compensation and often muddying interpretation of studies in which the maximum number of colors is required. Recently, new techniques have been developed to address the growing need for a higher fidelity flow cytometry-based analysis.

The Nolan group has popularized flow based single-cell mass spectrometry, nicknamed CyTOF (cytometry time of flight), to confront these open questions. Unlike traditional fluorescence-based flow, CyTOF utilizes antibodies conjugated to transition non-elemental isotopes [24]. When ran through an inductively coupled plasma time of flight mass spectrometer, the non-elemental mass tags allow to discrete peak detection in up to 100 different channels. Because these non-elemental conjugated are used, no

compensation is required and the dynamic range is substantial (10^9). CyTOF technology can simultaneously analyze 60 makers per cell; 20 for barcoding and therefore up to 20 samples at a time [25,26,27]. In sum, CyTOF is a powerful new tool that could allow for construction of an extremely high-complexity hierarchy of mammary epithelial cells.

CyTOF experiments have already yielded tremendous insights about the hematopoietic continuum. In Bendall et al., CyTOF allowed for simultaneous analysis of 34 separate parameters on each single cell in human bone marrow [24]. Unstimulated hematopoietic cells were overlaid with cells treated with an extensive panel of stimuli, generating a system-wide view of signaling behaviors. CyTOF allowed visualization of each marker's expression throughout hematopoiesis. Scientific computation software (SPADE, viSNE, Wanderlust, DREMI/DREVI, and Citrus) generates graphical depictions of developmental lineages, can identify rare populations of cells, and can draw comparisons between functional markers in the presence or absence of perturbations [28,29,30,31,32]. Cell activation, clusters of receptor expression, and previously known hierarchical relationships combined to generate not only a better understanding of hematopoiesis broadly, it also allowed high resolution visualization that stimulatory signals enact upon the developmental continuum (adapted for **Figure 6.5**). CyTOF can therefore be used throughout development, across a wide-range of markers, and across a wide-range of stimuli to build an unprecedented understanding of the mammary epithelial hierarchy.

We propose CyTOF using total MECs, luminal, and basal cell populations. We have generated a table of antibodies (**Table 6.4**) historically used to differentiation MECs that

will be chelated with non-elemental isotopes. MEC samples from wild type, *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef} will be stained with the CyTOF antibodies. Single-cell suspensions will then be ran through the mass spectrometer and analyzed. Mammary epithelial cell types will then be algorithmically assembled into groups based on surface antigen expression, to act as a superimposable map for later signaling conditions (**Figure 6.5**).

We will then perform CyTOF experiments using a variety of stimulating conditions, including EGF, AREG, Wnt3a, R-spondin1. The addition of stimulating factors will shift the identity of the mammary epithelial cells. For example, we believe that the addition of EGF to *Rasgrp1* perturbed cells drives basal / MaSC expansion. This can be visualized by CyTOF software, as the population found within the discrete hierarchical groups will be shifted towards a progenitor cell fate. For all stimulating factors, signaling within the MECs will be altered and therefore the resultant hierarchy (determined computationally) will change. We believe our findings will generate unprecedented insights into both the mammary epithelial hierarchy during pubertal development, as well as how perturbations to *Rasgrp1* resonate throughout distinct MEC populations.

FUTURE DIRECTIONS

Our data suggests that *Rasgrp1* plays an unexpected and critically important role in the maintenance of mammary stem and progenitor cell populations. We believe that the above experiments will provide detailed further insights into the exact mechanism by which proper *Rasgrp1* expression balances proliferation and differentiation.

Fluorescently-based sorts using additional extracellular antigens will allow us to hone in on a more specific MEC group in which *Rasgrp1* plays its biggest role. Sorted basal cells placed in organoid, colony forming, and mammosphere culture with new MaSC-specific stimuli will provide new functional insights into *Rasgrp1*'s role. Single cell RNAseq and CyTOF will allow us to generate new lineage relationships between MECs and the role for *Rasgrp1* in regulating cell fate decisions.

A better understanding of the mammary developmental hierarchy opens the door for numerous avenues of future exploration. The use of a more specific MaSC population in *ex vivo* experiments could exacerbate phenotypes and present a more physiologically relevant picture that *Rasgrp1* perturbations cause. RNAseq and especially CyTOF will allow for the discovery of new distinct developmental nodes. CyTOF could reveal new agonists of MaSC self-renewal and lineage restriction. Ultimately, the new insights gained will have a tremendous impact on understanding MEC heterogeneity.

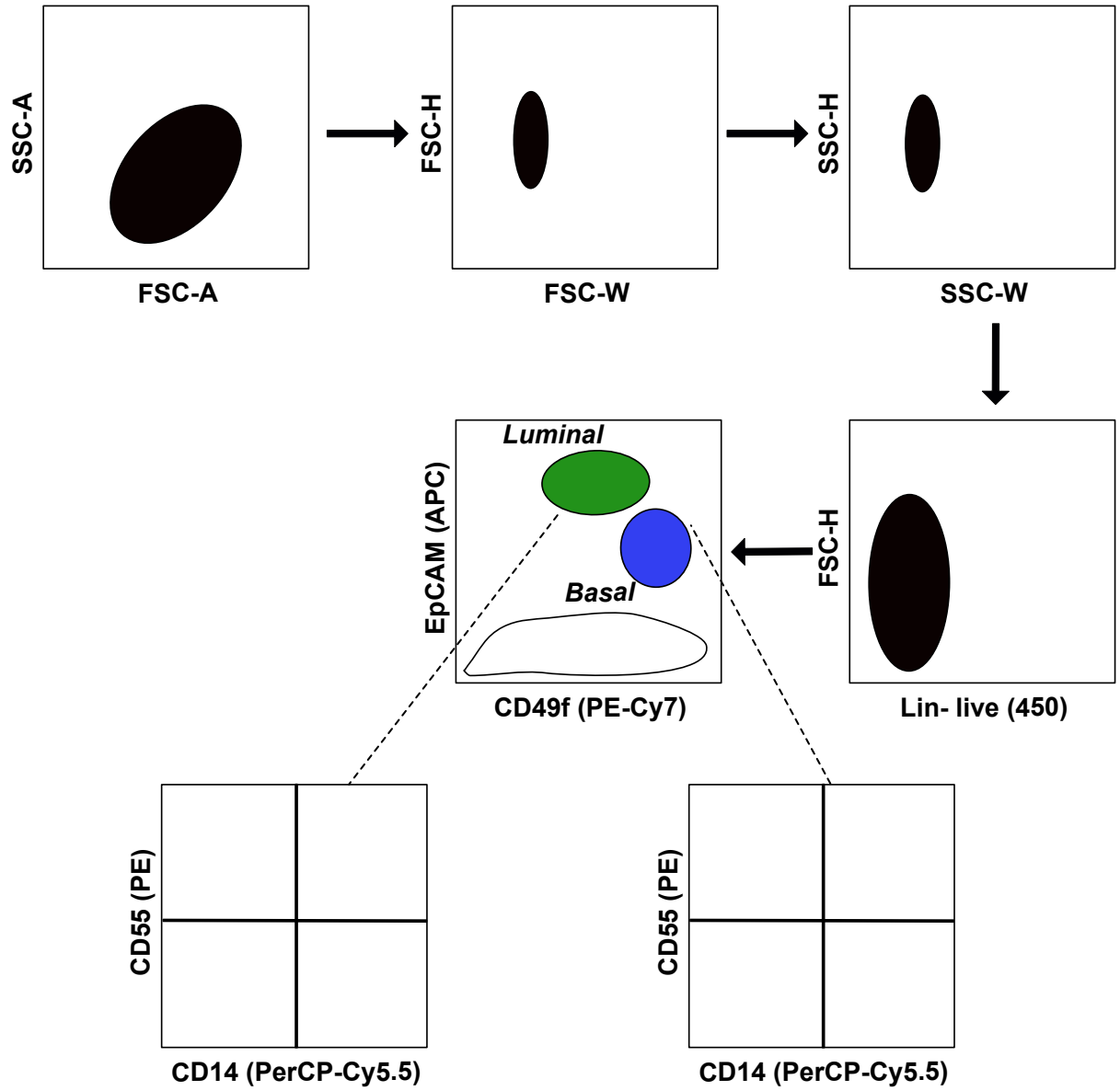


Figure 6.1. Identification of luminal and basal progenitor cell populations. Anticipated gate setup for FACS identification of new luminal and basal progenitor cell populations. Grouped cells used for each sequent phase of analysis depicted in black gate. Lineage negative live cells are separated into broad luminal ($CD49f^{+}EpCAM^{hi}$) and basal ($CD49f^{hi}EpCAM^{+}$) populations. $CD55^{+}CD14^{+}$ rare basal progenitors, and $CD55^{+}$ committed luminal progenitor populations gates depicted.

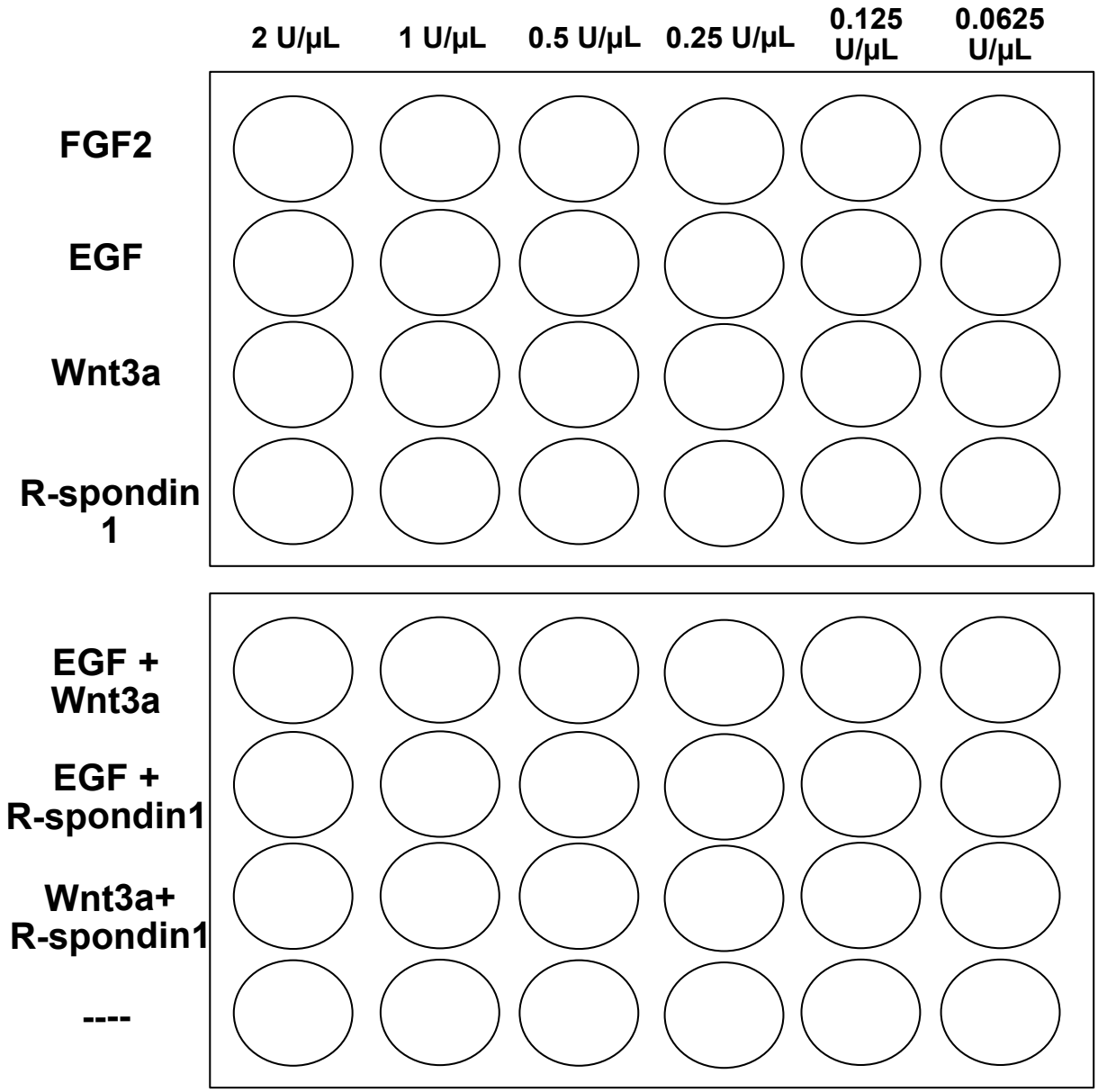
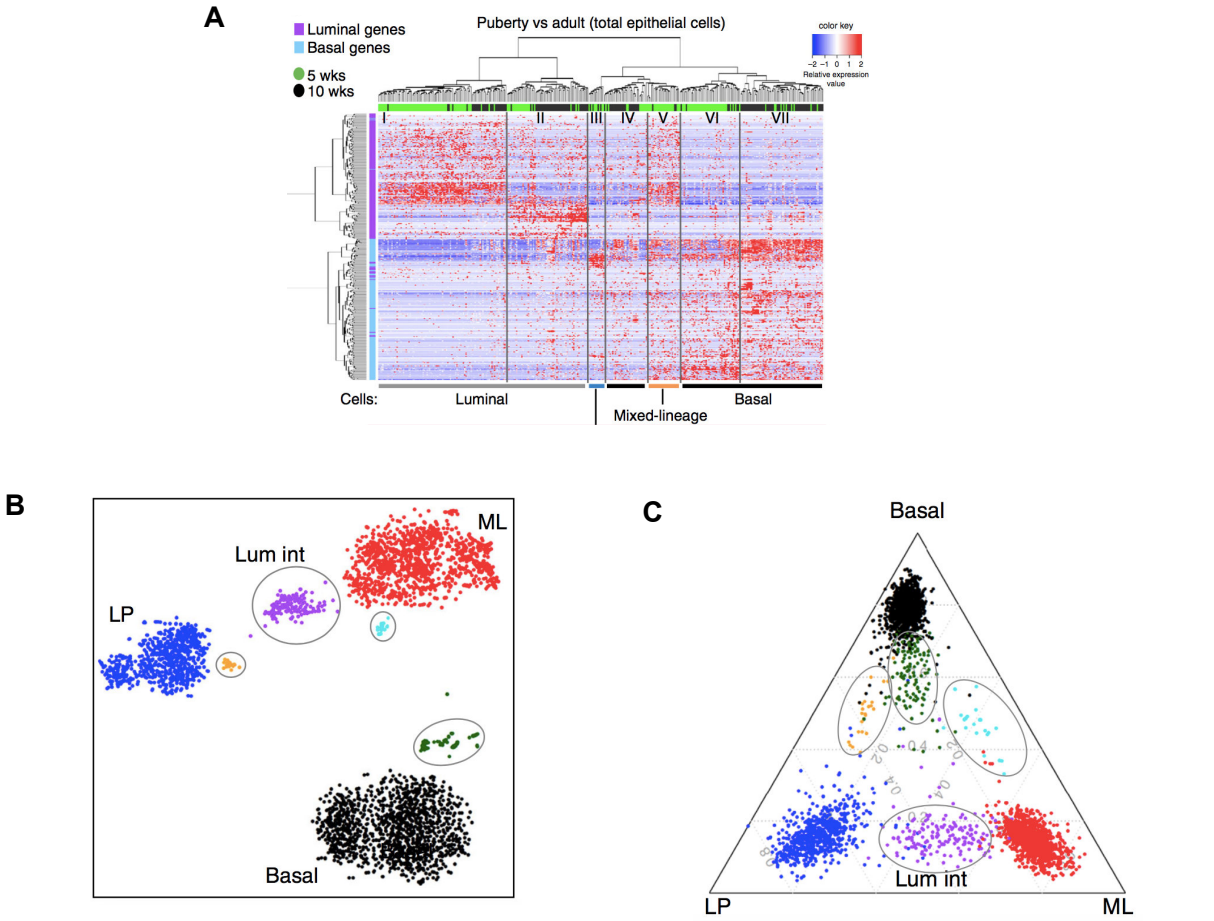


Figure 6.2. ***Rasgrp1* perturbation effects on MaSC activity in organoid, colony forming, and mammosphere assays**. Cartoon depicting the plate setup for organoid, colony forming, and mammosphere assays. Sorted basal cells from WT, *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef} are plated in FGF2-, EGF-, Wnt3a-, R-spondin1-, and combination stimulating conditions. Limiting dilutions from 2 units per ul to 0.0625 units per ul.



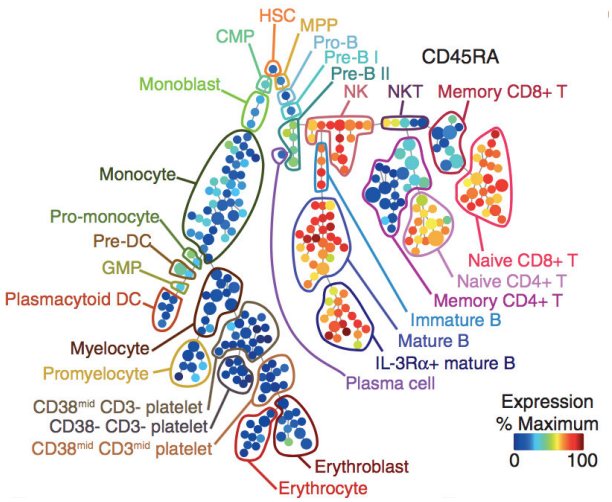
Adapted from Pal B et al. Nat Commun. 2017

Figure 6.3. Transcriptome analysis of wild type, *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef} MECs. (A) Representative heatmap and clustering of MECs from single-cell RNAseq analysis. Blue represents downregulated genes, red upregulated. MECs are group based upon their expression patterns. Adapted from data found in Pal et al. 2017. (B) t-SNE plot of transcriptomes of total mammary epithelial cells. Graphical depiction of RNA-based clustering of MECs. Adapted from data found in Pal et al. 2017. (C) Ternary plot classification of distinct epithelial cell subtypes, based on known lineage-specific signatures. Plotted based on relative proportion of genes expressed that matched the previously established profiles.

Mammary Epithelial Cells	Non-Epithelial Cells
CD49f	*CD45*
CD29	*CD31*
CD24	*Ter119*
EpCAM	*CD235a*
Sca1	
CD61	
c-Kit (CD117)	
CD49b	
CD14	
CD55	
CD133	
MUC1	
CD1d	
MFGM	
ALDH1	
Thy1	

Figure 6.4. Table of CyTOF antibodies for mammary epithelial cells. Table depicting antibodies selected for conjugation to non-elemental isotopes to be used for CyTOF. Markers seprated into columns for epithelial cells and non-epithelial cells, for removal of stroma from algorithmic analysis.

A



Adapted from Bendall, S.C. et al. *Science. 2011*

B

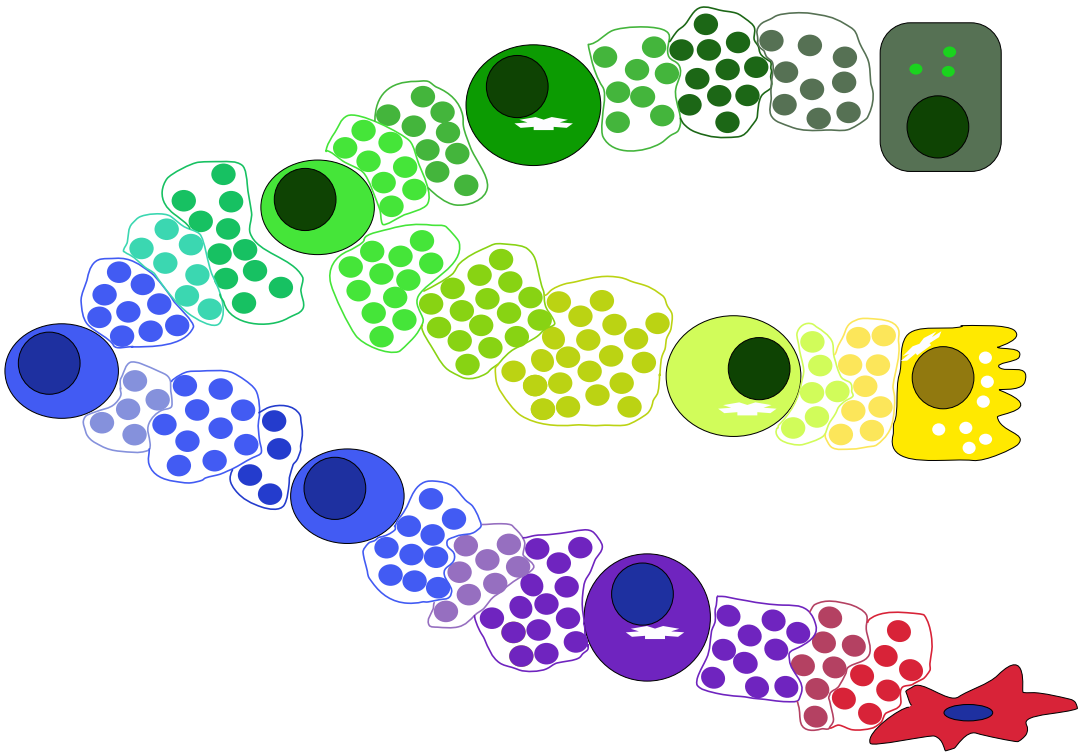


Figure 6.5. Algorithmically-determined developmental continuum of immune cells and model for mammary epithelium. (A) Graphical-depiction of hematopoietic continuum as determined through CyTOF analysis. Nodes are clustered by extracellular antigen expression. Adapted from Bendall et al. 2011. (B) Cartoon depiction of potential mammary epithelial developmental hierarchy following CyTOF.

METHODS

Single mammary epithelial cell prep for FACS

Single cell isolation for FACS adopted from a protocol provided by Kessenbrock K. and Lawson D (University of California, Irvine). #1- #5 fat pads were removed from 4- to 9-week old mice and placed in a dry 10-cm dish. The tissue was chopped with a razor until slurry-like in consistency. Mammary tissue was shaken in collagenase medium (2 mg /mL collagenase type IV, Sigma C5138, in DMEM / F12, Corning 10-090) at 37°C for 1 hour. Digested tissue is spun down at 1500 rpm for 5 minutes and then washed with PBS. MECs are freed using 0.05% Trypsin/EDTA (Corning 25-0520), and excess DNA is removed using DNAse (Worthington LS002139). Single MECs are counted and placed into FACS tubes.

Basal and luminal FACS stain on primary mouse MECs

We used flow cytometry to assess mature and progenitor luminal and basal cell populations. Luminal ($CD49f^{+}EpCAM^{hi}$), basal ($CD49f^{hi}EpCAM^{+}$), luminal progenitor ($CD49f^{+}EpCAM^{hi}CD14^{+}$), rare basal progenitor ($CD49f^{hi}EpCAM^{+}CD55^{+}CD14^{+}$), and MaSC ($CD49f^{hi}CD29^{hi}CD24^{+/med}Sca\text{-}1^{low}$) populations identified. Single MECs were isolated from WT, $Rasgrp1^{-/-}$, and $Rasgrp1^{Anaef}$ mice and placed into 500 ul DMEM / F12 FACS tubes (Falcon, 352235). Single color controls were made for CD49f, EpCAM, CD55, CD14, CD29, CD24, Sca-1, and Lin- (CD31, CD45, Ter119) in addition to a no stain control. FACS tubes are placed at 4°C in the dark for 20 minutes. Cells are spun down at 1500 rpm for 5 minutes and aliquoted into new FACS tubes through the cap filter. FACS tubes are kept on ice. FACS stains were performed on an LSRII and an FACS Aria

II machine. Sytox blue (ThermoFisher Scientific, S3457) is added to Lin- and ALL tubes just prior to their run, in order to differentiate lin-/live (MECs) from lin+/dead (stroma).

Mammary organoid prep

CO_2 euthanized mice were placed chest up and sprayed with 70% ethanol. We made a medial cut distal down the abdominal skin, which was peeled away from the peritoneum of the abdominal cavity. Inguinal (#4) fat pads were removed and cut with a scalpel until loosened. Mammary tissue and mammary epithelial cells (MECs) are transferred into 0.45 μM filtered collagenase solution; DMEM/F12 (UCSF CCF, CCFAA010-167201), 5% FBS, 50mg/mL gentamicin (Gibco, 83-50721M), 5 μg/mL insulin (Sigma, I5508), 2 mg/mL trypsin (Gibco, 27250-018) and type IV collagenase (Gibco,17104-019). Glands were shaken at 37°C for 35 min. The tissue then underwent differential centrifugation and treatment with 2 U/μl DNAse (Sigma D4263-1VL) to isolate MEC clusters. Organoid density was determined before placing MEC clusters into growth factor-reduced Matrigel (BD 354230). Organoids in Matrigel pellet are placed on a glass bottom chamber slide (Lab-Tek®, 177379) in the 37°C incubator for 20 min to solidify before DMEM/F12 media is added. Growth factors FGF2, EGF, Wnt3a, and R-spondin1 used. Growth and branching of organoids are followed for up to 10 days.

3D mammary epithelial colony forming cell assay

MEC clusters were first obtained following the protocol described in this manuscript. MEC clusters were then suspended in in 2mL of 0.05% trypsin/EDTA and placed in the 37°C incubator for 6 minutes. Cells were taken out every 2 minutes and pipetted up and down

several times to assist in the separate of clusters into individual cells. 5 ml of HBSS + 2% fetal bovine serum was added to stop trypsinization. Now single cells were repelleted and filtered over a 100 μM strainer. MECs were counted for the colony forming assay or frozen down in DMEM / F12 + 50% FBS + 10% DMSO. For Eph4 cells, 0.25% trypsin/EDTA was used to detach cells from the plate and inspected for single cellularity. 20-μL Matrigel platforms were created in a 48-well plate and let sit for 20 minutes at 37°C to solidify. Isolated MECs are resuspended in growth factor reduced Matrigel to give 2500 cells / 20μl. 20 μl of the Matrigel-cell suspension was added on top of the Matrigel platform in each well and placed in the 37°C incubator for 20 minutes to solidify. Growth factor and inhibitor medium were then added. Growth factors FGF2, EGF, Wnt3a and R-spondin1 used. Colonies were followed for 10 days.

Mammosphere formation assay

Mammosphere assays were carried out as described previously [2,3]. A single cell suspension was made from wild type, *Rasgrp1*$^{-/-}$ and *Rasgrp1*^{Anaef} mice. MECs were prepared in 1,000 and 5,000 cell dilutions in 100ul. Growth factor media was prepared as described above. Cells were then placed into 24-well ultra low adherence plates (Corning, 3473) and followed for 5 days. Growth factors FGF2, EGF, Wnt3a and R-spondin1 used.

Single-cell RNAseq

Mammary glands from 2- to 12-week old mice were isolated. Single MEC suspensions were generated, stained with fluorescent antibodies, and sorted for luminal and basal populations. Sorted cells were passed through a Fluidigm machine for single cell capture

and cDNA generation. We propose to utilize the sequencing library utilized in Pal et al. to generate RNAseq data. High throughput studies will utilize the 10X Genomics Chromium machine.

Mammary epithelial cell CyTOF

Mammary epithelial cells were isolated following the methods described as above. Antibodies chealated to non-elemental isotopes can be found in **Figure 6.4**. The experimental protocol for CyTOF used in Bendall et al. will be followed. In short, we will run the single-cell suspensions through an inductively coupled plasma time of flight mass spectrometer. We will algorithmically generate a MEC hierarchy based upon expression prolifes of extracellular antigen. Stimulation experiments will utilize EGF, Wnt3a, R-spond1. Stimulation and the subsequent effect it has on the mammary map will be graphically depicted.

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Chapter 7

Future Directions – A Role for Rasgrp1 in Breast Carcinoma

INTRODUCTION

In **Chapter 5** we initiated an investigation into the role for *Rasgrp1* expression in mammary epithelial cell (MEC) and mammary stem cell (MaSC) fate decisions. We observed substantial increases in the clonogenic potential of MaSCs when *Rasgrp1* is perturbed. We confirmed through flow cytometry analysis as well as matrigel-embedded colony forming assays that *Rasgrp1*$^{/-}$ and *Rasgrp1*^{Anaef}-derived MECs exhibit significant shifts in the number of total number of luminal and basal cells. When looking *in vitro*, we observed increases in the proliferative pattern of MECs during development. Instead of localizing exclusively in the terminal end buds (TEBs), we observed high levels of Ki67 staining in 'mature' epithelial ducts distant from the TEB. In **Chapter 6**, we hypothesized that *Rasgrp1* expression is essential for the maintenance of MaSC population. In sum, *Rasgrp1* plays a key role in balancing proliferation with differentiation in progenitor MEC populations. Given the cell-of-origin hypotheses about breast carcinomas, changes in the progenitor cell pool as a result of *Rasgrp1* may drive the formation and progression of distinct breast carcinomas.

Understanding the mechanistic details of MEC progenitor proliferation and differentiation is critically important for the study of breast cancer. Breast cancer is an extremely heterogeneous disease, from both a historical and molecular perspective [1]. Gene profiling has revealed at least five distinct subtypes: luminal A, luminal B, HER2-positive, basal-like and claudin-low [2,3,4]. Each subtype can be further split into several subgroups [5]. Breast carcinoma metastases also feature significant heterogeneity [6]. It

is thought that the heterogeneity predominantly derives from distinct breast epithelial cells serving as the cell of origin [7]. For example, “basal” breast cancers appear to be the malignant expansion of luminal progenitor cells. Her2 breast cancers are thought to arise from a non-fully differentiated luminal-restricted cell [8]. Patient outcome across these breast tumor subtypes varies as well – with increased aggressiveness and fewer targeted therapies for cancers arising from less differentiated MECs [9]. Breast cancer can also be divided on the basis of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression. Epidermal growth factor receptors (EGFRs) are of particular importance in breast cancer, discussed below. A summary of the molecular and genetic heterogeneity of breast cancers as it relates to the MEC hierarchy is shown in **Figure 7.1.**

Cancer is a genetic disease. Breast cancer, like all cancers, is defined by the genetic alterations that underlie the cell proliferative, angiogenic, and metastatic progression. Hereditary mutants account for roughly 10% of total breast cancers, and the most well studies of those are germline changes in breast cancer associated gene-1 (*BRCA1*) and p53/PTEN [10,11]. The remaining 90% of patients with breast cancer have lesions that resulted from sporadic somatic mutations. The EGFR family is frequent found mutated in human breast cancers. The EGFR family is frequent found mutated in human breast cancers.

The most EGFR family member most associated with breast cancer is Her2 (*neu*). Her2 is found significantly amplified in roughly 25-30% of human breast cancer patients [12].

Her2 is found increased as much as 200-fold in some patients, producing nearly 2 million copies of the receptor per MEC, leading to the generation of ductal carcinoma *in situ* [13]. This huge amplification of Her2 has been demonstrated to lead to augmented downstream effector pathway signaling. The high degree of Her2 expression is associated with poor prognosis in patients, and describes one of the five major subtypes of breast cancer [14]. The substantial difference in expression level between healthy and Her2+ patients has offered a few unique avenues for therapy, including enhanced sensitivity to chemotherapeutics and targeted approaches against the Her2 receptor itself.

Gene and functional profiling revealed that EGFR plays a key role in specific subsets of breast cancer—both of which are particularly malignant [15]. Mutants to EGFR are rare, but the gene is found highly expressed in basal and triple-negative breast cancers (TNBCs) [16]. Basal and TNBCs progress rapidly, are frequently metastatic, and generally have reduced patient outcome when compared to the other breast cancer subtypes [17]. *In vitro* studies showed that induction of EGFR expression alongside a second hit bolstered stem-cell activity and provided a significant growth advantage [18]. EGFR (and ErbB2) are also linked to an aggressive inflammatory type of the disease [19]. Finally, amplified EGFR is associated with an increase in cancer cell motility and invasion. Targeted therapies haven so far proven ineffective, but that could be due in large part to the functional overlap between EGFR family proteins [16].

The remaining two EGFR family members, ErbB3 and ErbB4, play important albeit smaller roles across the breast cancer landscape. ErB3 is perhaps the most common binding partner for Her2 in cancers driven by the amplification of Her2 [20]. Though it lacks catalytic activity itself, ErbB3 when phosphorylated is able to strongly recruit PI3K. PI3K is one of the pathways most frequently amplified in breast cancer [21]. ErbB4 is perhaps the most poorly understood EGFR family member in regards to cancer. ErbB4 is a driver of luminal cell differentiation, and therefore has been linked to the luminal A subtype of breast cancer [22]. Inhibition of ErbB4 can inhibit proliferation in breast carcinoma cells lines, but stimulation of ErB4 has also been shown to drive apoptosis.

In **Chapter 5**, we observed a strong gain-of-function proliferative MaSC phenotype in response to EGF stimulation of the EGFR-Ras pathway when *Rasgrp1* perturbed. The EGFR family of receptors plays roles across several breast cancer subtypes. One of the most common types of breast cancer, HER2, results from the overexpression or amplification of the EGFR family protein Her2/ErbB2 [23]. EGFR, HER3, and HER4 expression has been recently identified as potential therapeutic targets in basal-like tumors [24,25]. EGFR expression is commonly amplified in triple-negative and basal-like breast cancers [26]. This is of particular importance as triple-negative breast cancers have traditionally been difficult to treat, due to the lack of targeted therapies. HER2 expression is associated with regulating MaSCs, both in healthy tissue and during tumorigenesis and invasion [8]. Factors that act downstream of EGFR-signals in MECs, like *Ragrp1*, could play a huge and understudied role in regulating the onset and progression of many breast tumor types.

Changes in *Rasgrp1* have already been shown to affect a wide variety of cancer types. Transgenic overexpression of *Rasgrp1* can induce skin carcinomas, thymic lymphomas and T-ALL [27,28,29,30,31]. Loss of Rasgrp1 augments the cancer phenotype in KRAS mutant and APC^{min} colorectal models [32]. Other Rasgrp family members have been implicated in breast cancer, too. For example, gene-silencing, downregulation, and inhibition of expression of *Rasgrp3* was shown to decrease proliferation in a panel of human breast cancer cell lines [33]. The exact nature of *Rasgrp1* in mammary tumors, however, is still an open question. We are interested in deciphering how *Ragrp1*'s regulation of MaSC and progenitor cell populations during pubertal development could play a role in human breast cancers.

We hypothesize, given *Rasgrp1*'s ability to limit self-renewal and proliferation in MaSC populations, that loss of *Rasgrp1* will be associated to breast tumor-subtypes that resemble stem- and progenitor MECs. Reduced *Rasgrp1* expression may be associated with decreased breast-cancer specific survival in patients. We performed the following pilot experiments to begin addressing these questions.

PRELIMINARY RESULTS

Rasgrp1 expression varies in human breast cancer

To elucidate the role for *Rasgrp1*, we first set out to determine its expression in human breast cancer and its potential effects on downstream EGFR-Ras pathways. We initiated our study by interrogating the Rasgrp1 protein expression across a selection of breast carcinoma cell lines by using Western blots. We observed a wide range of Rasgrp1 expression in the 11 cell lines we surveyed. High Rasgrp1 levels were observed in three samples, moderate expression in two, and low levels in the remaining six. In cell lines in which Rasgrp1 expression was moderate to low, we observed augmented levels of phosphorylated ERK (**Figure 7.2A**). Two of the cell lines with the lowest Rasgrp1 expression, HCC1143 and HCC1008, are invasive ductal carcinomas. The two cell lines with the highest Rasgrp1 expression, HCC1599 and HCC2157, are triple-negative basal breast cancers. We further wanted to confirm the EGF-EGFR-Rasgrp1-Ras axis, so we stimulated the HCC2157 line with EGF. Rasgrp1 is phosphorylated at T184 in response to EGF stimulation, which has been observed to provide an enhancing effect on exchange activity [34] (**Figure 7.2B**).

As a pilot study to corroborate the above-mentioned cell line studies, we analyzed *Rasgrp1* mRNA expression from breast cancer patients. We performed an analysis of RNAseq data from 858 breast cancer patients from The Cancer Genome Atlas (TCGA). Normalized *Rasgrp1* and estrogen receptor (*ESR1*) mRNA levels were compared.

(**Figure 7.3**). No correlation was found between the two genes. *Rasgrp1* mRNA levels in breast cancers from patients resemble what was observed from breast cancer-derived cell lines, in which roughly half the total sample display low total *Rasgrp1* expression. In sum, we found that Rasgrp1 protein and RNA are expressed by a subset of human breast cancers. While our findings are preliminary, we believe these expression data indicate a potential role for *Rasgrp1* in the context of human breast tumors.

Low *Rasgrp1* expression is modestly correlated with poor breast-cancer specific survival probability

We performed an analysis on breast cancer patient TCGA RNAseq data looking at level of patient mammary tumor-specific *Rasgrp1* mRNA expression. We segregated total *Rasgrp1* into four groups based on percentile expression: 0-25th, 25-50th, 50-75th, and 75-100th. We plotted those groups against the patients' breast cancer-specific survival probability. Patients in the lowest percentile of *Rasgrp1* mRNA expression have the lowest breast cancer-specific survival probability, with p (logrank) = 0.0159 (**Figure 7.4)**. These data indicate that patients with the lowest *Rasgrp1* expression, including those in which *Rasgrp1* expression is absent within their breast tumors, have decreased breast cancer-specific survival when compared to their high *Rasgrp1*-expressing counterparts. These results resemble colorectal cancer studies from the Roose Lab. When *Rasgrp1* is genetically ablated in Ras-activating mutant and Apc^{Min} colorectal cancer mouse models, we observed increased size and frequency of colonic tumors and increased mortality [32].

A low level of *Rasgrp1* in patient colorectal cancer tumors is associated with poor clinical outcome [35,36].

FUTURE DIRECTIONS

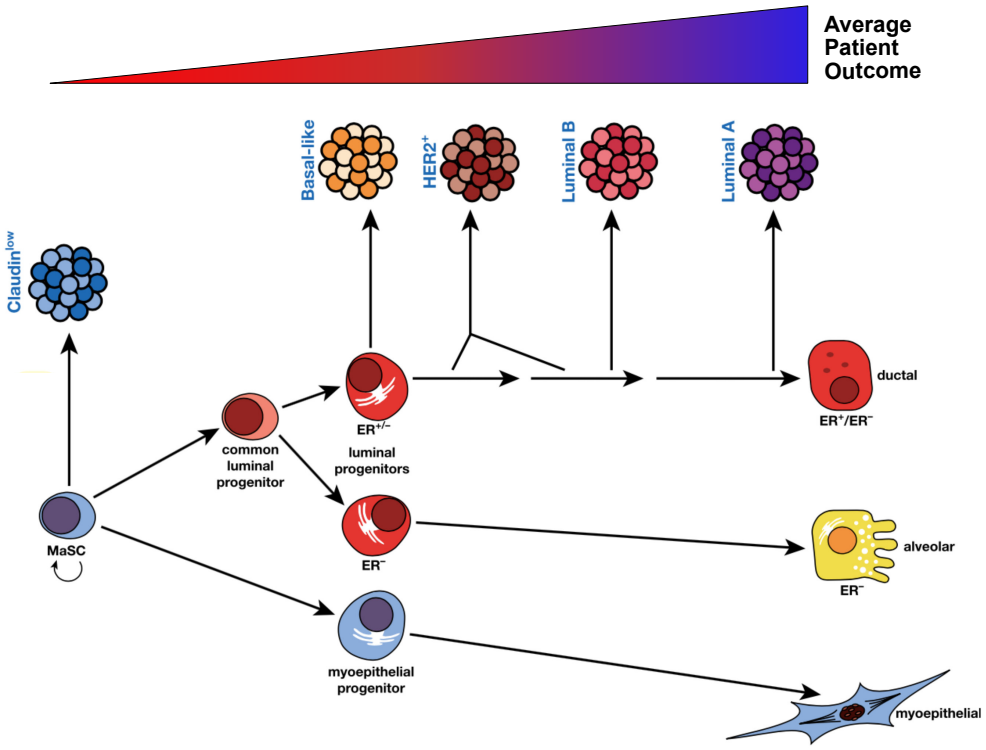
Our preliminary findings suggest that there may be an interesting link between *Rasgrp1* expression and the biology and progression of breast cancer in patients. Whereas it is pure speculation as of now, it is interesting to entertain the hypothesis that by altering the cell fate and proliferation of progenitor cell populations during development, *Rasgrp1* is poised to influence the onset and progression of human breast carcinomas.

Rasgrp1 mRNA expression varies in patient samples from TCGA. We believe that subdividing the patients based upon the tumor's molecular subtype will deconvolute *Rasgrp1*'s expression pattern. We suspect *Rasgrp1* expression may be higher in basal-like and claudin-low tumor given their cell of origin, given the observed effects of *Rasgrp1* perturbation on mammary progenitor cells. Further study is needed to better understand *Rasgrp1*'s role during oncogenesis and disease progression.

We plan to use CyTOF as described in **Chapter 6** to characterize Rasgrp1 expression in patient tumor samples. CyTOF provides an ideal platform to correlate the MEC hierarchy and MEC identity to distinct breast cancer subtypes. Organoids can be created from patient tumors to assay for *ex vivo Rasgrp1* expression and response to growth factor

stimuli. Rasgrp1-status may prove indispensable in the characterization of organoid stability and growth.

Finally, we made the observation that patients with the lowest percentile expression of *Rasgrp1* (0-25th) had the lowest breast cancer-specific survival probably. While the trend is modest, it is important to note that these data are not segregated by tumor type or disease-progression status. We suspect that when broken down into the five major subgroups of breast cancer (claudin-low, basal-like, HER2+, Luminal A, Luminal B), *Rasgrp1*'s importance will be highlighted. In conclusion, we believe *Rasgrp1* loss will promote oncogenesis and enhance disease progression in breast cancer. A better understanding of *Rasgrp1* in human tumors will lead to significant advancements in the study of breast cancer.



Adapted from Visvader J.E and Stingl, J.. *Genes Dev. 2014.*

Figure 7.1. Mammary epithelial hierarchy is associated with breast tumor subtypes and patient outcome. Gene profiling of mature and progenitor luminal and basal cells revealed the potential relationships with mammary tumor subtypes. Each of the five major subtypes are associated with their closest normal epithelial match. Difficulty in finding targets for specific-therapy and the likelihood of disease progression leads to worse patient outcome in breast cancer patients whose tumor cells resemble MaSC and progenitor cells.

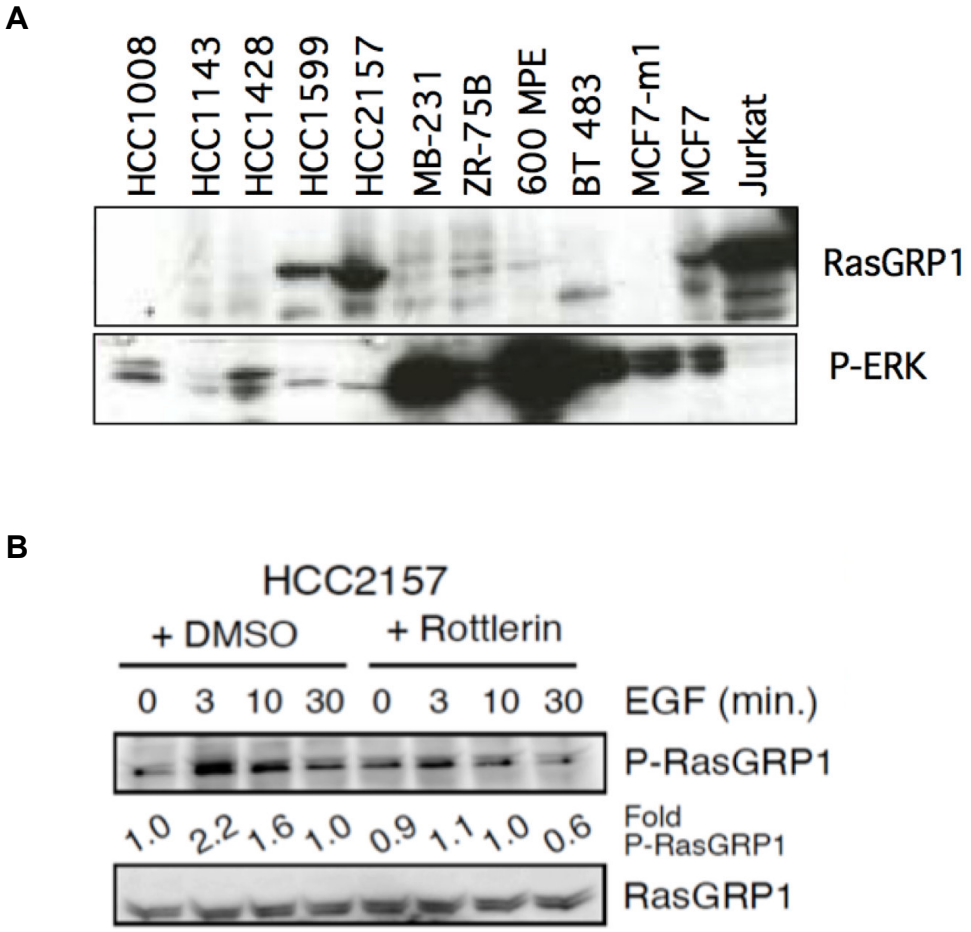


Figure 7.2. Rasgrp1 expression varies in human breast cancer cell lines and patient samples. (A) Rasgrp1 protein levels in a panel of human-derived breast cancer cell lines. Total Rasgrp1 is detected. Jurkat cells used as a positive control for Rasgrp1 expression. P-ERK levels are measured in each cell line. Samples with moderate to low Rasgrp1 expression display the highest levels of P-ERK.(B) P-Rasgrp1 levels in HCC2157 cells following EGF stimulation in the presence or absence of PKC inhibitor Rottelin. EGF stimulation leads to an increase in activation of Rasgrp1 protein, which can be blocked when a PCK inhibitor is present. Total Rasgrp1 is unaffected by the inhibitor.

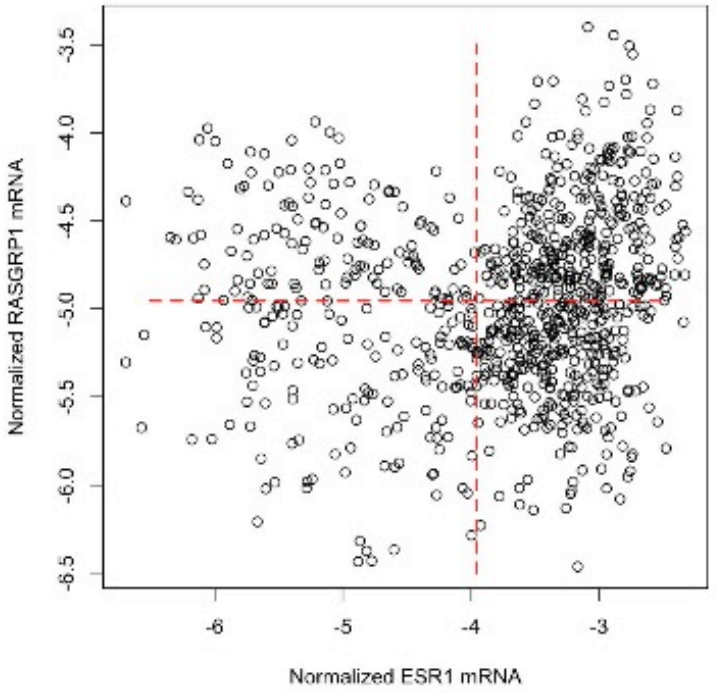


Figure 7.3. *Rasgrp1* is expressed in human breast cancer but does not correlate with *ESR1* expression. RNAseq data from 858 patient samples from TCGA. *Rasgrp1* and *ESR1* mRNA levels plotted for level of expression. *ESR1* segregates approximately into high and low expression. *Rasgrp1* expression is found in more of a continuum in human patient samples.

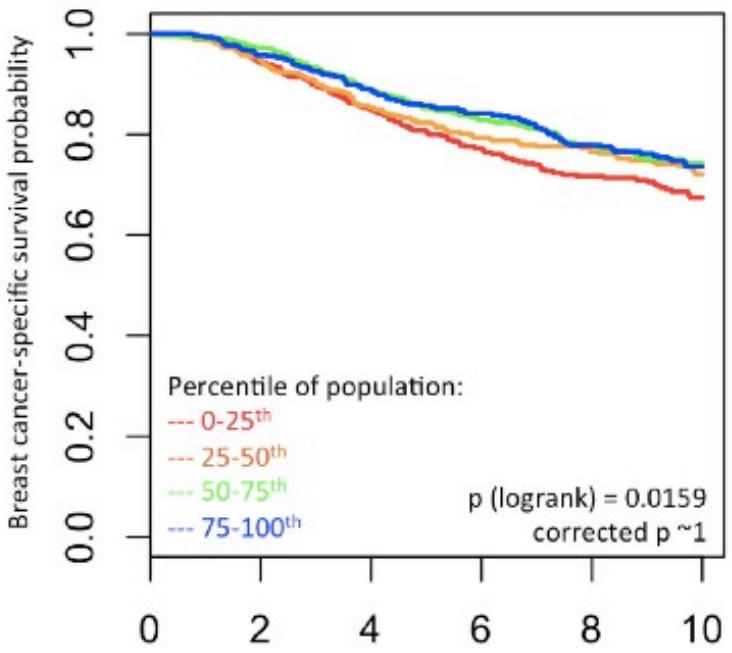


Figure 7.4. Low *Rasgrp1* modestly correlates with reduced breast cancer-specific survival. Graph depicting percentile *Rasgrp1* expression versus breast-cancer specific survival probability. TGCA patients with the lowest relative expression of *Rasgrp1* have the lowest breast-cancer specific survival probability. P = 0.0159, corrected p ~ 1.

METHODS

Breast carcinoma cell lines

A panel of commonly studied human breast cancer cell lines were selected for our research. HCC1008 (ductal carcinoma, metastatic, HER2 OE, ER/PR-), HCC1143 (ductal carcinoma, invasive, HER2/ER/PR-), HCC1428 (adenocarcinoma, FHIT tumor suppressor mutant), HCC1599 (ductal carcinoma, HER2/ER/PR-, basal), HCC2157 (ductal carcinoma, HER/ER/PR-, basal), MB-231 (adenocarcinoma, Wnt7b oncogene), ZR-75B (ductal carcinoma, invasive, ER+), BT 483 (ductal carcinoma, invasive), MCF7 (adenocarcinoma, Wnt7b, ER+), Jurkat (T cell leukemia, high Rasgrp1). All cell lines cultured following vendor protocols.

Western blot

The panel of human breast cancer cell lines were grown and then isolated for protein. HCC2157 cells were grown, rested in 1X PBS, and treated with EGF in the presence or absence of DMSO and Rottelin. Cells were lysed with ice-cold NP40 with added protease inhibitors (10 mM sodium fluoride, 2 mM sodium orthovanadate, 0.5 mM EDTA, 2 mM phenylmethylsulphonyl fluoride, 1 mM sodium molybdate, aprotinin (10 mg/mL), leupeptin (10 mg/mL), pepstatin (1 mg/mL)). After lysing for 30 minutes, lysate was centrifuged at 4°C and resuspended in 2X sample buffer. Lysates were run on pre-cast NuPAGE™ 4-12% Bis-Tris gel (NP0335BOX, Invitrogen) and transferred to PVDF membranes. Protein was incubated with primary antibodies overnight. Western blots were visualized with enhanced chemo-luminescence substrate (Thermo Scientific, 32106) and imaged on a

Fuji LAS 4000 image station (GE Healthcare). Bands were quantified with Multi Gauge software, and densitometry was determined within linear range of the exposure. Values were normalized to loading controls. Intensity was reported as fold difference from control sample.

TCGA RNAseq Analysis

Data from 858 breast cancer patients were obtained from The Cancer Genome Atlas. RNAseq data was obtained for *Rasgrp1* expression, *ESR1* expression, and breast cancer-specific survival probability. PCA analysis was performed to determine significance and clustering. *Rasgrp1* and *ESR1* data was normalized to internal controls.

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