THE ROLE OF P120 CATENIN IN MAMMARY DEVELOPMENT AND BREAST CANCER

By

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To those who question the world around them and seek to understand it…
You are eternal inspiration.
&
To my loving family and friends…
You are the greatest joy.

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LIST OF ABBREVIATIONS

AIG	anchorage independent growth
APC	adenomatous polyposis coli
BTB	bric-a-brac, tramtrack, broad-complex
BSA	bovine serum albumin
CAF	carcinoma associated fibroblast
CBD	catenin binding domain
CCL5	cc chemokine ligand 5
ChIP	chromatin immunoprecipitation
CTC	circulating tumor cell
E-cadherin	epithelial-cadherin
ECM	extracellular matrix
EGF	epidermal growth factor
EMT	epithelial to mesenchymal transition
ESRP	epithelial splicing regulatory protein
FBS	fetal bovine serum
FGF	fibroblast growth factor
GAP	GTPase associating/activating protein
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GI	gastrointestinal
GTP	guanosine triphosphate

IBC	inflammatory breast cancer
IF	immunofluorescence
IHC	immunohistochemistry
IRES	internal ribosome entry site
JMD	juxtamembrane domain
K8	keratin 8
KD	knockdown
KO	knockout
LPS	lipopolysaccharide
MET	mesenchymal to epithelial transition
MMP9	matrix metalloproteinase 9
MMTV	mouse mammary tumor virus
N-cadherin	neuronal-cadherin
NFkB	nuclear factor kappa B
PBS	phospho-buffered saline
PCR	polymerase chain reaction
PMEC	primary mammary epithelial cells
PyMT	Polyoma middle T
qRT-PCR	qualitative real-time PCR
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
RTK	receptor tyrosine kinase
shRNA	short hairpin RNA

SMA	smooth muscle actin
TAM	tumor associated macrophages
TEB	terminal end bud
UTR	untranslated region
WT	wildtype
ZF	zinc finger

CHAPTER I

INTRODUCTION

General Introduction to p120 and its cellular functions

p120 was originally described as a Src substrate and subsequently designated a catenin, a protein that interacts with and modulates function of cadherins (Reynolds et al., 1989, Reynolds et al., 1994, Daniel and Reynolds, 1995, Shibamoto et al., 1995). Cadherins are a critical family of proteins involved in tissue homeostasis, morphogenesis, and cancer that mediate cell-cell adhesion by forming homodimers between adjacent cells (reviewed in Takeichi, 1995, in Yap, 1998, in Gumbiner, 2005). p120 is the prototypical member of a family of armadillo-repeat proteins that includes δ-catenin (δ-ctn), ARVCF, p0071, and plakophilins (Anastasiadis and Reynolds, 2000). p120 lacks enzymatic activity and primarily acts as a scaffolding protein by bringing together and modulating dynamic protein complexes (Figure 1). p120 binds and stabilizes cadherins at the plasma membrane, while α catenin and β catenin (β-ctn) physically and/or functionally link cadherins to the actin cytoskeleton (Rimm et al., 1995, Yamada et al, 2005). p120 is also known to modulate actin cytoskeleton dynamics by regulating the activity of RhoGTPases and may mediate transcriptional response through interactions with Kaiso and Wnt signaling (reviewed in van Roy and McCrea, 2005, in Anastasiadis 2007, in Daniel, 2007).

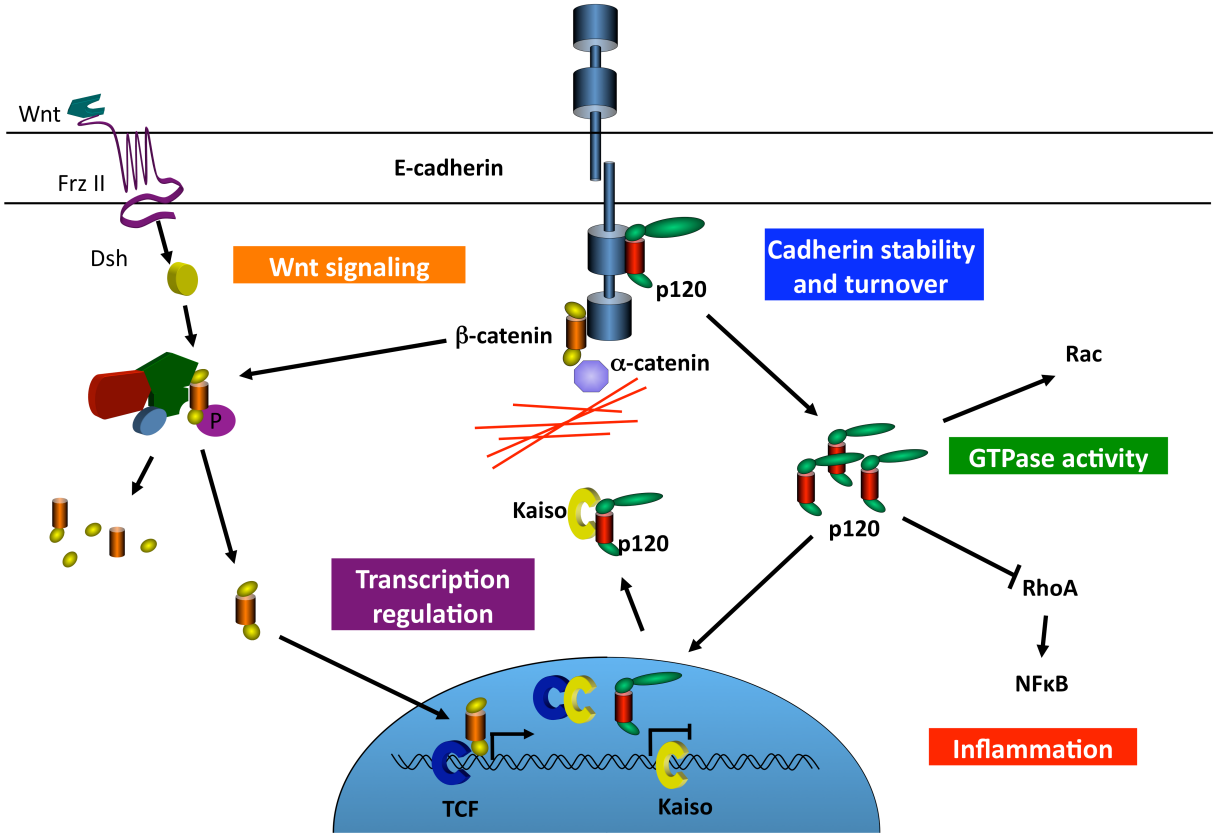


Figure 1. Cellular functions of p120

p120 binds to classical cadherins, such as E-cadherin, and stabilizes them at the membrane. p120 modulates the activity of Rho GTPases most commonly by inhibiting RhoA and activating Rac1. In the absence of p120, RhoA activation leads to NFkB activation and a subsequent inflammatory response. p120 plays a role in transcription through interactions with its binding partner, Kaiso. p120 is proposed to function in Wnt signaling at a variety of levels of the pathway.

Cadherin stabilizing function of p120

p120 functionally interacts with the entire cadherin complex by physically binding to and stabilizing classical cadherins. p120 binds to the juxtamembrane domain (JMD) of classical cadherins (e.g. E-, N-, and VE-cadherin) (Thoreson et al., 2000). Indeed, a triple-alanine mutation in the JMD leads to uncoupling of this interaction. Conversely, cadherins interact with armadillo repeats 1-5 of p120 (Ireton et al., 2002). Experiments with p120-deficient SW48 cells demonstrate that expression of mutant p120 lacking any one of these arm domains results in destabilized cadherins and reduced cell-cell adhesion. E-cadherin protein half-life is doubled when p120 is re-expressed, without changes to mRNA levels, indicating the mechanism of p120 control of cadherin involves protein stabilization (Ireton et al., 2002). Direct evidence for the cadherin stabilizing function comes from work utilizing shRNA depletion of p120 (Davis et al, 2003). Resident cadherin (e.g. E-cadherin in epithelial cells, VE-cadherin in endothelial cells) depletion is the direct result of p120 depletion, thus p120 acts as a rheostat of cadherin levels. This concept is strongly supported *in vivo* as most tissues in which p120 is experimentally ablated demonstrate significant reduction of junctional cadherins (Elia et al., 2006, Davis et al., 2006, Perez-Moreno et al., 2006, Bartlett et al., 2010, Oas et al., 2010, Smalley-Freed et al., 2010, Smalley-Freed et al., 2011, Marciano et al., 2011, Stairs et al., 2011, Chacon-Heszele et al., 2012). The mechanism likely involves inhibition of endocytosis, as p120 binding specifically prevents clathrin-dependent endocytosis of cadherins (Xiao et al., 2005) and masks a dileucine motif in E-cadherin that is necessary for this process (Miyashita and Ozawa, 2007). Recently, the crystal structure of p120 binding to the JMD of E-cadherin was resolved (Ishiyama et al., 2010), revealing the

molecular basis for uncoupling by the triple alanine mutations in E-cadherin (above) as well as individual amino acids in p120 that disrupt interaction with cadherins when mutated (i.e. K401, K444, N478).

Activity modulation of Rho family GTPases by p120

Rho family small GTPases function as binary switches that control important cellular processes, such as adhesion and motility. They are active or inactive when bound to GTP or GDP, respectively (reviewed in Jaffe and Hall, 2005). p120 modulates the activity of one such GTPase, RhoA, by a variety of non-mutually exclusive mechanisms. In NIH-3T3 fibroblasts, Rho inhibition leads to a striking branching phenotype that is phenocopied by overexpression of wildtype p120, but not by a 622-628 amino acid deletion mutant (Anastasiadis et al., 2000). p120 is proposed to bind directly to RhoA through p120 tyrosine 112 and/or amino acids 622-628 (Castano et al., 2007, Yanagisawa et al., 2008). These data suggest a model wherein p120 binds RhoA and keeps it in an inactive GDP-bound state (i.e. a guanosine dissociation inhibitor role). Alternatively, p120 binds p190 Rho GTPase activating protein (GAP), an inactivator of RhoA, thereby recruiting it to the cadherin complex to mediate local Rho inhibition (Wildenberg et al., 2006). While multiple mechanisms have been identified, p120 clearly inhibits RhoA *in vitro* and *in vivo* (Perez-Moreno et al., 2006). Interestingly, p120 may also promote local RhoA activity by its physical interaction with ROCK, a downstream effector of Rho (Smith et al., 2011, 2012). In turn, ROCK directly phosphorylates p120 on serine 268 (Smith et al., 2012- dissertation). While the significance of this observation remains

unclear, it indicates that this signaling is bidirectional and suggests a possible feedback loop.

p120 also modulates the activity of another Rho family member, Rac1, typically leading to its activation (Noren et al., 2000, Goodwin et al., 2003, Yanagisawa and Anastasiadis, 2006, Soto et al., 2008, Johnson et al., 2010). In MBA-MD-231 and Neu-transformed MCF10A cells, p120 activates Rac1 to promote cell growth and invasion (Soto et al., 2008, Johnston et al., 2010). However, in another transformed breast cell line, MCF7, p120 suppresses Rac1 activation (Soto et al., 2008). The authors propose that the difference in signaling between 231 and MCF7 cells is the cadherin present in the cell (i.e. cadherin-11 in 231 cells and E-cadherin in MCF7 cells) but other possibilities were not ruled out.

p120 is important for not only the control of Rac and Rho individually, but likely plays a critical role in the crosstalk between the two GTPases. Rac inhibits Rho through a well-characterized signaling pathway, known as the Bar-Sagi pathway (Nimnual et al., 2003). We have shown that this pathway is also dependent on p120 and p190RhoGAP (Wildenberg et al., 2006), and the interaction between p120 and p190RhoGAP requires Rac activation (Birukova et al., 2010). Based on these data, it has been suggested that p120 functions as a nexus for Rac1 and RhoA signaling.

p120 binding partner Kaiso and its connections to Wnt signaling

The p120 binding partner, Kaiso, is a member of the BTB/POZ-ZF (broad-complex, tramtrack, bric-a-brac/poxvirus and zinc finger) subfamily of transcription factors (Daniel and Reynolds, 1999). The interaction occurs via p120 ARM repeats 1-5

and the ZF domain of Kaiso (Daniel and Reynolds, 1999), but its significance is unclear. p120 has been proposed to sequester Kaiso in the cytoplasm, thus preventing its transcriptional action in the nucleus (reviewed in Daniel, 2007) and several variations on this theme have been suggested (Kim et al., 2004, Spring et al., 2005, Park et al., 2006, Del Valle-Perez et al., 2011). Kaiso-mediated repression of transcription is proposed to occur through bimodal DNA binding: either through a Kaiso consensus sequence or CpG methylation sites (Prokhortchouk et al., 2001, Daniel et al., 2002, Yoon et al., 2003). Moreover, Kaiso physically interacts with a number of proteins via its N-terminal POZ domain, including those involved with mediating transcription (Yoon et al., 2003, Defossez et al., 2005). For example, Kaiso directly binds nuclear receptor co-repressor as part of its mechanism of interacting with CpG islands (Yoon et al., 2003). Together, these data show that Kaiso generally functions in transcriptional repression.

Fellow adherens junction member, β-catenin, is a key player in Wnt signaling, and recent work suggests a role for p120 in this pathway as well. Wnt ligand activation of receptor complexes (e.g. Frizzled/LRP) leads to β-catenin stabilization, translocation into the nucleus, and interaction with transcription factors (e.g. TCFs) that activate β-catenin target genes (e.g. c-Myc)(reviewed in Gordon and Nusse, 2006). This pathway is essential for development and is often misregulated in cancer (reviewed in Nusse, 2005 and in Logan and Nusse, 2004).

p120 is suggested to modulate Wnt signaling via its binding partner Kaiso, which is reported in a *Xenopus laevis* model to directly repress both canonical and non-canonical Wnt signaling (Kim et al., 2004, Park et al., 2005, 2006). Kaiso is a repressor of non-canonical Wnt-11 and overexpression of p120 relieves this repression (Kim et al.,

2004). Similarly, Kaiso represses canonical Wnt target genes (e.g. siamois, cyclin-D1, and c-Myc) by binding to and sequestering TCF (Park et al., 2005). In a mechanism involving Frodo and Dishevelled, two Wnt signaling proteins, p120 prevents Kaiso from entering the nucleus and acting to suppress Wnt target genes (Park et al., 2006). However, the mechanism of Kaiso control of Wnt signaling, and Kaiso function during Xenopus gastrulation in general, has been called into question (Ruzov et al., 2009a, 2009b). Recent evidence suggests that rather than directly interacting with Wnt target gene promoters, Kaiso binds and sequesters TCF3 away from its promoter targets (Ruzov et al., 2009a, 2009b). In human cell lines, a similar model of mutual exclusion has been proposed wherein TCF4 or β-ctn binds to Kaiso to sequester them away from their respective promoter binding sites (Del Valle-Perez et al., 2011). Therefore, the exact mechanism of Kaiso-p120 interaction in Wnt signaling remains to be fully understood.

p120 is also proposed to participate in Wnt signaling by Kaiso-independent mechanisms. In sw480 colon carcinoma cells upon Wnt activation, p120 binds to and is phosphorylated by casein-kinase 1ε at serines 268/269. Disruption of this interaction by p120 depletion prevents numerous downstream aspects of Wnt signaling suggesting that p120 is required for Wnt signaling (Casagolda et al., 2010). While preliminary, these findings suggest a role for p120 in the initial steps of Wnt pathway activation.

p120 versus its family members

p120 family members are proposed to execute functions redundant to p120. For example, all four core family members, p120, ARVCF, p0071 and δ-ctn, bind and stabilize cadherins *in vitro* (Thoreson et al., 2000, Ireton et al., 2002, Davis et al., 2003,

Hatzfeld et al., 2003, Calkins et al., 2003, Setzer et al., 2004). If p120 is depleted in A431 cells using shRNA, expression of either ARVCF or δ-ctn can rescue cadherin stability (Davis et al., 2003). However, the scenario *in vivo* may be more complicated. While redundancy has been proposed in some organ systems to compensate for p120 loss (Perez-Moreno et al., 2006), the issue of redundancy has been complicated by a lack of good antibodies. High quality antibodies that are compatible with IHC have been recently developed and are beginning to clarify the specific expression patterns and contribution of each family member *in vivo* (Walter et al., 2008, 2009, 2010).

Evidence is mounting that p120 and its core family members perform non-redundant functions. The global p120 null mouse is embryonic lethal suggesting that p120 is essential for mammalian life, and family members do not overcome its loss (Davis et al., 2006). On the flip side, ARVCF, p0071, and δ-ctn perform functions and are involved in disease states in ways not attributed to p120 (Hatzfeld and Nachtsheim 1996, Sirotkin et al., 1997, Medina et al., 2000, Izawa et al., 2002, Jaulin-Bastard et al., 2002, Laura et al., 2002, Martinez et al., 2003, Kausalyn et al., 2004, Wolf et al., 2006). Most notably, the PDZ domain in the C-terminus of these family members confers physical and functional interaction with other PDZ domain containing proteins. ARVCF binds to tight junction protein ZO-1 (Kausalyn et al., 2004) and p0071, δ-ctn, and ARVCF bind erbin, a polarity protein (Izawa et al., 2002, Jaulin-Bastard et al., 2002, Laura et al., 2002). Thus, the presence of their PDZ domain links them to polarity complexes and tight junctions. This difference in p120, is hypothesized to allow p120 to have evolved unique and specified functions (reviewed in McCrea and Park, 2006,

Carnahan et al., 2010). However, the significance of this difference, as well as others (e.g. splicing in only p120 and ARVCF), has yet to be elucidated.

p120 Ablation in Model Organisms

Ablation studies in model organisms provide important perspective into the roles of p120 in different species and organ systems. *In vitro*, p120 depletion causes cell-cell adhesion defects and alterations to key signaling nodes (e.g. Rho family GTPases). Similar effects are observed *in vivo*, but the physiological effects are more complex (Table 1).

Surprisingly, the effects of p120 ablation in invertebrates are relatively mild, despite the presence of only one p120 family member (Table 1). For example, loss of Jac-1, the closest homologues of p120 in *Caenorhabditis elegans*, does not affect viability or generate a discernable phenotype. Likewise, the only p120 homologue present in *Drosophila* is not essential for life (Myster et al., 2003, Magie et al., 2002). However, ablation of these homologues exacerbates phenotypes driven by mutations in other adherens junction proteins suggesting that, despite its overall non-essential function, it serves a function in invertebrates (e.g. positive regulation of adhesion) (Pettitt et al., 2003, Myster et al., 2003). Evolutionary analysis of p120 and its family members suggests that the invertebrate p120 genes are functionally analogous to δ-catenin, rather than the gene duplication product that is vertebrate p120 (Carnahan et al., 2010). Therefore, while the minor consequences of invertebrate p120 loss seem unexpected, these data likely reflect specific evolution-driven divergence of p120 structure and function only acquired by vertebrates.

Organism	Member	Tissue	Phenotype	Citation
Mus	p120	Global null	Embryonic lethal	unpublished
		Salivary gland	Blocked development of acini, contorted ducts, intraepithelial neoplasia	Davis et al., 2006
		Skin	Chronic inflammation, hyperplasia, no barriar defects	Perez-Moreno et al., 2006, 2008
		Dorsal forebrain	Reduced spine density and synapse formation on dendrites	Elia et al., 2006
		Schwann cells	Lack of Schmidt–Lanterman incisures and thinner cells, reduced myelination	Perrin-Tricaud et al., 2007
		GI tract	Terminal bleeding, inflammation, neutrophil recruitment, adenoma formation	Smalley-Freed et al., 2010, 2011
		Teeth	Ameloblast detachment, reduced mineralization of enamal, sinusoid formation	Bartlett et al., 2010
		Vasculature	Lethal e11.5, decreased pericyte recruitment, angiogenic remodeling defects, hemorrhaging	Oas et al., 2010
		Esophagus	Inflammation and invasive squamous cell cancer	Stairs et al., 2011
		Kidney	Hypoplastic cystic kidneys, disrupted glomerulogenesis	Marciano et al., 2011
		Mammary gland	Delayed pubertal development, disrupted TEBs	ABR unpublished
		Prostate	No phenotype detected in aged adults	ABR unpublished
		Cochlea	Convergent extension defects, crosstalk with planar cell polarity genes	Chacon-Heszele et al., 2012
	δ-ctn	Global null	Disrupted cognitive function, hippocampal synaptic plasticity defects	Israely et al., 2004
		Global null	Decreased motility of endothelial cells, reduced pathological angiogenesis	Debusk et al., 2010
		Global null	Increased spine density and excitatory synaptic activity	Arikkath et al., 2009
Xenopus	xp120	Ectodermal structures	Reduced eye formation and craniofacial cartilidge and head width	Ciesiolka et al., 2004
		4-cell embryo	Gastrulation defects, inhibition of convergent extension	Fang et al., 2004
	xARVCF	4-cell embryo	Gastrulation defects, inhibition of convergent extension	Fang et al., 2004
Caenorhabditis	Jac-1	Global null	No embryonic or post-embryonic defects; potentiates a hmp1 hypomorphic mutation	Pettitt et al., 2003
Drosophila	p120/δ-ctn	Global null	Non-essential for life, fertility, or AJs; potentiates mutations (shg, arm); head involution defects	Myster et al., 2003, Magie et al., 2002

Table 1: Effects of p120 ablation in model organisms

In contrast to invertebrates, global loss of p120 in mice is embryonic lethal, and phenotypes associated with tissue-specific ablation are quite severe (Table 1). In fact, tissue-specific knockout of p120 in vasculature, salivary gland, or the GI tract leads to death (Oas et al., 2010, Davis et al., 2006, Smalley-freed et al., 2010). Mice die *in utero* due to p120-ablation in the vasculature and subsequent hemorrhaging (Oas et al., 2010). Tissue-specific ablation of p120 in the salivary gland prevents acini development and ultimately leads to perinatal death (Davis et al., 2006). In the GI tract, p120 loss induces severe deterioration of the gut epithelium and terminal bleeding (Smalley-Freed et al., 2010). While ablation of p120 in the teeth and skin does not induce death, these mice exhibit considerable wasting, tooth erosion, and epidermal hyperplasia (Bartlett et al., 2010, Perez-Moreno et al., 2006). A notable exception to the severity of p120 ablation is in the adult prostate gland, which does not demonstrate a phenotype, despite induction of extensive p120 ablation (ABR unpublished results). In general, the absence of p120 leads to cell-cell adhesion defects and disruption of tissue homeostasis, however tissue-specific differences exist.

Numerous factors contribute to the tissue-specific phenotypes caused by p120 ablation. For example, tissue structure plays a role in the outcome of p120 loss. Simple and bilayered epithelia exhibit significant adhesion defects in the absence of p120, whereas stratified epithelia without p120 are protected from cell sloughing. Even within a given tissue, different structural constraints influence the outcome of p120 ablation. In the mammary gland, ducts without p120 are partially malformed but exhibit some degree of cell-cell adhesion. In contrast, p120 null cells localized to the terminal end buds are

wholly dissociated from the functional unit likely stemming from an inability of the null cells to participate in the dynamic rearrangements of this structure (Chapter 3). In the GI tract, cell sloughing is more extensive once p120 null cells reach the tips of the villi where sheer stress is more prominent, further reflecting the role of tissue structure on the fate of cells lacking p120 (Smalley-Freed et al., 2010). The degree of environmental exposure is also likely to mediate tissue specific effects. In the GI tract, destruction induced by p120 ablation coincides with the transition from a relatively sterile environment to colonization by the gut flora. Prior to this transition, the epithelium remains relatively intact suggesting that exposure to external factors like the bacteria ridden GI lumen contribute to the consequence of p120 loss. Finally, the timing and context of the knockout may explain tissue specific differences. p120 ablation is catastrophic to the developing salivary and mammary glands, whereas loss in the adult prostate does not generate a detectable phenotype (Davis et al., 2006, chapter 3). These findings suggest that p120 loss during key morphogenetic processes is highly detrimental while loss in a more stable adult tissue is better tolerated.

p120 stabilizes endogenous classical cadherins in all cell and tissue types examined to date, however the subsequent effects on cell-cell adhesion are variable. The effect of p120 ablation is specific to classical cadherins and the adherens junction, as other junctions (e.g. desmosomes and tight junctions) are not significantly affected by p120 depletion (Smalley-freed et al., 2020, Davis et al., 2006). Ablation of p120 in the epidermis, GI tract, or salivary gland reduces cadherin levels by approximately 50%, whereas in the prostate and mammary glands, cadherins are near absent (Smalley-freed et al., 2006, Perez-Moreno et al., 2006, Davis et al., 2006, Chapter 3). Despite similar

reduction in cadherins, the p120-ablated gut suffers from adhesion and barrier defects, whereas the skin maintains barrier function. Furthermore, near complete downregulation of cadherins in the absence of p120 appears to be responsible for major adhesion defects in the mammary gland but has no effect on cell-cell attachment in the prostate. Thus, the degree of cadherin destabilization in the absence of p120 *in vivo* and its consequences are variable.

The contributions of cadherin-dependent and -independent functions of p120 *in vivo* are beginning to be elucidated. In many organ systems, such as the mammary gland and the vasculature, the loss of cadherin appears to be the dominant driver of p120 ablation-induced phenotypes (Davis et al., 2006, Oas et al., 2010, Bartlett et al., 2010, Chapter 3). However, cadherin-independent functions also contribute to p120 ablation-induced phenotypes. For example, loss of p120 in the epidermis relieves inhibition of Rho, consistent with *in vitro* data, and causes NFkB activation and inflammation (Perez-Moreno et al., 2006). Activation of Rho is also attributed to phenotypes in other p120 knockout tissues (Stairs et al., 2011, Elia et al., 2006). As more sophisticated methods are employed to study p120 *in vivo*, the role of cadherin-independent functions of p120 (e.g. modulation of binding partners) will be addressed.

Despite extensive pathology literature demonstrating a correlation between p120 loss and tumor progression (reviewed in Thoreson and Reynolds, 2002), only recent studies in mice have provided definitive evidence for p120 as a *bona fide* tumor suppressor. In the GI tract and esophagus, p120 knockout induces the formation of adenomas and invasive cancer, respectively (Smalley-freed et al. 2011, Stairs et al. 2011). Ablation of p120 also induces precancerous intraepithelial neoplasia and hyperplasia in

the salivary gland and skin, respectively (Davis et al., 2006, Perez-Moreno et al., 2006). *In vitro* studies using a tumorigenesis assay, anchorage-independent growth in soft agar, demonstrate that p120 is required for tumor formation driven by oncogenic Src or Rac1 (Dohn et al., 2009). These findings may be relevant to some *in vivo* systems since adenomas formed in p120-ablated GI tracts retain p120 (Smalley-Freed et al., 2011). On the other hand, tumors lacking p120 form in the esophagus, oral cavity, and squamous forestomach suggesting that there are organ specific requirements for p120 during tumor initiation (Stairs et al., 2011).

Inflammation, albeit derived by different mechanisms, is common to all these tumor models (Perez-Moreno et al., 2006, Smalley-Freed et al., 2010, Stairs et al., 2011). Cell autonomous activation of NFkB induces infiltration of immune cells in the skin and esophagus, whereas this inflammatory response may be the secondary effect of a barrier defect in the GI tract. Interestingly, knockout epithelia (e.g. kidney and mammary) that do not exhibit an immune cell infiltration, also not do not develop tumors (Marcinao et al., 2011, chapter 3). Furthermore, the common feature of tumor formation in p120-depleted mice is an inflammatory response suggesting that stromal events may contribute to tumor initiation or progression. Indeed, a causative role for this immune cell response was demonstrated with p120 ablation in the epidermis and esophagus (Perez-Moreno et al., 2006, 2008, Stairs et al., 2011). Knockout mice treated with dexamethasone, an immune suppressant, exhibit blockade of neoplasia formation or progression to invasion in the epidermis and esophagus, respectively. Thus, p120 loss may induce tumor formation by regulating the microenvironment, specifically the immune system.

Collectively, these data highlight the importance of p120 in maintaining tissue homeostasis and in developmental processes. However, dynamic regulation of p120, not simply its presence, is likely necessary to adapt to a wide variety of tissue-specific scenarios. The precise role of p120 levels in the context of mouse models of diseases, such as cancer, remains to be addressed.

Importance and regulation of p120 levels

A wealth of recent evidence indicates that p120 levels are critical for proper tissue development and homeostasis (Figure 2). It is increasingly clear that precise control of p120 levels is multifaceted with regulation occurring at many stages of p120 production (Figure 3). Furthermore, disease states, such as cancer, manipulate p120 status during their initiation, maintenance, and progression.

In vitro, altered p120 expression disrupts normal cellular function (Figure 2). For example, over-expression of p120 in NIH-3T3 cells induces a striking branching phenotype, while p120 depletion induces extensive actin stress fiber formation (Anastasiadis et al., 2000, Wildenberg et al., 2006). Similarly in epithelial cells, over-expression or knockdown of p120 has dramatic effects on morphology and actin distribution (Aho et al. 2002, Boguslavsky et al. 2007). Clearly, proper p120 levels, while context dependent, are directly responsible for maintenance of the normal cellular state.

Dysregulation of p120 expression in a variety of cancer types is well documented (reviewed in Thoreson and Reynolds, 2002, van Hengel and van Roy, 2007). For example, modulation of p120 levels in often associated with transition to or progression

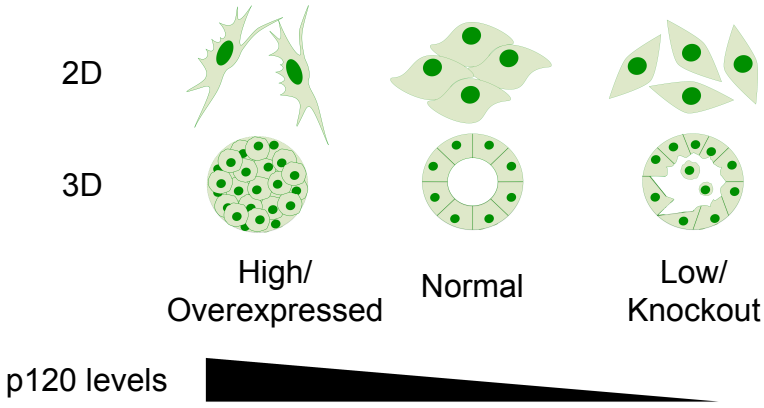


Figure 2. The importance of p120 levels
A schematic of the consequences of p120 expression levels in 2-D and 3-D. Normal p120 levels support cell-cell adhesion and tissue architecture (middle), over-expression leads to cell arborization on plastic and emboli formation in some cases (left), low p120 levels lead to a loss of cell-cell adhesion and a breakdown of tissue architecture (right).

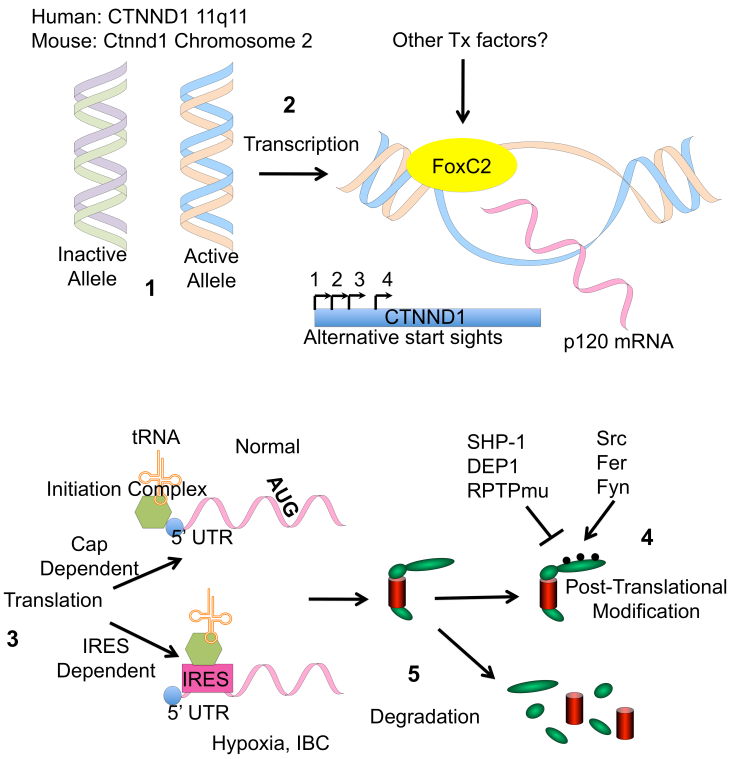


Figure 3. The life cycle of p120

A schematic of the life-cycle of p120. One the genomic level, p120 expression is controlled by monoallelic inactivation (1). p120 transcription is mediated by FoxC2 and possibly other transcription factors (2). Multiple alternative start sites regulate the isoform of p120 to be expressed. Translation of p120 mRNA occurs via cap-dependent translation under normal conditions, or by IRES-dependent mechanisms under stress conditions (3). At the post-translational stage, p120 is extensively modified by a number of kinases and phosphatases (4), while protein stability is regulated by degradation mechanisms (5).

of breast cancer. 10% of ductal breast cancers exhibit regions of complete loss, whereas p120 is relegated to the cytoplasm in most lobular breast cancers due to E-cadherin loss (Dillon et al., 1998, Nakopoulou et al., 2002, Sarrio et al., 2004). On the other hand, upregulated p120 is required for inflammatory breast cancer emboli formation suggesting that tumors differently alter p120 status to accommodate unique environmental requirements (Silvera et al., 2009). Interestingly, p120 is hijacked by *N. meningitidis* to form ectopic early junction-like domains with the endothelial cells, allowing the bacterium to cross the blood brain barrier (Coureuli et al. 2009). Given the importance of cellular amounts of p120, regulation of p120 at a variety of stages is likely to be advantageous.

Monoallelic inactivation

The p120 gene is located on human chromosome 11q11 (CTNND1) and mouse chromosome 2 (ctnnd1) (Reynolds et al., 1996, Mo and Reynolds, 1996, and Keirsebilck et al., 1998). Interestingly, a recent study using asynchronous DNA replication identified p120 as subject to random monoallelic inactivation, a process whereby a single allele of a gene is silenced by epigenetic means (Gimelbrant et al., 2005). This effect on p120 appears to be widespread as fibroblasts, B-cells, and embryonic stem cells all exhibit inactivation events (Gimelbrant et al., 2005, Dutta et al., 2009). Monoallelic inactivation of p120 leads to decreased protein levels suggesting that the active allele does not compensate for inactivation of the other. However, this does not rule out the possibility that alterations to post transcriptional processes can compensate for total protein levels.

This finding not only adds to the understanding of p120 level regulation but also presents a potentially useful model for studying drivers and modifiers of monoallelic inactivation.

Transcription, splicing, and translation

There is a high degree of complexity to p120 at the mRNA transcript level (Figure 4). In mammals, four alternative translational start sites as well as three alternatively spliced exons (i.e. A, B, and C) in the 3' region suggest a potential 32 different isoforms (Mo and Reynolds et al. 1996, Keirseblick et al. 1998). Isoform 1 is the predominant p120 isoform in mesenchymal cells (e.g. fibroblasts) while epithelial cells predominantly express isoform 3 (Mo and Reynolds, 1996). However, this is likely an oversimplification as multiple isoforms of p120 are expressed in cancer cell lines and tumor samples (Sarrio et al., 2004).

Evidence for distinct roles of p120 isoforms is emerging. Interestingly, a switch from p120 isoform 3 to isoform 1 is observed concomitant with cadherin switching and/or loss during epithelial-to-mesenchymal transition (EMT) (Sarrio et al., 2004, Slorach et al., 2011). This finding likely relates to invasive potential as isoform 3A blocks where as 1A facilitates invasion in 3D cultures (Slorach et al., 2011). This is echoed in NIH-3T3 and MDA-MB-231 cells where expression of isoform 1A, but not 4A, inhibits RhoA and ultimately promotes invasion (Yanagisawa et al., 2008). In contrast, in lung cancer cells, isoform 1A inhibits invasion and 3A does not alter invasion but rather inhibits cell-cycle progression (Liu et al., 2009). While there are notably cell specific differences, it is clear that p120 isoforms are not equivalent in their intracellular functions.

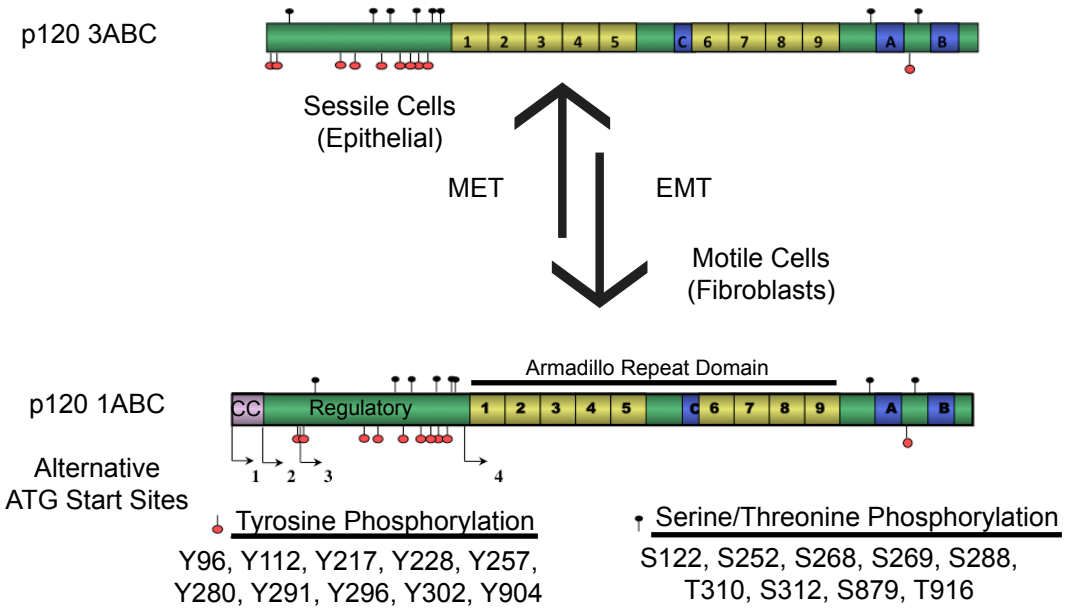


Figure 4. p120 isoforms and phosphorylation

The schematic of full length p120 isoform 1ABC and shorter isoform 3ABC are shown. The structural elements are as follows: coiled-coiled domain (pink), regulatory domain (green), armadillo repeats (yellow), exons (blue). Note the 4 isoforms formed by alternative ATG start sites. Tyrosine and serine/threonine sites are depicted as red and black circles, respectively. Cells undergoing EMT switch from isoform 3 to 1 and those undergoing MET from 1 to 3.

The presence of C-terminal alternatively spliced exons in p120 adds to transcript complexity but the advantage and/or function of these events is largely unknown. Most ectopic expression studies have used p120 cDNAs containing only exon A. While this is the most common exon found in transcripts (Keirseblick et al., 1998), such experimental designs necessarily prevent the understanding of other exons. Exon B contains a functional nuclear export signal and p120 phosphorylation at S879 occurs only in the presence of exon A (van Hengel et al., 1999, Vaughan et al., 2007).

Novel mechanisms of p120 transcription, splicing, and translation are beginning to be identified. The first, and currently only, published study of the p120 promoter identified putative *cis*-acting transcriptional elements, including FOXC2, by *in silico* analysis (Mortazavi et al., 2010). RNAi-mediated depletion of FOXC2 increases p120 mRNA transcripts and protein levels, and ChIP analysis indicates direct binding of FOXC2 to the endogenous p120 promoter, thus confirming its role as a transcriptional repressor of p120. Interestingly, FOXC2 also promotes EMT, which, as noted above, is associated with p120 isoform 1 to 3 switch (Mani et al., 2007, Slorach et al., 2011). Conversely, recent studies identify splicing factors, ESRP1 and ESPR2, as keepers of the epithelial phenotype. When present, these factors promote splicing of a group of transcripts, including p120, to generate the epithelial form of that protein (e.g. p120 isoform 3) (Warzecha et al. 2009, 2010).

Adding to the complexity, p120 transcripts contain internal ribosomal entry sites (IRES) in their 5' UTRs that are utilized for translation during cellular stress, nutrient deprivation, or hypoxia (reviewed in Silvera et al., 2010). Using a model of inflammatory breast cancer (IBC), Silvera and colleagues demonstrate that up-regulation

of p120 in emboli is necessary for increased E-cadherin stability, which provides a survival signal crucial for the etiology of IBC (Silvera et al., 2010). p120 is up-regulated by increased expression of the translation initiation factor eIF4G, which drives a switch from cap-dependent to IRES-mediated, cap-independent translation of p120 mRNA (Silvera et al., 2009). p120 over-expression is necessary for the development of IBC emboli suggesting that p120 does not act as a classical tumor suppressor in all contexts. Collectively, this work provides the first insights into control of p120 at the levels of transcription, splicing, and translation while demonstrating the intricacy of p120 regulation that continues to emerge.

Post-translational modifications and protein stability

p120 is extensively phosphorlyated but the role of this modification is not well understood (reviewed in Reynolds and Rocniak-Ferguson, 2004). Phosphorylation events on a multitude of tyrosine, serine, and threonine residues of p120 have been mapped and are shown in figure 4 (Mariner et al., Xia et al., Luo et al., 2008, Casagolda et al., 2010). p120 is known to directly bind a number of kinases and phosphatases (Keilhack et al., 2000, Holsinger et al., 2002, Piedra et al., 2003, Castano et al., 2007, Casagolda et al., 2010, Wong et al., 2010, Smith et al., 2012). Most of the phosphorylation sites of p120 are housed in the N-terminal regulatory domain. This domain is known to coordinate receptor tyrosine kinase signaling regulation of motility, thus intimating a role for phosphorylation (Cozzolino et al., 2003). Furthermore, specific phosphorylation events in this domain have been shown to affect cell-cell adhesion, although these findings are context and cell line specific (Kim and Wong, 1995, Piedra et

al., 2003, Xu et al., 2004, Calautti et al., 1998, 2002). Interestingly, no defects in cell-cell adhesion or alteration to the actin cytoskeleton are present when either all the serine/threonine or tyrosine sites are mutated to be phosphorylation-deficient (Xia et al., 2006, Mariner et al., 2001).

The degradation pathways that modulate p120 levels are beginning to be identified. As with β-ctn, p120 levels may be modulated by Wnt signaling and the proteasome (Hong et al., 2010). Work in *Xenopus laevis* suggest GSK3β and Axin facilitate a cascade of events, including phosphorylation and ubiquitylation of p120, which ultimately lead to proteasome-mediated degradation of p120 (Hong et al., 2010). Furthermore, Frodo, part of the Wnt pathway, binds p120, resulting in its stabilization (Park et al., 2006). Overall, Wnt signaling may control the levels of more than just β-catenin (Park et al., 2006, Oh et al., 2009, Hong et al., 2010).

The protease calpain is emerging as a key player in p120 degradation. Culture of neuroblastoma cells under hypoxic and hypoglycemic conditions rapidly induces activation of calpain and reduction of p120 protein (Ohno et al., 2007). In human bronchial cells and mouse lungs, induction of inflammation by lipopolysaccharide (LPS) treatment results in rapid loss of p120 protein without changes to p120 mRNA, suggesting that LPS induces degradation of p120 (Wang et al., 2010, Wang et al., 2011). Interestingly, tyrosine phosphorylation may play a role in LPS-induced degradation (Gong et al., 2008). Since LPS is known to induce calpain activity, it is possible that calpain mediates the effect on p120 (Li…Peng et al., 2009). In another scenario, exogenous expression of δ-catenin induces redistribution of p120 to the cytoplasm where it may be degraded (Yang et al., 2010). This effect can be subtly reduced by a calpain

inhibitor, which also blocks the proteasome at these amounts, suggesting that either factor may be contributing. Whether these effects are mediated by direct interaction with and cleavage by calpain, as well as the role the proteasome in this process, has yet to be fully addressed.

p120 effectors in mammary development

General mammary development

The life cycle of the mouse mammary gland involves a complex orchestration of events and processes, including proliferation, apoptosis, differentiation, and motility. The mammary gland begins development at approximately embryonic day 10.5, starts sprouting into the local mesenchyme at embryonic day 16, and continues isometric growth with elongation and branching until puberty begins at 3 weeks of age. This rudimentary bud responds to the hormonal milieu of puberty by generating terminal end buds (TEBs), multilayered structures composed of body and cap cells. The TEBs invade the mammary fat pad using collective migration and provide the material for subtending ducts. By 8-10 weeks, the TEBs have traversed the fat pad leaving in their wake an extensively branched mammary gland. The fully developed gland contains ducts and acini lined with luminal epithelial and loosely encapsulated by basal myoepithelial cells. Proliferation, differentiation, and tissue remodeling during pregnancy and lactation are utilized to form alveolar clusters of milk producing cells. Upon removal of pups, the mammary gland is reverted to a virgin-like state through involution, a process involving apoptosis and further tissue remodeling (reviewed in Cardiff and Wellings, 1999, in Hennighausen and Robinson, 2001, in Hennighausen and Robinson, 2005). The dynamic

life cycle of the mammary gland is ideal for studying the processes involved in normal development and tissue homeostasis, as well as the processes that malfunction in cancer. Furthermore, most of the development of this non-essential organ occurs after birth allowing for ease of manipulation.

Cadherins and mammary development

Cadherins are key players in mammary morphogenesis (reviewed in Knudsen and Wheelock, 2005 and in Andrews et al., 2012). They are differentially expressed in the mammary gland with E- and P-cadherin being the most prominent version in body and cap cells of the TEB, respectively (Daniel et al., 1995). This is echoed in the developed gland where E-cadherin is expressed in the luminal epithelial cells of ducts and acini, while P-cadherin predominates in the myoepithelial layers. Addition of functional blocking antibodies against E- and P-cadherin to developing glands completely abolishes cell-cell adhesion and decreases cell proliferation of the body and cap cells, respectively. While these striking results indicate an essential role for both cadherins during pubertal development, interestingly, genetic E-cadherin ablation models give far more subtle outcomes. Ablation of E-cadherin using a variety of different Cre-drivers leads to no effect on pubertal development, however null cells are also undetectable at this time point (Boussadia et al., 2002, Derksen et al., 2006, Derksen et al., 2011). Some authors merely pass over this subtle finding to focus on the more pronounced findings at later stages (Boussadia et al., 2002). Others conclude that E-cadherin ablation is not tolerated during development but do not explore it further (Derksen et al., 2006, Derksen et al., 2011). Without further examination of these mice, it is difficult to make solid conclusions about

the pubertal phase, however these data likely reflect selective pressures stemming from cell adhesion defects first observed in blocking antibody experiments.

Cadherin loss and misexpression also affect the lactation phase of mammary gland function. During lactation, E-cadherin acts as a survival factor since knockout mice demonstrate early onset involution (Boussadia et al., 2002). However, expression of the cytoplasmic domain of E-cadherin during lactation, which has a dominant-negative effect on adhesion, gives rise to the opposite effect: precocious alveolar formation (Delmas et al., 1999). These findings, while seemingly contradictory, may highlight functions of the cytoplasmic domain (e.g. p120 binding and sequestration). P-cadherin is also important for lactation as P-cadherin null females exhibit premature alveolar differentiation and develop hyperplasia when aged (Radice et al., 1997). Finally, ectopic N-cadherin under the endogenous E-cadherin promoter exacerbates the precocious involution induced by E-cadherin ablation alone (Kotb et al., 2011). Collectively, these studies suggest a vital role of cadherins during the mammary morphogenesis associated with lactation.

Rho signaling and mammary development

Dynamic control of RhoA activity levels is essential for mammary gland development. p190B RhoGTPase activating protein (GAP), a negative regulator of RhoA, is required for mammary gland morphogenesis and embryonic mammary bud formation (Chakravarty et al., 2003, Heckman et al., 2007). Overexpression of p190B Rho GAP results in increased branching, delayed ductal elongation, and tissue disorganization (Vargo-gogola et al., 2006). p190A RhoGAP is also required for TEB

architecture and ductal outgrowth during puberty (Heckman-Stoddard et al., 2011). Since both p190A and B RhoGAPs act to inhibit Rho activity, experiments with their absence suggest that hyperactive Rho signaling is incompatible with pubertal development. Interestingly, inhibition of Rho also blocks the processes necessary for branching morphogenesis (Ewald et al., 2008). In an organotypic model, ROCK, a downstream RhoA target, is required for restoration of the epithelial bilayer and its inhibition impairs proper luminal and myoepithelial cell motility. In this same system, Rac1 activation is required for duct initiation. Collectively, these studies suggest that a proper balance and temporal regulation of Rho and Rac are necessary for mammary morphogenesis.

p120 and its effectors in breast cancer

The American Cancer Society estimates that 229,060 new cases and 39,920 deaths due to breast cancer will occur in 2012 alone (ACS, Facts and Figures, 2012). Worldwide, breast cancer is the most common malignancy among women, resulting in approximately half a million deaths a year due to metastatic disease. Progression to metastasis, the main cause of mortality in patients, is achieved when cancer cells are capable of performing all steps of the metastatic cascade: local invasion; intravasation; survival in the circulation; extravasation; and colonization of the secondary site (Reviewed in Valastyan and Weinberg, 2011). These acquired features of cancer cells are rooted in changes to cell-cell adhesion and survival and likely involve p120 and its effectors.

p120 in breast cancer

p120 is often dysregulated in human cancer (reviewed in Thoreson and Reynolds, 2002). Specifically in breast cancer, abnormal p120 levels and/or localization are commonly observed (i.e. range 69%-91% of cases) (Dillon et al., 1998, Nakopoulou et al., 2002, Sarrio et al., 2004, Paredes et al., 2008, Talvinen et al., 2010). In ductal carcinoma, p120 downregulation is reproducibly observed in at least 50% of samples and regions of p120 loss are observed in approximately 10% of cases. In a data set consisting of mostly invasive ductal cases, low p120 expression correlates with poor patient outcome (Talvinen et al., 2010). These findings are mimicked in the MMTV-Polyoma Middle T (PyMT) mouse model of breast cancer wherein p120 is one of the most downregulated genes in the transition from adenoma to carcinoma (Kouros-Mehr et al., 2008). However, the relevance of these observations to ductal breast cancer progression has not been directly tested.

In lobular carcinoma, p120 is often cytoplasmic, reflecting the commonly occurring downregulation/silencing of E-cadherin that typifies this kind of breast cancer (Sarrio et al., 2004). In fact, this mislocalization is so prevalent that it provides clinicians with a diagnostic tool to distinguish lobular from ductal carcinomas (Dabbs et al., 2007). Cytoplasmic localization of p120 is correlated with decreased overall survival (Paredes et al., 2008). Recently, the contribution of this cytoplasmic pool to disease progression has been directly tested in a mouse model of lobular breast cancer (Derksen et al., 2006, Schackmann et al., 2011). p120 is required for *in vivo* growth and metastasis because it facilitates resistance to detachment induced death, anoikis, in this model (Schackmann et

al., 2011). Thus, the cytoplasmic pool of p120 observed in the pathology literature is truly part of the etiology of lobular breast cancer.

In contrast to ductal carcinoma, p120 is upregulated in Her2-driven and inflammatory breast cancer (IBC) (Landis et al., 2005, Silvera et al., 2009, Johnson et al., 2010). Upregulation of p120 mRNA is observed in the MMTV-neu mouse model and these findings are recapitulated when MCF10A cells are transformed with Neu (Landis et al., 2005, and Johnson et al., 2010). p120 upregulation is proposed to activate Rac1 and Cdc42 downstream of src activation by Her2 and, ultimately, facilitate metastasis (Johnson et al., 2010). As described above, IRES-dependent translation leads to upregulation of p120 in IBC (Silvera et al., 2009). Survival of IBC emboli in the lymphatic system requires upregulation of p120 (and subsequent stabilization of E-cadherin) that is the direct result of IRES-dependent translation of p120 mRNA. These latest findings specifically contribute to the understanding of two specific types of breast cancer, however the mechanisms contained within these studies may be more globally applicable. Clearly, p120 levels are differentially altered in specific types of breast cancer and the importance of these levels is under continued investigation.

p120 effectors in breast cancer

E-cadherin is a widely accepted tumor and metastasis suppressor (Perl et al., 1998, Derksen et al., 2006, reviewed in Birchmeier and Behrens 1994, in Jeanes et al., 2008). In lobular carcinoma, E-cadherin loss occurs in the vast majority of cases and is thought to occur through mutation or epigenetic silencing of CDH1 combined with loss of heterozygosity (Moll et al., 1993, Gamallo et al., 1993, De Leeuw et al., 1997, Vos et

al., 1997, Berx et al., 1998, Lehr et al., 2000, Palacios et al., 2003). In a breast cancer mouse model, E-cadherin loss is a direct mediator of tumor formation and causes metastatic disease (Derksen et al., 2006). Thus, E-cadherin, in this setting, is indeed a tumor and metastasis suppressor. However, in ductal carcinomas, E-cadherin expression is maintained or modulated rather than silenced (Moll et al., 1993, Rasbridge et al., 1993, Dillon et al., 1998, Nakopoulou et al., 2002). Generally, E-cadherin reduction is correlated with metastasis and poor patient outcome, however many studies did not observe this correlation and some even suggest the opposite (Lipponen et al., 1994, Siitonen et al., 1996, Maguire et al., 1997, Charpin et al., 1998, Tan et al., 1999, Asgeirsson et al., 2000, Heimann et al., 2000).

These discrepancies in the pathology literature carry over into the experimental world, where the necessity and function of E-cadherin during metastasis of breast cancer cells is also inconsistent. Ectopic E-cadherin expression in E-cadherin-null breast cancer cells prevents invasion and metastasis (Vleminckx et al., 1991, Mbalaviele et al., 1996, Meiners et al., 1998). Similarly, depletion of twist, a transcriptional repressor of E-cadherin, in the highly metastatic 4T1 mouse mammary carcinoma cell line prevents pulmonary metastasis (Yang et al., 2004). Taken together, these studies indicate that E-cadherin is metastasis suppressor. However, there is also strong evidence for a requirement for E-cadherin during metastases (Lou et al., 2008, Dykxhoorn et al., 2009, Silvera et al., 2009, Korpal et al, 2011). Of the members of a commonly used isogenic panel of mammary carcinoma cell lines, only the line that highly expresses E-cadherin (i.e. 4T1 cells) is fully competent at metastasis (Lou et al., 2008). Another member of this panel, 4T07 cells, which are typically unable to colonize the lung, become

colonization-competent when forced to express miR200, an inhibitor of the transcriptional repressor of E-cadherin, Zeb2 (Lou et al., 2008, Dykxhoorn et al., 2009). Thus, E-cadherin expression also conveys metastatic capacity. Furthermore, recent attention has been paid to the role of collective invasion, a process that is cadherin-dependent, on its contribution to cancer metastasis (Giampieri et al., 2009, reviewed in Friedl and Wolf, 2003, in Friedl, 2004, in Sahai, 2005).

These seemingly contradictory findings may reflect the need for cellular plasticity in ductal carcinoma to achieve metastases, rather than just the presence or absence of E-cadherin. By being adaptable and plastic, cancer cells are able to overcome the unique challenges presented to them at each stage of the metastatic cascade: local invasion; intravasation; survival in the circulation; extravasation; and colonization (reviewed in Friedl, 2004, in Valastyan and Weinberg, 2011). While there are a variety of methods by which cancer cells invade the surrounding stroma (reviewed in Sahai, 2005, in Friedl and Wolf, 2003), one of the most discussed is epithelial-to-mesenchymal transition (EMT), whereby a carcinoma cell takes on the attributes of a mesenchymal cell (reviewed in Kalluri and Weinberg, 2009). While the specific molecular changes associated with EMT are multifaceted and often debated, the gold standard alteration during EMT is reduction of E-cadherin, and this is often accompanied by switching to expression of mesenchymal cadherins (Wheelock et al., 2008). EMT confers a variety of abilities to cancer cells including increased motility and invasion, stemness, resistance to chemotherapy, and immune evasion (reviewed in Micalizzi et al., 2010). However, the reverse process, mesenchymal-to-epithelial transition (MET), may be required to complete the entire metastatic cascade (reviewed in Hugo et al., 2007). MET provides

cancer cells with key signals which may be critical for overcoming anoikis, survival in the circulation, and growth at the secondary site (Reddy et al., 2005, Kang et al., 2007, Wells et al., 2008, Chao et al., 2010, Chao et al., 2012). Thus, while E-cadherin depletion during EMT may facilitate early stages of the metastatic cascade, E-cadherin expression during MET may support later steps in the cascade.

Given their participation in EMT, the role of mesenchymal cadherins in breast cancer metastasis is an active area of study. In contrast to E-cadherin, mesenchymal cadherins are not expressed in the normal mammary epithelium, however they are upregulated in human breast tumors and likely facilitate progression to metastasis (Niemann et al., 1999, Pishvaian et al., 1999, Hazan et al., 2000, Kim et al., 2000, Feltes et al., 2002, Nagi et al., 2005, Yanagisawa and Anastasiadis 2006, Hulit et al., 2007). Forced expression of N-cadherin in either human breast cancer cells or a mouse model of breast cancer leads to increased invasion *in vitro* and metastasis *in vivo* (Hazan et al., 2000, Suyama et al., 2002, Hulit et al., 2007). N-cadherin promotion of metastasis is thought to occur by two non-mutually exclusive mechanisms. In one scenario, N-cadherin expression facilitates carcinoma-stroma interactions and thus invasion (Hazan et al., 1997). In another scenario, N-cadherin protects FGFR-1 from ligand-induced downregulation by physically interacting with this RTK. This leads to activation of MAP kinase signaling and secretion of MMP9, thus providing a mechanism for the previously described phenotypes (Suyama et al., 2002, Hulit et al., 2007). Thus, expression and stabilization of mesenchymal cadherins, such as N-cadherin, likely promote breast cancer metastasis.

Another p120 effector, RhoA, is central to breast cancer progression and metastasis. RhoA is not mutated in breast cancer (Moscow et al., 1994) but rather is overexpressed or hyperactivated (Fritz et al., 1999, 2002). This overexpression likely directly effects breast cancer progression as expression of dominant-negative Rho in MTLn3 invasive breast cancer cells reduces circulating tumor cells and completely prevents pulmonary metastases (Bouzahzah et al., 2001). As such, direct targeting of RhoGTPase is thought be a promising new breast cancer therapy, however the quest for such inhibitors without non-specific toxicity is thus far unsuccessful (reviewed in Sahai and Marshall 2002, in Berndt et al., 2011). Intratumoral injections of siRNA against RhoA in pre-clinical models stunt tumor growth without the toxicity observed with other methods (e.g. prenyltransferase inhibitors) (Pille et al., 2005). Thus, RhoA is directly involved in breast cancer progression and may be a target for future breast cancer therapies.

Hypothesis

I hypothesize that p120 plays a critical role in mammary development and breast cancer progression. Specifically, I propose that p120 coordinates cadherins and Rho GTPase signaling during the dynamic and complex process of mammary morphogenesis. During breast cancer, loss or dysfunction of p120 likely potentiates metastasis given its role as a stabilizer of metastasis suppressor, E-cadherin. However, a strong argument can be made for the opposite outcome. Since p120 facilitates pro-metastatic events (e.g. mesenchymal cadherin stabilization and collective invasion), its absence may in fact prevent metastasis. Furthermore, p120 isoforms 1 and 3 have unequal effects on key

parts of metastasis (e.g. invasion). Thus, whether p120 ablation will inhibit or promote metastasis is far less predictable than originally thought and will be directly addressed here. The primary objective of my work is to test these hypotheses and more importantly, contribute an understanding of the relevance of the observed changes to p120 in human breast cancer

CHAPTER II

MATERIALS AND METHODS

Animals

To generate mammary-specific p120 KO mice, $p120^{f/f}$ mice were backcrossed onto an FVB/NJ background and crossed with MMTV-cre#7 obtained from Dr. Muller on an FVB background (Andrechek et al., 2000; Andrechek et al., 2005; Davis and Reynolds, 2006). p120 ablation in a mouse model of breast cancer was achieved by crossing the mammary-specific p120 KO mice with the MMTV-Polyoma Middle T (PyMT) model (Guy et al., 1992). Kaiso loss in this model was accomplished by crossing the PyMT mice with a global Kaiso null mouse (Prokhortchouk et al., 2006). Genotyping was accomplished as previously described (Andrechek et al., 2000; Davis and Reynolds, 2006, Prokhortchouk et al., 2006). All experiments involving animals were approved by the Vanderbilt University Institutional Animal Care and Use Committee.

Whole Mount Mammary Gland Analysis

Inguinal mammary glands were fixed overnight in Carnoy's II [1:3:6 glacial acetic acid: chloroform: ethanol] fixative and gradually rehydrated. Glands were stained with carmine alum, washed, and dehydrated. Clearing was performed using Histoclear (National diagnostics, Atlanta, Georgia, USA). Whole mount images were acquired using an Olympus QColor 3TM digital camera and assessed using Metamorph software (Molecular Devices, Sunnyvale, California, USA). To quantify outgrowth, the average

distance of the three longest ducts was measured relative to a line tangential to the nipple-proximal face of the lymph node. Outgrowths beyond and before the lymph node were quantified with positive and negative values, respectively. To quantify TEB area, the average area of the six largest TEBs per whole mount was obtained. For both assessments, glands from at least five mice per genotype were analyzed.

Immunohistochemistry

Immunostaining on tissue was performed as previously described (Davis and Reynolds, 2006). Briefly, tissues were fixed in 10% formalin. Paraffin-embedded tissue sections were deparaffinized and rehydrated. Antigen retrieval was performed by boiling slides in 10 mM Sodium Citrate pH 6.0 for 10 minutes. After blocking, slides were incubated in primary and secondary antibody overnight and for 2 hours, respectively. All antibodies are listed in Table 2. Sections were mounted with Prolong Gold Antifade Mounting Medium (Invitrogen, Carlsbad, California, USA). Tissue processing and hematoxylin and eosin (H&E) staining was performed by the Vanderbilt Translational Pathology Shared Resource Core using standard techniques. TUNEL staining was performed as per the manufacturer's instructions with the following modification: antigen retrieval for 10 minutes in proteinase K (Millipore, Danvers, Massachusetts, USA). Staining was visualized using an Axioplan 2 microscope (Zeiss, Oberkochen, Germany). Images were collected with either an Olympus QColor 3TM digital camera or a Hamamatsu Orca ER fluorescent camera and processed using MetaMorph software.

Antibody	Company/Catalog #	Species/Isotype	Concentration (in µg/ml)
pp120 (p120)	BD 610134	Mouse IgG1	0.8
F1αSH (p120)	Reynolds Lab	Rabbit	1:500
6H11 (p120 N-term)	Reynolds Lab	Mouse	
E-cadherin	BD 610181	Mouse IgG2a	0.5
β-catenin	Sigma C2206	Rabbit	1:800
mARVCF	Hofmann Lab	Guinea Pig	1:100
mp0071	Hofmann Lab	Guinea Pig	1:300
δ-catenin	EMD Millipore 07-259	Rabbit	1
Kaiso	Reynolds Lab	Rabbit	1:3000
Kaiso (6F)	Reynolds Lab	Mouse	1:500
Crumbs3	Margolis Lab	Rabbit	1:500
Desmoglein-1 (H-290)	Santa Cruz sc-2014	Rabbit	1
F4/80	Serotec MCA497R	Rat IgG2b	2
Ly6B.2 (neutrophil)	Serotec MCA771G	Rat IgG2a	5
CD3 (b-cells)	Santa Cruz sc-1127	Goat	1:600
CD31 (endothelium)	Dianova dia310	Rat IgG2a	1:100
Meca-32 (endothelium)	BD 550563	Rat	
Smooth muscle actin	Covance MMS-466S	Mouse IgG2a	1:1000
p63 (H-129)	Santa Cruz sc-8344	Rabbit	1
Keratin 8/TROMA I	U. of Iowa Hybridoma	Rat	0.2
Cleaved Caspase-3 (Asp175)	Cell Signaling 9661	Rabbit	1:400
Phosphorylated Histone H3	EMD Millipore 06-570	Rabbit	2

Table 2: List of antibodies used for immunostaining of tissue

Cell culture and Manipulation of MCF10A cells

CommaD-beta (CDβ) cells, a gift from Dr. Medina at Baylor University, were cultured as previously described (Zhan et al., 2008). Primary mammary epithelial cells were isolated and cultured as previously described (Vaught et al., 2009). NMuMG and Phoenix293 cells were cultured in DMEM supplemented with antibiotics and 10% heat-inactivated fetal bovine serum. MCF10A cells were cultured as previously described (Debnath et al., 2003). For MCF10A manipulation, pRetroSuper-puromycin vectors expressing shRNA against human p120 and pLZRS (neomycin) vectors expressing mouse p120 isoforms 1A or 3A were utilized to deplete or add back p120, respectively (Davis et. al, 2003). pLZRS-neomycin vector expressing mouse p120 isoform 3A Δarm1.CAAX was generated as previously described (Wildenberg et al. 2006). Production virus for protein expression and shRNA expression was conducted in Phoenix293 cells as previously described (Ireton et al., 2002). MCF10A cells were selected for expression of pRetroSuper and pLZRS constructs by addition of 2 μg/mL puromycin and 500 μg/mL G418, respectively. After transduction and selection, monoclonal cell lines of MCF10A p120 shRNA cells were generated using limiting dilution. For rescue experiments, clonal cell lines were transduced with empty vector or vectors expressing mouse p120 isoform 1A, 3A, or 3A Δarm1.CAAX.

Generation and Manipulation of PyMT-derived Cell Lines

Tumor tissue (as indicated) was collected under sterile conditions, minced into 1mm size pieces, and incubated in digest media [DMEM:F12, 1% antibiotic/antimycotic, 100 units/mL hyaluronidase, 3 mg/mL collagenase A, gentimicin] for 3-4 hours with agitation

at 37°C. Cells were pelleted at 1000 RPM and washed 5 times in PBS supplemented with 5% adult bovine serum. Fibroblasts were removed by resuspending the pellet in low serum media and plating the material on a sterile petri dish. After 1 hour in the incubator, cells not attached to the dish were spun down and resuspended in full growth media [DMEM:F12, 5% adult bovine serum, insulin, progesterone, 17-b-estradiol, EGF, 1% antibiotic/antimycotic, gentimicin]. Cells were plated onto collagen-coated plates and allowed to adhere for 48 hours before media changing. Cell lines were established by passaging cells at least 15 times prior to experimental use after which point they were grown in DMEM supplemented with 10% FBS. A variety of lines were generated using this method and their tissue sources were as follows: 177 (primary tumor), 584.4 (primary tumor), 783.4A (lung metastasis), 986M2 (lung metastasis). To enrich for metastatic potential, cell line 584.4A was generated by isolating a single lung met the from orthotopic transplant of cell line 584.4 (originally derived from a primary tumor). The PyMT-derived cell lines were used in the following figures: 177 (Fig.25A-D); 986M2 (poly Fig.25E,F&27); 783.4A (mono #1 Fig.25F); 584.4A (mono #2 Fig.26,25,&27). To generate matched cell lines with and without p120 or E-cadherin, lentiviral constructs expressing non-targeting shRNA (control), cre recombinase (p120 KO), or shRNA against mouse E-cadherin (E-cad KD). Infected cells were selected for by puromycin treatment. To make clonal cell lines ("mono" as listed in figures), cells were plated at low density and single clones were selected by cloning cylinders. After upsizing, clones were screened for expression of keratin 8, p120, and E-cadherin. For Kaiso experiments, 17L3C cells, derived from PyMT tumors by Dr. B. Fingleton, were utilized. Kaiso depletion was achieved by lentiviral infection with Sigma Mission

construct #1009, 1659, and 2352 expressing shRNA against mouse Kaiso. Infected cells were selected for by puromycin treatment.

Cell Immunofluorescence

Cells were plated on glass coverslips and fixed in 3% paraformaldehyde for 30 minutes. After PBS washes, cells were permeabilized in PBS with 0.2% TritonX-100 for 5 minutes. After more PBS washes, non-specific binding was blocked using PBS with 3% nonfat milk. Cells were incubated in primary antibody for 30 minutes, washed, and incubated in secondary antibody for 30 minutes. After 3 PBS washes, nuclei were stained using Hoechst dye. Coverslips were coated in ProLong gold (invitrogen) and mounted on glass slides.

Wound Healing Assays

MCF10A or PyMT-derived cells were plated to confluence and scratched with a P200 tip to generate the wound. Cells were rinsed with PBS, covered in growth media, and imaged at 6 regions per scratch every 6 hours. Cell migration was calculated as percent closure of the original wound. For time-lapse microscopy, the above procedure was performed and cells were imaged every 10 minutes. Images were acquired using an Axiovert 200M microscope (Zeiss) and processed using MetaMorph software.

Western Blot Analysis

Protein was isolated as previously described (Mariner et al., 2004). Briefly, cells were washed with PBS, lysed in RIPA buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1%

Nonidet P-40, 0.5% Deoxycholic Acid, 0.1% sodium dodecyl sulfate] containing inhibitors [1mM phenylmethylsulfonyl fluoride, 5 μg/mL leupeptin, 2 μg/mL aprotinin, 1mM sodium orthovanadate, 1mM EDTA, 50mM NaF, 40mM B-glycerophasphate] and spun at 14,000g at 4°C for 5 minutes. Cleared total protein was quantified using a bicinchoninic acid assay (Pierce, Rockford, Illinois, USA). 20 μg of protein per sample were boiled in 2X laemmli sample buffer and separated by SDS-polyacrlamide gel electrophoresis. Proteins were transferred to nitrocellulose (PerkinElmer, Waltham, Massachusetts, USA). Non-specific binding was blocked by incubating membranes in 3% nonfat milk in Tris-buffered saline and odyssesy blocking buffer (LI-COR, Lincoln, Nebraska USA) prior to addition of primary and secondary antibodies, respectively. Anti-p120/pp120 (0.1μg/mL, BD Biosciences), anti-E-cadherin (0.1μg/mL, BD Biosciences), anti-tubulin/DM1α (1:1000, Sigma-Aldrich), anti-N-cadherin (0.8μg/mL 13A9, Millipore), and anti-P-cadherin (1:250, BD Biosciences) antibodies were used. The odyssey system was used for detection of secondary goat anti-mouse IgG IRDye 800CW antibodies (1:10,000 LI-COR).

3D Branching Assays and Mammary Transplants

Primary Mammary Epithelial Cells (PMECs) were isolated as previously described (McCaffrey and Macara 2009). Lentivirus was generated by transfecting HEK293T cells with pLL5.0-GFP expressing shRNA against human (control) or mouse p120 (p120i) and psPAX2 and pMD2.G (Addgene). PMECs were infected with lentivirus (MOI=100) for 3 hours during centrifugation at 300g. Cells were grown in suspension on low adhesion plates (Corning) for 5-7 days in mammosphere media (DMEM:F12; 20 ng/mL EGF, 20

ng/mL FGF2, and 2% B27 supplement) (Dontu et al. 2003). For 3D branching assays, 100 mammospheres were suspended in 50 μL Matrigel per 96-well plate well and equilibrated in minimal media (DMEM:F12, 1% v/v insulin/transferring/selenium, 1% penicillin/streptomycin)(Ewald et al. 2008). Branching was induced with 2.5 nM FGF2 in minimal media changed twice. Quantification of percent branched and number and fluorescence status of branches was performed after 7 days (>30 mammospheres per experiment). For mammary transplantation assays, PMECs were infected and grown in suspension. After 7 days, cells were trypsinized and flow sorted for GFP by the Vanderbilt Flow Cytometry Laboratory. $1x10^5$ cells in 10 μL of PBS with 10% Matrigel expressing control GFP or p120i GFP virus were injected into contralateral cleared fat pads of 3-week-old FVB mice. After 6 weeks, glands were removed and analyzed for GFP positive outgrowth relative to gland size using a Nikon AZ 100M fluorescent widefield microscope.

Tail Vein Injections and Lung Whole Mount

PyMT-derived cells were trypsinized and pelleted at 1000g. After 2-3 washes in PBS, cells were passed through a 70 μM pore cell strainer. $1x10^6$ cells per 100 μL were injected into the tail vein of mice. After 4 weeks, mice were sacrificed and lungs were collected. Lungs were analyzed by whole mount and tumor burden analysis. Tumor burden was quantified as a percent of metastases area to total lung area at 3 depths per lung. For lung whole mount, lungs were inflated with formalin and fixed overnight. Lungs were then dehydrated in progressive increasing amounts of ethanol and cleared overnight in Histoclear. Rehydration the next day was followed by staining in Mayer's

hematoxylin. After destain steps in 1% HCl solution and water, lungs were dehydrated and cleared once again. Analysis of lung metastases was performed using a dissecting scope and an Olympus QColor 3TM digital camera.

Transwell Invasion Assays

Matrigel-coated transwells were equilibrated by the manufacturer's instructions. Cells were then plated in serum free media in the top well and promoted to invade toward 10% media in the bottom chamber. After 24-48 hours, transwells were fixed and stained using a Diff-Quik staining kit (Allegiance). 10 random fields of view per transwell were analyzed.

Macrophage Invasion Assays

Intraperitoneal injections with 2 mL thioglycolate were performed using a 27G needle. After 4 days, the abdominal cavity was filled with PBS and peritoneal macrophages were isolated. Cells were pelleted and resuspended in serum free media and immediately used for invasion assays as described above. To generate the stimulus for macrophage invasion, PyMT-derived cells were grown in 3D matrigel cultures for at least 7 days. Serum free media was added to the cultures and collected after 24 hours. This media was then used for invasion induction of macrophages.

Whole Tumor and Spleen Flow Cytometry

Tumors were collected, minced, and incubated in digest media [RPMI with 1 mg/mL collagenase I, 1 mg/mL dispase II] for 2 hours at 37°C. Tumor material was then pressed

through a 70 μM cell strainer in 10 mL cold PBS and repeated for a total of 50 mL PBS. Tumor cells were then treated with 10 μL of 5MU/mL DNAseI for 5 minutes at room temperature. Cells were pelleted at 300g to remove DNAse treatment. Spleens were minced and pressed through a 70 μM cell strainer in 10 mL cold PBS and repeated for a total of 50 mL PBS. Red blood cells were removed from tumor and spleen preparations using lysis buffer. Cells were washed with PBS, strained, and then counted in the presence of trypan blue. Each sample of 5x10^6 cells per 100 μL flow buffer [PBS, 0.5% BSA, 2mM EDTA] was treated with Fc block and then incubated in antibodies conjugated to compatible fluorophore combinations for 30 minutes on ice. All antibodies were purchased through eBioscience. Antibodies combinations are listed in Table 3. After 2 washes with flow buffer, cells were analyzed by flow cytometry by the Vanderbilt Flow Cytometry Shared Resource Core.

Anchorage Independent and 3D Matrigel Growth

For anchorage independent growth assays, 6 well-dishes were coated with a bottom layer of 0.7% low gelling temperature agarose. 5x10^3 PyMT-derived cells suspended in 0.35% agarose were plated per well and covered in 2 mL growth media. After 3.5 weeks of growth, colonies were imaged using an inverted microscope. For 3D growth assays, 35 mm tissue culture dishes were coated with 500 μL of Matrigel. 7x10^5 PyMT-derived cells were plated in growth media supplemented with 2% Matrigel.

Antibody Combination	Cell Type Detected
CD45+	Leukocytes
CD45+ Gr1+ CD11b+	Myeloid derived suppressor cells
CD45+ Gr1+ CD11b- F4/80+	Macrophages
CD45+ CD3+	B-cells
CD45+ CD19+	T-cells
CD45+ CD11b+ CD11c+ MHCclassII +	Dendritic cells
CD45+ CD11b+ F4/80+ CD80+ CD86+	M1 Macrophages
CD45+ CD11b+ F4/80+ CD204+ CD206+	M2 Macrophages

Table 3: Antibody combinations used for flow cytometry

Cytokine Arrays and Microarrays

PyMT-derived cells were grown in 3D matrigel as described above. For cytokine arrays, cultures were rinsed once with serum free media and secreted factors allowed to accumulate in serum free media overnight. Secreted media collected from 9 and 19 day control and p120 KO cultures was added to the RayBio Mouse G3 Cytokine Array. Arrays were performed as per the manufacturer's instructions. For microarrays, total RNA was isolated using RNAStat60 (Amsbio) and RNeasy Mini Kit (Qiagen). RNA was hybridized to Affymetrix Mouse Gene 1.1 ST arrays. Targets changed consistently at least 1.6 fold over control in each sample were considered meaningful. Analysis for cytokine arrays and microarrays was performed with assistance by the Functional Genomics Shared Resource Core.

Contractility Assays

Contractility assays were performed as extensively described by Wozniak and Keely (Wozniak and Keely, 2005). Briefly, 1X10^5 cells were suspended in 1 mL of 1:1 type I collagen (1.19 mg/mL final concentration) to neutralization solution [100mM Hepes in 2X PBS pH 7.3]. 1 mL was added per 6-well and each cell line was plated in triplicate. After overnight incubation, gels were floated using a P200 tip. Two days post floating, gel contraction was measured for each cell type.

Orthotopic Transplant

1X0^6 cells were suspended in 50 μL of 1:1 type I collagen (1.19 mg/mL final concentration) to neutralization solution [100mM Hepes in 2X PBS pH 7.3]. Plugs were

allowed to solidify for 1 hour in the incubator and then covered in growth media. The following day plugs were transplanted into the cleared fat mammary fat pads of mice.

qRT-PCR on Macrophages

Total RNA was extracted from sorted CD45+CD11b+F4/80+ cells from tumors of d54 PyMT control and p120 KO mice (n=3 per genotype) using QIAshredder columns and RNeasy mini kit (Qiagen). cDNA was synthesized using Invitrogen Superscript First-strand synthesis system for RT-PCR (Invitrogen). Primers specific for iNOS, TNFα, IL-12, IL-6, IL-1b, VEGF, MMP2, MMP9, MMP13, CXCL1, and IL-10 were used and the relative gene expression was determined using ABI PRISM 7900HT Sequence Detection System (PE Applied Biosystems). The comparative threshold cycle method was used to calculate gene expression normalized to β–actin.

Circulating Tumor Cell Colonization Assay

Mice 54 days after initial palpation were euthanized (n=4 per genotype) and whole blood was collected in a heparinized needle. Each well of a 6-well dish was coated in 1:1 mix of matrigel and DMEM supplemented with 10% FBS. After Matrigel solidification, 500 μL of whole blood was plated per 6-well dish and covered in 2 mL media. After 48 hours, cultures were washed with PBS. Red blood cells were removed with lysis buffer [155 mM NH_4Cl, 12mM $NaHCO_3$, 0.1 mM EDTA] and then washed away with PBS. Colonies were then allowed to grow for 7-10 days with media changes every 3-4 days. Colonies were counted in 5 random 2.5x fields and 10 random 10x fields.

Statistical Analysis

Statistical analyses were preformed using Prism (GraphPad La Jolla, California, USA) as described in figure legends. For assays with or without normal distribution, two-tailed Student's t-tests or Mann-Whitney tests were performed, respectively.

CHAPTER III

P120-CATENIN IS ESSENTIAL FOR TERMINAL END BUD FUNCTION AND MAMMARY MORPHOGENESIS

Introduction

Cell-cell adhesion plays a key role in development, tissue maintenance and cancer (Birchmeier, 1995; Gumbiner, 2005; Takeichi, 1995; Yap, 1998). In vertebrates, the classical cadherins (i.e., Type I and Type II cadherins) comprise a large family (26 members) of transmembrane glycoproteins found in essentially all adhesive tissues (Gallin, 1998; Hulpiau and van Roy, 2009). Epithelial-cadherin (E-cadherin) is the main cadherin in epithelial tissues and plays an important role in morphogenesis and homeostasis in most glandular tissues, including the mammary gland. Although the importance of cadherins in mammary morphogenesis is widely accepted, the role of p120 catenin in this process remains to be investigated.

The extracellular domains of cadherins connect adjacent cells via homophillic interaction, while the cytoplasmic domains form a complex with a group of proteins, known as catenins (Gumbiner, 2005; Takeichi, 1991). p120-catenin (hereafter p120) and β-catenin are armadillo repeat domain proteins that bind directly to distinct regions of the cytoplasmic domain (Davis et al., 2003; Hulsken et al., 1994; Ireton et al., 2002; McCrea and Gumbiner, 1991; Reynolds et al., 1996; Takeichi et al., 1989; Thoreson et al., 2000; Yap et al., 1998). β-catenin connects the cadherins physically and/or functionally to the actin cytoskeleton through a mechanism involving α-catenin (Herrenknecht et al., 1991; Nagafuchi et al., 1991; Rimm et al., 1995; Yamada et al., 2005). In contrast, p120

appears to regulate the strength of cell-cell adhesion by modulating cadherin retention at the cell surface (Davis et al., 2003; Ireton et al., 2002; Xiao et al., 2003). In its absence, cadherins are internalized and degraded, thus defining p120 as a master regulator of cadherin stability (Davis et al., 2003). p120 is also thought to modulate actin dynamics via Rho-GTPases, -GEFs, and GAPs (Anastasiadis et al., 2000; Noren et al., 2000; Wildenberg et al., 2006). Together, these observations suggest that the catenins play a central role in regulating functional interactions between cadherins and the actin cytoskeleton.

Phenotypes associated with p120 ablation *in vivo* appear to be largely tissue dependent and surprisingly unpredictable (Bartlett et al., 2010; Davis and Reynolds, 2006; Elia et al., 2006; Marciano et al., 2011; Oas et al., 2010; Perez-Moreno et al., 2006; Smalley-Freed et al., 2010; Stairs et al., 2011). For example, in the developing salivary gland, p120 ablation completely blocks acini formation (Davis and Reynolds, 2006). Ducts are grossly distorted and characterized by cell-cell adhesion defects reminiscent of those observed in intraepithelial neoplasia. On the other hand, p120 knockout (KO) in the epidermis induces a massive inflammatory response despite essentially normal adhesion and barrier function (Perez-Moreno et al., 2006). In the intestine, p120 KO causes a prominent barrier defect along with cell-cell adhesion abnormalities and inflammation (Smalley-Freed et al., 2010). These animals die from gastrointestinal bleeding within 3 weeks of birth. Other p120 KO-associated defects include reduced vessel density and anomalies in dendritic spine and synapse development in hippocampal neurons (Elia et al., 2006; Oas et al., 2010). Surprisingly, p120 KO in the prostate has no detectable effect on either cell morphology or adhesion despite near complete downregulation of E-

cadherin (A.B. Reynolds, unpublished). These studies, for the most part, reflect dramatic phenotypes, although consequences of p120 ablation differ markedly from one organ system to the next. However, the effects of p120 loss in the mammary gland have not been formally addressed.

The mammary gland provides an outstanding *in vivo* system for studying morphogenetic events (e.g. invasion and differentiation), since the majority of the development of this non-vital organ occurs after birth. Prior to puberty, the mammary gland exists as a rudimentary ductal tree. At the onset of puberty around 3 weeks of age, proliferative structures at the tips of ducts, known as terminal end buds (TEBs), develop and begin to invade the surrounding stroma (Hinck and Silberstein, 2005). TEBs are comprised of a dynamic mass of E-cadherin positive luminal body cells surrounded by a motile cap cell layer expressing P-cadherin (Daniel et al., 1995; Ewald et al., 2008; Hinck and Silberstein, 2005). The TEBs bifurcate repeatedly to form the ductal tree and ultimately, the mature gland. This process, termed branching morphogenesis, concludes around weeks 10-12 when the TEBs have traversed the length of the fat pad and a fully developed ductal tree has formed (Cardiff and Wellings, 1999; Hennighausen and Robinson, 2005; Richert et al., 2000; Sternlicht, 2006). Disruption of TEBs is often associated with delayed ductal outgrowth and impaired branching morphogenesis thus suggesting an essential function of TEBs in the overall development of the mammary gland (Jackson-Fisher et al., 2004; Kouros-Mehr et al., 2006; Lu et al., 2008; Parsa et al., 2008; Srinivasan et al., 2003; Sternlicht et al., 2006).

Here, we examine the role of p120 in the developing mammary epithelium. MMTV promoter-driven Cre recombinase expression in $p120^{f/f}$ mice was used to induce

p120 ablation at the onset of puberty. In week 4, developing epithelial structures exhibited mosaic p120 ablation, the extent of which varied widely between mice. p120 loss in nascent ducts caused severe morphological defects (e.g. cell rounding and sloughing into the lumen), despite the presence of family members, which were unable to compensate for p120 loss. p120 null cells were observed less frequently in the TEB itself due to rapid shedding from TEBs. *In vitro* two- and three-dimensional modeling suggest that TEB function is compromised in the absence of p120, most likely due to defects in cell-cell adhesion and collective cell migration. At the whole organ level the phenotype manifested as a transient delay in ductal outgrowth due to selective loss of p120-null cells and preferential outgrowth of the p120-positive cell population. Reconstitution with pure populations of p120-depleted cells blocked mammary gland formation completely. These data reveal an essential, non-redundant role for p120 in mammary gland development.

Results

Characterization of p120 expression in the developing mammary gland

To characterize baseline p120 expression patterns in the developing mammary gland, sections of glands from 4-week-old control mice were co-immunostained with antibodies to p120 along with the basal and luminal cell markers smooth muscle actin (SMA) and keratin 8 (K8), respectively (Fig.5A). Figure 5A illustrates diffuse p120 staining of stromal cells (arrows) and sharp junctional staining in the epithelium of ducts (top panels) and terminal end buds (TEBs) (bottom panels). Note that p120 staining in the basal compartment of the TEB is markedly reduced relative to the very strong staining in the luminal compartment. These patterns of p120 localization were the same over all stages of pubertal development (data not shown).

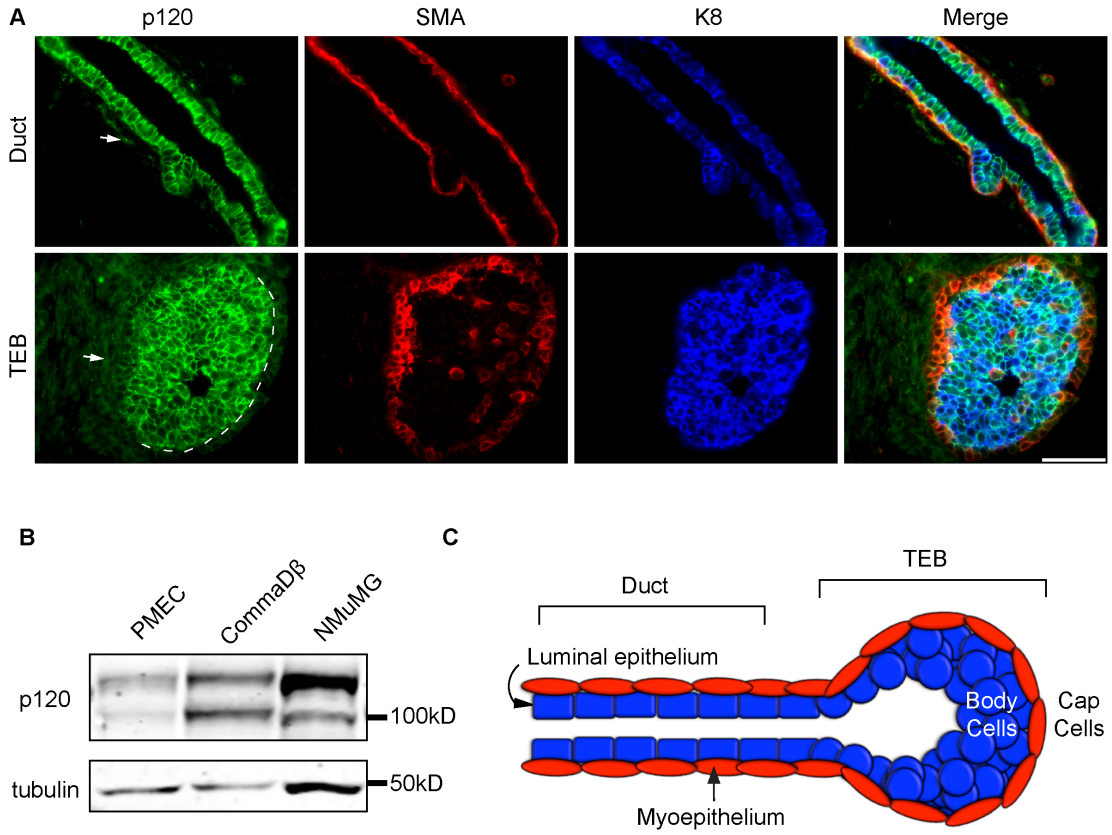


Figure 5. p120 is ubiquitously expressed in the mouse mammary gland (A) Immunostaining for p120, SMA, and K8 on sections from glands of 4 week-old females. SMA and K8 mark the cap and body cells, respectively. Representative images for ducts and terminal end buds (TEB) are shown. Arrow denotes diffuse stromal p120 staining. Dotted line indicates the division between body and cap cells. Scale bar = 50 μM. (B) Immunoblots for p120 and tubulin in a panel of normal mammary epithelial cells. (C) Diagram of the mammary TEB and duct.

Typically, epithelial cells predominantly express p120 isoform 3, whereas fibroblasts express isoform 1. However, figure 5B illustrates biochemically that primary mammary epithelial cells (PMECs) and the untransformed mouse mammary cell lines CDβ and NMuMG express both p120 isoforms 1 and 3. Furthermore, both layers of the mammary epithelium demonstrated positive immunostaining using an antibody that only recognizes p120 isoform 1 and 2 suggesting that isoforms other than 3 are also expressed in the epithelium *in vivo* (data not shown). Collectively, these results demonstrate expression of p120 in the mature basal and luminal mammary epithelium, as well as in the body and cap layers of the TEB.

Mosaic p120 knockout at puberty induces transient delay of ductal outgrowth

To target p120 knockout to the mammary gland, MMTV-Cre;$p120^{fl/fl}$ mice were generated by crossing MMTV-Cre #7 mice on an FVB background (Andrechek et al., 2000; Andrechek et al., 2005) to floxed p120 mice, which was backcrossed to a FVB background (Davis and Reynolds, 2006). Effects of p120 ablation were examined initially by whole mount analysis at time points spanning pubertal development (Fig.6). At 3 weeks, knockout and control rudimentary mammary trees were grossly indistinguishable (Fig.6A). In contrast, ductal outgrowth was significantly reduced at weeks 4, 5, and 6 (Fig.6A,2B). By week 9, however, control and knockout glands were again indistinguishable. Thus, p120 ablation induces a transient delay in ductal outgrowth that is ultimately resolved by week 9.

Further analysis of the glands by immunostaining revealed mosaic, epithelium-specific p120 ablation in all experimental animals starting at week 3. Significant p120

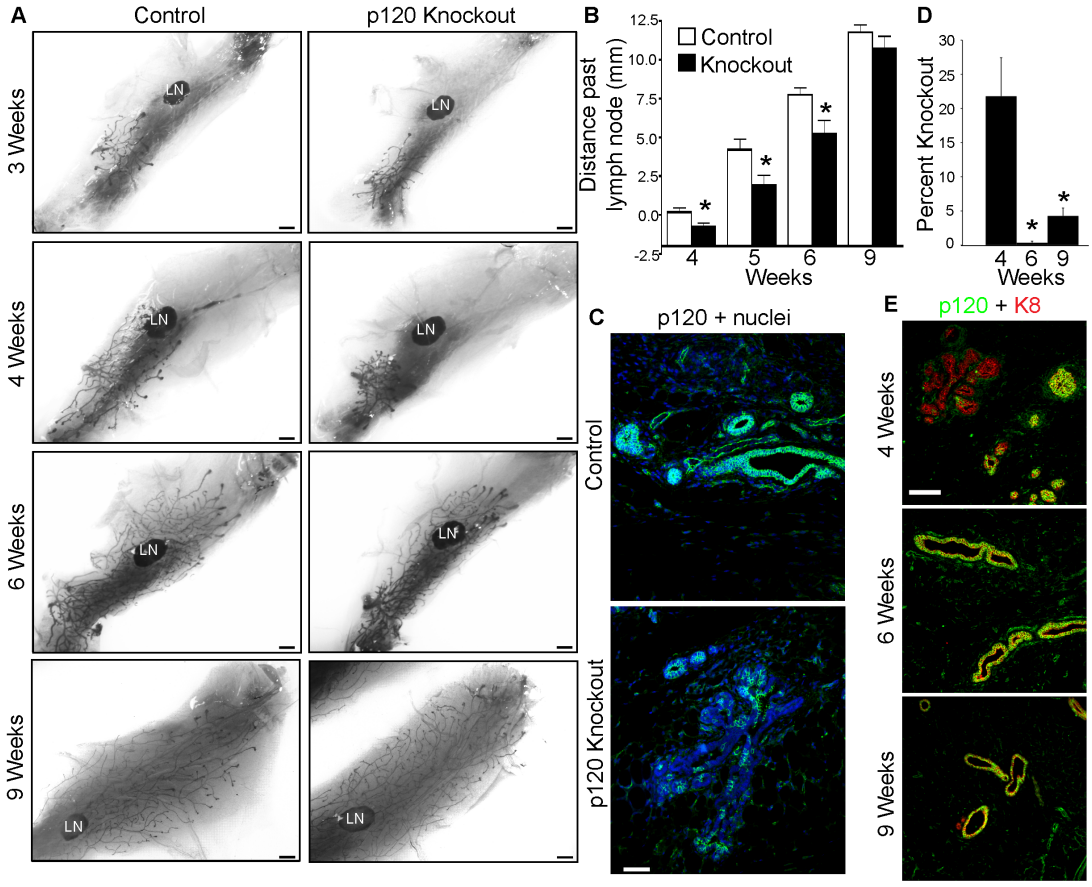


Figure 6. p120 ablation in the developing mammary gland delays ductal outgrowth

(A) Images of virgin mammary gland whole mounts from control and p120 KO animals. LN denotes lymph node. Scale bars = 1 mm (B) Quantitative comparisons of ductal outgrowth. Graph depicts mean and s.e.m. Mann-Whitney test *p<0.05. (C) Representative images of immunostaining for p120 in 4-week-old glands. Arrowheads denote areas of knockout. Scale bar = 50 μ M. (D) Quantification of p120 ablation in glands from mice at indicated ages. Graph depicts mean and s.e.m. Mann-Whitney *p<0.05. (E) Representative images for p120 and K8 of glands at indicated ages. Scale bar = 50 μM.

knockout was observed at week 4 (Fig.6C). The overall percentage of knockout cells varied widely (range: 38-6.7%, n=6) but averaged 22% of the nascent epithelium following puberty-induced expression of Cre (Fig.6D,E). Thereafter, p120 null cells were increasingly scarce and almost completely absent by week 6 (range: 0-0.9% n=5) (Fig.6D,E). Thus, from week 6 on, glands were essentially p120 positive (despite the MMTV-Cre;$p120^{fl/fl}$ genotype) and further development (including pregnancy and lactation) was indistinguishable from that of $p120^{fl/fl}$ controls.

The rapid loss of p120 null cells between weeks 3 and 6 suggested the possibility of reduced cell proliferation or elevated cell death. We broadly assessed cell death by TUNEL staining, which marks cells undergoing apoptosis, necrosis, or lysosome-mediated death (Grasl-Kraupp et al., 1995; McIlroy et al., 2000; Overholtzer et al., 2007). There was no statistically significant change in global cell death in p120 KO ducts or TEBs (Fig.7A,B a-b). However, the percent of cells TUNEL positive increased 3-fold when analyzing only those detached from the body of the TEB in knockout mice (Fig.7A,B a-b arrows). Similarly, detached cells demonstrated a 4-fold increase in the percent positive for cleaved caspase 3 further suggesting that p120 null cells are dying by anoikis, detachment-induced cell death (Fig.7AB, c-d arrows). This cell detachment was rarely seen in control TEBs. Cell proliferation in ducts and TEBs was unaffected by p120 ablation as monitored by phosphorylated histone H3 staining (Fig.7A,B e-f).

We also examined the possibility that p120-null cells might be removed or engulfed by elements of the immune system. In several other organ systems (e.g., intestine, esophagus, epidermis), p120 ablation is associated with significant immune cell infiltration, which could facilitate rapid clearance of malfunctioning cells. However,

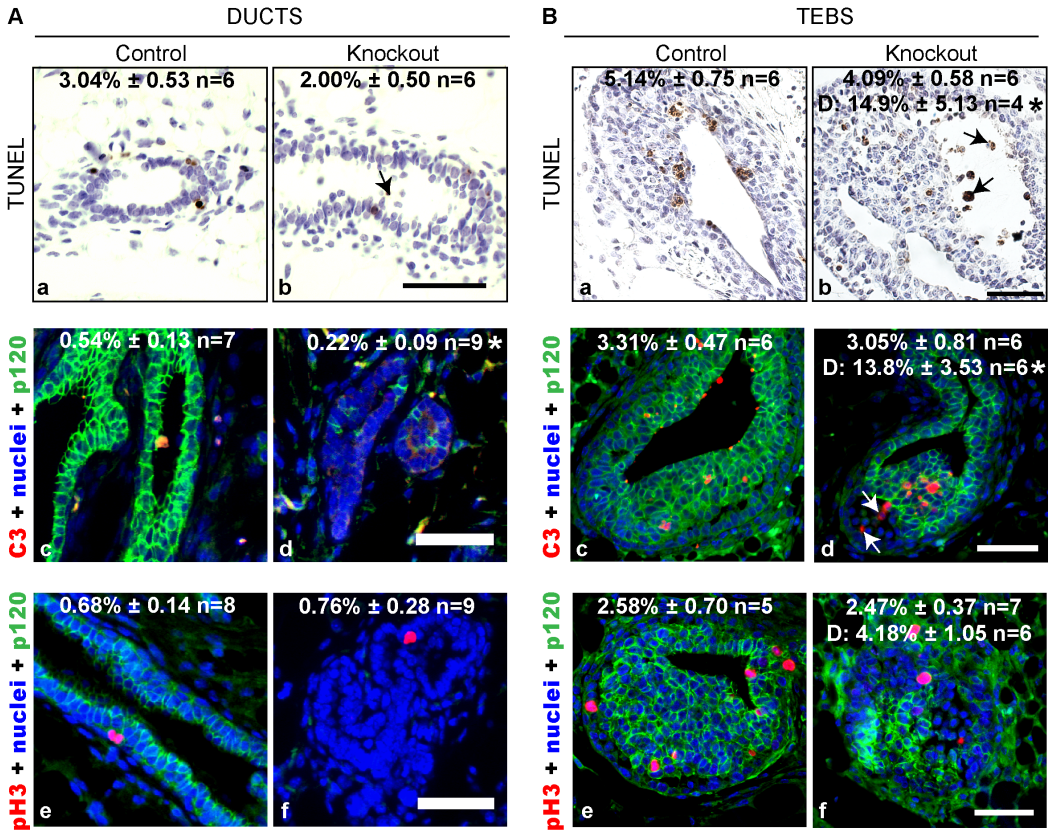


Figure 7. Analysis of proliferation and cell death in the absence of p120
Analysis of cell death and proliferation was performed on ducts (A) and TEBs (B). (a-b) Representative TUNEL stained sections from glands of 4-week-old control and p120 knockout mice. Arrows denote detached TUNEL positive cells. (c-f) Analysis of apoptosis and proliferation was performed using immunostaining for cleaved caspase-3 (c-d) and phosphorylated histone H3 (e-f), respectively. Values are mean +/- s.e.m. and n= number of animals. D denotes analysis of detached cells. Student's t-test *p<0.05. All scale bars = 50 μM.

immunostaining for macrophage/eosinophil- (anti-F4/80) and neutrophil- (anti-Ly-6B.2) markers showed little, if any, evidence for unusual recruitment of these cell types (data not shown). Collectively, these experiments suggest that p120 null cells are being selectively lost by anoikis and subsequent clearance by mechanisms that do not involve obvious inflammation.

p120 is required and non-redundant for ductal architecture

To determine the immediate consequences of p120 loss on ductal architecture, sections from week 4 glands were analyzed by hematoxylin and eosin (H&E) staining and immunofluorescence microscopy (Fig.8). H&E analyses revealed cell-cell adhesion defects manifested by dramatic cell sloughing and frequent partial occlusions of the lumens (Fig.8A). Immunostaining for the apical marker Crumbs 3 illustrated obvious rounding of the apical cell surface, presumably reflecting poor basolateral cell-cell adhesions (Fig.8B). On the other hand, basal cells (marked by p63) were never displaced from their normal position at the basement membrane, but appeared more sparse than in the wildtype glands (Fig.8C).

As observed in several other organ systems, p120 ablation selectively affected the adherens junction as shown by decreased expression of E-cadherin and β-catenin in p120 null cells (Fig.9A,B). Loss of p120 did not affect ZO-1 expressing tight junctions (Fig.9C) or desmosomes (data not shown). Since immunostaining of E-cadherin and β-catenin was dramatically decreased in the absence of p120, these data suggest that p120 family members, if present, are unable to compensate in this tissue. Therefore, we analyzed the expression of p120 family members, ARVCF, p0071, and δ-catenin, using

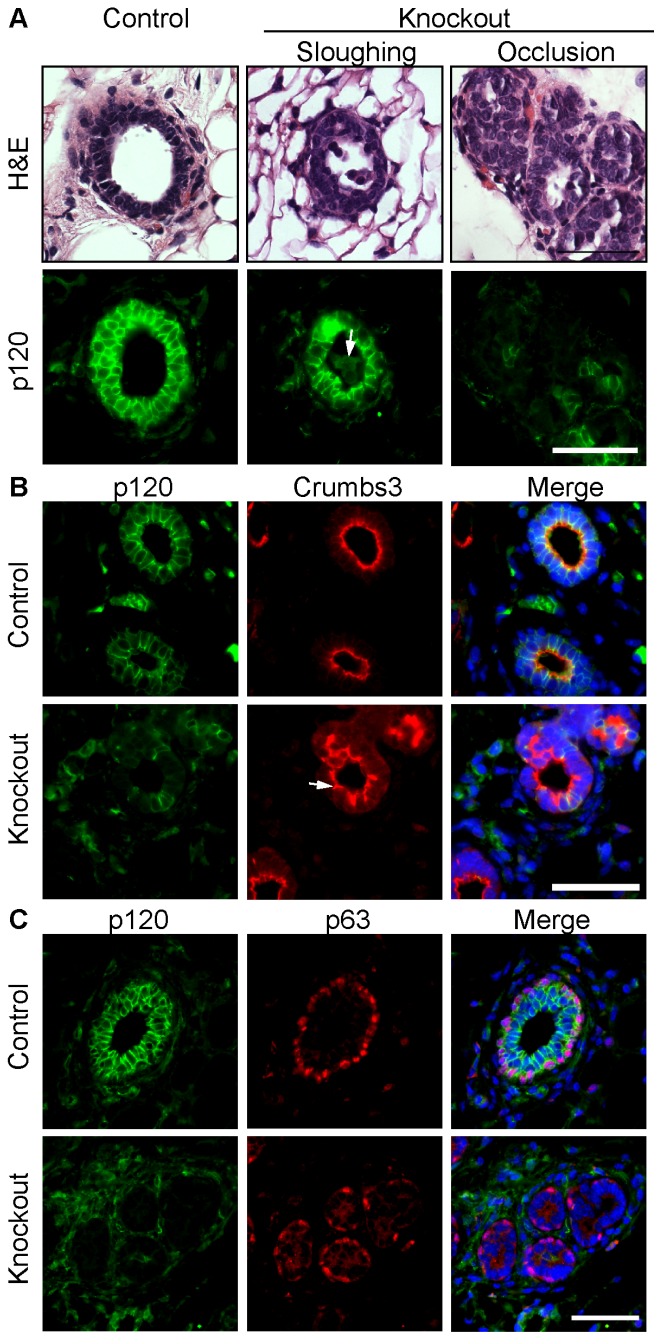


Figure 8. p120 ablation disrupts ductal architecture

(A) Serial sections of mammary glands from 4 week-old mice were stained with H&E and with antibodies against p120. Representative images of the two phenotypes observed, luminal sloughing and partial occlusions, are shown. Arrow denotes sloughed knockout cells. (B) 4-week-old glands were co-immunostained for p120 and Crumbs3. Arrows indicate the mislocalized apical marker and severe disruption ductal morphology in areas of p120 ablation. (C) 4-week-old glands were co-immunostained for p120 and p63. All scale bars = 50 μM.

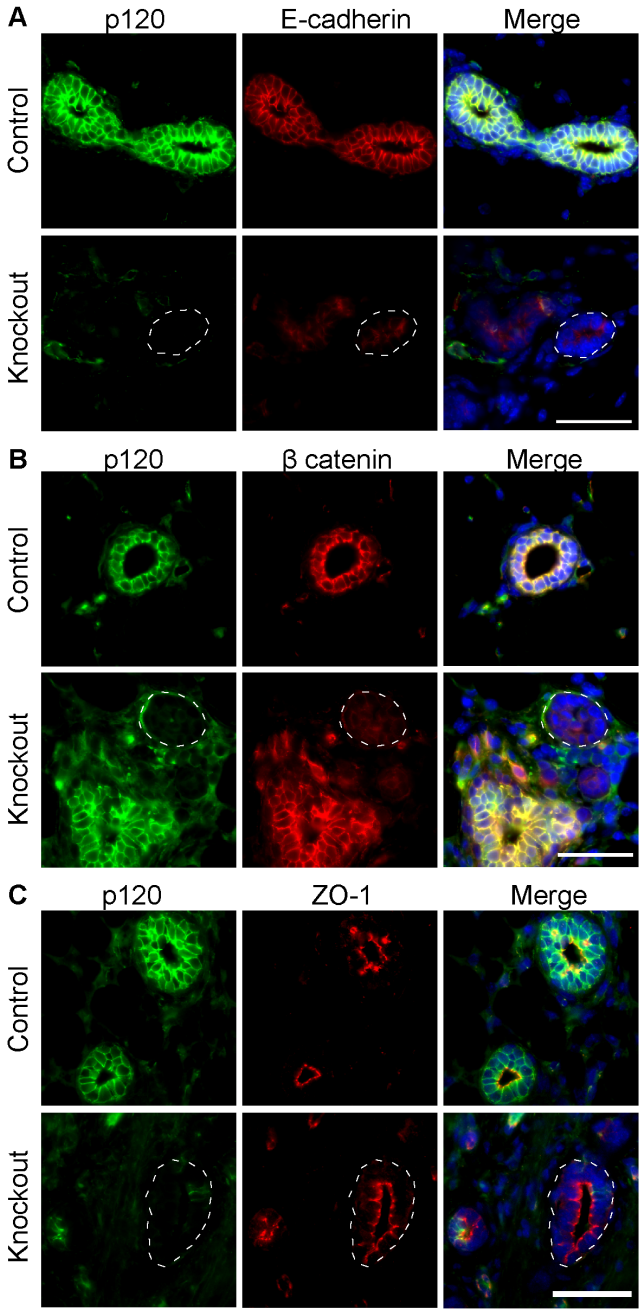


Figure 9. Dysregulation of the mammary gland cadherin complexes in the absence of p120

Sections of mammary glands from control and p120 knockout mice were co-immunostained for the following: (A) p120 and E-cadherin; (B) p120 and β-catenin; (C) p120 and ZO-1. Dotted circles denote areas of p120 ablation and consequent downregulation of E-cadherin and β-catenin, but not ZO-1. All scale bars = 50 μM.

well-characterized antibodies (Hofmann et al. 2009, Marciano et al. 2011, Walter et al. 2008, Walter et al. 2010). Family members demonstrated membranous and cytoplasmic localization in the mammary epithelium, which was not altered in the absence of p120 (Fig.10). Thus, p120 provides a non-redundant function in cell-cell adhesion of mammary ductal cells.

p120 null cells are rapidly sorted and eliminated from nascent TEBs

The driving force behind development of the mammary gland during puberty is the TEB, where the vast majority of cell growth, death, and invasion occur. To determine the role of p120 in the TEB, we examined the effects of p120 ablation on TEB size and morphology (Fig.11). While total TEB number was not altered in p120 knockout mice (data not shown), average TEB size was significantly reduced at week 4 (Fig.11A,B). However, TEB size normalized by week 5, well before the knockout gland outgrowth caught up with that of the wildtype gland (Fig.11A,B and Fig.6). Thus, the early delay in ductal elongation due to p120 ablation may occur in response to events taking place with the TEBs, which rely on p120 expression.

To understand the nature of the defect at week 4, the histological morphology of TEBs from 4 week-old mice was examined. Figures 11C & 11D show examples of typical TEB phenotypes in cross and longitudinal sections, respectively. Interestingly, the majority of TEBs from KO mice lacked p120 null cells altogether, suggesting that the early size discrepancy might reflect very rapid clearing and/or loss of p120 null cells from these structures. In TEBs retaining significant numbers of p120 null cells, the cells invariably were rounded, non-adhesive, and likely unable to participate in endbud

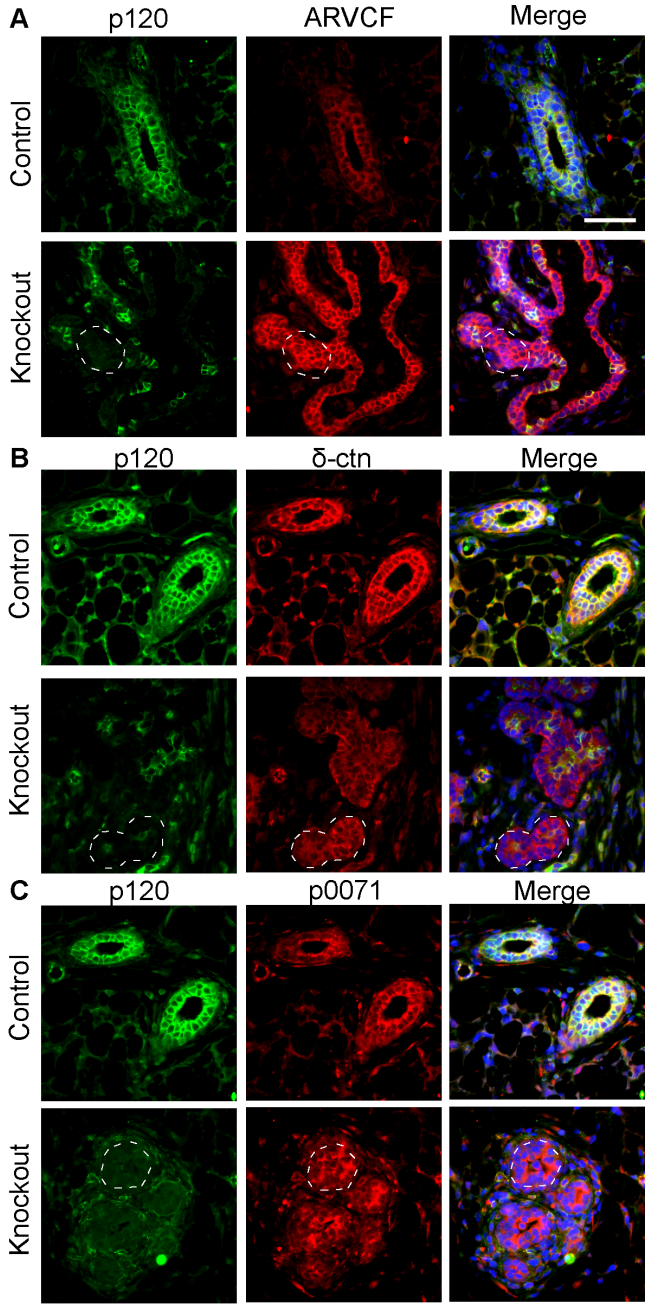


Figure 10. p120 family members cannot compensate for p120 loss. Sections from mammary glands of 4-week-old control and p120 knockout mice were co-immunostained with antibodies against p120 and ARVCF (A) or δ-catenin (B) or p0071 (C). Dotted regions denote areas of p120 ablation. Scale bar = 50 μM.

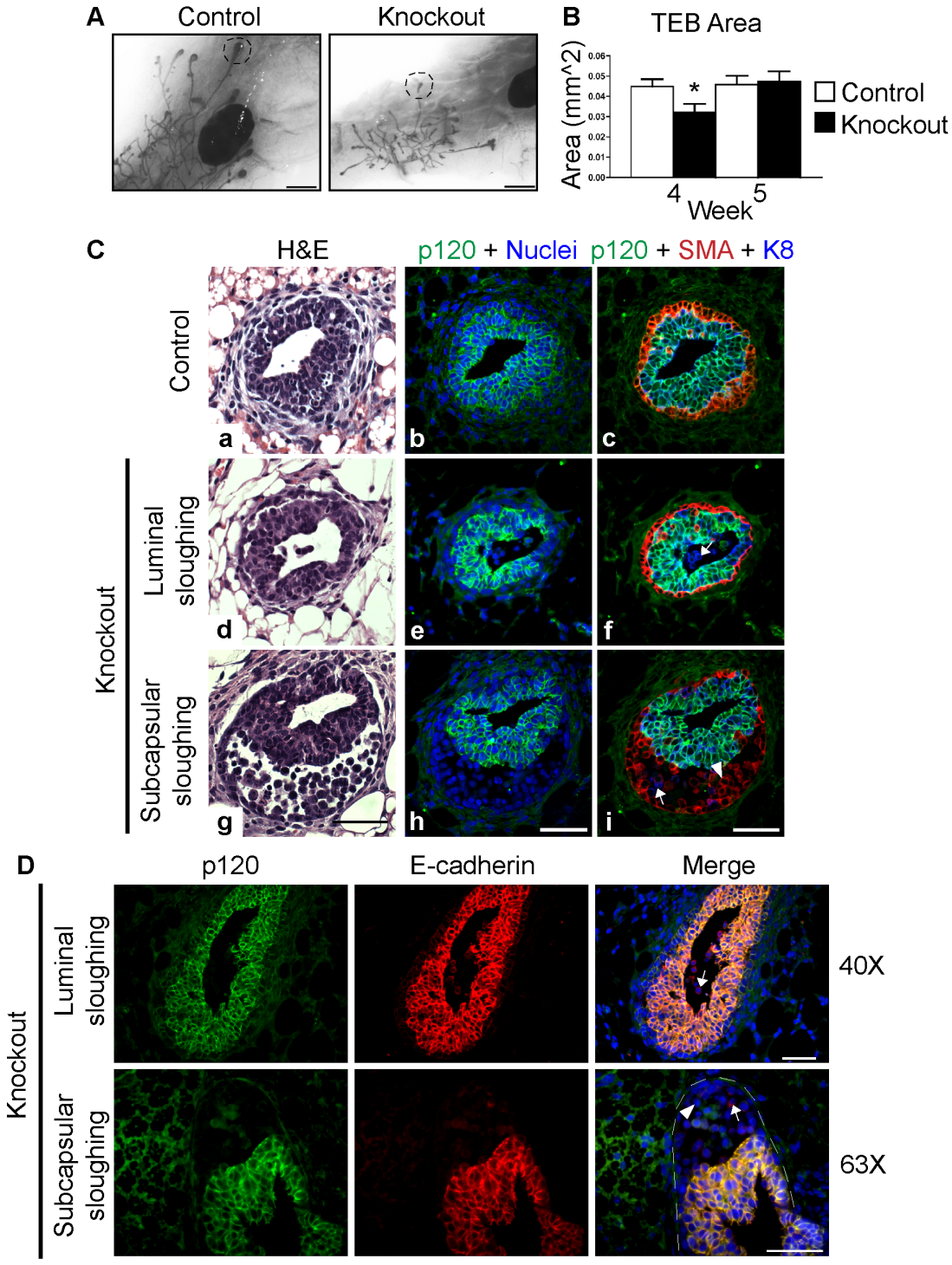


Figure 11. p120 null cells are rapidly shed and fail to participate in TEB development.

Figure 11. p120 null cells are rapidly shed and fail to participate in TEB development.

(A) Whole mount images of control and p120 knockout mammary glands. LN denotes the lymph node. Dotted circles highlight representative TEBs. Scale bars = 1 mm (B) Analysis of TEB size. Knockout mice exhibited statistically significant decrease in TEB size at 4 weeks but not 5 weeks of age. Graph depicts mean and s.e.m. Student's t-test *$p<0.05$. (C) Cross-sections of TEBs from inguinal mammary glands harvested at 4 weeks. Serial sections were stained with H&E (a, d, g) or immunostained for p120, SMA, and K8. Nuclei were costained with Hoechst dye. Examples of luminal (d-f) and subcapsular (g-i) sloughing are shown. All scale bars = 50 μM. (D) Longitudinal sections of TEBs from inguinal mammary glands harvested at 4 weeks. Sections were immunostained for p120 and E-cadherin. Examples show luminal (top) and subcapsular (bottom) cell sloughing. All scale bars = 50 μM.

activity. In general, such structures segregated into one of two distinct scenarios based on where the p120 null cells accumulated. Figure 11C (d-f) illustrates sloughing of p120 null cells to the lumen. Alternatively, TEBs from p120 KO mice frequently contained aberrantly large subcapsular spaces, and these also were found to accumulate significant numbers of p120 null cells (Fig.11C g-i). The presence of p120 family members was insufficient to support TEB morphology in the absence of p120 (data not shown).

To identify the origin of the sloughed p120 null cells (i.e. cap versus body cells), we analyzed the images for basal (SMA) or luminal (K8) markers (Fig.11C). Cross-section of TEBs from control mice demonstrated a multi-layered, K8+/p120+ body, surrounded by a single-cell SMA+ cap cell layer, that also expressed p120, albeit at lower levels (Fig.11C). p120 null cells shed into the lumen were K8+/SMA- (Fig.11Cf arrow). Whereas, p120 null cells accumulating in the subcapsular compartment were predominantly K8-/SMA+ suggesting that they were derived from the cap layer or myoepithelial cells (Fig.11Ci arrowhead). Occasional examples of K8+/SMA-/p120- cells were detected in the subcapsular region (Fig.11Ci arrow). Note that p120 expressing cells were rarely seen in the lumen or in the subcapsular space. Similar results were observed in longitudinal TEB sections stained with antibodies against p120 and E-cadherin (Fig.11D). Together, these observations show that p120 null mammary epithelial cells generated at puberty in the nascent TEBs were derived from both body and cap cell components, underscoring the requirement for p120 in both epithelial cell populations for adhesion and TEB morphogenesis.

p120 loss *in vitro* disables collective migration required for branching morphogenesis *in vivo*

To clarify mechanism, we utilized RNA interference to stably knock down p120 in the nontumorigenic human mammary cell line, MCF10A (Fig.12). Although experiments *in vitro* do not necessarily recapitulate the complexity of *in vivo* morphogenesis, MCF10A cells have nonetheless been used frequently for mechanistic modeling of collective migration and other phenomena associated with mammary development (Debnath et al., 2002; Simpson et al., 2008). Figure 12A illustrates the extent of p120 knockdown in two independently derived MCF10A clones. Similar to what was seen in the mammary epithelium, E-cadherin levels are significantly reduced by p120 depletion and efficiently rescued by forced expression of either p120 isoform 1A or 3A (Fig.12A).

In 2D cell cultures, parental MCF10A cells formed tightly adherent colonies, whereas cell-cell adhesion was completely disrupted by p120 knockdown (Fig.12B). The phenotype was efficiently rescued by either p120 isoform 1A or 3A, as expected if the cell-cell adhesion defects are the result of p120 depletion (Fig.12B).

We then examined the effects of p120 depletion on 3D acinar morphogenesis using a previously described matrigel system (Fig.13) (Debnath et al., 2003). While parental MCF10A cells formed simple lumen-containing acini, loss p120 resulted in disorganization of acinar structure and poorly defined lumens (Fig.13). In general, these structures closely resembled the images of p120 null ducts illustrated in figure 8.

Collective cell migration is required for TEB invasion through the mammary fat pad. To assess the role of p120 in collective cell migration, we conducted p120

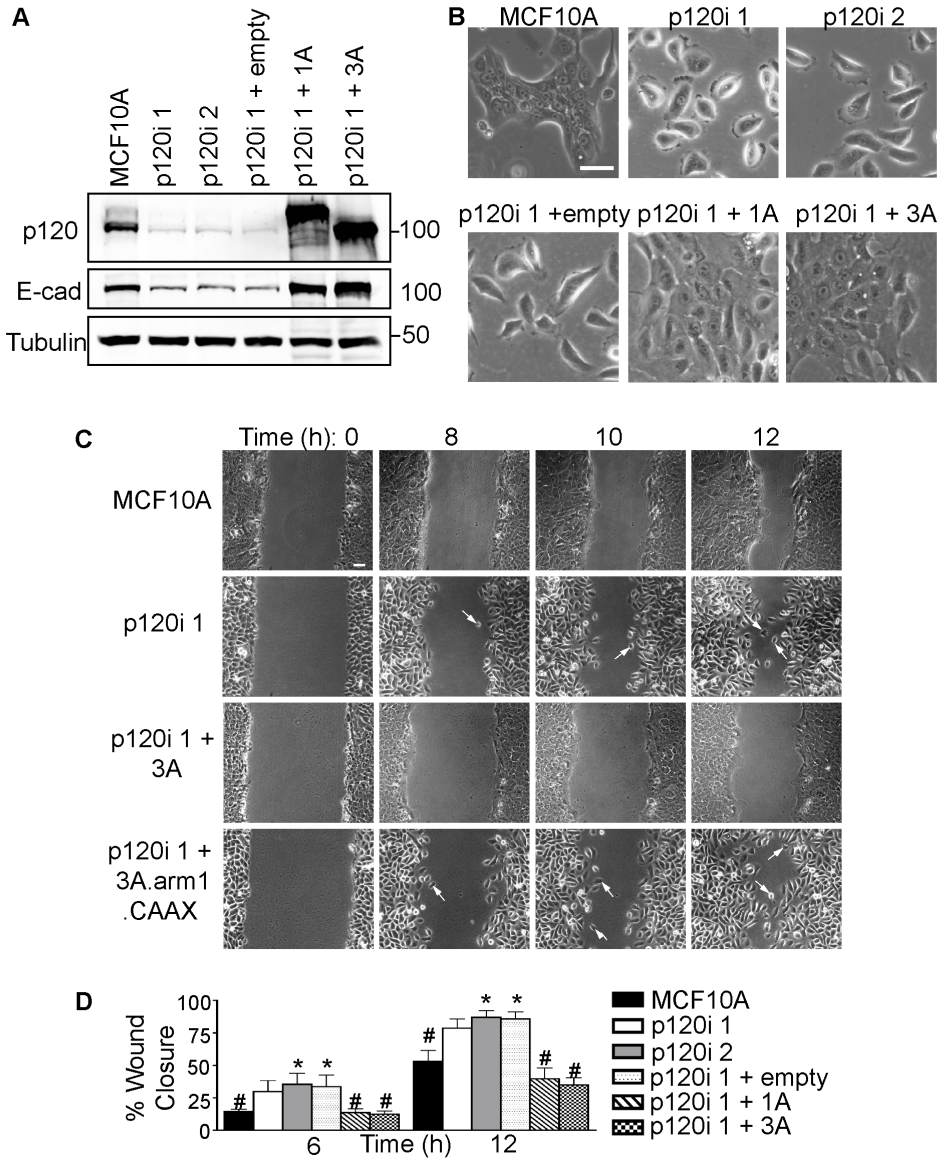


Figure 12. p120 is required for collective migration

(A) Lysates from parental MCF10A cells, p120 knockdown monoclonal lines (p120i 1 and 2) and polyclonal cells expressing control, human p120 isoform 1A, or 3A vectors were analyzed by immunoblotting as indicated (tubulin loading control). (B) 2D morphology. Subconfluent cells were imaged by bright field microscopy (C) Wound healing assays. Images are from time-lapse videos using indicated cell lines. Scale bar = 50 μM. Arrows denote single cell migration events. (D) Quantification of wound healing. Cells from (A) were assayed at 6 or 12 hours. Graph depicts mean and s.e.m. of 5 independent experiments. Mann-Whitney tests: *p<0.05 compared to parental MCF10A cells and #p<0.05 compared to p120i+empty vector.

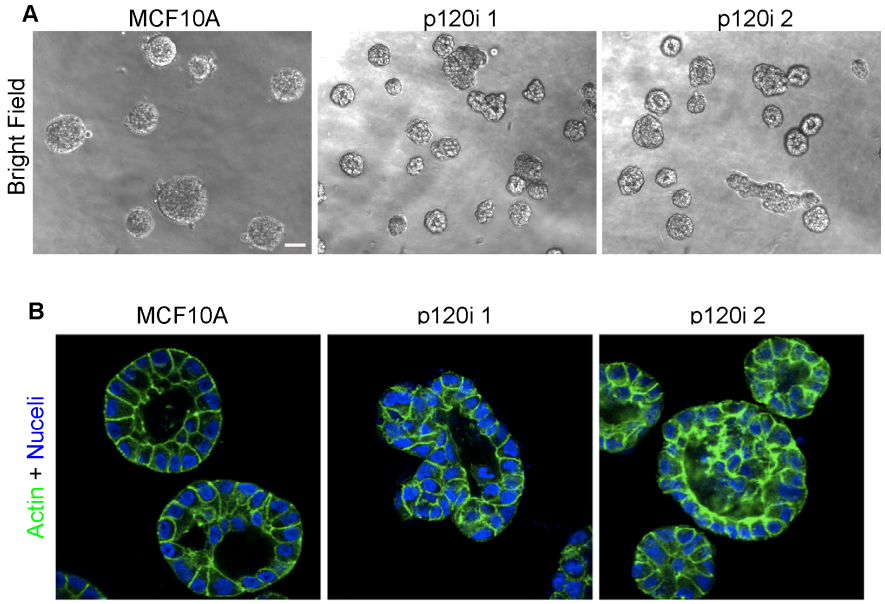


Figure 13. 3D morphology of p120-depeleted MCF10A cells is disrupted
(A) MCF10A cells were grown as mammospheres as described in the methods and imaged using bright field microscopy after 12 days.
(B) Confocal images of mammospheres with and without p120. Mammospheres were stained with Hoechst dye and fluorescently labeld phalloidin to mark actin.

knockdown/addback experiments using wound-healing assays as a read out. Figure 12C shows selected images from time-lapse movies (Fig.12C). As also observed *in vivo*, p120 depletion did not alter cell proliferation (data not shown). However, while TEB outgrowth was delayed in the p120 KO gland, wound closure *in vitro* by p120 KD MCF10A cells was not impaired, and individual p120 KD cells in fact migrated faster than their parental counterparts (Fig.12D). Thus, the delay in TEB outgrowth is not likely to stem from a migration defect per se. Instead, the data suggest that p120-deficient cells simply fail to participate in the collective migration process due to loss of cell-cell adhesion (Fig. 12C).

To determine whether p120's role in collective migration is dependent on cadherin association, we tested a mutant form of p120 isoform 3 that is driven to the membrane by a CAAX box but cannot bind cadherins (3A Δarm1.CAAX) (Wildenberg et al. 2006, Fig.15). In contrast to p120-3A, the mutant did not rescue collective migration, suggesting that p120's role in this process is cadherin-dependent (Fig. 12C). Depletion of either E-cadherin or N-cadherin individually from MCF10A cells did not disrupt collective migration, suggesting that one can stand in for the other. p120 depletion, on the other hand, is effective because it reduces all classical cadherins (Fig.14).

Collectively, these observations predict that branching morphogenesis will be impaired or altogether blocked if p120 is unavailable. Thus, we examined the effects of p120 depletion *in vitro* and *in vivo* on branching morphogenesis (Fig. 16). PMECs were formed into mammospheres and induced to branch using FGF2 (McCaffrey and Macara 2009, Ewald et al., 2008). In the absence of p120, branching was reduced, and often failed entirely (Fig.16A-C). p120 depletion is illustrated in figure 17. Note that when

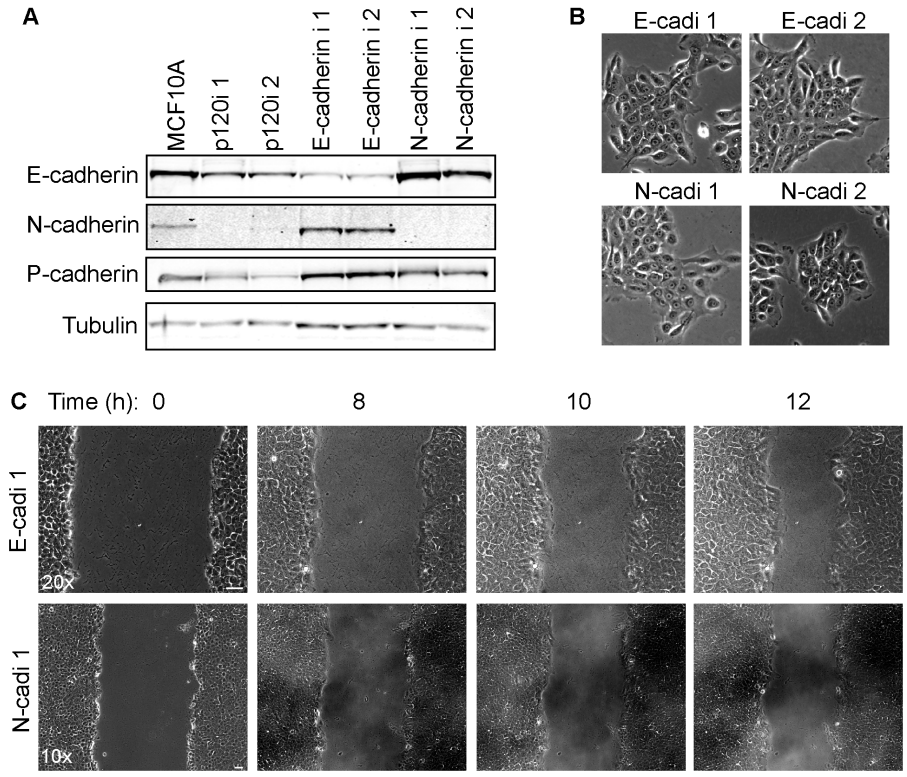


Figure 14. Depletion of either E- or N-cadherin does not disrupt collective migration

(A) Lysates from parental MCF10A cells and two representative monoclonal cells lines from p120, E-cadherin, or N-cadherin knockdown were analyzed by immunoblotting for E-cadherin, N-cadherin, and P-cadherin. Tubulin levels were used as a loading control. Note that while p120 depletion reduces all classical cadherins present, depletion of individual cadherins does not reduce other cadherins. (B) Colony formation is maintained in E- or N-cadherin depleted MCF10A cells. (C) Representative images from time-lapse microscopy of wound healing assays of E-cadherin or N-cadherin depleted MCF10A cells. Scale bar = 50 μM.

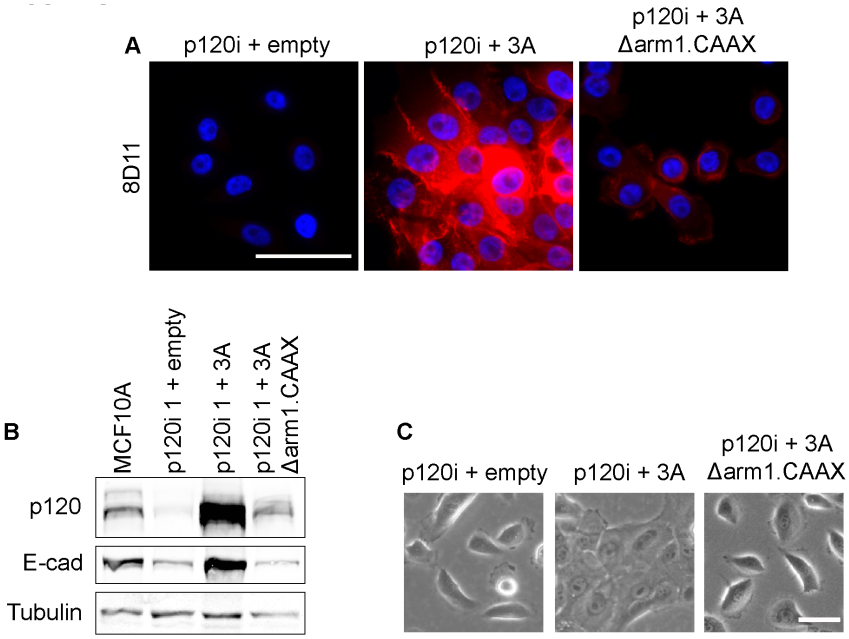


Figure 15. Expression of mutant p120 3A without armadillo repeat 1 in p120 depleted cells does not stabilize endogenous cadherin

A mutant form of p120 that cannot bind cadherins but is driven to the membrane because of CAAX sequences (3A.Δarm1.CAAX) was expressed in MCF10A p120i cells. (A) Expression was confirmed by immunofluorescence with 8D11, an antibody that recognizes only mouse p120. (B) Lysates from parental MCF10A, p120i, p120i expressing isoform 3A, and p120i expressing mutant 3A cells were analyzed by immunoblotting for p120 and E-cadherin. Tubulin levels were used as a loading control. Note that E-cadherin is not stabilized by mutant p120 3A. (C) Colony formation is not rescued by cadherin binding defective mutants. All scale bars = 50 μM.

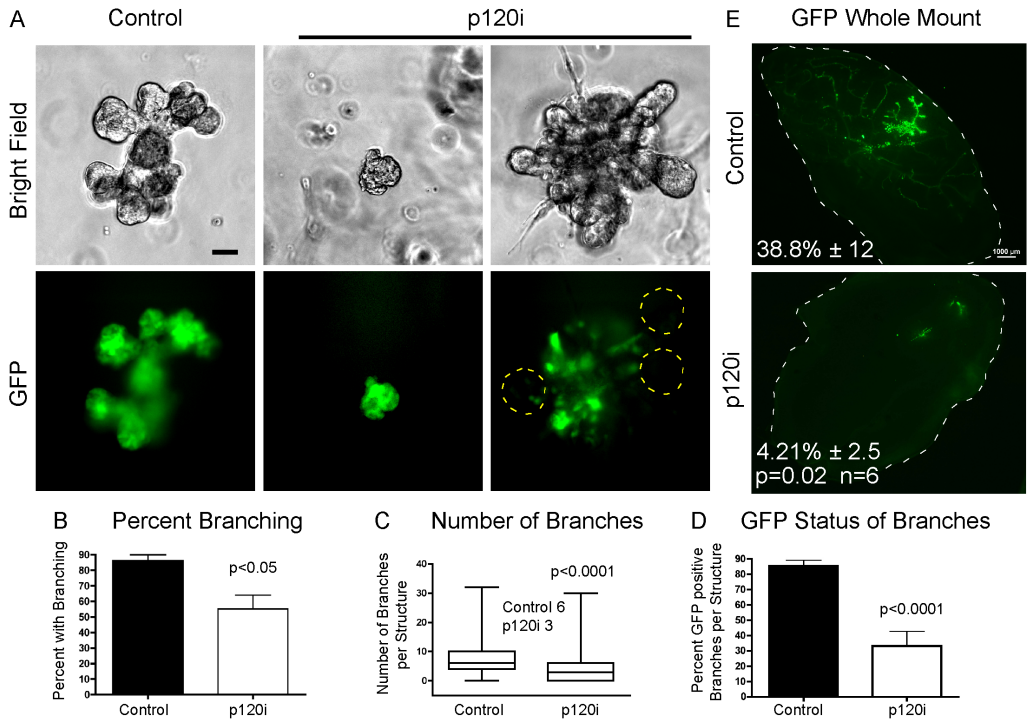


Figure 16. p120 is necessary for in vitro mammary branching and in vivo gland reconstitution

(A-D) PMECs infected with either control GFP or p120i GFP virus were subjected to branching assays as described in the materials and methods. (A) Representative images of control and p120-depleted branched mammospheres. Yellow dotted lines depict absence of p120-depleted cells in TEB-like structures. (B) Quantification of percent branched mammospheres from 5 independent experiments. Student's t-test. (C) Representative quantification of branches per mammosphere. Median values are listed. 3 independent experiments were performed. Student's t-test. (D) Representative graph of the percent of GFP-positive branches per mammosphere. 3 independent experiments were performed. Student's t-test. (E) In vivo mammary reconstitution assays. Representative fluorescent images of whole mounts are shown. Data are mean percent outgrowth +/- s.e.m... Paired t-test n=6.

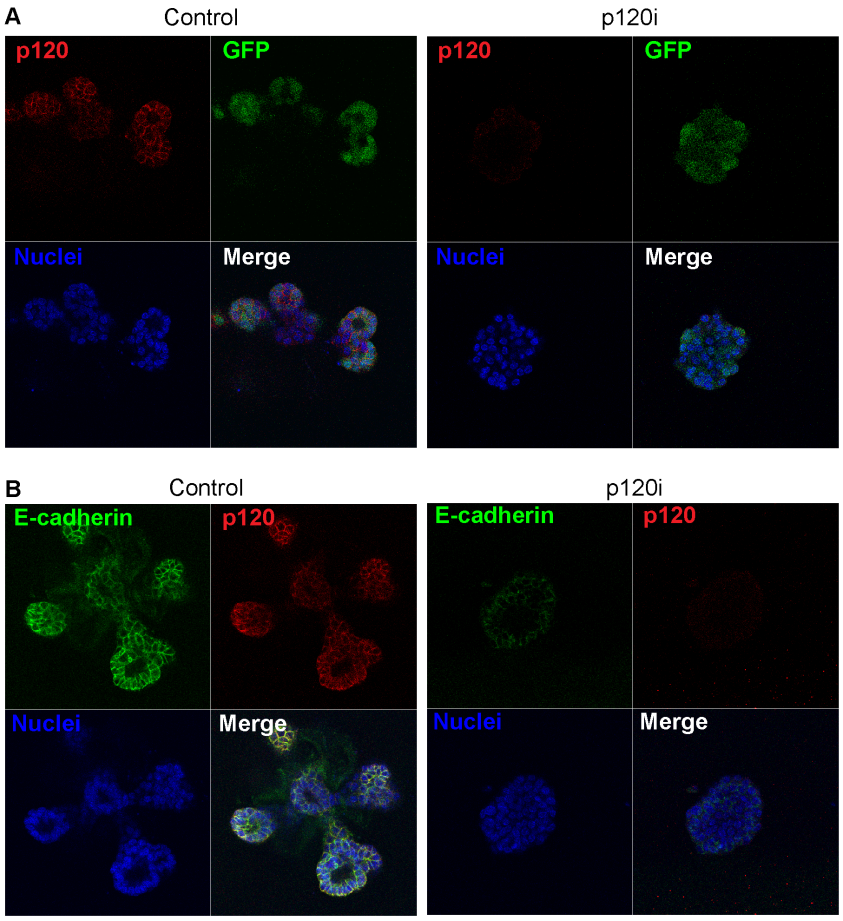


Figure. 17. Depletion of p120 in 3D branching assays of primary mammary epithelial cells

Immunostaining for p120 and E-cadherin was performed on mammospheres induced to branch with FGF2. (A) Confocal images of p120 and GFP in mammospheres expressing control and p120 depletion constructs. (B) Confocal images of p120 and E-cadherin in mammospheres.

branching was observed, p120 was invariably retained (i.e. GFP negative)(Fig.16D). When transplanted into cleared fat pads, p120i PMECs were unable to reconstitute the gland (Fig.16E). Control cell formed clearly identifiable ducts and TEBs, whereas p120-depleted cells manifested as thin strands and small cell groups without discernable structure.

Discussion

The effects of p120 knockout in different organ systems are highly variable. Here, we show that p120 plays an essential role in the morphogenesis of the mammary gland. Ductal architecture is rapidly compromised and p120-null cells disappear altogether within a few weeks. In the TEB, p120 null cells are virtually unable to participate in the dynamic rearrangements required for invasion and morphogenesis. Functional analyses *in vitro* reveal severe defects in cell-cell adhesion and a striking failure of collective migration. Thus, it appears that mammary gland development depends on p120 because the TEB, the main functional unit of mammary development, is effectively disabled by p120 ablation.

In our current mouse model, the severity of the phenotype is largely masked by the mosaic nature of the p120 knockout. The phenotype ultimately manifests as little more than a delay in ductal penetration, but in fact reflects massive sorting and elimination of p120 null cells such that very few remain 3 weeks after p120 ablation. From there on out, the "knockout" gland is essentially p120 positive and morphogenesis proceeds normally. The strong selective pressure for cells that have retained p120 suggests that if the knockout had been complete, the gland would not have formed at all.

Anecdotal evidence from previous studies of p120 ablation in the salivary gland using a different MMTV-Cre mouse suggests that this is, in fact, the case. Indeed, although the vast majority of these animals died shortly after birth, females that survived into adulthood were completely devoid of mammary ductal trees (Davis and Reynolds, 2006). Our *in vivo* PMEC assays confirm these findings, as p120i cells are unable to form a mammary gland (Fig.16E).

Interestingly, *in vitro* p120 depletion in different epithelial cell types results in a wide spectrum of adhesion phenotypes. For example, mammary MCF10A cells separate completely from one another in 2D cultures, whereas similarly cultured MDCK cells lacking p120 form normal appearing colonies that are essentially indistinguishable from parental controls (Fig.12B, Dohn et al., 2009; Simpson et al., 2008). More common is a spectrum of adhesive defects that fall between these extremes (Davis et al., 2003). Similarly, *in vivo* p120 KO phenotypes are surprisingly diverse. For example, although intercellular adhesive defects are not observed after p120 KO in the epidermis, a massive inflammatory response is induced by cell autonomous signaling defects associated with NFkB activity (Perez-Moreno et al., 2006). In the prostatic epithelium, cadherin expression is nearly eliminated by p120 ablation and glandular morphology appears to be virtually unaffected (A.B. Reynolds, unpublished). In salivary gland and intestinal epithelium, cadherin-depletion is more moderate following p120 KO (i.e. ~50% depletion relative to control epithelium), but nonetheless causes obvious adhesion defects with extensive cell shedding (Davis and Reynolds, 2006; Smalley-Freed et al., 2010). Cell- and tissue-specific contexts are clearly critical and contribute along with other factors to the ultimate effect of p120 ablation.

Our *in vivo* data reveal that the TEB is extraordinarily sensitive to p120 ablation. Interestingly, an unbiased *in vitro* RNAi screen for proteins affecting MCF10A cell motility identified both p120 and P-cadherin as central mediators of collective migration (Simpson et al., 2008). The result highlights the often overlooked fact that p120 stablizes all classical cadherins, and implies an activity for P-cadherin that might not be shared by E- and/or N-cadherin (at least in MCF10A cells). Similarly, p120 is required for cadherin-dependent collective invasion in an A431 squamous carcinoma cell model (Macpherson et al., 2007). Eric Sahai's group has recently proposed that collective migration is controlled in part by an E-cadherin/DDR1/Par3-Par6 complex that functions to limit actomyosin contractility as needed at adherens junctions through mechanisms involving p190ARhoGAP and RhoE (Hidalgo-Carcedo et al., 2011). Although not directly included as part of the Sahai model, p120 is likely to play a role. We have previously demonstrated that interactions between p120, RhoA and p190RhoGAP function to limit contractility at N-cadherin-based adherens junctions in NIH3T3 cells (Wildenberg et al., 2006). Thus, one possibility is that p120 functions in the Sahai model as part of the machinery that enables collective migration by suppressing RhoA. Indeed, p120-depleted MCF10A cells are highly contractile and demonstrate readouts indicative of high Rho activity (data not shown). Alternatively, p120-ablation may simply override the normal mechanisms for modulating collective migration by depleting E-cadherin to levels that cannot sustain cell-cell adhesion. These models are not necessarily mutually exclusive. Exactly how E-cadherin levels are controlled by p120 is not well understood and could conceivably be related to novel concepts proposed by Sahai and colleagues.

Although TEB defects associated with p120 ablation could in principle stem from events unrelated to loss of E-cadherin (e.g., dysregulation of Kaiso activity) (Daniel and Reynolds, 1999), the evidence overall points strongly to E-cadherin depletion as the dominant, if not the sole driver of the phenotype. p120 is required for the stability of all classical cadherins, including the E- and P-cadherins found in luminal and basal cells, respectively. Accordingly, E-cadherin neutralizing antibodies selectively disrupt the body cell layer, whereas those for P-cadherin disrupt only the cap cell layer (Daniel et al., 1995). TEB activity stalls in either case, indicating that both layers must be intact for the TEB to function normally. Notably, the cell-cell adhesion defects associated with cadherin blocking are morphologically almost indistinguishable from those induced by p120 ablation, and both mechanisms clearly act through disabling cadherins. Thus, the effects of p120 ablation on cadherin loss are sufficiently severe in the TEB that secondary and/or less obvious consequences of p120 ablation, if present, go undetected. For example, cell polarity proteins interact functionally with cadherin complexes (Qin et al., 2005, Navarro et al., 2005, Zhan et al., 2008), but may be largely disabled in the context of severely compromised cell-cell adhesion.

Surprisingly, p120 family members were unable to compensate for loss of p120, despite evidence that they can effectively rescue cadherin stability and cell adhesion *in vitro* (Davis et al. 2003). Figure 10 illustrates clearly the significant presence of all three family members in p120 KO tissue. It is unclear whether this failure to rescue p120-ablation extends to other organs. In most epithelial tissues, including the epidermis, GI tract and salivary glands, cadherin levels are reduced but not decimated to the extent observed in the mammary gland. In fact, on average, p120 ablated tissues tend to retain

between 25 and 50% of cadherin levels found in control tissue (Davis and Reynolds, 2006; Perez-Moreno et al., 2006; Smalley-Freed et al., 2010). *In vivo* correlations where available appear to support the *in vitro* data in that p120 family members have been found in tissues where p120-ablation does not result in complete cadherin loss (Marciano et al., 2011; Perez-Moreno et al., 2006). However, whether endogenous p120 family members do in fact compensate for p120 loss *in vivo* has yet to be directly demonstrated in any tissue (e.g., by *in vivo* double KO). In the TEB, the near complete absence of both cadherins and junctional β-catenin following p120 ablation indicate that these potential compensatory mechanisms are either insufficient or inactive.

Although p120 knockdown *in vitro* induced severe distortions in MCF10A mammosphere morphology, the cells themselves were healthy and persisted indefinitely. In contrast, p120 ablated cells in the developing mammary gland were rapidly lost and rarely observed past week 6. Interestingly, detached cells were frequently TUNEL and cleaved caspase-3 positive. Thus, while the exact mechanism of cell death is not clear, our detachment and apoptosis data imply a form of anoikis (Wang et al. 2003, Gilmore 2005).

In contrast to several other tissues (Perez-Moreno et al., 2006; Smalley-Freed et al., 2010), we did not observe significant inflammation. It may be that recognition and removal of p120 null cells does not require de novo influx of immune cells. Rather, in the greater scheme of active TEB invasion, efficient removal of p120 null cells by already present tissue-resident macrophages may be sufficiently routine to go largely unnoticed. Tissue-resident macrophages are known to actively participate in TEB-proximal stromal remodeling and were in fact detected at normal levels (Gouon-Evans et

al., 2002; Ingman et al., 2006). Additionally, these cells may also be cleared by neighboring mammary epithelial cells via efferocytosis, a phagocytic process recently shown to be important during involution of the mammary gland (Monks et al., 2008; Sandahl et al. 2010).

In conclusion, we demonstrate for the first time that p120 is essential for mammary gland development. The explanation likely lies in the extraordinary sensitivity of the TEB to p120 loss and the dependence of TEB function on collective migration, a phenomenon based on dynamically regulated cell-cell adhesion. Our work extends previous observations on the role of p120 in collective migration (Hidalgo-Carcedo et al., 2011; Macpherson et al., 2007; Simpson et al., 2008) to a highly relevant *in vivo* setting and is in line with prior anecdotal evidence that mammary development essentially fails altogether in the absence of p120 (Davis and Reynolds, 2006). Given the unique morphogenetic status of the TEB, it will be interesting in future work to extend these studies to p120 KO in breast cancer models as well as fully developed mammary epithelium.

CHAPTER IV

P120 ABLATION POTENTIATES A MOUSE MODEL OF BREAST CANCER, BUT IS PARADOXICALLY REQUIRED FOR METASTASIS

Introduction

p120 is critical for tissue integrity and cell-cell adhesion, disruption of which is associated with diseased states of inflammation and cancer. p120 performs a variety of cancer-relevant tasks in the cell, including cadherin stabilization and modulation of RhoGTPase activity, yet the exact mechanisms of p120 function in the transformed epithelium remains unclear and is likely tissue and context specific. As such, the role of p120 in tumorigenesis and progression is an active area of research.

Alterations in cadherins, the central components of adherens junctions, play a role in breast tumor initiation, progression, and metastasis (reviewed Wheelock et al., 2001). E-cadherin, the predominant cadherin expressed in mammary epithelial cells, is well established as a tumor and metastasis suppressor (Perl et al., 1998, Derksen et al., 2006, reviewed in Birchmeier and Behrens 1994, and in Jeanes et al., 2008). Experimental ablation of E-cadherin leads to increased metastasis in a mouse model of lobular breast cancer and re-expression in E-cadherin-deficient cell lines frequently blocks metastases (Vleminckx et al., 1991, Mbalaviele et al., 1996, Derksen et al., 2006). In contrast, mesenchymal cadherins, such as N-cadherin and cadherin-11, are not expressed in the normal mammary epithelium. They are, however, upregulated in human breast tumors and are likely involved in progression to metastasis (Niemann et al., 1999, Hazan et al.,

2000, Kim et al., 2000, Nagi et al., 2005, Yanagisawa and Anastasiadis, 200, Hulit et al., 2007). For example, forced expression of N-cadherin in either human breast cancer cells or a mouse model of breast cancer leads to increased invasion *in vitro* and metastasis *in vivo* (Hazan et al., 2000, Hulit et al., 2007). This dichotomy between E-cadherin and mesenchymal cadherins is at the heart of epithelial plasticity, namely EMT and the reverse process, MET (reviewed in Kalluri and Weinberg 2009). Recent work has linked EMT to breast cancer stemness and has started to elucidate the specific pathways that control the ability of these cells to colonize distant tissues (Mani et al., 2008, Malanchi et al., 2012). Cadherins are also essential for collective invasion, adding yet another dimension to their role(s) in the metastatic cascade (Reviewed in Friedl and Wolf, 2003). Thus, cadherins, their control of cell-cell adhesion and migration processes, and their regulation of cellular signaling are critical mediators of breast cancer metastasis.

Changes to p120 behavior in carcinomas, including breast cancer, are well documented in the literature (reviewed in Thoreson and Reynolds 2002). In lobular breast carcinomas, p120 is characteristically mislocalized to the cytoplasm in response to E-cadherin loss (Sarrio et al., 2004, others), and instances of p120 upregulation have been documented in Her2 positive and inflammatory breast cancer (Landis et al., 2005, Silvera et al., 2009, Johnson et al., 2010). The most frequent change, however is downregulation, observed in over 50% of human breast ductal carcinomas (Sarrio et al., 2004), and regions of complete p120 loss were reported in approximately 10% of ductal carcinomas (Dillon et al., 1998, Nakopoulou et al., 2002). This downregulation is recapitulated during the progression from adenoma to carcinoma in the MMTV-PyMT mouse model of breast cancer (Kouros-Mehr et al., 2008). In humans, downregulation of

p120 is correlated with poor outcome in patients with invasive breast cancer (Talvinen et al., 2010). However, the importance of p120 downregulation in the overall scheme of breast cancer progression remains unclear.

Evidence exists for p120 acting as both a metastasis promoter and suppressor in the context of breast cancer. Since p120 stabilizes a known metastasis suppressor, E-cadherin, p120 downreguation may promote invasion and metastasis by destabilizing E-cadherin (Behrens et al., 1989, Davis et al., 2003). However, Anastasiadis and others have proposed that the stability of mesenchymal cadherins (e.g. N-cadherin and cadherin-11) is required for acquisition of true invasiveness (Yanagisawa and Anastasiadis 2006, Yanagisawa et al., 2008). According to this model, p120 ablation also reduces mesenchymal cadherin levels, which in turn are essential for invasion and metastasis.

Interestingly, inflammatory breast cancer (IBC) is unique in that its highly metastatic behavior depends on upregulation of p120. In IBC, p120 levels are aberrantly elevated by an IRES-dependent translational mechanism induced by inflammatory stress (Silvera et al., 2009). E-cadherin is upregulated in tandem and resulting in formation of tumor emboli and a highly aggressive form of breast cancer that spreads by a process known as passive metastasis. Tumor emboli colonize other tissues via diffusion through lymphovascular spaces. *In vitro* and *in vivo* work demonstrates that the ability of p120 to stabilize E-cadherin is essential for both metastatic emboli formation and tumor cell survival (Silvera et al., 2009). The IBC phenomenon highlights the fact that disregulation of p120 levels in either direction leads to adverse effects relevant to cancer.

Cadherin-independent functions of p120 may also contribute to metastatic outcome. For example, E-cadherin loss in lobular breast cancer leads to cytoplasmic

localization of p120, which is critical for growth and metastasis by mechanisms involving control of small GTPases (Schackmann et al., 2011). In MDA-MD-231 cells, abrogation of invasion following p120 loss is rescued by expression of constitutively-active Rho, suggesting that p120's inhibition of Rho promotes invasiveness (Yanagisawa and Anastasiadis, 2006). This finding is possibly clinically relevant as overexpression and increased activity of Rho proteins is associated with malignancy in breast (Fritz et al., 1999, Fritz et al., 2002). Thus, p120 can inhibit Rho by several mechanisms and this activity appears to play a role in breast cancer metastasis (Anastasiadis et al., 2000, Wildenberg et al., 2006, Smith et al., 2012).

Non-cell autonomous consequences of p120 ablation (e.g. inflammation) have also been implicated in metastasis. For example, ablation of p120 in the epidermis induces activation of NFkB, secretion of cytokines, and increased infiltration of immune cells (Perez-Moreno et al., 2006, Smalley-Freed et al., 2010, Stairs et al., 2011). These observations illustrate multiple context and tissue-specific roles for p120. Clearly, the consequences of p120 ablation in the context of breast cancer are not entirely predictably and will have to be determined empirically.

Here, we addressed this question directly through mammary-specific p120 knockout in a mouse model of metastatic breast cancer, MMTV-PyMT (Guy et al., 1992, other citations). We find that p120 ablation did not alter tumor growth or latency, but markedly affected tumor morphology. Control tumors were consistently a mix of a compact/solid morphology and pseudopapillary, however, regions of p120 ablation were uniformly pseudopapillary in nature. Furthermore, these areas exhibited macrophage and myofibroblast infiltration, collagen deposition, and reduction of E-cadherin and

junctional β-catenin. p120 ablation directly induced macrophage recruitment and promoted the M2 phenotype, a pro-tumor/metastasis state, likely through a mechanism involving Ccl5 secretion. Although PyMT p120 KO mice demonstrated a significant increase in number and size of pulmonary metastases, p120 null cells were rarely observed in pulmonary metastases, suggesting that p120 null cells in the primary tumor were potentiating metastases in an indirect manner. Indeed, we find that p120 is in fact required for a late stage event in the metastatic cascade, likely colonization. Overall, our data suggest that p120 ablation facilitates metastasis by non-cell autonomous mechanisms, but paradoxically completely blocks cell-autonomous metastasis.

Results

Loss of p120 does not alter latency or tumor growth but affects tumor histology

To directly test the function of p120 during mammary tumorigenesis and progression to metastasis, we analyzed the consequences of p120 ablation on the MMTV-PyMT transgenic mouse line, a well-characterized model of breast cancer known to exhibit consistent pulmonary metastases (Guy et al., 1992, reviewed in Lin et al., 2003). To this end, we crossed our mammary-specific p120 knockout mice to the MMTV-PyMT line ($p120^{f/f}$; MMTV-Cre; MMTV-PyMT hereafter p120 KO PyMT) (Davis et al., 2006). Control mice were defined as floxed p120 mice expressing the Middle T antigen in the absence of Cre recombinase (hereafter PyMT Control). Ablation of p120 in PyMT p120 KO mice was confirmed by immunofluorescence and immunoblot analysis of primary tumors (Fig.18A-B). Expression of MMTV-driven cre recombinase yielded an average of 38.3% p120 negative cells per mouse (Range: 22.6-58.3%) (Fig.18C). All tumors

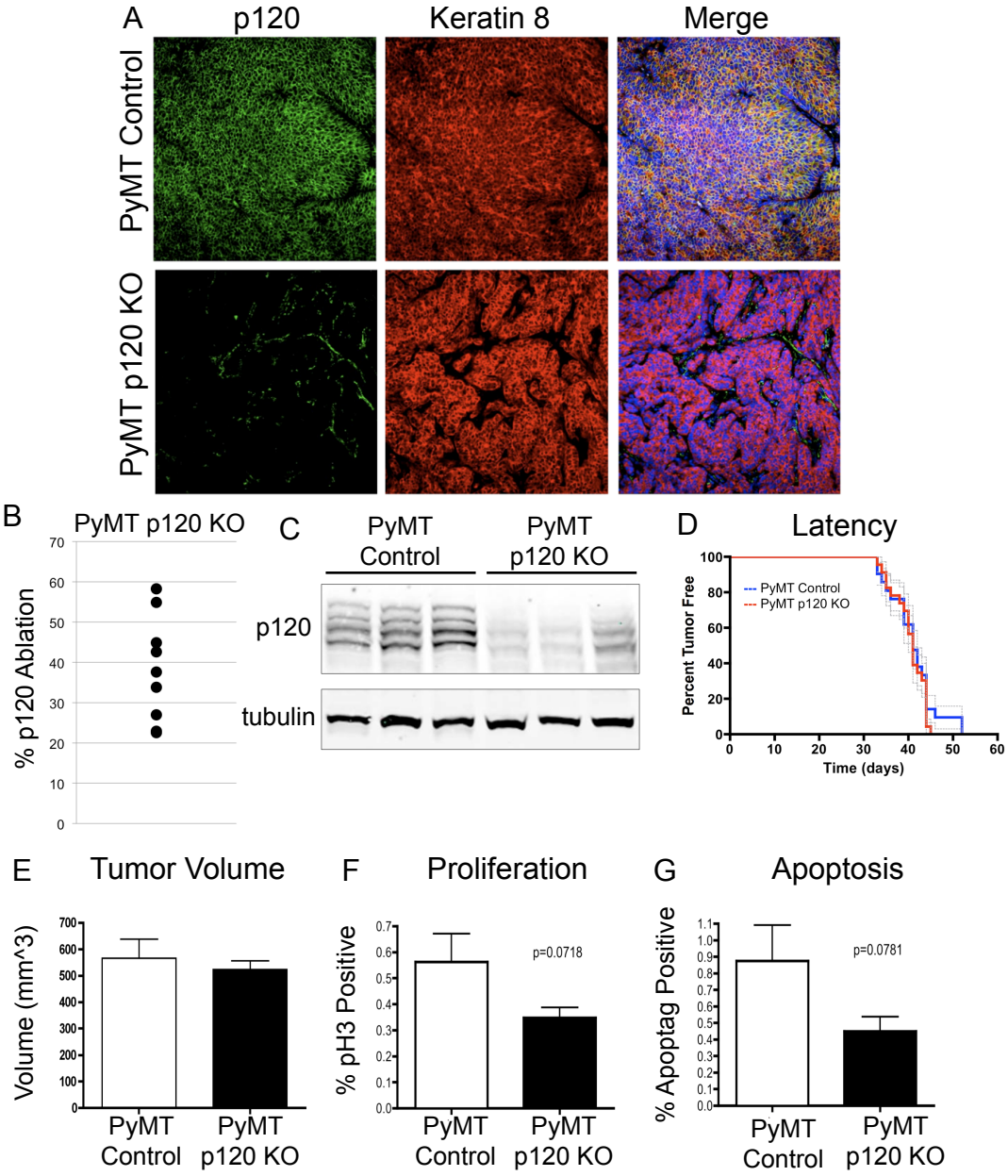


Figure 18: p120 ablation in MMTV-PyMT does not affect tumor latency and volume p120 null cells in knockout but not control mice were identified by both immunoblot (A) and immunofluorescence (B). (C) The amount of p120 knockout per mouse was estimated by immunofluorescent staining of a random sampling of primary tumors from a given mouse. (D) Control and p120 ablated mice were monitored for tumors by palpation. (E) The volume of all ten mammary tumors per mouse were approximated by calipering (h*l*w=approx. volume) 54 days post palpation. No significant difference was observed using a logrank test. (F) To assess proliferation, PyMT tumors were stained for phosphorylated histone H3 by immunofluorescence. (G) To assess apoptosis, TUNEL was performed on PyMT tumors. The percent of positive nuclei was assessed in 3 different pseudopapillary regions per n=6 tumor of each genotype. No significant difference in rates of proliferation or apoptosis was observed.

exhibited some degree of knockout at both early and late stages suggesting that the Middle T antigen is sufficient to rescue the survival of p120 null mammary epithelial cells, which typically do not persist in the context of the developing gland (See Chapter 3).

Primary tumor morphology, but not overall growth rate, was altered by p120 ablation. No differences were observed in tumor latency or final total tumor volume (Fig.18D-E). Furthermore, while there was a trend toward both a decrease in proliferation and apoptosis in regions of p120 negativity, these data were not statistically significant (Fig.18F-G). Interestingly, while there were no gross changes to tumor onset or volume, the morphological characteristics of the p120 null regions were dramatically altered in comparison to controls. In hyperplastic lesions , the differences manifested as cell rounding and sloughing into the lumen (Fig.19A). In later stages (54 days post palpation), p120 null regions were exclusively pseudopapillary, despite the equal distribution of compact and pseudopapillary lesions in PyMT control tumors (Fig.19B).

Given the known role of p120 in adherens junction stability and tissue architecture, we analyzed a number of junctional components by immunostaining. Figure 20 depicts a striking reduction in adherens junction components, E-cadherin and β-catenin, in regions of p120 ablation (Fig.20A-B). Given the ability of p120 family members, ARVCF, p0071, and δ-catenin, to rescue cadherin stability in the absence of p120 *in vitro* (Davis et al. 2003), we hypothesized that family members were completely absent in PyMT tumors (Fig.20C-E). However, immunofluorescence demonstrated that all three family members were indeed expressed. δ-catenin immunostaining appeared to be reduced in regions of p120 ablation, however separate analyses using other available

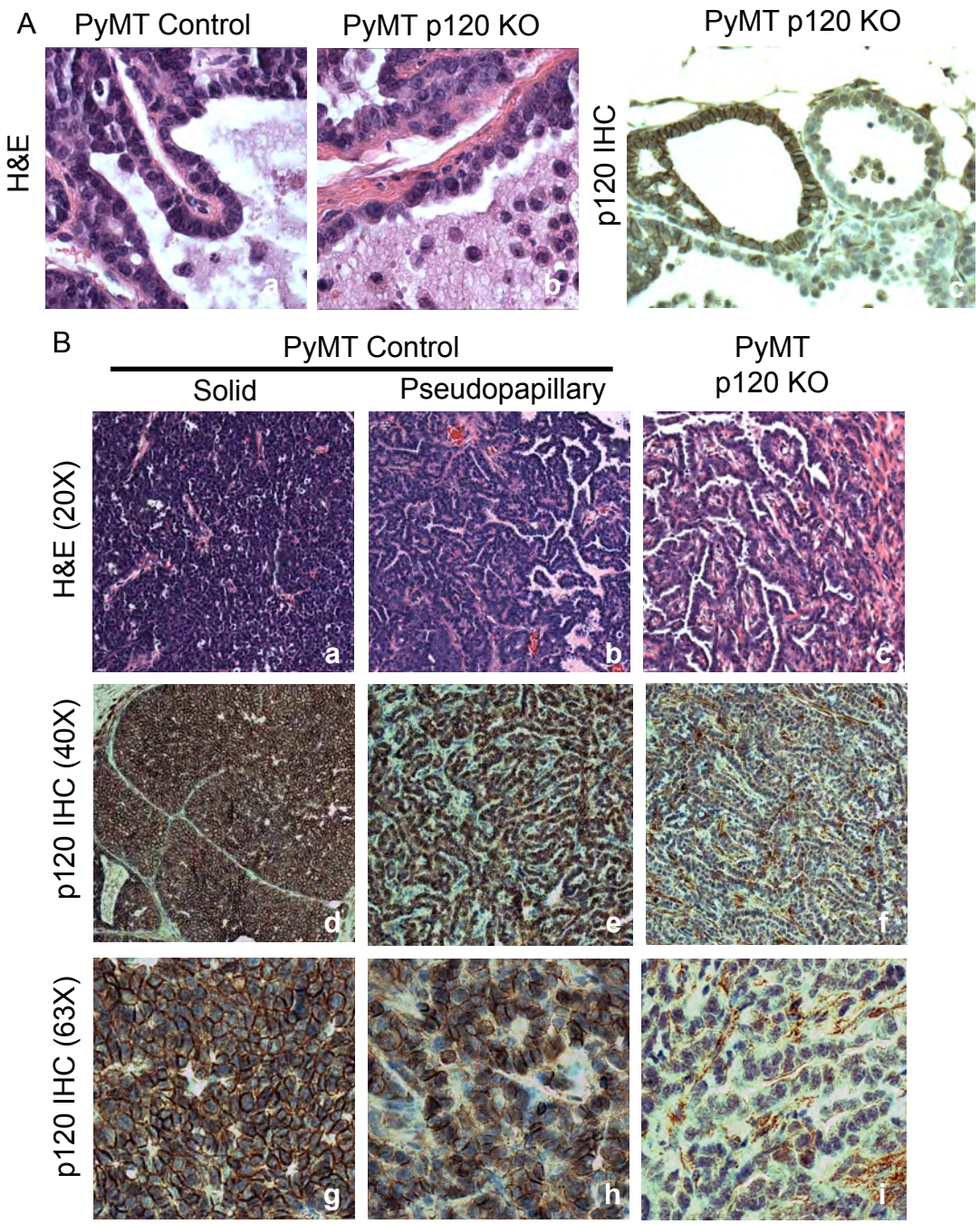


Figure 19: p120 ablation results in cell rounding in hyperplastic ducts and a pseudopapillary phenotype

(A) Hyperplastic ducts from p120 KO PyMT tumors were imaged after hematoxylin and eosin staining or IHC for p120. (B) PyMT tumors were imaged after hematoxylin and eosin staining or IHC for p120. While control mice exhibited both solid and pseudopapillary regions, p120 loss was confined to areas of pseudopapillary or papillary phenotypes

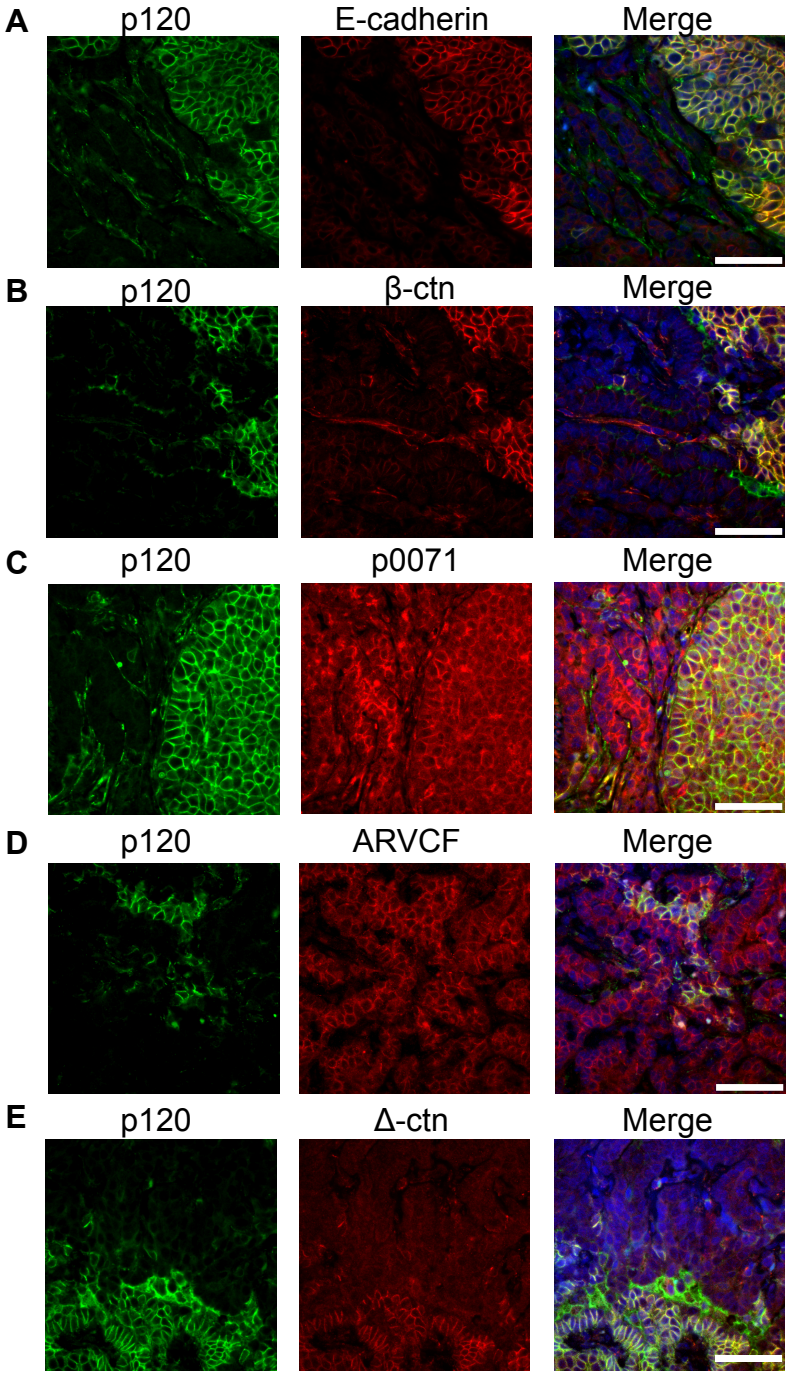


Figure 20. Adherens junctions are disrupted by p120 ablation and family members do not compensate

To assess adherens junctions, PyMT p120 knockout sections were stained for E-cadherin and β-catenin (A-B). Representative images of regions of p120 ablation depict loss of junctional E-cadherin and β-catenin. To assess the presence and localization of p120 family members p0071, ARVCF, and δ-catenin, immunofluorscence using antibodies against these proteins was performed (C-E). Representative images of regions of p120 ablation depict no changes to p0071 or ARVCF. Scale bars = 50 uM

antibodies did not confirm this downregulation (Fig.20E, data not shown). Overall, these data suggest that in the context of PyMT-induced tumorigenesis p120 acts to stabilize cadherin-based structures using a mechanism unable to be performed by other family members.

p120 ablation induces alterations to the tumor microenvironment

Besides changes to the epithelium itself, p120 ablation induced dramatic changes to the tumor microenvironment. To thoroughly analyze the stromal changes first observed via H&E analysis, we utilized flow cytometry and immunohistochemistry to globally and regionally assess different populations of immune cells. Flow cytometry analysis of whole tumor or spleen yielded no statistically significant change in numbers of myeloid derived suppressor, T-, B-, or dendritic cells (Fig.21). However, regions of p120 ablation exhibited increased myofibroblasts and macrophages as monitored by SMA and F4/80 immunostaining (Fig.22A-B). SMA marks many cell types, thus we also analyzed these SMA positive cells for expression of keratin 5. Given that keratin 5 rarely stained positive in SMA positive cells (data not shown), we reasoned that these SMA+ cells were likely myofibroblasts, rather than myoepithelial cells. To assess the endothelium, we utilized antibodies against Meca32 (pan-endothelial), VonWillebrand Factor (macrovasculature) and CD31 (microvasculature). None showed a significant change in regions of p120 loss (Fig.22C-D, data not shown). T-cell numbers were also unchanged as assessed by IHC for CD3 (Fig.22E). Regions of p120 ablation also demonstrated a striking upregulation of collagen as monitored by trichrome blue staining

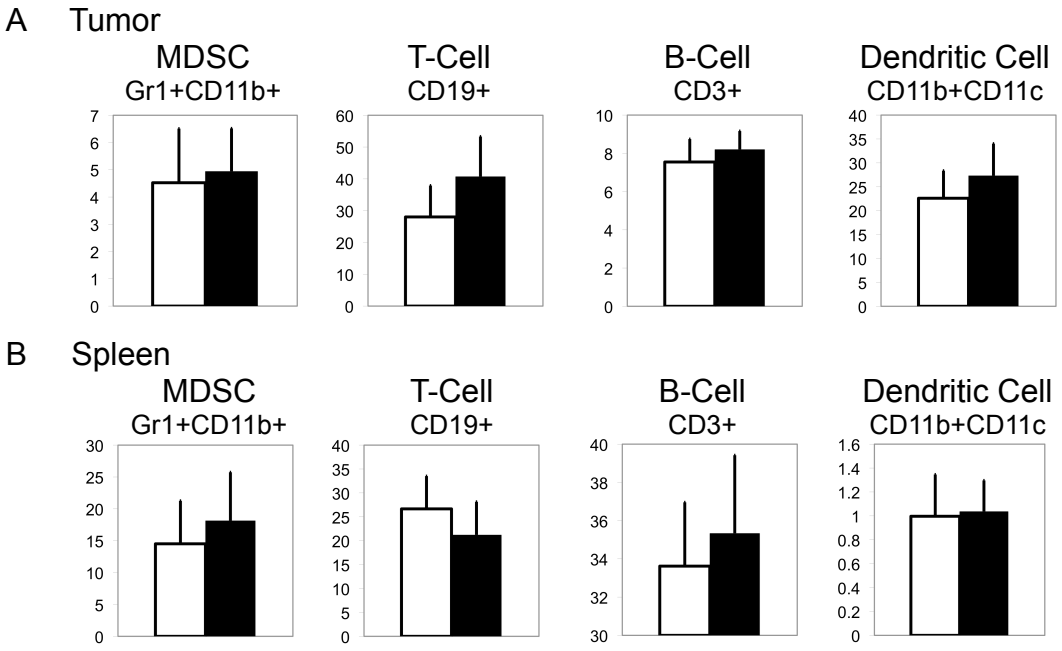


Figure 21. Flow cytometric analysis of PyMT tumors and spleens
Tumor (A) and spleens (B) from PyMT Control (white) and p120 KO (black) mice 30 days post palpation (n=5 per genotype) were analyzed by flow cytometry. All analyses are shown as % positive of DAPI-CD45+ and cell type specific markers listed in Table 3. Mann-Whitney tests comparing control to p120 KO for each cell type yielded no significant differences. Graphs depict mean +/- SEM.

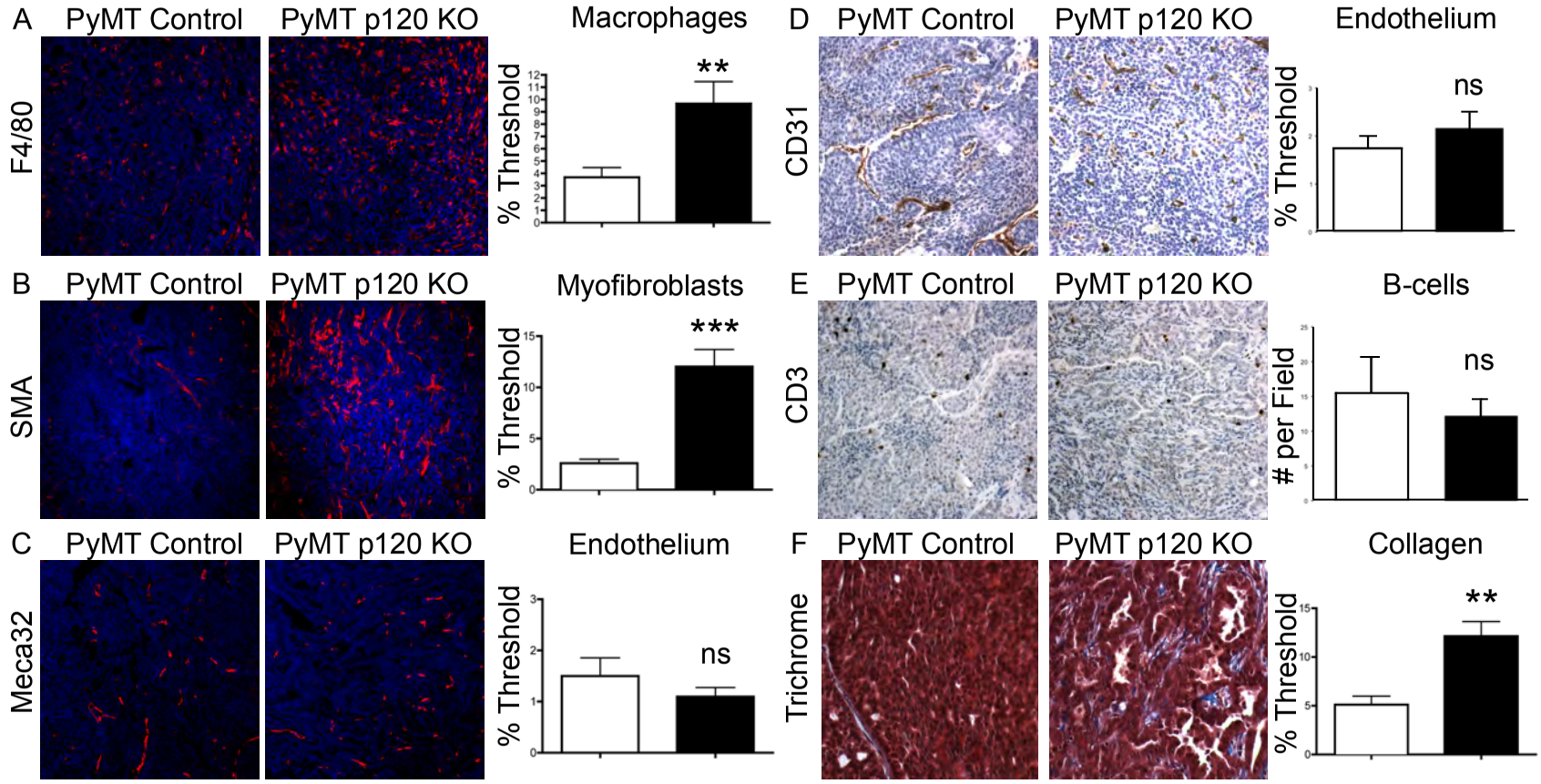


Figure 22: p120 ablation induces changes to the primary tumor microenvironment

PyMT tumors were immunostained for F4/80 (A), SMA (B), or Meca-32 (C) to detect macrophages, myofibroblasts, and endothelial cells, respectively. IHC for CD31 (D) and CD3 (E) was used to detect endothelial and T-cells, respectively. To detect collagen, sections were stained with trichrome blue (F). Mann-Whitney test: ***p<0.0001, **p<0.01, ns= no significance. White bars are PyMT Control and black bars are PyMT p120 KO.

(Fig.22F). Thus, p120 ablation induced specific changes to the mircoenvironment and these alterations occurred in regions of p120 loss rather than globally/systemically.

Macrophages are known to play a role in tumor progression (reviewed in Condeelis and Pollard, 2006, in Joyce and Pollard, 2009), thus, we next analyzed macrophage infiltration and phenotype. To test the effects of p120 ablation on total macrophage recruitment, whole tumors were analyzed by flow cytometry (CD45+, CD11b+, F4/80+). By this method, total macrophage number was unaltered in PyMT p120 KO mice (Fig.23A). Given that macrophage phenotypes of M1 and M2 are associated with anti- and pro-tumor progression, respectively (reviewed in DeNardo and Coussens, 2007), we assessed their phenotype by two different methods. Using flow cytometry of known cell surface markers, there was no change in the number of M1 macrophages, however PyMT p120 KO tumors contained a significant increase in the number of M2 macrophages (Fig.23B). To assess transcript levels associated with macrophage phenotype, RNA from triple positive, CD45+, CD11b+, and F4/80+, tumor macrophages was collected and subjected to real-time PCR analyses. These data demonstrated a statistically significant increase in MMP2 and MMP9, two well-defined markers of M2 phenotype, with a decrease to M1 markers, TNFα, and IL12b (Fig.23B). Thus, by two independent methods, p120 ablation increased the prevalence of an M2 phenotype in tumor-associated macrophages.

To test the possibility that p120 null cells directly recruit macrophages, we analyzed the ability of isolated macrophages to invade through Matrigel-coated filters in response to secreted media from PyMT-derived cell lines manipulated *ex vivo* to express control or Cre vectors. 3- to 10-fold more peritoneal macrophages invaded through

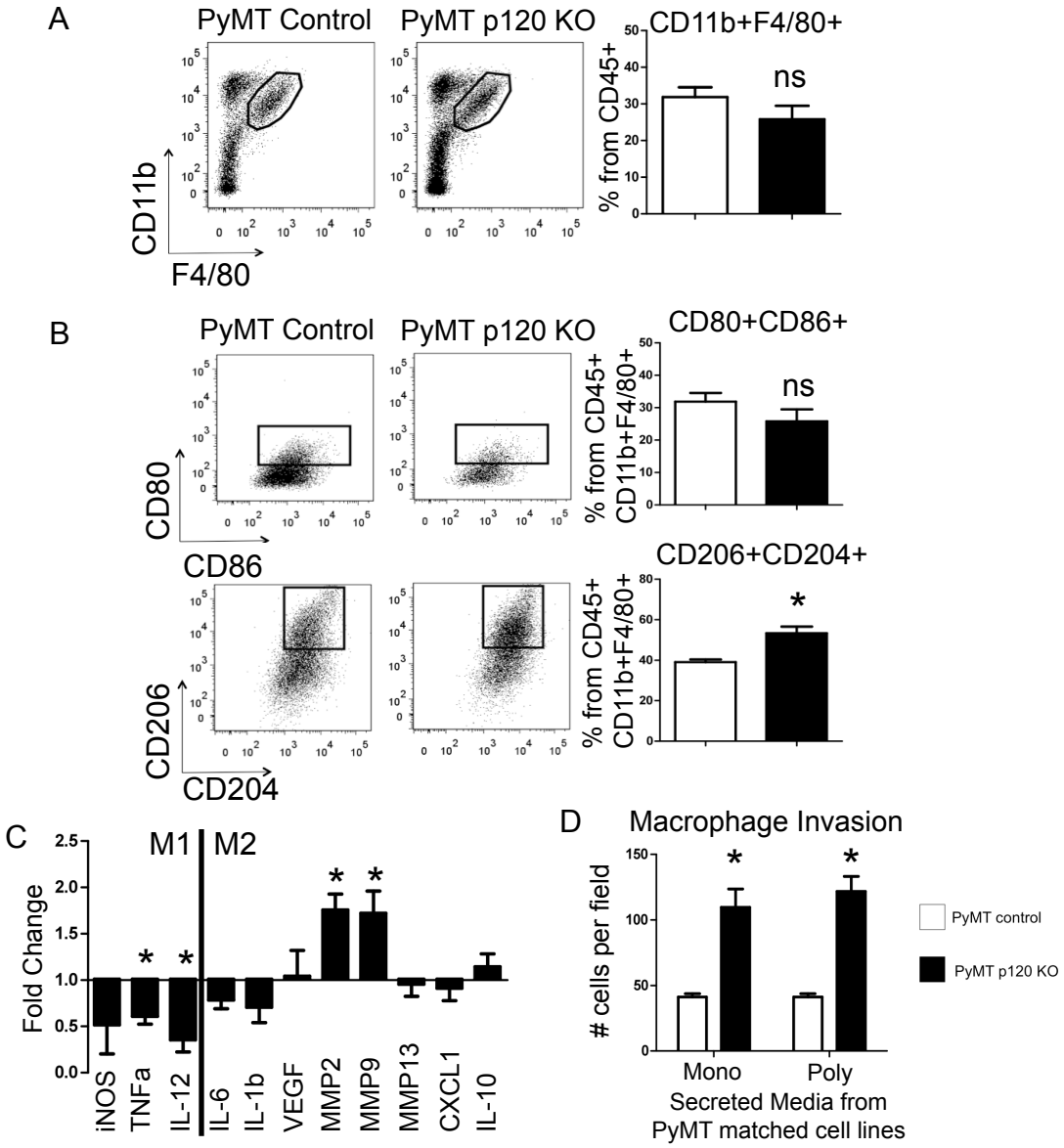


Figure 23. p120 ablation induces M2 macrophage phenotype and macrophage invasion

(A) Representative FACS plots of macrophage numbers in tumor tissue. Plots are gated as CD45+DAPI-, (B) Flow cytometry analysis of CD45+CD11b+F4/80+ cells in tumor tissue. (C) Quantitative RT-PCR on CD45+CD11b+F4/80+ cells from PyMT Control and PyMT p120 KO tumor tissues sorted by FACSAria and converted to cDNA. Data are shown as fold over control. (D) Monoclonal or polyclonal matched PyMT-derived cell lines were grown in 3D cultures. After establishment of colonies, serum free media was added and collected after 24 hours. Peritoneal macrophages were derived and plated onto Matrigel-coated transwells. Macrophages were allowed to invade for 48 hours toward control or p120 KO cell secreted media. This graph is representative of two replicates. 10 fields per transwell were assessed for number of cells per field. p value for Student's t-test.

Matrigel in response to secreted media from PyMT p120 KO cells which suggested that *in vivo*, p120 null cells were more proficient at recruiting macrophages than their wildtype counterparts (Fig.23C). Cytokine arrays performed on secreted factors from PyMT control or p120 KO cells grown in 3D provided a list of candidates for this direct recruitment (Fig.27C). One cytokine identified as upregulated in PyMT KO cells, Ccl5/Rantes, is known to increase monocyte recruitment and facilitate M2 skewing (Azenshtein et al. 2002, Mantovani et al. 2007, Robinson et al. 2003). Thus, these arrays provided likely candidates for the direct effects of p120 ablation on macrophage recruitment and M2 phenotype. In summary, p120 ablation and subsequent changes in cytokine production appeared to directly increase infiltration of macrophages, which are enriched for M2 phenotype, a pro-progression/pro-metastasis state.

p120 ablation potentiates PyMT-induced metastasis, yet its expression is required for pulmonary colonization

Given the striking changes to the epithelium and microenvironment induced by p120 ablation, we hypothesized that p120 ablation alters metastasis. To directly test the effects of p120 loss on breast cancer metastasis, pulmonary tissue from mice 54 days after initial tumor palpation was analyzed for tumor burden. A statistically significant increase in number of pulmonary metastases as measured by lung whole mount was observed in PyMT p120 KO mice (median of 3 in controls compared to 12.5 in KO) (Fig.24A). There was also a striking increase in lung metastasis size in the PyMT p120 KO mice as quantified in H&E stained sections of lung (Fig.24B-C). If p120 ablation potentiates metastases in a cell autonomous manner, it follows that p120 null cells would

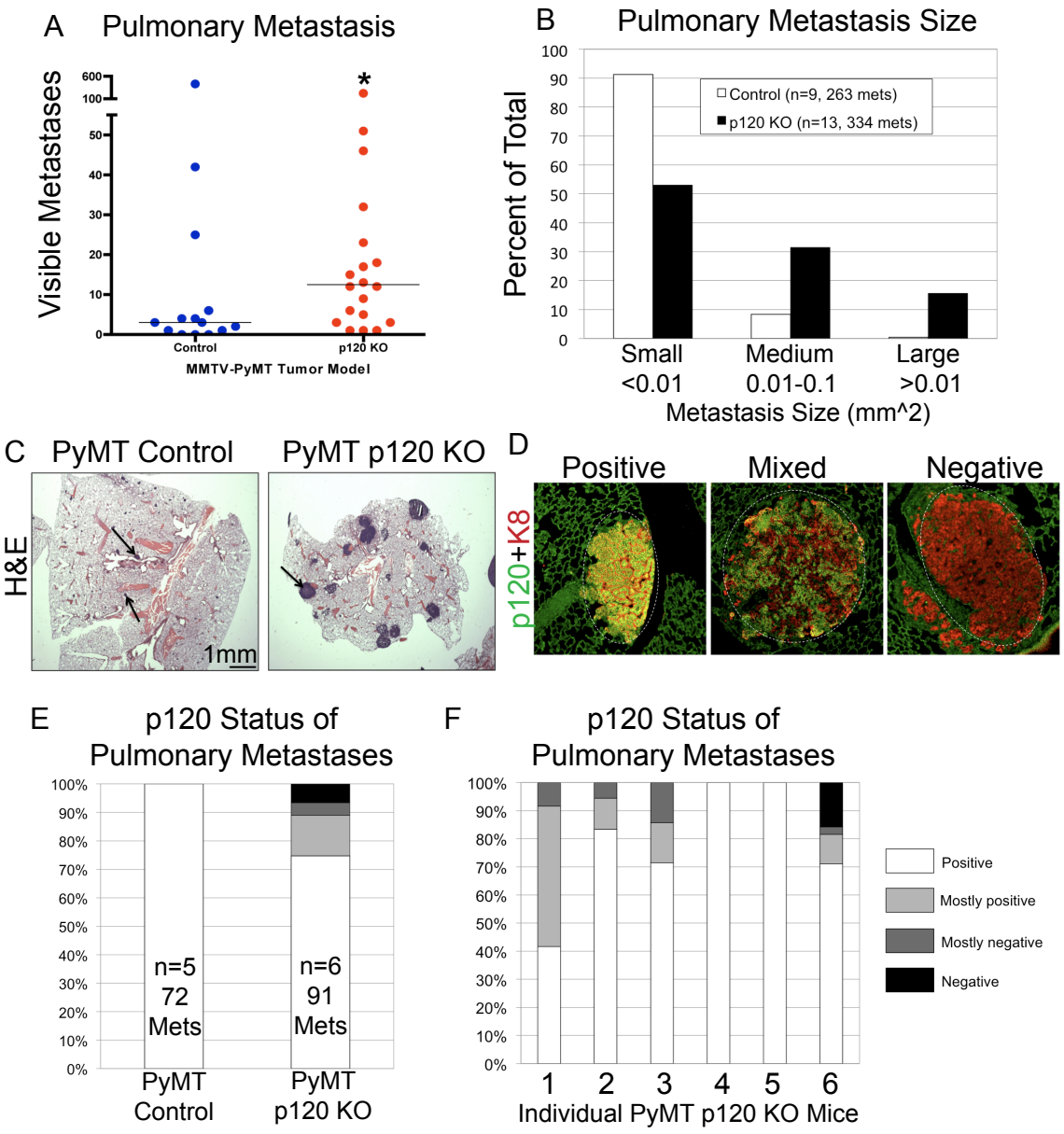


Figure 24. p120 ablation increases number and size of pulmonary metastases

(A) Hematoxylin whole mount analysis and counts of surface lung metastases were performed on mice 54 days post tumor palpation. Metastasis was compared using a Mann-Whitney test *p<0.05 (B) Metastases size was measured on lung sections stained with H&E. (C) Representative sections are shown. Lungs from PyMT p120 knock out mice were stained for p120 and keratin 8 by immunofluorescence. After identification by keratin 8, lung metastases were analyzed for p120 status. (D) Representative examples of pulmonary metastases stained for p120 and keratin 8.(E) Collective data (F) p120 status of lung metastases from individual mice. Almost all metastases from KO mice retained p120.

dominate the lung metastases of PyMT p120 KO mice. In fact, we observed the opposite. PyMT control metastases always retained p120, however PyMT p120 KO mice rarely exhibited wholly p120 null metastases (Fig24D-F). By co-immunostaining for keratin 8 and p120, only 1 in 6 PyMT p120 KO mice demonstrated any completely null pulmonary tumors, and 2 of 6 demonstrated only completely p120 positive tumors. These data suggested that the increase in metastases observed in PyMT p120 KO mice occurred via a non-cell autonomous mechanism, and p120 null cells are themselves metastasis deficient.

To directly test the metastatic capacity of PyMT p120 null cells, we generated matched panels of PyMT-derived cell lines. While variable, a high percentage of cells with total p120 ablation was achieved using retrovirus expressed Cre recombinase (example of 1 such polyclonal line in Fig.25A). This matched pair demonstrated indistinguishable tumor growth kinetics when injected into the orthotopic site (Fig.25B). However, the absence of p120 prevented pulmonary metastases (0% of mice injected with KO cells compared to 77.8% of those with control cells), despite both lines being equally locally invasive (Fig.24C-D). To assess the motility and invasion *in vitro*, wound healing and Matrigel invasion assays were performed with 3 different matched sets of PyMT-derived cell lines. While control cells closed the wound utilizing a mixture of collective and single cell migration, p120 KO cells migrated solely as single cells (Fig.25E). No differences in migratory rate were observed. However, invasion was increased in the absence of p120 thus providing further evidence that p120 is not required for early stages of the metastatic cascade (Fig.25F). In fact, its absence apparently promoted the first steps of metastasis.

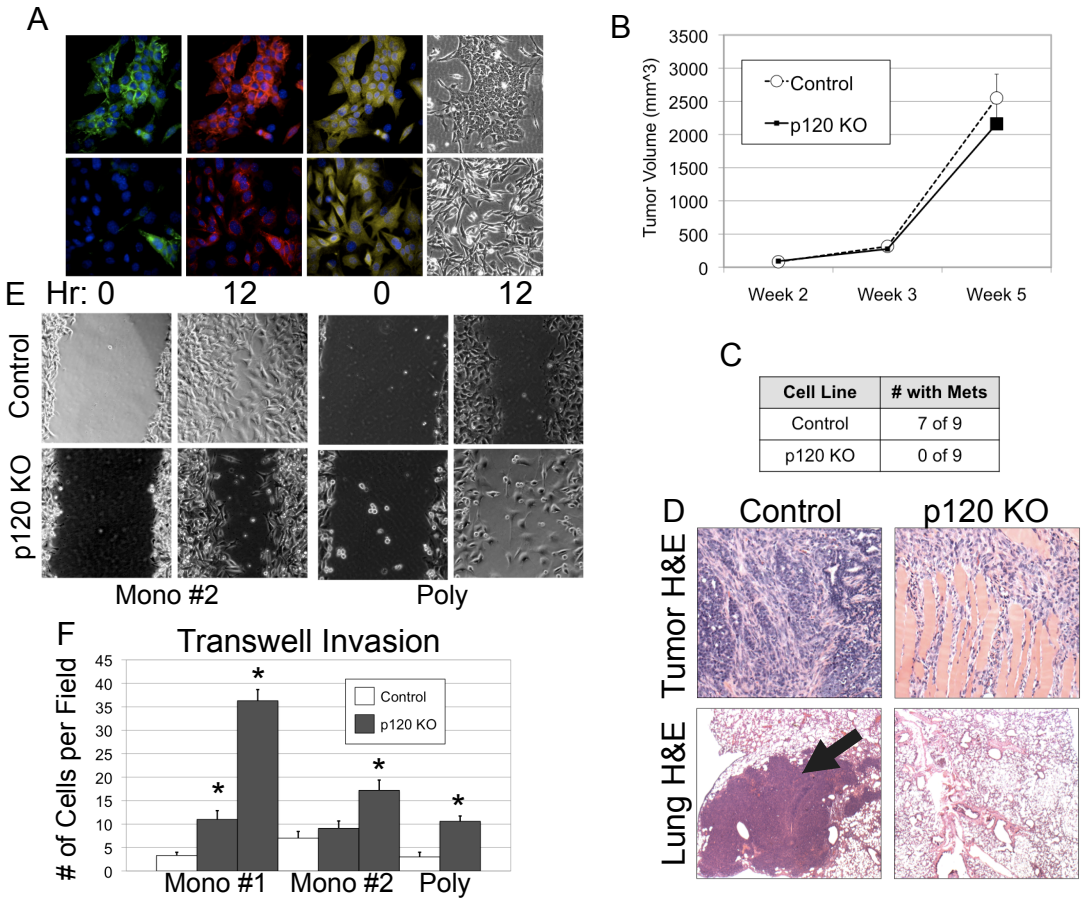


Figure 25. p120 loss induces single cell migration and increased invasion, but inhibits metastasis

(A) PyMT-derived cells were infected with empty or Cre expressing retrovirus to generate control or KO cells. (A) Co-immunofluorescence for p120, E-cadherin, and Keratin 8 confirms a predominantly p120 negative, keratin positive cell line. KO cells are fibroblastic on plastic compared to cobblestone, epithelial control cells. (B) Tumor growth of control and p120 KO cells transplanted into the orthotopic site of nude mice was monitored by calipering (C-D) Pulmonary metastases were assessed by H&E of lung sections. Both control and KO cells are locally invasive when transplanted into the orthotopic site of nude mice. However, Control cells, but not KO cells metastasize to the lungs when transplanted into mammary glands of nude mice. Representative H&E stained sections of pulmonary tissue are shown above. The arrow denotes a metastasis. (E) Matched monoclonal and polyclonal PyMT-derived cell lines were plated to confluence and subjected to wound healing assays. Time-lapse microscopy demonstrates that while control cells migrated with a mixture of collective and single cell motility, p120 KO cells moved only as single cells. Representative images at 0 and 12 hours are shown. (F) Invasion assays were performed using these cells and Matrigel coated transwells. Assays were performed in duplicate and a representative graph is shown. 10 random fields of view per transwell were assessed after 36 hours. Student's t-test *p<0.001

Metastatic deficiency in the absence of p120 could occur at a variety of steps in the metastatic cascade. To identify the exact stage of metastatic deficiency, we performed a battery of experiments to dissect other aspects of the metastatic cascade. First, equal colonies were formed from blood of late stage PyMT control or p120 KO mice which suggested that p120 ablation did not significantly alter circulating tumor cell number (Fig.26C). Furthermore, when assayed for survival in suspension, one requirement of circulating tumor cells, control and p120 KO cells exhibited similar low levels of death suggesting that p120 ablation did not prevent survival in the blood stream (data not shown). To test the ability of p120 null cells to grow in the absence of extracellular matrix, PyMT cells were grown in 3 dimensional agarose. Here, p120 was required for PyMT-mediated anchorage-independent growth. As a positive control, cells were grown in Matrigel and, consistent with our *in vivo* data, exhibited structural but not growth differences in the presence of ECM (Fig.26B). These data suggested that a certain threshold of ECM is required for p120 null cells to grow. Furthermore, the inhibition of anchorage-independent growth, a stringent assay of tumorigenicity, in p120 ablated cells suggested that p120 was required for lung colonization, a form of tumorigenesis. To directly test this hypothesis, tail vein injections were performed with two different control and p120 KO clones. Control PyMT-derived cells reproducibly generated pulmonary tumors indicating that they recapitulated the colonization of the original mouse model (Fig.26D-F). However, p120 KO clones were completely deficient in colonization (Fig.26D-F). Coupled with the observation of a preference for p120 positive cells in the genetic model pulmonary tissue, these assays suggested that p120 was required for the late stages of the metastatic cascade, specifically colonization.

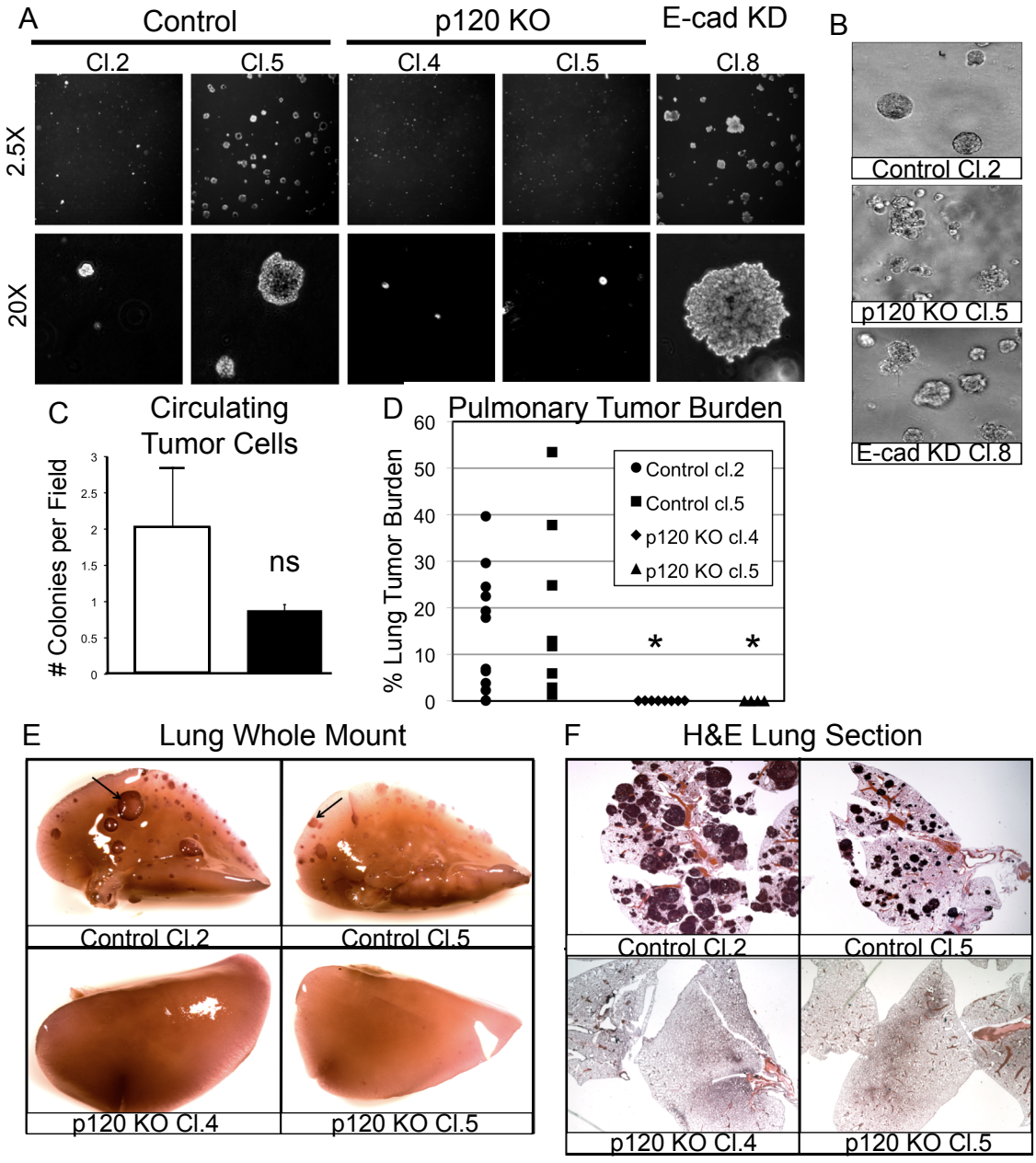


Figure. 26 p120 is required for anchorage independent growth and pulmonary colonization

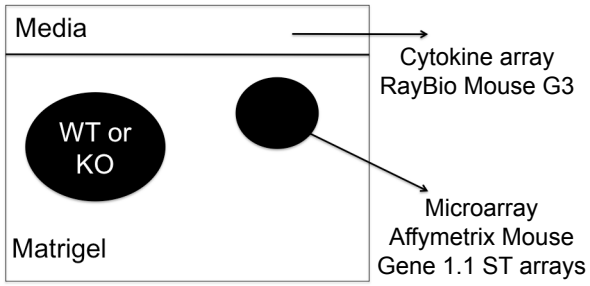
(A) PyMT-derived cell lines were grown in agar for 3.5 weeks. Representative images at 2.5x and 20x magnification are shown. (B) Cells were grown in Matrigel as described in the materials and methods. Representative images are show. (C) Whole blood was plated on matrigel and colony formation from circulating tumor cells was assessed. Graph depicts number of colonies per 2.5x field. Mann-Whitney test n.s. (D-F) PyMT-derived cells were injected into the tail veins of female mice and lung tumor burden was quantified after 4 weeks. (D) Quantification of tumor burden. Mann-Whitney *p<0.01. Representative lung whole mount images (E) and H&E stained lung sections (F).

Finally, we sought to indentify mechanisms for the above-described alterations in microenvironment and metastasis. To identify the changes induced by p120 ablation, mircoarray and cytokine array analyses were performed on RNA and secreted factors from PyMT-derived cells grown in 3 dimensions, respectively (Fig.27A). A list of transcripts that were similarly up or downregulated in 3 different PyMT p120 KO cell lines (2 monoclonal and 1 polyclonal) compared to their respective control cell line is supplied in figure 27B. Independent Kegg Pathway analysis of either the polyclonal or monoclonal set of matched cell lines identified chemokine signaling pathways and cytokine-cytokine receptor interactions as networks whose components are altered in the absence of p120 (Fig.27D-E) was conducted. Furthermore, pathways involved in cell motility and growth, such as axon guidance and ErbB signaling, were identified as being enriched for transcripts. Overall, these data suggested possible mechanisms of p120 ablation-induced phenotypes. Whether these changes are specific to p120 ablation and recapitulate the changes *in vivo* remains to be seen.

Discussion

Here, for the first time, we directly tested whether p120 acts as a metastasis-promoting or -suppressing agent *in vivo* by analyzing p120 ablation in the context of PyMT-driven tumorigenesis. While tumor growth and latency were unchanged (Fig.18), p120 loss significantly increased pulmonary metastases suggesting p120 acts as a metastasis-suppressing agent (Fig.24). However, PyMT-derived p120 null cells were not intrinsically metastatic but rather facilitated the metastasis of cells retaining p120 by a non-cell autonomous mechanism (Fig.25&26). The increased metastasis was likely the indirect result of changes to the microenvironment, namely increased collagen,

A



B

Target	Fold Change for Each Biological Replicate 1	2	3
angptl2	2.68	2.45	5.08
bmp2	2.25	2.94	6.71
bnc1	3.56	2.12	4.04
chchd7	2.16	2.15	2.74
clip4	4.88	3.21	9.92
cpm	5.09	2.15	3.77
cxcl16	3.98	2.02	2.01
dsg3	8.56	3.68	5.95
fgfbp1	7.89	2.27	3.76
Foxq1	4.11	2.31	2.08
jag2	2.25	2.16	2.54
klk14	3.26	2.9	3.4
mmp13	5.83	2.02	16.52
msx2	5.11	3.4	3.13
nav1	3.01	2.83	2.18
nkd2	6.17	3.06	2.17
nrarp	2.7	2.12	4.42
plk3	4.1	2.02	4.22
plxna2	3.49	3.1	2.24
ppic	2.81	2.05	3.69
sdc1	4.37	2.08	2.53
tead4	3.28	2.34	3.48
zswim5	4.51	2.9	2.22
Arhgef6	-7.17	-4.71	-3.58
dcn	-9.97	-7.13	-7.32
foxi1	-3.94	-2.05	-5.75

C

Decreased	Increased
IL12 p40/p70	CCL5
MIP-1a	IGFBP-3
SDF-1a	

D

Pathway	Hits	%	P-Value
Cytokine-Cytokine Receptor Interaction	11	0.5	7.3E-6
PPAR Signaling Pathway	6	0.4	3.5E-5
Chemokine Signaling Pathway	8	0.3	4.2E-4
Prion Diseases	4	0.2	5.8E-4
Hematopoietic Cell Lineage	5	0.2	1.4E-3
Small Cell Lung Cancer	5	0.2	1.7E-3
ErbB Signaling Pathway	5	0.2	1.8E-3
TGF-β Signaling Pathway	5	0.2	1.1E-2
Glioma	4	0.2	2.0E-2
Pathways in Cancer	9	0.4	3.0E-2

E

Pathway	Hits	%	P-Value
Axon Guidance	20	2.2	7.3E-6
Focal Adhesion	24	2.7	3.5E-5
Hematopoietic Cell Lineage	13	1.4	4.2E-4
Jak-STAT Signaling Pathway	18	2.0	5.8E-4
ECM-Receptor Interaction	12	1.3	1.4E-3
Chemokine Signaling Pathway	19	2.1	1.7E-3
Pathways in Cancer	28	3.1	1.8E-3
Regulation of Actin Cytoskeleton	19	2.1	1.1E-2
Nitrogen Metabolism	5	0.6	2.0E-2
B Cell Receptor Signaling Pathway	9	1.0	3.0E-2
Arginine and Proline Metabolism	7	0.8	3.4E-2
GnRH Signaling Pathway	10	1.1	3.4E-2
Melanogenesis	10	1.1	4.1E-2
Gap Junction	9	1.0	4.4E-2
ErbB Signaling Pathway	9	1.0	4.7E-2
Vascular Smooth Muscle Contraction	11	1.2	5.0E-2

Figure 27. List of transcripts and cytokines altered by p120 ablation
(A) Schematic for cytokine and microarray studies with matched PyMT-derived cell lines (B) List of microarray targets consistently altered at least 2-fold in 3 biological replicates. (C) Changes to cytokine secretion in p120-ablated cells compared to control. (D) Kegg pathway analysis of transcripts altered in two monoclonal KO clones compared to their matched control. (E) Kegg pathway analysis of transcripts altered in a polyclonal KO line compared to its matched control.

macrophages, and myofibroblasts (Fig.22&23). Surprisingly, p120 was required for metastasis, specifically colonization, indicating that p120 is actually a metastasis-promoting agent, cell autonomously.

p120 loss is insufficient to disrupt PyMT-driven tumorigenesis

The seemingly contradictory role of p120 in tumorigenesis likely reflects cell-autonomous and non-cell autonomous functions of p120. p120 ablation is sufficient to produce neoplasia in a variety of organ systems and, in some cases, frank carcinoma suggesting that p120 is a *bone-fide* tumor suppressor (Davis and Reynolds, 2006, Perez-Moreno et al., 2006, Perez-Moreno et al., 2008, Smalley-Freed et al., 2011, Stairs et al., 2011). The concomitant inflammation induced by p120 loss in these models likely reflects one underlying mechanism of tumor formation (Perez-Moreno et al., 2006, Perez-Moreno et al., 2008, Smalley-Freed et al., 2011, Stairs et al., 2011). Indeed, inhibition of inflammation by a variety of methods blocks hyperplasia or transition to invasiveness, depending on the model (Perez-Moreno et al., 2008, Stairs et al., 2011). On the other hand, p120 is required for tumorigenesis in other contexts (Soto et al., 2008, Dohn et al., 2009, Silvera et al., 2009, Johnson et al., 2010, Schackmann et al., 2011). p120 depletion in models of lobular, inflammatory, and Her2-driven breast cancer reduces tumor growth (Silvera et al., 2009, Johnson et al., 2010, Schackmann et al., 2011). Furthermore, work from our laboratory and others indicates that p120 is required for anchorage-independent growth, an *in vitro* surrogate for tumorigenesis, in the context of transformation by specific oncogenes (e.g. Src and Rac)(Soto et al., 2008, Dohn et al., 2009).

Despite these previously published works suggesting a role for p120 in tumorigenesis, we observed no alterations to overall tumorigenesis or growth in the absence of p120. Our data suggest that expression of the PyMT oncogene, which activates a multitude of pathways (Dilworth, 2002, Lin et al., 2003), sufficiently overcomes any growth disadvantages that may be posed by p120 loss. However, there was a noticeable absence of morphologically solid/compact tumors without p120. Whether these different lesions (i.e. solid and pseudopapillary) arise from the same cell-of-origin or if their progression is similar has yet to be elucidated. These data may reflect a requirement for p120 in the formation of a specific type of lesion (i.e. morphologically solid/compact carcinoma). On the other hand, p120 ablation may simply lead to the type of tissue organization reflected in pseudopapillary lesions. Depletion of E-cadherin or Scribble, a polarity protein intimately linked to cadherin complexes, also leads to winding, linearized lesions resembling the pseudopapillary regions of PyMT tumors (Qin et al., 2005, Derksen et al. 2006, Zhan et al., 2008). However, no correlation between E-cadherin status and compact versus scattered invasive ductal breast carcinoma was observed in a small set of patient samples suggesting that this finding might be context dependent (Hashizume et al., 1996). Overall, while p120 ablation did not alter PyMT-driven tumor growth, use of other models will be necessary to understand the full breadth of p120's roles in diverse forms and pathways to breast cancer.

p120 ablation facilitates metastasis by potentiating a pro-metastatic microenvironment

Despite the cell-autonomous requirement of p120 for cells to colonize, its ablation potentiates metastasis by fueling a pro-metastatic microenvironment. In a variety of

tissues, inflammation and immune cell infiltration are the consequence of p120 ablation (Perez-Moreno et al., 2006, Perez-Moreno et al., 2008, Smalley-Freed et al., 2010, Smalley-Freed et al., 2011, Stairs et al., 2011). Consistently, in our system, increased collagen, fibroblasts, and macrophages were observed in regions of p120 ablation (Fig.22), changes generally associated with metastatic progression (Reviewed in Joyce and Pollard 2009). For example, p120 ablation in the esophagus and subsequent inflammatory response are sufficient to induce invasive tumors (Stairs et al., 2011). Dexamethasone treatment, an anti-inflammatory and immunosuppressant compound, is able to maintain the lesions as hyperplasias indicating that the immune cells and inflammation directly contribute to progression to invasiveness (Stairs et al., 2011). Thus, it is highly likely that p120-ablation induced changes to the microenvironment in the PyMT model are directly contributing to increased metastasis.

Regions of p120 ablation are associated with carcinoma associated fibroblasts (CAFs) and increased collagen, which are associated with reactive stroma and facilitate metastasis (reviewed in Ronnov-Jessen et al., 1996, in Bhowmick et al., 2004, in Kalluri and Zeisberg, 2006). CAFs promote metastasis through numerous direct and indirect mechanisms (Dimanche-Boitrel et al., 1994, Lochter et al., 1997, Orimo et al., 2005, Grum-Schewensen et al., 2005, 2010). For example, ablation of S100A4, which is produced by fibroblasts, reduces the number of pulmonary metastases of PyMT mice, indicating that factors produced by fibroblasts are able to promote metastasis (Grum-Schewensen et al., 2005, 2010). CAFs also secrete cxcl12/SDF-1, which stimulates angiogenesis and promotes carcinoma cell proliferation, thus contributing to progression (Orimo et al., 2005). CAFs are known to be active fibroblasts, which by definition

produce increased amounts of ECM components, including collagen (Tomesek et al., 2002). A direct link between collagen fibers and *in vivo* carcinoma cell invasion has been identified using elegant intravital imaging studies (Wyckoff et al., 2007). Furthermore, increased tissue stiffness, one consequence of increased collagen and fibrotic stroma, promotes progression to malignancy (Paszek et al., 2005). Thus, the increase in collagen and myofibroblasts (i.e. CAFs) caused by p120 ablation likely contribute both directly and indirectly to metastasis.

Tumor-associated macrophages (TAMs), also increased by p120 ablation, are known to mediate tumor progression by affecting: tumor cell invasion; inflammation; matrix remodeling; intravasation; seeding at distant sites; and angiogenesis (reviewed in Pollard, 2004, Condeelis and Pollard, 2007 and in Joyce and Pollard, 2009). In human breast cancer, TAMs are correlated with poor prognosis (Lin et al., 2002, Bingle et al. 2002). Indeed, elimination of macrophages from the PyMT model abrogates metastasis suggesting that these macrophages are directly involved in disease progression (Lin et al., 2001). Our data indicate that p120 ablation leads not only to the direct recruitment of macrophages, but also their switching to an M2 phenotype (Fig.23D). This provides an likely explanation for the increased metastases in KO mice, as macrophages skewed toward the M2 side of the M1/M2 spectrum are widely accepted to potentiate tumor progression and facilitate metastasis (DeNardo et al., 2009, Reviewed in Mantovani et al., 2007, in DeNardo and Coussens, 2007, in Sica et al., 2008).

p120 ablation stimulates these changes to macrophages, and ultimately metastases, by a mechanism that likely involves NFkB activation, ccl5 secretion, and MMP9 production. Increased *in vitro* invasion by macrophages suggests that ablation of

p120 results in secretion of specific factors that facilitate this process. While several cytokines are altered *in vitro* by p120 ablation (Fig.27), ccl5, one such upregulated cytokine, is known to facilitate macrophage recruitment providing a probable candidate for induction of our observed phenotype (Azenshtein et al. 2002, Mantovani et al. 2007, Robinson et al. 2003). Furthermore, NFkB, a transcriptional activator of ccl5, is itself activated downstream of p120 loss *in vivo* (Wickremasinghe et al., 2004, Perez-Moreno et al., 2006, Stairs et al., 2011), thus suggesting a mechanism of upregulation of ccl5 in our system. In addition to recruitment, promotion of M2 macrophage phenotype may also be the result of ccl5 secretion. Ccl5 treatment of monocyte cell lines upregulates the M2 marker, MMP9 (Robinson et al., 2002) suggesting that macrophage phenotype switching is a direct result of factors secreted by p120 null cells. While we have not directly tested the functions of ccl5 and MMP9 in our p120 knockout mice, previously published work strongly supports their contribution to increased metastases. For example, MMP9 contributes to carcinoma EMT, invasion, and pulmonary metastasis (Hiratsuka et al., 2002, Rizki et al., 2008, Wang et al., 2011- oncogene), and increased MMP9 secretion from either macrophages or endothelial cells may prime the pre-metastatic niche for tumor cell colonization (Hiratsuka et al., 2002). Ccl5 is also able to directly stimulate invasion and metastasis of breast cancer cells (Karnoub et al., 2007). In patients, high CCL5 is part of a signature associated with poor-outcome in IBC patients and is associated with progressive malignancy in breast and cervical cancer (Niwa et al., 2001, Bieche et al., 2004). Treatment with MetCCL5, an antagonist of CCR1 and CCR5, reduces tumor growth and immune cell infiltration (Robinson et al. 2003). Collectively, our data support a model wherein p120 ablation leads to

macrophage recruitment and M2 phenotype, including upregulation of MMP9, by intracellular activation of NFkB and secretion of ccl5 by KO cells (Figure 28). These changes to the macrophage population coupled with increased CAFs and collagen ultimately exacerbate metastasis.

p120 is required for pulmonary colonization

The metastatic cascade describes the steps cancer cells take to achieve metastasis at secondary sites, namely: local invasion; intravasation; survival in the lymphatic or blood stream; extravasation; and growth at the secondary site/colonization (Reviewed in Valastyan and Weinberg 2001). Overall, our data suggest that p120 is necessary for metastasis (Figs.25&26). In some contexts, p120 depletion completely blocks motility and invasion (Wildenberg et al., 2006, Macpherson et al., 2007). However, we observe that p120 ablation increases *in vitro* invasion of mammary carcinoma cells (Fig.25F). The PyMT-derived p120 KO cells are mesenchymal in morphology and behavior while demonstrating decreased E-cadherin, which are markers of EMT (Fig.25). However, this EMT appears to be incomplete since several other EMT markers (e.g., SMA, vimentin, and mesenchymal cadherins) are not altered in the KO cells (data not shown). Despite the lack of complete EMT, p120 ablation is sufficient for, and in fact promotes, early steps of the metastatic cascade (e.g., invasion) in this experimental setting.

p120 loss is clearly incompatible with colonization, the rate-limiting step of metastasis (Fig.25&26)(Luzzi et al., 1998, Aguirre-Ghiso, 2007). While the fate of these cells once in the lung has yet to be identified, recent work challenging the understanding of dormancy and cell survival at secondary sites may provide an answer (Podsypanina et

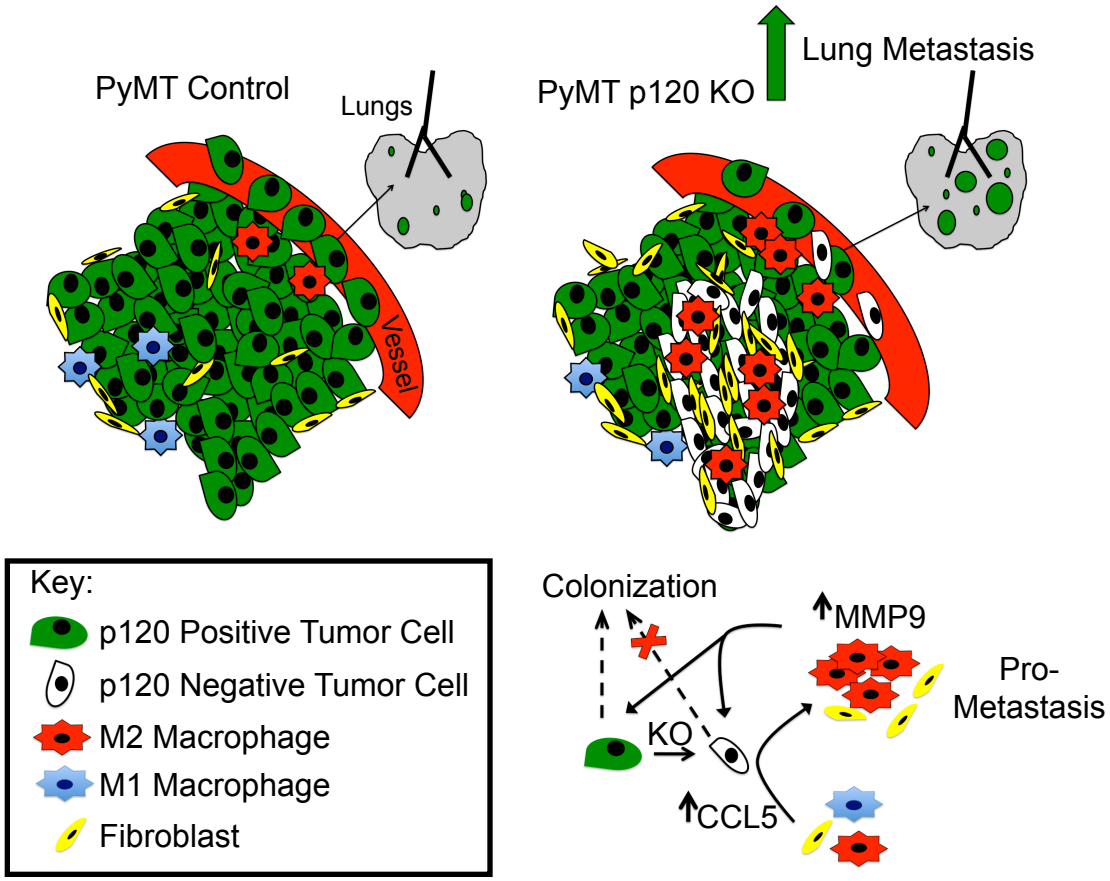


Figure 28. Model of p120 ablation in PyMT-driven metastasis
A schematic for changes induced by p120 ablation. All cell types are labeled in the key. An increase in fibroblasts and M2 macrophages facilitates increased metastases of p120 positive cancer cells in the PyMT p120 KO mice. In the absence of p120, CCL5 secretion is increased leading to more macrophages and phenotype switching to a M2 state. Subsequently, MMP9 production by macrophages is increased leading to increased invasion of cancer cells. However, p120 negative cancer cells are colonization-defective. Overall, while p120 ablation promotes metastases, it is itself required for the final steps in the metastatic cascade.

al., 2008). Surprisingly, normal mammary epithelial cells, if injected intravenously, are able to reside and survive in the lungs for weeks (Podsypanina et al., 2008). Consistently, there is evidence that integrin-mediated proliferation signals are necessary for escape from dormancy by tumor cells in secondary sites (Barkan et al., 2008, Barkan et al., 2010). Thus, given the well-established crosstalk between integrins and cadherins, one possible fate of p120 null cells is dormancy. However, it is also possible that in the absence of adhesion mediated survival signals (Boussadia et al., 2002, Silver et al., 2009), p120 null cells are more susceptible to death once in the lung. A more complex analysis of individual p120 null cells will be necessary to understand their ultimate fate in the lungs. Whether the p120 null PyMT cells are actively dying/being cleared or are simply dormant in the lungs has yet to be directly tested.

Our data highlight an emerging concept that cancer cell plasticity, rather than simply the irreversible acquisition of a particular state (e.g., mesenchymal), may be essential for metastasis. Indeed, many metastases exhibit the same degree of epithelial characteristics as the primary tumor (Jeschke et al., 2007, Park et al., 2007) suggesting that either only epithelial-like cells contribute to the metastases, or MET is taking place. While the former may exist in some contexts (e.g., inflammatory breast cancer and collective invasion), there is a wealth of direct and indirect evidence for the latter in mouse models and humans, respectively (Ahmed et al., 2002, Wyckoff et al., 2004, Jeschke et al., 2007, Park et al., 2007, Wyckoff et al., 2007, Korpal et al., 2011). Cellular plasticity provides the cancer the ability to overcome different obstacles faced during the metastatic cascade.

Given its cadherin stabilizing function (Thoreson et al., 2000, Ireton et al., 2002, Davis et al., 2003), p120 is uniquely suited to be a key mediator of cellular plasticity. In one cadherin-centric scenario, the lack of mesenchymal cadherin stability (e.g. N-cadherin and cadherin-11), and thus incomplete EMT, prevents lodging/outgrowth at the secondary site. In another scenario, a requirement for re-epithelialization (including E-cadherin stability) makes p120 null cells unable to colonize. E-cadherin depletion in our PyMT-derived cells blocks colonization in tail vein assays (data not shown) thus phenocopying p120 ablation and supporting this second scenario. Recent evidence using an isogenic panel of mammary carcinoma lines suggests that only the cells able to re-express E-cadherin (i.e. undergo MET) are colonization-competent (Lou et al., 2008, Dykxhoorn et al., 2009). However, simply overexpressing E-cadherin may be insufficient for MET in the context of p120 loss, as multiple pathways are known to contribute to the epithelial plasticity necessary for colonization (Korpal et al., 2011). Overall, our data suggest that p120 is required for late stages in the metastatic cascade and the inability of null cells to undergo MET prevents their outgrowth at the secondary site.

In this study, we have directly tested the relevance of the observed regions of p120 ablation in 10% of ductal carcinomas. Our findings suggest that these regions are detrimental to overall patient health due to the microenvironmental changes that accompany them. Here we have provided the beginnings of likely mechanism for these alterations (i.e. NFkB and ccl5). However, downregulation and loss may not have the same consequences on breast cancer progression and patient outcome. Perhaps the more clinically relevant discussion is what leads to the downregulation of p120 in over 50% of

ductal carcinoma case and more generally, how cellular p120 levels are regulated. A scattering of reports has started to reveal the mechanisms of p120 level control (see p120 levels section of Chapter 1), however much remains to be elucidated. Future studies will need to focus on understanding regulation of p120 levels and its contribution to breast cancer metastasis. Moreover, these studies will also need to address the cues, both cellular and environmental, that result in the variability of p120's role in pathological processes.

CHAPTER V

FUTURE DIRECTIONS

Introduction

The objective of this work was to test if p120 matters in mammary development and breast cancer. First, I have shown that p120 is essential for mammary development and terminal end bud function. Second, I have demonstrated that p120 is required for PyMT-driven metastasis. These findings have provided foundational observations for the Reynolds laboratory in mammary development and breast cancer research. As such, this research has established the importance of p120 in these areas of research and provided impetus for continued work. Indeed, these initial insights have generated a number of ideas yet to be explored.

Mammary methods for identification of p120 binding partner functions

Branching of mammary glands requires coordination of a variety of processes including, proliferation, apoptosis, and cell motility. These events are closely modeled *in vitro* with PMEC 3D matrigel cultures and perfectly recapitulated *in vivo* with gland reconstitution assays. Moreover, coupled with lentivirus systems, PMECs are easily manipulated to repress or express whatever the target of interest. Overall, this system provides a robust tool to study normal cellular function and dynamic 3D processes.

A particularly interesting application of these techniques is discovery of functions for novel p120 binding partners. To identify novel functions of p120, the Reynolds laboratory has performed a number p120 binding partner screens. One set was performed utilizing cutting edge yeast two-hybrid techniques in collaboration with Hybrigenics and the other using a new technique developed by Dr. Andrew Smith in the Reynolds laboratory, RE-CLIP. After putative binding partners have been confirmed by traditional techniques, one of the main challenges of such data sets is where to begin to look for function. I propose that *in vitro* branching assays provide a good first screen of binding partner function in a normal (i.e., untransformed) setting. Our laboratory possesses a variety of lentiviral systems that, with minimal manipulation, allow for knockdown and addback of any protein of interest and are easily coupled with branching assays. For example, our p120-depleteion experiments shown in Figure 16 are proof-of-concept experiments showing the power of this system. Examination of the effects of binding partner reduction and overexpression, as well as these effects in the absence of presence of p120, is fairly straightforward. If *in vitro* readouts are altered, *in vivo* assays are then performed without the full costs (both financial and temporal) of traditional mouse genetics. By bringing this technology to our laboratory, we are now able to explore and identify the functions of these novel p120 binding partners in relevant, easily manipulated *in vitro*/*in vivo* system.

<u>p120 isoforms and their importance during metastasis and epithelial plasticity</u>

p120 isoform switching often occurs concomitantly with cadherin switching during EMT (Figure 4). In fact, p120 alternative splicing (i.e. p120 isoform switch) is

one of the main targets of the ESRPs, key splicing-regulators of cellular plasticity (Warzecha et al., 2009). FGFR2 switching events occur in parallel with p120 isoform switching and a mouse line that marks the FGFR2 splicing events with fluorescence has been generated (Bonano et al., 2006). Importantly, similar splicing reporter constructs have also been used in prostate cancer cells to monitor epithelial plasticity during metastasis (Oltean et al., 2006). This raises the exciting possibility that the reporter mice and constructs, while directly monitoring FGFR2, could serve as a surrogate for monitoring spatial and temporal changes in p120 isoforms expression patterns.

Some aspects of p120 biology are isoform independent (e.g. cadherin binding), however emerging data suggests that isoform 1 and isoform 3 have different effects on cancer cell function. For example, overexpression of isoform 1A or 3A in lung cancer cells leads to differential effects on invasion, Rho family of GTPase activation, and tumor growth (Liu et al., 2009). Furthermore, expression of isoform 3A in mammary cells is able to repress Zeppo1-induced invasion (and switching to isoform 1) suggesting that this isoform is able to block one downstream effect of EMT driven by Zeppo1 (Slorach et al., 2011). Work in our laboratory by Dr. Michael Dohn indicates that only isoform 1, but not 3, rescues anchorage independent growth, normally blocked by p120 depletion (ABR unpublished results). Collectively, these data indicate that p120 isoforms play different roles in the cell and suggest changes in p120 isoforms is tightly linked with epithelial plasticity. One of the logical next steps in our metastasis project is test whether individual isoforms are able to rescue colonization defects observed in p120 KO cells. Furthermore, cells expressing exogenous isoform 1 or 3 can also be examined by other assays to interrogate the isoform-specific functions during each step of the metastatic

cascade (e.g. transwell assays). Once these experiments have identified isoform-specific roles, we can begin to address a variety of downstream questions, such as: What is the minimal structural requirement to confer isoform 1-specific functions? Are isoform 1-specific binding partners, recently discovered in the Reynolds laboratory, part of the isoform 1-driven event? Do isoform-specific events require specific cadherins (e.g. isoform 1 and mesenchymal cadherin pairing)?. Ultimately, our understanding of isoform-specific functions would benefit from a mouse expressing only isoform 1 or 3 downstream of the endogenous p120 promoter. Mammary-specific knockin mice expressing N-cadherin under the endogenous E-cadherin promoter have been generated by the Kemler group and could serve as a paradigm to achieve isoform specific expression (Kotb et al., 2011).

Regulation of p120 levels

It is increasingly clear that p120 levels matter for normal cellular function and are usurped by disease states (see Introduction). However, studies identifying the mechanisms surrounding these level changes are a rarity. As demonstrated by Silvera and colleagues, the first step to identifying novel mechanisms of regulation is to find a scenario where p120 levels are altered (e.g. IBC) (Silvera et al., 2009). Therefore, I propose using two systems to monitor p120 expression changes during both normal mammary branching and the different steps of the metastatic cascade. First, to monitor changes in transcription, I suggest using reporter constructs with luciferase or GFP expression downstream of the p120 promoter. Second, to monitor type and relative amount of translation of p120, I suggest employing previously described bicistronic

reporter constructs that express cap-dependent Renilla luciferase and firefly luciferase downstream of the p120 5'UTR (Silvera et al., 2009). These two systems can be monitored during a variety of assays described herein. Establishing the times or events (e.g. during branch budding) when changes in transcription and translation of p120 occur will allow for study of the mechanisms driving these changes.

Clinical Relevance

p120 itself is a poor drug target as it is ubiquitously expressed and lacks enzymatic activity or cell-surface expression. However, p120 status of the breast tumor is likely to prove useful for clinical decisions. Low levels of p120 are correlated with poor outcome of breast cancer patients (Talvinen et al., 2010) and regions of p120 loss in mouse models induce a pro-metastatic microenvironment (chapter 4) suggesting that patients with low p120 expression should be monitored for progression more closely. Furthermore, understanding the pathways both up and downstream of p120 and how they are altered in specific types of breast cancer may also lead to identification of new drug targets for breast cancer. Finally, since metastasis is the leading cause of mortality in patients, it is critical that we understand the processes that cause this endpoint. p120 is at the heart of all of the known ways that cancer cells metastasize. As such, by understanding p120, we begin to discover at least part of how metastasis occurs and, perhaps, we can identify new ways to specifically inhibit it.

APPENDIX A

KAISO IN THE MMTV POLYOMA MIDDLE T MOUSE MODEL OF BREAST CANCER

Introduction

Kaiso family members, ZBTB4, BCL-6, PLZF, and HIC-1, are implicated in initiation and progression of cancer (reviewed in van Roy and McCrea, 2005). For example, transgenic mouse models that force the same translocations of Bcl-6 observed in humans, exhibit B cell lymphomas, thus presenting direct evidence that Bcl-6 translocations are causative in B cell lymphomas (Pasqualucci et al., 2003, Baron et al., 2004, Cattoretti et al., 2005). Furthermore, inhibition of Bcl-6 successfully treats a mouse model of chronic myeloic leukemia by eradicating leukemia initiating cells (Hurtz et al., 2011). Translocations involving PLZF are also associated with leukemia (Chen et al., 1993). Kaiso family member, HIC-1 is also proposed to act during tumor progression, as it is hypermethylated and underexpressed in tumor cells resulting in a state of resistance to DNA damage-induced p53-mediated apoptosis (Wales et al., 1995, Fujii et al., 1998, Chen et al., 2005). Heterozygous knock out of HIC-1 and subsequent epigenetic silencing of the other allele results in carcinomas in male mice and sarcomas or lymphomas in female mice suggesting a tumor suppressive role for HIC-1 (Chen et al., 2005). Finally, Kaiso's closest family member, ZBTB4 is decreased in human tumors and its high expression is correlated with cancer patient survival (Weber et al., 2008, Kim et al., 2011). ZBTB4 transcriptional control of p21 expression likely contributes to its

effects on tumor progression but the exact mechanism is unclear (Weber et al., 2008, Yamada et al., 2009).

Kaiso itself is also postulated to be involved in tumor progression, however the exact role of Kaiso in cancer remains unclear. Kaiso represses targets known to facilitate metastasis (e.g. matrilysin) and to promote tumor formation (e.g. non-canonical Wnts) suggesting a tumor suppressive role for Kaiso. However, Kaiso null mice exhibit reduced tumorigeneicity in the context of the APC^{Min} mouse model of colon cancer suggesting a tumor-promoting role for Kaiso (Prokhortchouk et al., 2006). Furthermore, a recent study shows that Kaiso represses tumor suppressor genes in a methylation-dependent manner, thus Kaiso may be selectively acting as an oncogene only in areas of hypermethylation (Lopes et al., 2008). Collectively, while these data provide hints that Kaiso and its family members are important for tumor progression, their exact functions in this process have yet to be discovered.

Results

Kaiso, a p120-binding partner, was found to be upregulated in mouse models of colon cancer (ABR unpublished results, Prokhortchouk et al., 2006). Therefore, we tested whether this upregulation was observed in other mouse models of cancer. Indeed, immunofluorescent analysis for Kaiso in the PyMT mouse model of breast cancer demonstrated a striking upregulation of Kaiso in early hyperplastic lesions compared to normal mammary ducts (Fig.29A). This strong nuclear staining was upheld in late carcinoma lesions (Fig.29B). The polyclonal antibody utilized was shown to be specific to Kaiso since null tissue does not produce a detectable signal (data not shown). Given

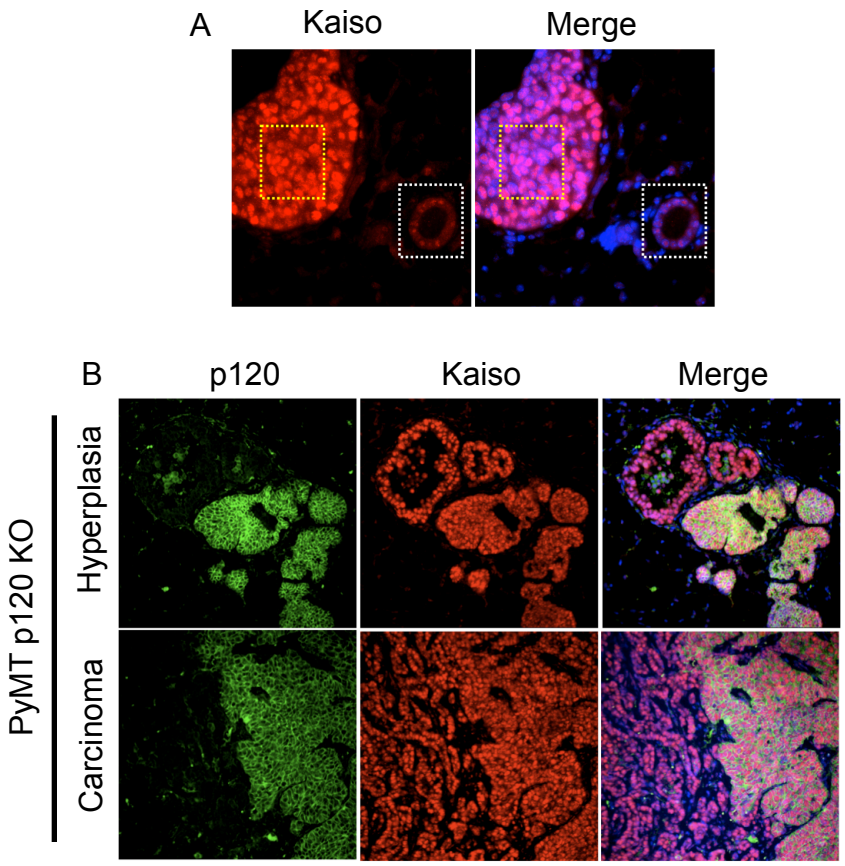


Figure 29. Kaiso is upregulated in the PyMT mouse model
(A) Immunofluorescence for Kaiso demonstrates an increase in PyMT-induced lesions. Yellow=hyperplasia White=normal duct
(B) Immunofluorescence staing for p120 and Kaiso in PyMT p120 KO tissue. p120 ablation does not alter localization or intensity of Kaiso staining

that Kaiso is a p120 binding partner, we analyzed PyMT p120 knockout tissue to test if p120 ablation affected the pronounced nuclear Kaiso signal see in wildtype lesions. However, loss of p120 does not affect Kaiso localization or strength of immunofluorescent signal (Fig. 29B).

To directly test the effect of Kaiso loss on PyMT tumor progression, we utilized a PyMT-derived cell line, 17L3C (a kind gift from Dr. Barbara Fingleton), *in vitro* and *in vivo*. First, we confirmed that the strong nuclear Kaiso observed in the mouse model was recapitulated in 17L3C (Fig.30A). Then, Kaiso depletion was achieved *in vitro* using three different shRNA constructs targeted to mouse Kaiso (Fig.30B). Growth capacity of these cells was assessed *in vitro* and *in vivo*. Loss of Kaiso did not alter cell growth on tissue culture plastic and tumor growth in the mammary fat pad was reduced in one of three lines (Fig.30C). At first glance, these findings suggested that Kaiso loss did not consistently reduce growth. However, analysis of tumor lysates demonstrated re-expression of Kaiso *in vivo*, thus making it difficult to come to strong conclusions.

Given this selection for re-expression of Kaiso, we needed to switch to a system unable to revert back to wildtype. Therefore, to achieve this, we crossed Kaiso null mice with PyMT mice (Guy et al., 1992, Prokhortchouk et al., 2006). These crosses gave rise to PyMT;Kaiso$^{+/+}$ (Control), PyMT;Kaiso$^{+/-}$ (Kaiso Het), and PyMT;Kaiso$^{-/-}$ (Kaiso null) mice. Bi-weekly palpation of these cohorts revealed a trend toward increased time to tumor onset with decreased Kaiso suggesting an inverse relationship between Kaiso and days until detectable tumor formation (Fig.31A). However, these data were not statistically significant. Furthermore, final tumor volume at 54 days post palpation was unchanged in either Kasio heterozygous or null mice (Fig.31B). Consistently, there was

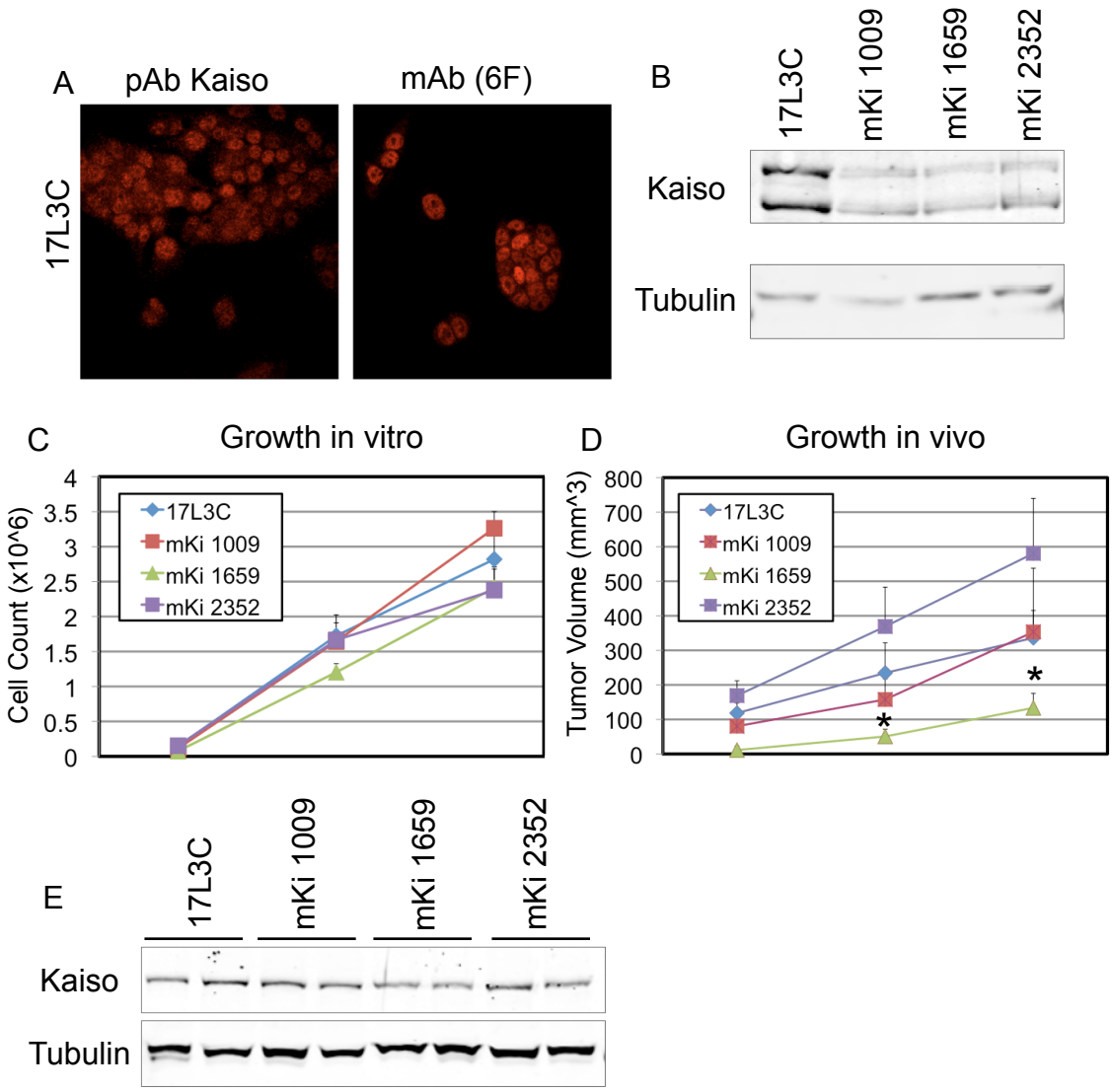


Figure 30. Kaiso depletion is selected against in vivo

(A) Immunostaining for Kaiso in PyMT-derived 17L3C cells using two different antibodies. (B) Immunoblotting for Kaiso. Depletion of Kaiso was achieved using three different shRNA constructs. (C) Cell growth in vitro. Cell number was assessed in triplicate. No differences in overall growth were observed. (D) Cell growth in vivo. Cells were transplanted into mammary fat pads and calipered at the time points indicated. Mann-Whitney test *p>0.05. (E) Immunoblotting for Kaiso from tumor protein isolates. Note the re-expression of Kaiso in tumors in vivo.

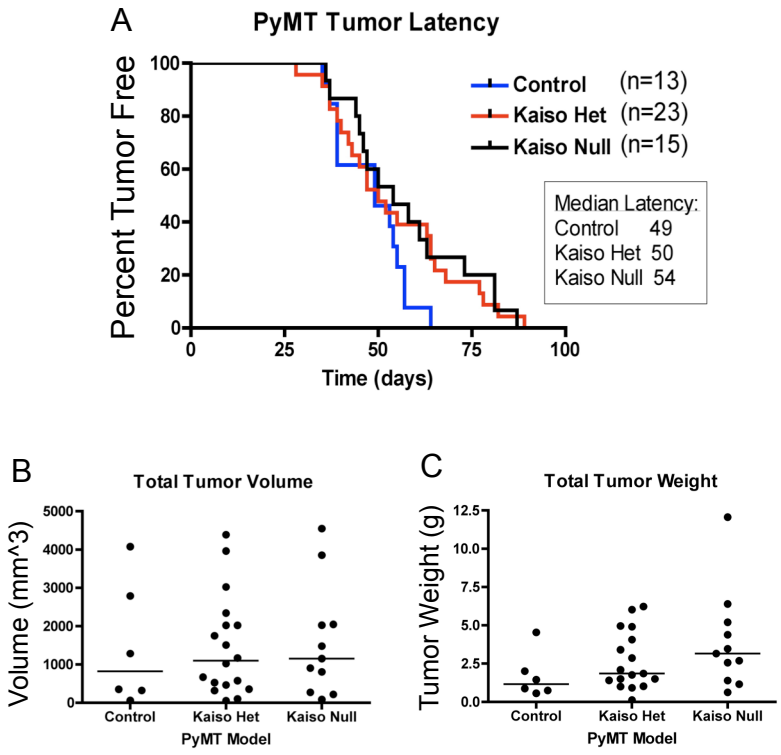


Figure 31. Kaiso is not required for PyMT-driven tumorigenesis
(A) Wildtype, Kaiso heterozygous, and Kaiso null PyMT mice were monitored for tumors by palpation. No significant difference was observed using a logrank test. (B) The volume of all ten mammary tumors per mouse were approximated by calipering (0.52*(w^2)*l=approx. volume) 54 days post palpation. (C) Total tumor weight was assessed. No significant difference was observed by either calculation of tumor size.

no statistically significant change in final tumor weight at this time point (Fig.31C). Thus, indicating that global Kaiso loss is insufficient to disrupt PyMT-driven tumor formation.

Discussion

Despite the significant upregulation of Kaiso in even the earliest PyMT lesions, global Kaiso ablation did not affect tumor formation. We postulate that this negative result is explained by the following possibilities. One such possibility is that the mixed genetic background on which these studies were performed (i.e. part FVB, part C57/bl6) generated enough variability to conceal statistically significant results. Indeed, the latency and metastatic outcome of the PyMT model is highly dependent on genetic background (Lifsted et al., 1998, Le Voyer et al., 2000, Davie et al., 2007). However, if the observed upregulation of Kaiso is truly necessary for tumorigenesis, background differences would likely not mask the complete blockade of tumor formation in the absence of Kaiso. A more likely scenario involves compensatory mechanisms by Kaiso family members. Similar to Kaiso, ZBTB4 binds methylated DNA and acts as a transcriptional repressor (Weber et al., 2008, Yamada et al., 2009). However, rather than the upregulation of Kaiso, ZBTB4 downregulation occurs in breast cancer (Weber et al., 2008, Kim et al., 2011). This downregulation appears to directly contribute to breast cancer progression as overexpression of ZBTB4 blocks proliferation and invasion of breast cancer cell lines (Kim et al., 2011). Whether there is crosstalk between these events (e.g. upregulation of Kaiso and downregulation of ZBTB4) and whether disruption of Kaiso leads to changes in ZBTB4 signaling has yet to be addressed.

While Kaiso appears to be upregulated in all PyMT cells, the physiologically importance of this upregulation may only occur in a specific population (e.g. cancer stem cells). PyMT-derived cancer stem cell maintenance and outgrowth in the lung require fibroblasts in the pre-metastatic to initiate a set of signaling events, including Wnt signaling (Malanchi et al., 2012). Without this stroma-initiated Wnt signaling in cancer stem cells, colonization is blocked. In a GI cancer model driven by the Wnt pathway, Kaiso ablation slows its progression suggesting that Kaiso facilitates Wnt signaling in some contexts (Prokhortchouk et al., 2006). Indeed, ongoing work in the Reynolds lab is focused on Wnt-Kaiso connections in this model system. Whether these events in GI cancer crossover into breast cancer has yet to be tested. The next area of work on Kaiso in breast cancer should focus on the cancer stem cell and Wnt signaling-driven colonization.

APPENDIX B

CHARACTERIZATION OF P120-DEPLETED MCF10A CELLS

Introduction

MCF10A cells are widely used to model untransformed mammary epithelial cell behavior (Debnath et al., 2002, Overholtzer et al., 2007, Mailleux et al., 2008, Simpson et al., 2008, Zhan et al., 2008). We utilized this model to study the mechanism(s) underlying the phenotypes observed in the p120 KO mammary mice (Chapter 3). In fact, use of this cell line was instrumental in identifying one of the main defects in the absence of p120, disrupted collective migration (Fig.12C). Herein, we characterized our previously described MCF10A p120-depleted cells (Fig.12A) in a other assays not described in Chapter 3, namely proliferation, contractility, and stemness.

Results

As part of the initial characterization of p120-depleted MCF10A cells, growth curves were generated. No difference in growth rates were observed between control MCF10A cells and p120-depleted cells (Fig.32). Interestingly, p120-depleted MCF10A cells exhibited serum-independent growth (Fig.32). While parental MCF10A cells are growth halted in the absence of serum, p120 knockdown cells continue to proliferate. While interesting, the relevance of this finding is discussed below.

In p120 KO mammary glands, subtle but present alterations to the ductal basal layer (Fig.8C) and striking dissociations of the TEB cap cell layer (Fig.11C) were

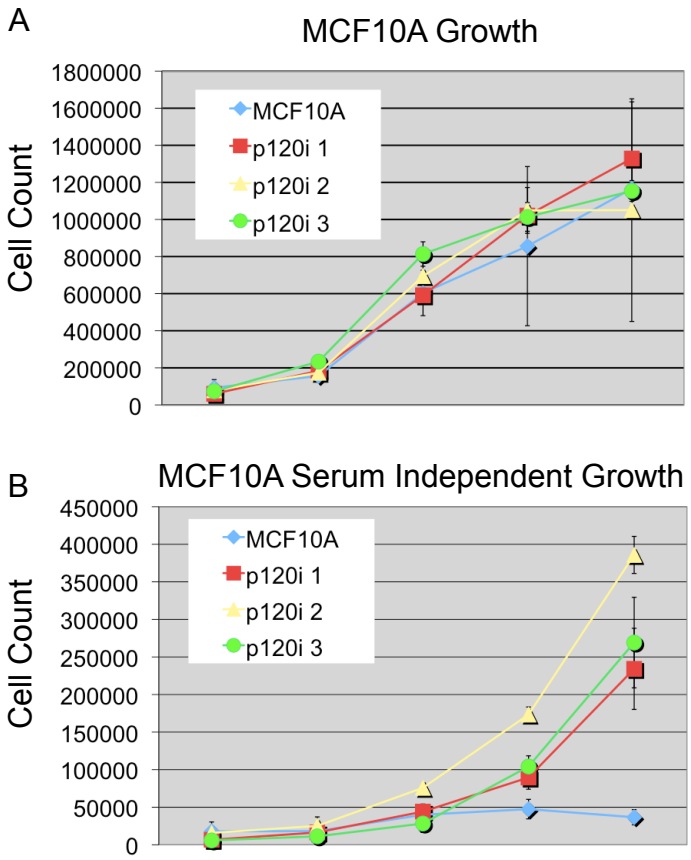


Figure 32. p120-depletion in MCF10A cells promotes serum-independent growth

Parental and p120-depleted MCF10A cells were grown in the presence (A) or absence (B) of serum and growth factors. Cell counts were taken every day for four days.

observed. Since this layer is thought to contain mammary progenitor cells, we wanted to test if p120 loss altered this population. Previous flow cytometry analysis demonstrated that CD44+CD24- cells correspond to cells expressing mesenchymal or basal markers (i.e. myoepithelial cells)(Sheridan et al., 2006) and this population exhibits stem cell properties (Mani et al., 2008). In contrast, the CD44-CD24+ cells were more differentiated and exhibited features of luminal cells (Mani et al., 2008). As such, we used the CD44+CD24- surface marker analysis as a cursory surrogate for stemness. To directly test the effects of p120-depletion on this population, we performed this flow cytometry analysis of MCF10A cells with and without p120. MDA-MB-231 cells were used as a positive control, as these cells are reported to have a high percent of this population (Sheridan et al., 2006). Indeed, 231 cells demonstrated 98.2% of cells as CD44+CD24- phenotype (Fig.33). Furthermore, MCF7 cells, a negative control, exhibited a similar pattern to that previously published (i.e., mostly CD44-CD24+)(data not shown), indicating that not every cell line assessed non-discriminately mimicked MDA-MB-231 cells. In our hands, MCF10A cells had a large basal population of CD44+CD24- cells (~70%) (Fig.33). This is in contrast to amounts reported previously (17±4%)(Sheridan et al., 2006). p120-depleted MCF10A cells had a similar amount of CD44+CD24- cells as control MCF10A cells suggesting that p120 depletion alone is insufficient to disrupt this population of basal/stem cells.

Since p120 is known to inhibit Rho activity (Anastasiadis et al., 2000, Perez-Moreno et al., 2006, Wildenberg et al., 2006), we next tested the effects of p120 reduction on readouts of Rho activity, stress fiber formation and contractility. p120 depletion induced a switch from cortical actin to stress fibers, a phenotype that could be

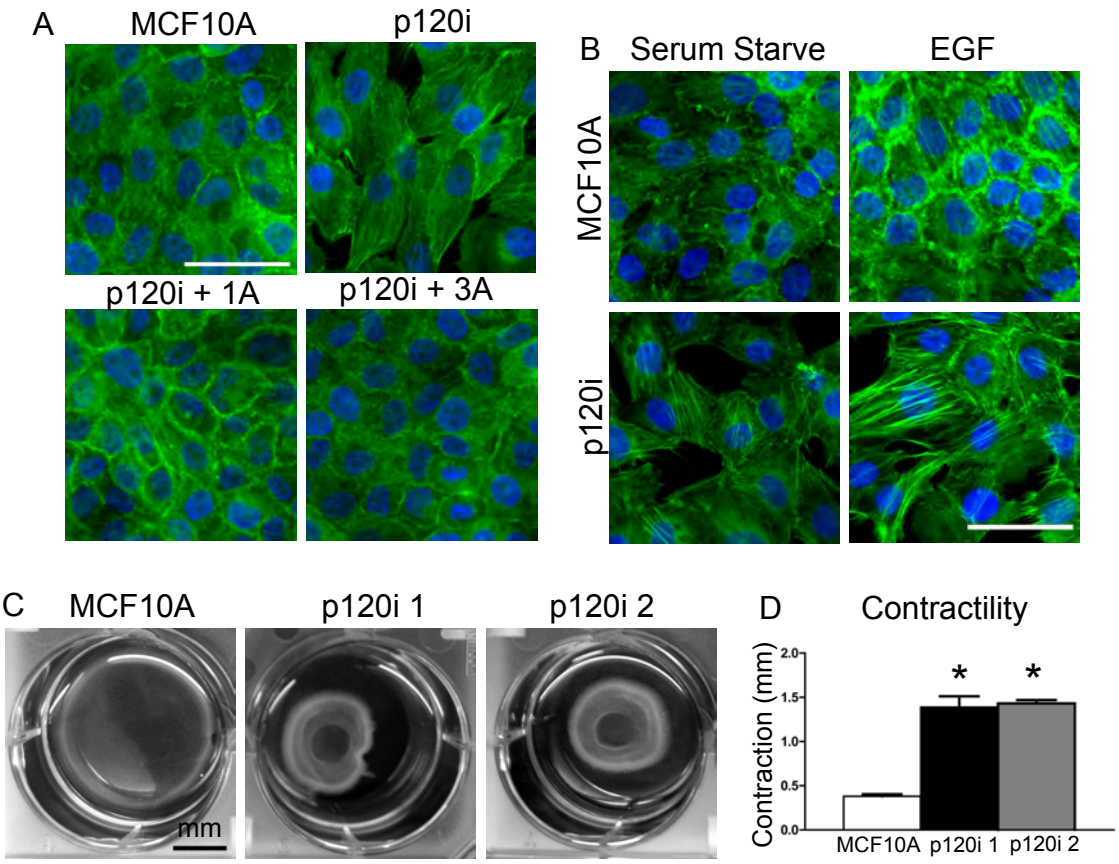


Figure 33. p120-depletion in MCF10A cells induces contractility
(A-B) Actin staining. (A) MCF10A cells, p120-depleted cells, and those with isoform 1A or 3A added, back were stained for actin. (B) Cells were then subjected to overnight serum starvation and treatment with 20 ng/mL EGF. Images depict the actin cytoskeleton (green) and nuclei stained with Hoecscht dye (blue). All scale bars = 50 μM. (C-D) Contractility assays. (C) Representative images of gels 2 days after floating are shown. (D) A representative graph for the assay performed in triplicate is depicted. Student's t-test *p<0.05

rescued with either p120 isoform 1A or 3A expression (Fig. 34A). Even after treatment with EGF, a factor that drives cortical actin formation in MCF10A cells, p120-depleted cells retain actin stress fibers, again indicating high Rho activity (Fig.34B). As a second method of monitoring of Rho activation, MCF10A cells were subjected to contractility assays wherein cells are embedded in floating collagen cells and the amount of contraction is monitored over time (Wozniak and Keely, 2005). In this assay, p120-depleted cells were approximately 3 times as contractile as parental MCF10A cells (Fig.34C,D). Collectively, these data suggest that p120 acts to inhibit Rho in MCF10A cells.

Discussion

Self-sufficiency in growth signals (e.g. serum/growth factor independent-growth), observed in p120-depleted MCF10A cells, is one of the hallmarks of cancer (Hanahan and Weinberg, 2000). Thus, the phenotype shown in figure 33 may prime p120-depleted cells for tumor formation. Indeed, in fibroblasts, p120 facilitates contact inhibition and in its absence foci form (Wildenberg et al., 2006). This growth may result from unimpeded receptor tyrosine kinase (RTK) signaling since in some settings E-cadherin acts an inhibitor of RTKs (Qian et al., 2004). However, p120 is required for tumor growth in a variety of settings (Soto et al., 2008, Dohn et al., 2009) and E-cadherin is also able to active RTK signaling in some contexts (Pece and Gutkind, 2000). In point of fact, tumors do not form in p120 mammary-specific knockout mice, and instead, p120 null cells are rapidly lost via anoikis (Fig.6&7). Thus, while MCF10As and other immortalized cell lines exhibit growth factor independent growth in the absence of p120,

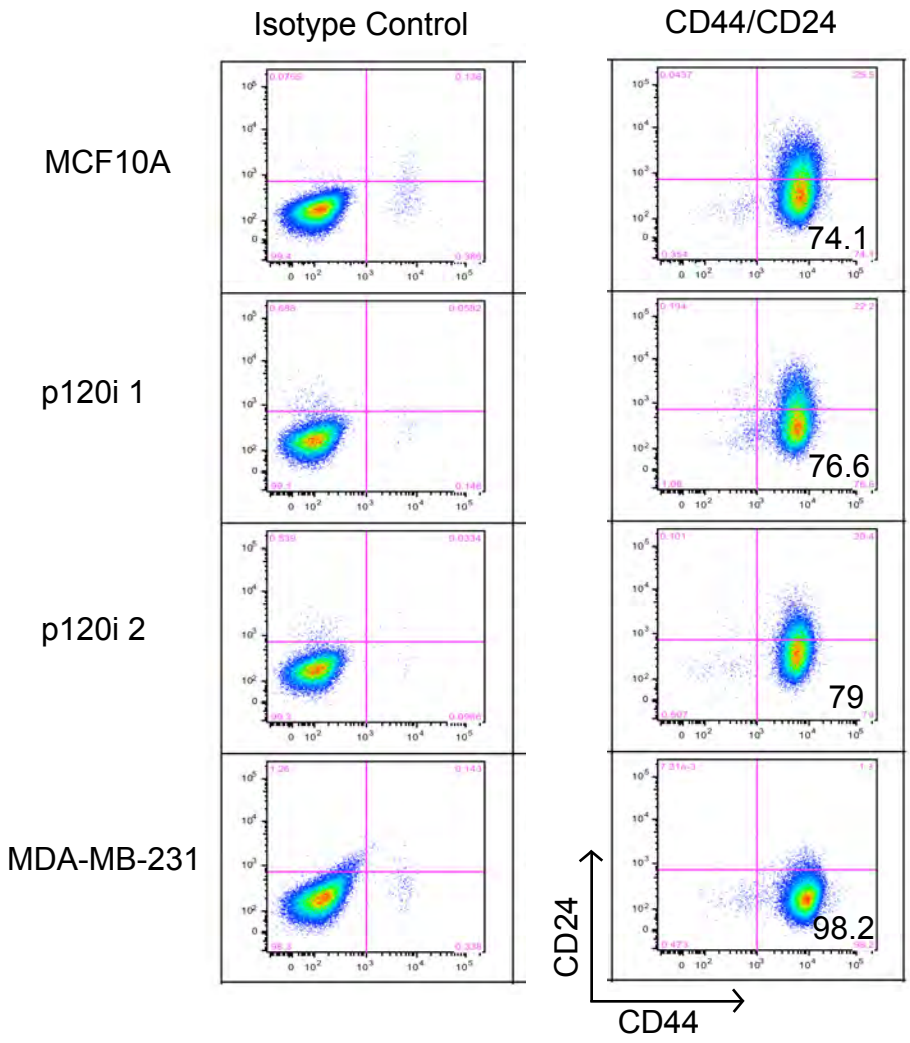


Figure 34. p120-depletion does not alter differentiation status of MCF10A cells

Parental and p120-depleted MCF10A cells and MDA-MB-231 cells were analyzed by flow cytometry for surface expression of CD44 and CD24 (right panels). Isotype controls were utilized to set gating (left panels).

primary cells *in vivo* are likely to behave differently. The very cues that allow for immortalization of tissue culture cells, as well as the signals derived from plating cells on rigid plastic, may cloud or alter some of the endogenous signaling in primary cells. For example, Dohn et al. discuss this concept in regards to tension-dependent death, a process whereby cells sense high tension and induce death (Dohn et al., 2009). p120-depleted MDCK cells transformed by dominant active Src are susceptible to death in the context of reduced external tension (e.g. in soft agar), but not while on plastic (i.e. under conditions of high external tension) suggesting that signals that are normally properly processed are not correctly interpreted when cells are on tissue culture plastic. Thus, 3D *in vitro* or *in vivo* follow up experiments are necessary to interpret the relevance of the observed serum independent growth.

Similarly, while cursory studies of stemness in the context of p120-depletion in MCF10A cells revealed no changes, our *in vivo* data intimate that the progenitor population is reduced and/or disrupted by p120 ablation (Fig.8&11). The Netrin and neogenin adhesion cues that function between body and cap cells are necessary for maintenance of the progenitor cells (Srinivasan et al., 2003). Interestingly, both the netrin and neogenin null mammary glands phenocopy our p120 knockout TEBs. Thus, while MCF10A cells provide a great starting point and indeed, some of its phenotypes are mimicked *in vivo* (e.g. disruption of collective migration), ultimately, it does not do a sufficient job recapitulating our *in vivo* findings. As such, any conclusions reached using MCF10A cells would need to be repeated with PMECs in a 3D or *in vivo* assay.

In contrast to growth alterations and stemness assays, the increased Rho activity observed in p120-depleted MCF10A cells is likely directly relevant to p120-ablation

induced phenotypes observed *in vivo*. Indeed, high levels of Rho activity driven by ablation of p190A RhoGAP phenocopies the TEB destruction observed with p120 ablation (Heckman-Stoddard et al., 2011). Thus, the observed increase in stress fiber formation and contractility, both indicative of heightened Rho activity, may be physiologically relevant and contribute to p120-ablation induced phenotypes. High Rho activity is known to disrupt adherens junctions suggesting that loss of p120 may promote the striking disruption of adhesion observed in the TEB by more than just decreased cadherin stability (Wildenberg et al., 2006, Hidalgo-Carcedo et al., 2011). Thus, while we still believe that loss of junctional cadherin is the dominant phenotype, augmentation of Rho signaling by p120 loss may also contribute to the disruption of the TEB.

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