TUMOR SUPPRESSOR MECHANISMS OF THE POLARITY PROTEIN PAR3

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DEDICATION

To Professor David Wessner of Davidson College, whose Biology 111 course

started my interest in medical research.

ACKNOWLEDGEMENTS

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Abbreviation	
2D	2-dimensional
3D	3-dimensional
aPKC	Atypical protein kinase C
aPKCi-CA	Constitutively active atypical protein kinase C-ι/λ
aPKCι/λ	Atypical protein kinase C-ι/λ
aPKCζ	Atypical protein kinase C-ζ
Baz	Bazooka
BSA	Bovine serum albumin
CAPE	Caffeic acid phenethylester
Cdc42	Cell division control protein 42 homologue
Clu	Clueless
Cora	Coracle
Crb	Crumbs
DAPI	4',6-diamidino-2-phenylindole
DaPKC	*Drosophila* atypical protein kinase C
Dlg	Discs large
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ErbB2/Neu	Receptor tyrosine-protein kinase erbB2/proto-oncogene Neu

Erk	Extracellular signal-regulated kinase
ESCC	Esophageal squamous cell carcinoma
GBM	Glioblastoma
gp130	Glycoprotein 130
HNSCC	Head and neck squamous cell carcinoma
HOOH	Hydrogen peroxide
HPV	Human papilloma virus
HRP	Horseradish peroxidase
HuMEC	Human mammary epithelial cell
IKK	IκB Kinase
IL-6	Interleukin-6
ILK	Integrin-linked kinase
kDa	Kilodalton
Lgl	Lethal giant larvae
Lkb1	Liver kinase B1
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KO	Knockout
MDCK	Madine-Darby canine kidney
mMEC	Mouse mammary epithelial cell
MOI	Multiplicity of infection
NICD1	Notch1-intracellular domain
NICD1-mMEC	NICD1-trasnformed mouse mammary epithelial cell

NF-κB	Nuclear factor κ-light chain-enhancer of activated B cells
NOD/SCID	Non-obese diabetic/Severe combined immunodeficiency
Nrx-IV	Neurexin-IV
Pals1	Protein Associated with Lin Seven-1
Par3	Partitioning defective 3 homologue
Par6	Partitioning defective 6 homologue
Patj	Pals1-associated tight junction protein
PBS	Phosphate-buffered saline
PDK1	Phosphoinositide-dependent protein kinase 1
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
Pins	Partner of inscuteable
PP2A	Protein phosphatase 2
PtdIns(3,4,5)P3	Phosphatidylinositol (3,4,5)-trisphosphate
PtdIns(4,5)P2	Phosphatidylinositol (4,5)-bisphosphate
PTEN	Phosphatase and tensin homologue
Scrib	Scribble
Sdt	Stardust
shRNA	Short hairpin RNA
Stat3	Signal transducer and activator of transcription 3
TBS-T	Tris-buffered saline-Tween 20
TNF-α	Tumor necrosis factor α
tRFP	Turbo red fluorescent protein
Upd	Unpaired

WT   Wild type

Yrt   Yurt

x

Chapter I

Introduction to Epithelial Polarity Signaling and Its Dysregulation in Cancer

Overview

All eukaryotic cells, including single-cell organisms such as yeast, must behave

in a polarized manner at some point in their life cycle. Polarization can be

defined as the asymmetric distribution of proteins, nucleic acids, or lipids within

cells. This segregation of components is necessary if different parts of the cell

are to perform different functions. For example, migrating cells must determine

their leading edge, neurons must segregate dendrites and cell bodies from

axons, epithelial cells must specify apical and basal membranes, and some cell

divisions require an asymmetric distribution of factors into the two daughter cells

(McCaffrey & Macara, 2012). Complex tissues could not be organized without

such spatial restriction of function. To establish and maintain functionally

polarized domains, cells activate complicated signaling networks.

Given the fundamental importance of these signaling networks to tissue

organization, it is not surprising that they are commonly disrupted in cancers

(Halaoui & McCaffrey, 2014). However, clear experimental evidence that polarity

genes act as tumor suppressors or oncogenes has only recently emerged, and

the mechanisms are largely unknown. In this chapter, I will review the current

understanding of the machinery of cell polarity, and discuss the relationship between polarity signaling and cancer.

Polarity in Budding Yeast

In budding yeast, the establishment of polarity is a necessary step in the transition from either spherical growth to division via budding or generation of a mating projection (Slaughter, Smith, & Li, 2009). These two varieties of polarity establishment are triggered differently. In the case of vegetative growth via budding, the cue for symmetry breaking is cell-autonomous, coming from the cell cycle machinery (Richman, Sawyer, & Johnson, 2002; Ziman et al., 1993). On the other hand, polarization cues for sexual reproduction come from pheromones secreted by cells of the opposite mating type (Pryciak & Huntress, 1998; Zhao, Leung, Manser, & Lim, 1995), proving that polarity signaling can be initiated cell-nonautonomously even in very primitive organisms. In both cases, the GTPase Cdc42 is the central player in symmetry breaking. An initial concentration of Cdc42-GTP at the plasma membrane triggers nucleation of actin cables (Lechler, Shevchenko, Shevchenko, & Li, 2000; Li, Zheng, & Drubin, 1995; Park & Bi, 2007), which then act as delivery routes for endocytic vesicles containing additional Cdc42 (Irazoqui, Howell, Theesfeld, & Lew, 2005; Wedlich-Soldner, Wai, Schmidt, & Li, 2004). The arrival of additional Cdc42 enhances actin cable nucleation, thus providing positive feedback. This mechanism is sufficient to break symmetry in the yeast (Slaughter et al., 2009). Though this process is not fully conserved in multicellular organisms, many of the proteins involved are. In

particular, Cdc42 is necessary for epithelial polarity in worms and mammals (McCaffrey & Macara, 2012).

Polarity Complexes in Epithelia of Higher Organisms

In higher organisms, there are three highly conserved polarity complexes: the apical Par complex, which contains Par3, Par6, and aPKC; the apical Crumbs complex, of which Crb, Pals1, and Patj are essential members; and the basolateral Scribble group, consisting of Scrib, Dlg, and Lgl (Assémat, Bazellières, Pallesi-Pocachard, Le Bivic, & Massey-Harroche, 2008). These three polarity groups are summarized in Figure 1. The components of these complexes, as well as many of their interaction partners, were initially discovered via genetics screens in *Caenorhabditis elegans* and *Drosophila melanogaster* models. In this section, I will introduce these three major groups, and discuss their role in maintaining epithelial polarity.

<u>The Crumbs Complex</u>

The gene for Crb was first identified in a screen for genes affecting the larval cuticle of *Drosophila* (Jürgens, Wieschaus, Nüsslein-Volhard, & Kluding, 1984). Because an underlying epithelium secretes the larval cuticle, changes in cuticle organization were used as a readout for defects in epithelial structure. Mutations in Crb caused numerous small holes to form in the cuticle, giving the Cuticle an appearance of bread crumbs on a plate. Several years after this initial discovery, Crb was shown to regulate cell polarity in *Drosophila* (Tepass, Theres, & Knust,
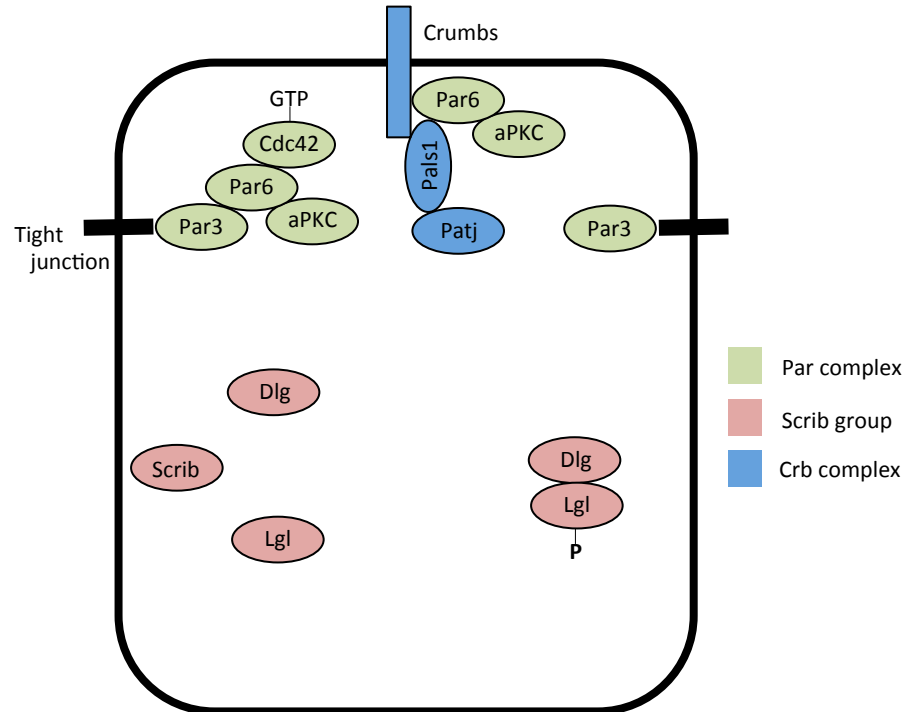
Figure 1. Localization of the major polarity regulators in polarized mammalian epithelial cells. The three major polarity groups are the apical Par complex, which contains Par3, Par6, and aPKC; the basolateral Scribble group, which consists of Scrib, Dlg, and Lgl; and the apical Crumbs complex, containing Crb, Pals1, and Patj. When Par6 binds to Cdc42-GTP, aPKC is activated and phosphorylates Par3, triggering dissociation of Par3 from aPKC. However, Par3 remains in a physical complex with Par6 (and thus indirectly with aPKC) until Crb displaces Par6. Par6 remains bound to aPKC, and interactions between Par6 and Crb and Pals1 anchor Par6/aPKC to the apical cortex. Par3 remains at the level of the tight junctions. The basolateral Scribble group proteins are not known to form a physical complex involving all three members. Recent data shows that when Lgl is phosphorylated on any of three highly-conserved serine residues, Dlg can bind directly to it.

1990). This study reported that Crb contains a large extracellular domain, which includes 30 EGF-like repeats, and a small intracellular tail. The transcript for this gene was shown to localize to the apical region on the blastoderm, and homozygous loss of function mutations lead to near-total loss of cuticle structure due to extensive death of the underlying epidermis. The authors speculated that Crb helps to establish epithelial polarity.

Despite its smallness, the intracellular domain of Crb is highly conserved between *Drosophila* and humans (Hollander et al., 2001; Klebes & Knust, 2000; Lemmers et al., 2002), and this region is essential for Crb polarizing activity (A Wodarz, Grawe, & Knust, 1993). This intracellular tail contains two domains that are highly conserved in all Crumbs isoforms thus far described: a FERM domain in the juxtamembrane region, and a PDZ domain at the C-terminus (Izaddoost, Nam, Bhat, Bellen, & Choi, 2002; Klebes & Knust, 2000; Makarova, Roh, Liu, Laurinec, & Margolis, 2003). The PDZ region interacts directly with the second component of the Crumbs complex, Pals1. Known as Sdt in *Drosophila*, it was first reported that mutations in this gene closely phenocopied Crb mutations in fly embryos (Tepass & Knust, 1993). Via its sole PDZ domain, Sdt interacts directly with the final 4 amino acids in the C-terminus of Crb (Bachmann, Schneider, Theilenberg, Grawe, & Knust, 2001; Hong, Stronach, Perrimon, Jan, & Jan, 2001). The final component of the Crumbs complex is a PDZ domain-containing protein known as Patj (Pielage, Stork, Bunse, & Klämbt, 2003), which induces polarity defects in *Drosophila* embryos when mutated (Nam & Choi, 2006). In both developing and mature *Drosophila*, Patj co-localizes with and interacts biochemically with the Crumbs complex (Klebes & Knust, 2000; Pellikka et al., 2002). In mammalian cells, the Pals1's C-terminus interacts with the intracellular tail of Crb, and an L27 domain on Pals1's N-terminus directly interacts with Patj (Roh, Makarova, et al., 2002; Roh, Liu, Laurinec, & Margolis, 2002). Pals1 thus mediates an indirect interaction between Crb and Patj, forming the Crumbs complex.

Although studies in *Drosophila* led to the initial discovery of the Crumbs complex, there is only one Crb gene in the fly. In contrast, mammals have three genes, known as *CRB1*, *2*, and *3*. These largely have distinct tissue expressions, with *CRB1* and *CRB2* mainly being found in neural tissues, and *CRB3* being highly expressed in epithelia (Assémat et al., 2008). Consistent with this distribution, *CRB1* mutations are common in human patients suffering from retinitis pigmentosa and leber congenital amaurosis. The phenotypes of these two diseases are thought to arise from defects in junction formation and apical surface specification in the retina (Beryozkin et al., 2013; Bujakowska et al., 2012; Corton et al., 2013; den Hollander et al., 1999; Richard et al., 2006). A mammalian function for *CRB1* in specifying the apical region of the retinal pigment epithelium would be consistent with Crb function in *Drosophila*, where Crb is an essential positional signal for the adherens junction in retinal cells (Izaddoost et al., 2002; Pellikka et al., 2002).

Crb plays a similar role in epithelial tissues as in the human and fly retinas. In *Drosophila* embryonic epithelium, Crb is necessary for adherens junction formation and proper E-cadherin localization to cell-cell contacts. Moreover, overexpression of Crb causes the apical surface to expand and bulge, suggesting that this protein may delineate the apical region of the plasma membrane (Klebes & Knust, 2000; Andreas Wodarz, Hinz, Engelbert, & Knust, 1995). Further evidence that Crb specifies the apical territory comes from

localization of *Drosophila* β-heavy spectrin. The spectrins are actin cross-linking proteins that function as tetramers, with two α-spectrins and two of either β-spectrin or β-heavy spectrin in each complex. αβ complexes are exclusively basolateral, while the αβ-heavy tetramer is found only at the apical surface. Following initial reports that overexpression of Crb increases expression of β-heavy spectrin on the apical surface (Andreas Wodarz et al., 1995), it was demonstrated that Crb co-precipitates with β-heavy spectrin and is required for its apical localization (Médina et al., 2002). *CRB3* is also necessary for cilia formation in MDCK cells, providing additional evidence that it is important for apical specification in mammalian cells (Fan et al., 2004).

CRB3 is essential for tight junction formation in mammalian epithelia. In a mammary epithelial cell line (MCF10A) that both lacks Crumbs expression and fails to form tight junctions, overexpression of Crumbs is sufficient to induce junctional formation (Fogg, Liu, & Margolis, 2005). This function clearly depends upon intracellular interactions, as both the FERM domain and the C-terminal PDZ domain are necessary. It seems that the Crb levels must be tightly controlled, however, because overexpression of Crumbs in MDCK cells – which normally display tight junctions – disrupts junction formation (Lemmers et al., 2004).

The Crumbs complex does not act in isolation, but interacts with other polarity complexes. The Par complex protein Par6 interacts directly with Pals1 (Q. Wang, Hurd, & Margolis, 2004), and this interaction is necessary for proper

localization of the complexes and tight junction formation (Hurd, Gao, Roh, Macara, & Margolis, 2003). These findings are corroborated by recent work in *Drosophila* models, which has demonstrated that interactions between Baz (the fly homologue of Par3) and Std are required for epithelial membrane polarization (Krahn, Bückers, Kastrup, & Wodarz, 2010), and that Crumbs acts in concert with aPKC to exclude Baz from the apical region during photoreceptor remodeling (Walther & Pichaud, 2010).

<div align="center">The Scribble Group</div>

The second major polarity group, which is positioned on the basolateral cortex, is the Scribble group. Though the Scribble group is often referred to as a complex, there is very limited evidence that its members physically interact, and it is better referred to as a group. The namesake of this group was identified in a screen for maternal effect genes that impact epithelial organization (Bilder & Perrimon, 2000). When the Scrib gene is mutated, the embryonic cuticle is corrugated and contains holes, giving the appearance of scribbled lines. The underlying epithelium becomes disorganized and multilayered. In mature epithelium in *Drosophila*, the product of this gene localizes to the lateral membrane, and its loss triggers mislocalization of apical proteins (Bilder & Perrimon, 2000). These findings imply that Scrib is a polarity regulator that acts to restrict apical determinants to the apical membrane. Shortly after this initial report, it was demonstrated that mutations in Scrib closely phenocopy mutations in two *Drosophila* tumor suppressor genes, Lgl and Dlg. Moreover, it was shown that

<div align="center">8</div>

these genes interact genetically with one another, suggesting that the three act in a common pathway that regulates epithelial polarity (Bilder, Li, & Perrimon, 2000).

The Lgl gene locus has been known for decades to be a tumor suppressor in *Drosophila* (E. Gateff & Schneiderman, 1974). Cloning of this locus revealed an open reading frame that encoded a protein with sequence similarities to genes involved in cell adhesion (Lutzelschwab, Klambt, Rossa, & Schmidt, 1987; Mechler, McGinnis, & Gehring, 1985). There are two mammalian versions of this protein, Lgl1 and 2 (Humbert et al., 2008). In mammalian cells, these proteins are found on the basolateral membrane, a localization that requires phosphorylation of serine residues by aPKC (Musch et al., 2002; Yamanaka et al., 2003). A requirement for aPKC-mediated phosphorylation of Lgl is also observed in *Drosophila* (Hutterer, Betschinger, Petronczki, & Knoblich, 2004).

Similarly to Lgl, Dlg is a tumor suppressor in *Drosophila* models. Early studies using *Drosophila* systems revealed that Dlg mutagenesis causes overgrowth and fusion of tissues in the wing imaginal disc (Daniel F. Woods & Bryant, 1989). The Dlg protein was found to localize to septate junctions, and to contain a guanylate kinase domain (Daniel F. Woods & Bryant, 1991). This made it the inaugural member of the membrane-associated guanylate kinases, or MAGUK, proteins (Roberts, Delury, & Marsh, 2012). In addition to this kinase domain, which has not been shown to function enzymatically, Dlg contains three PDZ

domains and a Src-homology domain (Hough, Woods, Park, & Bryant, 1997),

which act together to facilitate extensive interactions with other proteins. Further

studies revealed that Dlg was necessary for maintenance of septate junctions

and apical-basal polarity in *Drosophila* wing imaginal discs (D. F. Woods, Hough,

Peel, Callaini, & Bryant, 1996). In contrast to flies, the *C. elegans* homologue of

Dlg, DLG, is localized at adherens junctions rather than septate junctions

(Bossinger, Klebes, Segbert, Theres, & Knust, 2001). DLG is necessary for the

formation of the adherens junction in this organism, and overexpression of DLG

causes Crumbs to mislocalize in a manner that is reminiscent of the mislocalized

apical proteins observed in *Drosophila* Scribble mutants (Bossinger et al., 2001;

Firestein & Rongo, 2001).

In mammalian cells, there are four Dlg proteins, known as Dlg1, 2, 3 and 4

(Roberts et al., 2012). Intriguingly, these four genes do not localize identically.

Dlg1, like its *Drosophila* counterpart, is found on the basolateral membrane. In

contrast, Dlg2 and 3 are observed both along the basolateral membrane and in

the cytoplasm, while Dlg4 is found in all membrane domains (Van Campenhout

et al., 2011). Consistent with a highly conserved function for Dlg1, deficiency in

developing mice causes several gross defects, including palatal clefting and

excessive proliferation of the eye's lens epithelium (Caruana & Bernstein, 2001;

Nguyen et al., 2003). Congenital urogenital defects also arise, including

misalignment of the ureturic smooth muscle cells, suggesting that Dlg1 may be

involved in orienting cells in ways other than ensuring apical-basal polarity of

epithelial cells (Iizuka-Kogo, Ishidao, Akiyama, & Senda, 2007; Mahoney et al., 2006).

Despite the similar phenotypes seen upon mutations in Scrib complex genes, colocalization within cells, and the evidence that they act in a common genetic pathway, there is only limited data that they physically interact (Humbert et al., 2008). At synapses in *Drosophila*, the guanylate kinase domain of Dlg interacts with GUK-holder protein, which in turn interacts with the second PDZ domain of Scribble, thus forming a tripartite complex (Mathew et al., 2002). Nhs11b is a mammalian gene product homologous to GUK-holder that has been shown to co-precipitate with Scribble in human cells (Walsh, Grant, Morgan, & Moens, 2011), but it is neither clear that this interaction is direct nor that it is important for cell polarity. When Scribble is overexpressed in mammalian cells, it co-precipitates with Lgl1; however, this interaction also may be indirect (Kallay, McNickle, Brennwald, Hubbard, & Braiterman, 2006). More recently, structural and biochemical studies have shown that mammalian Lgl and Dlg directly interact (Zhu et al., 2014). This interaction requires aPKC phosphorylation of at least one of three highly-conserved serine residues on Lgl. Of note, aPKC-mediated phosphorylation of Lgl is necessary to exclude it from the apical surface in *Drosophila* epithelium, suggesting an important mechanism by which the Par complex regulates the localization and interactions of Scribble group members (Hutterer et al., 2004).

The Par Complex

The final polarity complex, called the Par complex, contains Par3, aPKC, and

Par6, and localizes to the apical surface and tight junctions of epithelial cells.

The proteins of this complex, as well as many of their interaction partners, were

first found through a screen in *C. elegans* embryos. Fertilization of the egg in this

organism triggers symmetry breaking and asymmetric distribution of polarity

proteins. When Priess and Kemphues screened this system for maternal effect

genes that were embryonic-lethal when mutated, they identified eight mutations

in four genes – *par-1*, *par-2*, *par-3*, and *par-4* – that caused cleavage defects

(Kemphues, Priess, Morton, & Cheng, 1988). Subsequent studies revealed

additional par genes. Par1, which has sequence homology with serine/threonine

kinases, is found in the posterior cortex of the one-cell embryo, where it is

required for asymmetric divisions (S. Guo & Kemphues, 1995). Cloning of the

Par2 gene identified ATP- and zinc-binding motifs, and subsequent functional

studies demonstrated that it is asymmetrically distributed at the posterior cortex

in the one-cell embryo, and that its localization depends upon Par3 (Boyd, Guo,

Levitan, Stinchcomb, & Kemphues, 1996; Levitan, Boyd, Mello, Kemphues, &

Stinchcomb, 1994). However, uniquely among the Par genes identified by

Kemphues and colleagues, Par2 has no known homologues in other organisms

(McCaffrey & Macara, 2012). Par3 contains conserved PDZ domains,

suggesting a scaffolding role (Kurzchalia & Hartmann, 1996), and is required for the localization of other polarity factors and for spindle orientation in the early embryo (Etemad-Moghadam, Guo, & Kemphues, 1995). Par4, also known as LKB1, encodes a serine/threonine kinase (Watts, Morton, Bestman, & Kemphues, 2000). Par5 is a 14-3-3 protein that is necessary for polarization of the embryo but which is not itself asymmetrically distributed (Morton et al., 2002). Like Par3, Par6 is a PDZ domain-containing protein. Early studies demonstrated that it colocalizes with Par3 at the anterior periphery of one-cell embryos, and that it is necessary for the proper distribution of Par3 (Hung & Kemphues, 1999). In addition to the Par proteins, Kemphues's lab also identified PKC-3 – known as aPKC in mammals – as being necessary for asymmetric embryonic divisions (Tabuse et al., 1998). The similar phenotypes and colocalization seen for Par3, Par6, and aPKC suggested that they act together to regulate cell polarity.

Par6 is a relatively small protein – approximately 37 kDa – with three important protein interaction regions (Assémat et al., 2008). Near its N-terminus, it contains a PB1 domain that interacts with aPKC (Hirano et al., 2005; Noda et al., 2003), a C-terminal PDZ domain that mediates interactions with Par3, Crumbs, and Pals1 (Hurd, Gao, et al., 2003; Kempkens et al., 2006; Lemmers et al., 2004; Penkert, DiVittorio, & Prehoda, 2004), and an intervening CRIB motif that mediates interactions with GTP-bound Cdc42 (Burbelo, Drechsel, & Hall, 1995; Noda et al., 2001). There are four Par6 genes in mammalian cells that share these structural features but have distinct subcellular localizations, implying

distinct functions (Gao & Macara, 2004). Based on data showing that Par6 interacts exclusively with either Lgl or Par3, the main role of Par6 may be to act as a scaffold that facilitates interactions between aPKC and its substrates (Yamanaka et al., 2003). Par6 also has a role in tight junction assembly, as overexpression of Par6B delays formation of tight junctions and disrupts mature junctions (Gao, Joberty, & Macara, 2002; Gao & Macara, 2004; Hurd, Gao, et al., 2003). The PDZ domain of Par6B is required to disrupt the junctions, suggesting that overexpression exerts these effects by disrupting interactions with Par6 binding partners. However, underscoring the functional divergence between Par6 isoforms, overexpression of either Par6A or Par6C does not disrupt tight junctions (Gao & Macara, 2004).

Atypical protein kinase C's (aPKCs) are members of the protein kinase C class, but unlike other members they are not regulated by calcium or diacylglycerol signaling (Garg et al., 2013). Two distinct aPKCs are found in mammalian genomes, called aPKCι/λ and aPKCζ. aPKC is a key member of the polarity machinery, and its worm homologue was identified in early studies of *C. elegans* embryos (Tabuse et al., 1998). Numerous proteins with roles in polarity signaling are substrates for aPKC, including LGN, Par3, Lgl, Crumbs, Numb, and NuMA (J. Chen & Zhang, 2013). In 2D cultures of epithelial cells, aPKC is heavily enriched at the tight junctions (X. Chen & Macara, 2005; Izumi et al., 1998), while in 3D cultures it is found above tight junctions at the apical surface (Hao et al., 2010). The situation in 3D cultures more closely resembles what is seen *in vivo*,

where aPKC is localized to the apical surface of healthy mammary epithelial cells (McCaffrey & Macara, 2009). Like other members of the Par3 complex, aPKC appears to have an important role in regulating tight junction formation (Gopalakrishnan, Hallett, Atkinson, & Marrs, 2007; Suzuki et al., 2001). aPKC helps to specify the apical membrane domain in epithelial cells by actively excluding basolateral factors (Betschinger, Mechtler, & Knoblich, 2003; Hao et al., 2010; Hurov, Watkins, & Piwnica-Worms, 2004).

Much like Par6, Par3 contains numerous conserved domains that mediate interactions with other polarity factors. At its N-terminal region, Par3 contains a conserved self-oligomerization motif that is necessary for its localization and enrichment at tight junctions (Benton & Johnston, 2003a; Feng, Wu, Chan, & Zhang, 2007; Mizuno et al., 2003). Beyond this self-oligomerization region, Par3 contains three PDZ domains. The first of these domains mediates interactions with Par6 (Joberty, Petersen, Gao, & Macara, 2000; Lin et al., 2000; Suzuki et al., 2001), while the second interacts with membrane lipids and the third with PTEN (Feng, Wu, Chan, & Zhang, 2008; H. Wu et al., 2007; C. G. Yu & Harris, 2012). These interactions with membrane lipids and PTEN are necessary for proper epithelial polarization, suggesting that Par3 might be a platform for PTEN to interact with its phospholipid substrates. Beyond the PDZ region, there is a small aPKC interaction motif (Nagai-Tamai, Mizuno, Hirose, Suzuki, & Ohno, 2002) and two conserved regions that bind to Numb, Tiam1, and microtubule

motors (X. Chen & Macara, 2005; Nishimura et al., 2004; Nishimura & Kaibuchi, 2007; H. Zhang & Macara, 2006).

Par3 is required for proper targeting of the Par complex to the apical surface of cells. Within an epithelium, Par3 is found at the tight junctions, while aPKC and Par6 are at the apical surface (Hao et al., 2010; Hayase et al., 2013; Izumi et al., 1998; Joberty et al., 2000; Lemmers et al., 2004; Morais-de-Sá, Mirouse, & St Johnston, 2010; Q. Wang et al., 2004). Par6 interacts with aPKC via their respective PB1 domains, and this interaction attenuates aPKC activity (Hirano et al., 2005; Yamanaka et al., 2001). Par6-mediated inhibition of aPKC is relieved when Par6 interacts with the GTP-bound form of Cdc42 (Yamanaka et al., 2001). In polarized cells, aPKC that is activated by binding of Cdc42-GTP to Par6 can phosphorylate Par3 on a residue within Par3's aPKC binding domain (Nagai-Tamai et al., 2002), triggering dissociation of Par3 from aPKC. Until Crumbs displaces Par3, Par3 will remain in a complex with Par6 and aPKC via binding of Par3's first PDZ domain to the PDZ domain of Par6 (Morais-de-Sá et al., 2010). In mature epithelium, this series of interactions ensures that most Par3 localizes to the tight junctions, and does not directly interact with apical Par6 and aPKC.

In addition to Par3, Pa6, and aPKC – which are referred to collectively as the Par Complex – the other Par proteins identified in *C. elegans* also help regulate mammalian epithelial polarity. Par1 is localized to the basolateral cell cortex, where it plays an important role in specifying membrane domains. It is a

serine/threonine kinase that phosphorylates Par3 on serine-144 if Par3 inappropriately localize to the basolateral region (Fig. 2). Following this phosphorylation, Par5 (also called 14-3-3) binds to Par3, causing it to fall off of the membrane (Benton & Johnston, 2003b; Hurd, Fan, et al., 2003). This active process thus excludes Par3 from the basolateral membrane regions where Par1 resides. A similar, reciprocal process mediated by aPKC ensures that Par1 is restricted to the basolateral surface (Hurov et al., 2004; Watkins et al., 2008). Par5 is diffuse in the cytoplasm, where it binds to substrate proteins when they are phosphorylated on specific serine or threonine residues (Hurd, Fan, et al., 2003). Par4, also called Lkb1, is also spread diffusely through the cytoplasm. Lkb1 is best studied as a tumor suppressor and regulator of stem cell function (Martin-Belmonte & Perez-Moreno, 2012), but overexpressing it in intestinal epithelial cells is sufficient to induce polarization, suggesting that it is a polarity factor (Baas et al., 2004; Martin-Belmonte & Perez-Moreno, 2012).
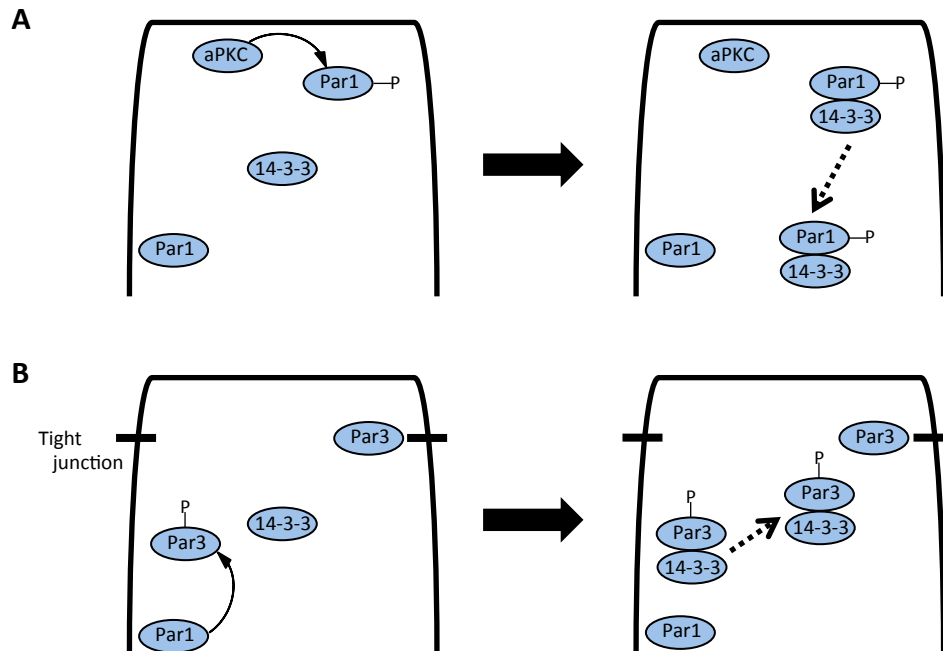
Figure 2. Active exclusion of polarity factors from inappropriate cortex regions via phosphorylation and binding of 14-3-3. (A) Par1 ordinarily localizes to the basolateral cortex. If it inappropriately arrives at the apical region, active aPKC (in a complex with Par6 and Cdc42-GTP, both not shown) will phosphorylate Par1 on a threonine residue. Threonine phosphorylation of Par1 creates a docking site for 14-3-3 (also called Par5). Binding of 14-3-3 causes Par1 to drop off of the cortex. A similar mechanism excludes Pins from the apical surface during mitosis. (B) In polarized mammalian epithelial cells, Par3 resides at the tight junction. If Par3 escapes to the basolateral cortex, it is phosphorylated on serine residues by Par1. This phosphorylation event generates a 14-3-3 binding site, and binding of 14-3-3 causes Par3 to fall off of the cortex. Par3 is later dephosphorylated by PP1α (not shown).

Other Factors Regulating Apical-Basal Cell Polarity

There is recent evidence that a fourth polarity system may exist in *Drosophila*, consisting of Yrt, Cora, Nrx-IV, and the Na$^+$,K$^+$-ATPase (Laprise et al., 2009). Like the Scrib group, these proteins are found on the basolateral membrane of epithelial cells, where they have a role in specifying the basolateral domain. However, the Yrt/Cora complex appears to act at different stages of development than the Scrib complex. Yrt/Cora is necessary for polarity during organogenesis

in the developing *Drosophila* larva but not for the initial establishment of polarity, at which time Scrib is indispensible (Laprise et al., 2009). Yrt interacts both biochemically and genetically with Crb, and counteracts Crb-mediated apical specification (Laprise et al., 2006). Consistent with this, loss of Yrt causes an expansion of the apical membrane that resembles the phenotype seen when Crb activity is lost (Laprise et al., 2006). More recently, it was shown in *Drosophila* that phosphorylation by aPKC regulates the localization and activity of Yrt. Mutants of Yrt that lack an aPKC phosphorylation site cause loss of the apical domain. Additionally, binding of Yrt to aPKC appears to restrict activity of aPKC to the apical surface (Gamblin, Hardy, Chartier, Bisson, & Laprise, 2014). The Yrt/Cora complex thus appears to be a novel polarity pathway that interacts with established polarity mechanisms. Whether the Yrt/Cora system is conserved in mammalian cells is unknown.

In addition to the protein factors discussed so far, there is accumulating evidence that membrane phospholipids help to specify polarized membrane domains in epithelial tissues (Krahn & Wodarz, 2012). Phosphoinositides are lipids that exist solely in the cytoplasmic side of the plasma membrane, and which can be phosphorylated at three locations on their head group. Of particular importance for cell polarity are the PtdIns(4,5)P2 and PtdIns(3,4,5)P3 species, with the former being enriched at the apical surface of epithelial cells, and the latter in the basolateral membrane. Based on to evidence that these lipid species have distinct compartmentalization in migrating leukocytes and in cultured neurons

(Shi, Jan, & Jan, 2003; F. Wang et al., 2002; Weiner et al., 2002), the Mostov lab studied the distribution and function of PtdIns(3,4,5)P3 in MDCK cells (Gassama-Diagne et al., 2006). They reported that PtdIns(3,4,5)P3 localizes to the basolateral membrane, and that ectopic expression at the apical surface triggers the formation of protrusions that contain baslolateral membrane proteins. Subsequent work showed that PTEN activity is necessary for PtdIns(4,5)P2 accumulation at the apical membrane, and that this lipid accumulation is necessary for the recruitment of Cdc42 and aPKC (Martin-Belmonte et al., 2007). These results were further supported by the observation that *Pseudomonas aeruginosa*, which infects cells more efficiently via the basolateral membrane, can generate apical entry routes into cells by enriching the PtdIns(3,4,5)P3 content at patches of apical membrane. This PtdIns(3,4,5)P3 enrichment causes the patches to acquire properties of the basolateral surface (Kierbel et al., 2007). Similarly, *Drosophila* epithelial cells have enrichment of PtdIns(4,5)P2 at the apical membrane, and PtdIns(3,4,5)P3 is found exclusively at the basolateral surface (Claret, Jouette, Benoit, Legent, & Guichet, 2014). However, the mechanism differs from MDCK cells, as PTEN is not involved. Finally, Par3/Baz associates with phosphoinositides in both mammalian and *Drosophila* cells, and this interaction may be important for targeting the protein to the cell cortex (Horikoshi, Hamada, Ohno, & Suetsugu, 2011; Krahn, Klopfenstein, Fischer, & Wodarz, 2010; Stein, Ramrath, Grimm, Müller-Borg, & Wodarz, 2005).

While the molecular pathways that organize cell polarity are understood in some

detail, they do not explain how cells determine which surface is apical and which

is basal. Although there is limited *in vivo* data, current evidence suggests that

interactions with basement membrane proteins serve to orient epithelial polarity

(Rodriguez-Boulan & Macara, 2014). MDCK cells in suspension cultures lacking

basement membrane proteins will form hollow cystic structures, with the outer

surface displaying apical features. However, addition of matrix proteins to the

culture environment causes the orientation to reverse, as apical proteins are

transcytosed to the inner surface (Ojakian & Schwimmer, 1994; A. Z. Wang,

Ojakian, & Nelson, 1990a, 1990b). Similarly, MDCK monolayers will internalize

their apical surface proteins if a collagen matrix is overlaid on top of the cells (W.

Yu et al., 2005). This process depends upon interactions between collagen in

the extracellular milieu and β1 integrins on the cell surface, as blocking this

interaction with an antibody prevents polarity reversal (Ojakian & Schwimmer,

1994; W. Yu et al., 2005, 2008). A similar process is observed in single MDCK

cells grown in collagen matrix, which must decide how to orient their polarity as

they divide to form multicellular cysts. At the two-cell stage, apical proteins are

localized to the cell periphery. Vesicle transport relocalizes these proteins to a

small patch of membrane between the two cells, around which future mitoses are

oriented (Bryant et al., 2010). The limited *in vivo* evidence comes from the

mammary gland, where deletion of β1 integrins causes defects in ductal

organization and inversion of ductal polarity (Akhtar & Streuli, 2013). However,

the process differs from what is observed in MDCK cultures at a molecular level,

since polarity orientation in MDCK cells depends upon Rac1 signaling (O'Brien et al., 2001; W. Yu et al., 2005, 2008), while *in vivo* in the mammary gland Rac1 is not involved, but ILK is required (Akhtar & Streuli, 2013). Future *in vivo* studies in other tissues will be required to determine whether interactions between the extracellular matrix and integrins are a common feature of epithelial polarity during animal organogenesis.

Polarity Signaling in Cancer

Given the fundamental importance of polarity signaling to cell structure, it seems intuitive that disruptions in polarity could be involved in tumorigenesis. However, much of the evidence for polarity genes as either tumor suppressors or oncogenes is correlative, and much of the experimental evidence thus far comes from fly models. To the extent that polarity genes have been clearly demonstrated to be tumor suppressors, the mechanisms remain largely unexplored. The work I present in later chapters helps uncover mechanisms by which the Par3 protein can act as a tumor suppressor. In this section, I will review the current evidence that polarity proteins impact tumor growth and dissemination, with an emphasis on experimental evidence.

The initial suggestion that polarity genes act as tumor suppressors came from observations in *Drosophila* models, where mutations in several polarity genes lead to tissue overgrowth (Elisabeth Gateff, 1978; E. Gateff & Schneiderman, 1974; Kurzik-Dumke, Phannavong, Gundacker, & Gateff, 1992). In fly models,

loss of polarity genes synergizes with activation of oncogenes to promote tumor growth and invasion (Cordero et al., 2010; Pagliarini & Xu, 2003; M. Wu, Pastor-Pareja, & Xu, 2010). Given the highly-conserved function of polarity proteins between flies and mammals (Bergstralh & St Johnston, 2012), these studies suggest that polarity genes may function as tumor suppressors in higher organisms. However, until recently there was only circumstantial data to support this hypothesis.

In mammalian tumor models, there is some evidence for involvement of all three major polarity complexes. HPV, which is a risk factor for several tumors (Mammas, Sourvinos, Giannoudis, & Spandidos, 2008), encodes for proteins that target cell polarity proteins for degradation (Javier, 2008). The Crb complex component Patj is one polarity protein that the E6 protein of HPV targets (Storrs & Silverstein, 2007), suggesting that Patj may have a tumor suppressor role. Other evidence for Crb complex involvement comes from studies showing that Crb3 expression is lost in mouse kidney epithelial cells that are selected *in vivo* for tumor formation (Karp et al., 2008). This study showed that reexpression of Crb3 reduces cell invasion and proliferation in culture assays, and prolongs animal survival by limiting metastasis *in vivo*. Studies in *Drosophila* have shown that Crb acts to restrain the Hippo pathway, and that loss of Crb leads to overgrowth in the wing imaginal disc due to Hippo activation (Ling et al., 2010). Although a tumor growth phenotype has not been shown, mammalian Pals1 and Patj have been shown to interact with the Hippo component Yap/Taz (Varelas et

al., 2010). This appears to function as a density-sensing mechanism, as this

interaction retains Yap/Taz in the cytoplasm and limits proliferation when cells

are cultured at high density (Varelas et al., 2010). Finally, there is evidence that

the EMT factors Snail and Zeb1 transcriptionally repress Crumbs expression

(Aigner et al., 2007; Spaderna et al., 2008; Whiteman, Liu, Fearon, & Margolis,

2008). Because expression of these EMT factors is associated with metastasis,

this implies that repression of Crumbs may be an important step in tumor

dissemination. Though largely correlative, this body of work suggests that the

Crumbs complex has tumor suppressor functions. However, definitive studies in

mouse models will be required to confirm this, and to determine the mechanisms

by which Crumbs proteins may restrain tumors *in vivo*.

Of the three major polarity complexes, the Scrib group is best studied as a tumor

suppressor, albeit mainly in flies. For four decades, it has been understood that

mutations in Lgl or Dlg can lead to tissue disorganization and overgrowth

(Elisabeth Gateff, 1978; E. Gateff & Schneiderman, 1974). Scrib was recognized

as a tumor suppressor in *Drosophila* at the same time as it was found to interact

genetically with Lgl and Dlg (Bilder et al., 2000). In this early study, homozygous

Scrib deletions led to overgrowth of the wing disc epithelium (Bilder et al., 2000).

A screen for genes that cooperate with activated Ras to promote metastasis in

flies identified numerous polarity proteins, including all three members of the

Scrib group (Pagliarini & Xu, 2003). This has been confirmed by numerous

experiments, which have found that loss of Scrib group genes cooperates with

activation of various oncogenes to promote tumor growth and invasion in flies (Brumby & Richardson, 2003; Cordero et al., 2010; Doggett, Grusche, Richardson, & Brumby, 2011; Froldi et al., 2010).

There is significant correlative evidence that the Scrib complex has tumor suppressor functions in mammals, as the *Drosophila* experiments would suggest. For example, Scrib and Dlg have decreased expression in cervical and colon cancers (Gardiol, Zacchi, Petrera, Stanta, & Banks, 2006; S. Nakagawa et al., 2004), and Scrib and Dlg are targeted for degradation by HPV E6 proteins (Gardiol et al., 1999; Shunsuke Nakagawa & Huibregtse, 2000; Thomas, Massimi, Navarro, Borg, & Banks, 2005). Lgl1 protein levels are markedly reduced in human melanomas, and Lgl2 is often lost or mislocalized in gastric tumors (Kuphal et al., 2005; Lisovsky et al., 2009). In tumors where Dlg expression is not affected, it is often mislocalized away from the basolateral cortex (Cavatorta et al., 2004), suggesting that proper localization of Scrib complex proteins is important for their tumor suppressor function.

To date, there is limited experimental data to support the Scrib complex as a tumor suppressor in mammals. Silencing of Scrib promotes intraepithelial neoplasia in the mouse prostate, and can cooperate with Ras to trigger frank prostate carcinoma (Pearson et al., 2011). Further, loss of Scrib cooperates with Myc overexpression to promote growth of mouse mammary tumors *in vivo* (Zhan et al., 2008). Together with the data from fly models and correlative evidence

from human tumor samples, these studies strongly implicate Scrib complex

proteins as clinically-relevant tumor suppressors. However, additional

experimental evidence will be necessary for confirmation and to elucidate

molecular mechanisms.

There is some correlative evidence that Par complex components are involved in

cancer. 15% of primary esophageal SCC cells examined in one study were

found to have copy number losses of the PARD3 gene, and reduced expression

of Par3 in clinical samples was found to correlate with lymph node metastasis

(Zen et al., 2009). Similarly, a screen of 684 cancer cell lines for genetic

microdeletions found frequent disruption of the PARD3 gene in human head and

neck SCC, esophageal tumors, and glioblastomas (Rothenberg et al., 2010), and

expression of Par3 in esophageal SCC cells that lack the PARD3 gene slowed

proliferation in culture. Amplification of the PRKCI gene has been found in

esophageal SCC, and correlates with lymph node metastasis (Yang et al., 2008).

Overexpression and mislocalization of the aPKCι/λ protein is seen in high-grade

breast (Kojima et al., 2008) and ovarian cancers (Eder et al., 2005; L. Zhang et

al., 2006). In gastric tumor samples, overexpression of aPKCι/λ is a negative

prognostic factor (Takagawa et al., 2010). Similarly, the Par6 protein is

overexpressed in many breast tumor samples as compared to normal tissue

(Nolan et al., 2008).

In *Drosophila* models, disruption of either Baz or the aPKC binding partner

Cdc42 does not cause tissue overgrowth (Genova, Jong, Camp, & Fehon, 2000;

Müller & Wieschaus, 1996). This contrasts with Scrib complex proteins, for

whom deletion throughout a tissue is sufficient to induce hyperplasia (Pagliarini &

Xu, 2003). However, when combined with Ras activation loss of Baz produces a

phenotype very similar to Scrib deletion, suggesting that Baz and Scrib act

similarly to restrain proliferation in the context of activated oncogenes. The only

other study to date of Baz's function in *Drosophila* tumors shows that wild-type

Baz is required for invasion of Dlg-deficient neoplasms of the ovarian follicular

epithelium (Abdelilah-Seyfried, Cox, & Jan, 2003). This suggests that Par

proteins may actually promote tumor progression in some contexts.

Cell culture and *in vivo* experiments in mammalian systems indicate that Par

complex proteins can act both to suppress and to promote tumor progression.

Par6 is generally implicated in promoting tumor progression via mechanisms that

require its interaction with aPKC. In 3D cultures of MDCK and MCF10A cells,

overexpression of the oncogene ErbB2 triggers proliferation, suppresses

apoptosis, and causes disorganization of multicellular structures (Aranda et al.,

2006). If interactions between ErbB2 and Par6/aPKC are prevented, the

disruption of epithelial architecture is inhibited and apoptosis is restored (Aranda

et al., 2006). Consistent with this result, *in vitro* and *in vivo* studies have found

that Par6 activity is required for TGFβ-induced metastasis of breast cancer cells

(Viloria-Petit et al., 2009). Further, overexpression of Par6 drives proliferation of

breast cancer cell lines, and Par6 is overexpressed in clinical breast tumor samples (Nolan et al., 2008). Notably, Par6 must interact with Cdc42 and aPKC, but not with Par3, to drive proliferation of tumor cells (Nolan et al., 2008).

There is considerable experimental evidence that the aPKCι/λ isoform is an oncogene. One of the earliest studies implicating it in human malignancy revealed that it was necessary for both drug resistance (Jamieson, Carpenter, Biden, & Fields, 1999) and NF-κB-mediated survival (Lu, Jamieson, Brasier, & Fields, 2001) in myelogenos leukemia cells. This was followed by the discovery that aPKCι/λ is overexpressed in lung tumors and promotes growth of lung cancer cells (Regala et al., 2009; Regala, Weems, Jamieson, Copland, et al., 2005; Regala, Weems, Jamieson, Khoor, et al., 2005), and that aPKCι/λ is required for Ras-mediated transformation of mouse colonic epithelium *in vivo* (Murray et al., 2004). aPKCι/λ also is necessary for maintaining the population of ovarian tumor-initiating cells (Y. Wang, Hill, & Fields, 2013). aPKCι/λ can promote tumors growth by activating other signaling pathways. There is considerable evidence that aPKCι/λ is involved in activating the NK-κB pathway (S. Ghosh & Baltimore, 1990; Lallena, Diaz-Meco, Bren, Payá, & Moscat, 1999; Win & Acevedo-Duncan, 2008), which is important for hormone-independent growth of prostate tumors (Ishiguro et al., 2009). It also triggers activation of hedgehog signaling, which is important for *in vivo* initiation of lung SCCs (Justilien et al., 2014) and drug resistance in basal cell carcinomas in the skin of mice (Atwood, Li, Lee, Tang, & Oro, 2013).

There are numerous studies examining the expression levels of either the

PRKCZ gene or its protein product, aPKCζ, in human tumors, and they have

been reported to be both increased and decreased (Garg et al., 2013). Cell

culture studies of this kinase are similarly conflicting, with several reports that

increased expression of aPKCζ inhibits growth and triggers apoptosis of tumor

cells (Mustafi, Cerda, Chumsangsri, Fichera, & Bissonnette, 2006; Nazarenko et

al., 2010), while others have shown a role for aPKCζ in proliferation and

protection against apoptosis (P. M. Ghosh, Bedolla, Mikhailova, & Kreisberg,

2002; Xin, Gao, May, Flagg, & Deng, 2007). On balance, the *in vivo* data

supports aPKCζ as a tumor suppressor. Although no effect was seen in primary

tumor burden, overexpression of aPKCζ in rat prostate tumor cells markedly

reduced metastasis to the lungs (Powell et al., 1996). More recently, it has been

reported that aPKCζ deficiency leads to increased production of IL-6, which

promotes tumorigenesis in a mouse model of Ras-induced lung cancer (Galvez

et al., 2009). There is evidence that aPKCζ directly phosphorylates the

oncogene c-Myc, thus inhibiting its nuclear translocation and preventing prostate

tumors in mice (J. Y. Kim et al., 2013). aPKCζ suppresses cancer through

metabolic mechanisms, since decreased expression permits tumors to produce

ATP via glutamine oxidation in the Krebs cycle rather than through glycolysis (Ma

et al., 2013). aPKCζ has been reported to inhibit the activation of NF-κB

signaling in response to reactive oxygen species (Banan et al., 2003). Since NF-

κB signaling is commonly activated in human tumors (Bassères & Baldwin,

2006), this hints at another mechanism for aPKCζ-mediated tumor suppression, albeit one lacking *in vivo* confirmation.

There is a small but compelling literature experimentally demonstrating that Par3 is a mammalian tumor suppressor. Iden *et al* (2013) have demonstrated that Par3 is a tumor suppressor in a model of chemical mutagen-driven keratoacanthomas. Although loss of Par3 dramatically increases the incidence of these tumors in mice, silencing it actually decreases incidence of Ras-driven papillomas. In both cases, aPKC immunoreactivity is lost at cell-cell junctions and is found diffuse in the cytoplasm. However, the significance of aPKC mislocalization in this study is unclear. Similar to the keratoacanthomas studied by Iden *et al*, Par3 appears to act as a suppressor of growth and metastasis in both Ras- and Notch-driven models of murine primary breast cancer (McCaffrey, Montalbano, Mihai, & Macara, 2012). These effects appear to be mediated by aPKC-dependent activation of Stat3 (McCaffrey et al., 2012), but signaling events downstream of aPKC have not been identified. Moreover, the role of aPKC has only been demonstrated via inhibition with pharmacologic compounds that have significant potential for off-target effects (Lee et al., 2013; Volk, Bachman, Johnson, Yu, & Huganir, 2013). Earlier work showed that *in vivo* knockdown of Par3 in the mouse mammary gland leads to disorganization of the mammary ducts, with increased proliferation offset by apoptosis (McCaffrey & Macara, 2009). This suggests that loss of Par3 is sufficient to trigger the tissue disorganization that is typical of cancers, but that a compensatory apoptotic

response prevents tumor formation unless an active oncogene suppresses cell death.

In addition to effects mediated by aPKC, a Tiam1-Rac1 axis appears to be active in Par3-deficient tumors. There is *in vitro* and *in vivo* evidence from the mouse mammary gland that loss of Par3 triggers proliferation via activation of Tiam1 and Rac1, but it is not clear whether this pathways is involved in activating Stat3 or promoting metastasis (Archibald, Mihai, Macara, & McCaffrey, 2014). Another recent study has shown that loss of Par3 promotes metastasis of human breast cancer cells in nude mice by altering E-cadherin dynamics and reducing cell-cell adhesion via Tiam1-mediated Rac1 activation, but no effect on primary tumor growth has been observed (Xue, Krishnamurthy, Allred, & Muthuswamy, 2013). The discrepancy in tumor growth phenoctypes may be due to the tumor models used. Archibald et al. (2014) used primary mammary epithelial cells transformed by NICD1, which is a weak oncogene with long tumor latency (McCaffrey et al., 2012). In contrast, Xue et al. (2012) used ErbB2-transformed MCF10A cells. These independent studies strongly implicate the Tiam1-Rac1 axis following Par3 silencing, but it is unclear if this pathway is involved in the Stat3 activation that has been reported (McCaffrey et al., 2012).

The Par3 protein is decreased in human breast tumor samples (McCaffrey et al., 2012; Xue et al., 2013), but the mechanism is not clear. Although genomic deletions have been found in a subset of SCCs and GBMs, a screen of 684

human cancer cell lines found no genomic loss of Par3 in any other type of cancer (Pickering et al., 2014; Rothenberg et al., 2010). This suggests that epigenetic or post-transcriptional mechanisms are predominant. A recent study demonstrated that the scaffold protein Amot2L is upregulated in human breast and colon cancers, and that it acts to sequester Par3 and Crb3 into large cytoplasmic vesicles, thus disrupting cell polarity (Mojallal et al., 2014). It is unclear whether this sequestration impacts the stability of the Par3 protein. Nevertheless, this demonstrates that post-translational mechanisms may limit Par3 activity in tumors.

While there is growing evidence that the mammalian polarity proteins impact tumor biology, the mechanisms are largely undiscovered. It remains unclear why loss of Par3 should promote tumor progression in some contexts, but restrain it in others (Iden et al., 2012). With regard to metastasis, data linking loss of Par3 to activation of Stat3 in the murine mammary gland (McCaffrey et al., 2012) represents a promising pathway, because Stat3 is widely implicated in cancer biology (H. Yu, Pardoll, & Jove, 2009). Despite these recent advances, numerous questions remain. How does loss of Par3 trigger Stat3 activation? Is restraining Stat signaling a general mechanism by which polarity regulators repress tumors? Evidence that loss of Scrib plus activation of Ras triggers Stat signaling and metastasis via the JNK pathway in *Drosophila* implies that this may be a highly-conserved mechanism (M. Wu et al., 2010). The studies reported here were undertaken to address this issue. In particular, I set out to test the

hypothesis that loss of Par3 triggers Stat3 signaling through activation of aPKCι/λ in mouse mammary cells (Chapter II). I additionally investigated whether loss of Par3 impacts human mammary epithelial cells and human tumor cell lines in the same way as it impacts murine mammary cells (Chapter III).

Chapter II

Loss of the Polarity Protein Par3 Activates Stat3 Signaling via an aPKC/NF-κB/IL-6 Axis in Mouse Mammary Cells

The contents of this chapter, with small experimental changes, have been accepted for publication in the Journal of Biological Chemistry *under the same title. Prof. Ian G. Macara is both the senior author and sole co-author on the accepted manuscript.*

Abstract

The machinery of epithelial apical-basal polarity plays a key role not only in cell polarization but also in tissue homeostasis and tumor suppression. In particular, the Par3 polarity protein is important for both growth- and metastasis-suppression in mammary tumor models. I propose that Par3 organizes and limits multiple signaling pathways, and that inappropriate activation of these pathways can occur upon depletion of Par3. In support of this concept, silencing Par3 in conjunction with oncogenic activation promotes invasion and metastasis via the constitutive activation of Stat3 in the murine mammary gland. However, the mechanism of Stat3 activation has not been identified. I now show that loss of Par3 triggers increased production of the cytokine IL-6, which acts in an autocrine fashion to induce Stat3 signaling. This process is mediated by activation of aPKCι/λ, which induces the NF-κB pathway and IL-6 production.

These results suggest that Par3 behaves as a tumor suppressor by restraining aPKCɩ/λ, and by preventing aPKCɩ/λ from inappropriately activating of an oncogenic signaling network.

Introduction

Cancer results from alterations in signaling networks that control cell growth and differentiation. Like most solid tumors, breast cancers arise from epithelial tissues, and often retain epithelial characteristics as they progress (Korpal et al., 2011). Although many of the signaling pathways involved in breast tumors have been elucidated, others are still being discovered. After many years of speculation that polarity disruption is fundamental to cancer, recent data have proven that defects in the epithelial polarity machinery accelerates solid tumor progression in mammals (Benhamouche et al., 2010; Galvez et al., 2009; Ling, Su, Zuo, & Muller, 2012; Zhan et al., 2008; Zhuang et al., 2013). Although the tumor suppressor functions of polarity pathways have been extensively studied in *Drosophila* models (Bergstralh & St Johnston, 2012), in mammalian systems the mechanisms by which polarity restrains tumor progression remain largely unknown. Understanding how polarity signaling impacts tumor biology will aid in the development of targeted therapies.

Three polarity networks are highly conserved in mammalian cells: the Par complex, consisting of Par3, Par6, aPKC, is situated at tight junctions and the apical surface; the Crumbs complex, containing Crb, Pals1, and Patj, is essential

for specifying the apical membrane; and a group of proteins that includes Scrib, Dlg, and Lgl that localizes to the basolateral membrane (Chatterjee & McCaffrey, 2014). Members of all three groups have been implicated in tumorigenesis, although mainly through correlative evidence. Amplification of the aPKC isoform aPKCι/λ is commonly observed in lung cancers (Regala, Weems, Jamieson, Copland, et al., 2005), and Par6b is often highly expressed in breast tumors (Nolan et al., 2008). In *Drosophila* models, Lgl and Scrib have been implicated as tumor suppressors (Agrawal, Kango, Mishra, & Sinha, 1995; Timmons et al., 1993; M. Wu et al., 2010). The human Lgl gene, HUGL2, may restrain growth and invasion of transformed mammary cells in 3D cultures (Russ, Louderbough, Zarnescu, & Schroeder, 2012), and deletion of Scrib cooperates with c-Myc to promote mammary tumor formation in a mouse model (Zhan et al., 2008). Finally, recent papers have directly implicated Par3 as a growth and metastasis suppressor in mouse models of mammary and skin tumors (Archibald et al., 2014; Iden et al., 2012; McCaffrey et al., 2012; Xue et al., 2013).

Par3 is a highly conserved regulator of cell polarity. It was first identified as a maternal effect gene required for proper cleavage of *Caenorhabditis elegans* embryos (Etemad-Moghadam et al., 1995; Kemphues et al., 1988), and has since been found to control metazoan cell polarity in many contexts (Goldstein & Macara, 2007). In mammalian epithelial cells, it self-oligomerizes and localizes to the tight junctions, where it is the key member of the Par complex (Benton & Johnston, 2003a; Feng et al., 2007; Mizuno et al., 2003). It acts to deliver aPKC

and Par6 to the apical membrane, where aPKC acts in some epithelia to phosphorylate Pins to restrict it the basolateral surface (Hao et al., 2010). Because Pins anchors the mitotic spindle to the cell cortex, loss of Par3 and subsequent mislocalization of aPKC in MDCK epithelial cells allows the axis of cell division to randomize, leading to epithelial disorganization (Hao et al., 2010). These functions make Par3 an important regulator of tissue structure, such as in the mammary gland.

Loss of Par3 in the developing mammary gland leads to disorganized ducts resembling atypical ductal hyperplasia, with increased proliferation offset by heightened apoptosis (McCaffrey & Macara, 2009). When loss of Par3 synergizes with an oncogene in mammary ducts, apoptosis is suppressed, growth of primary tumors is accelerated, and aggressive metastatic lesions arise in a Stat3-dependent manner (McCaffrey et al., 2012). Expression of the NICD1 oncogene alone does not induce Stat3 in mMECs (McCaffrey et al., 2012), showing that the effect depends on loss of Par3. Moreover, NICD1-mMECs properly localize polarity markers such as aPKC, but these markers become disrupted when Par3 is silenced (McCaffrey et al., 2012), recapitulating what is observed in other epithelial cells (Hao et al., 2010; Iden et al., 2012).

Stat3 is a transcription factor that has been implicated in the initiation, progression, and metastatic spread of numerous types of cancer, and may be a valuable therapeutic target (Bromberg et al., 1999; Johnston & Grandis, 2011).

In ErbB2/Neu-transformed mammary cells, constitutive activation of Stat3 increases local invasion both in culture and *in vivo* and enhances metastatic colonization of the lungs, highlighting its importance in breast cancer (Barbieri et al., 2010). In the contexts of inflammation and neoplasia, Stat3 can be activated by numerous pathways, including Src signaling, inflammatory cytokines, toll-like receptor activation, adrenergic signaling, and radiation damage (H. Yu et al., 2009). Preceding work has found that Stat3 becomes active following loss of Par3 in NICD1-transformed mammary cells, and that this activation mediates local invasion and lung metastasis (McCaffrey et al., 2012). However, the mechanism of Stat3 activation following loss of Par3 has not been identified. In the work presented in this chapter, I sought to determine how loss of Par3 activates Stat3 in a breast cancer cell model.

There is reason to suspect that aPKCι/λ mediates the oncogenic effects – such as Stat3 activation – that are observed when Par3 is lost. There are two mammalian aPKC genes, Prkci and Prkcz, which encode for aPKCι/λ and aPKCζ, respectively (Ono et al., 1989; Selbie, Schmitz-Peiffer, Sheng, & Biden, 1993). There is significant homology between these two isoforms (Selbie et al., 1993), and thus some functional overlap. Both are expressed in the brain, and aPKCζ is also abundant in the kidneys (Assémat et al., 2008). Although both are expressed in epithelial cells, aPKCι/λ is markedly more abundant in murine mammary cells. Numerous studies have shown that loss of Par3 leads to either mislocalization or mislocalization plus activation of aPKC in epithelial cells (Hao

et al., 2010; Iden et al., 2012; McCaffrey & Macara, 2009; McCaffrey et al., 2012). This observation, combined with growing evidence that aPKCι/λ has oncogenic functions (Fields & Regala, 2007), suggests that aPKCι/λ may favor tumor aggression when Par3 is silenced. While the literature suggests several mechanisms by which aPKC activation could promote Stat3 signaling – such as potentiating TNF-α signaling (Lallena et al., 1999), interacting with NF-κB (Ishiguro et al., 2009), promoting Erk activity (Chung, Uchida, Grammer, & Blenis, 1997; Litherland et al., 2010), and transducing signals downstream of EGFR or Ras (Iden et al., 2012; Kusne et al., 2014; Überall et al., 1999) – it is not obvious which is involved when Par3 is disrupted. In the present study, we use gene silencing to rigorously test the role of aPKCι/λ following Par3 knockdown in transformed mouse mammary cells and identify the mechanism through which it induces Stat3.

Results

<u>Stat3 activation following loss of Par3 in transformed mammary epithelial cells</u>

<u>requires gp130</u>

Stat3 activation commonly occurs by IL-6 family cytokines binding to the gp130 receptor (Jones, Scheller, & Rose-John, 2011). The *Drosophila* homologues of the IL-6 family, Upd genes, are induced when polarity is disrupted in ventral nerve cord tumors (M. Wu et al., 2010), and so I hypothesized that cytokine signaling via the gp130 receptor might activate Stat3 in our model. To silence the expression of gp130, I expressed shRNAs against the Il6st gene in primary murine mammary epithelial cells transformed by lentiviral transduction with NICD1 (Fig. 3A). Consistent with prior findings (McCaffrey et al., 2012), knock down of Par3 led to robust activation of Stat3, as assessed by tyrosine-705 (Y705) phosphorylation (Fig. 3A, B), but Stat3 activation returned to baseline levels when gp130 and Par3 were silenced together (Fig. 3A, B).

To determine if the gp130-mediated Stat3 activation was specific to transformed cells, I knocked down Par3 and gp130 in primary mammary epithelial cells isolated from mammary glands of C3H mice. The cells were grown for 5 days in 3D mammosphere culture, then lysed them and probed for Stat3 activation. Loss of Par3 in these primary cells activated Stat3, and simultaneous silencing of Par3 and gp130 prevented Stat3 phosphorylation (Fig. 3C), showing that this effect occurs in wild type primary MECs and is not restricted to NICD1-transformed cells. In addition, I observed activation of Stat3 when Par3 was silenced in two

widely-used murine mammary cell lines, NMuMG and Eph4, and knock down of gp130 again prevented Stat3 activation (Fig. S1). These results confirm that Stat3 activation following Par3 knock down occurs through a cytokine receptor-dependent pathway in multiple mammary epithelial cell lines, independently of oncogene activation. I pursued further characterization of the pathway using NICD1-mMECs, because unlike the primary WT cells they can be easily cultured in sufficient quantities for biochemical analysis. Additionally, the immortalized cell lines were not used subsequently because they might have unknown genetic or epigenetic changes associated with long-term growth in culture that could alter connectivity within signaling networks.
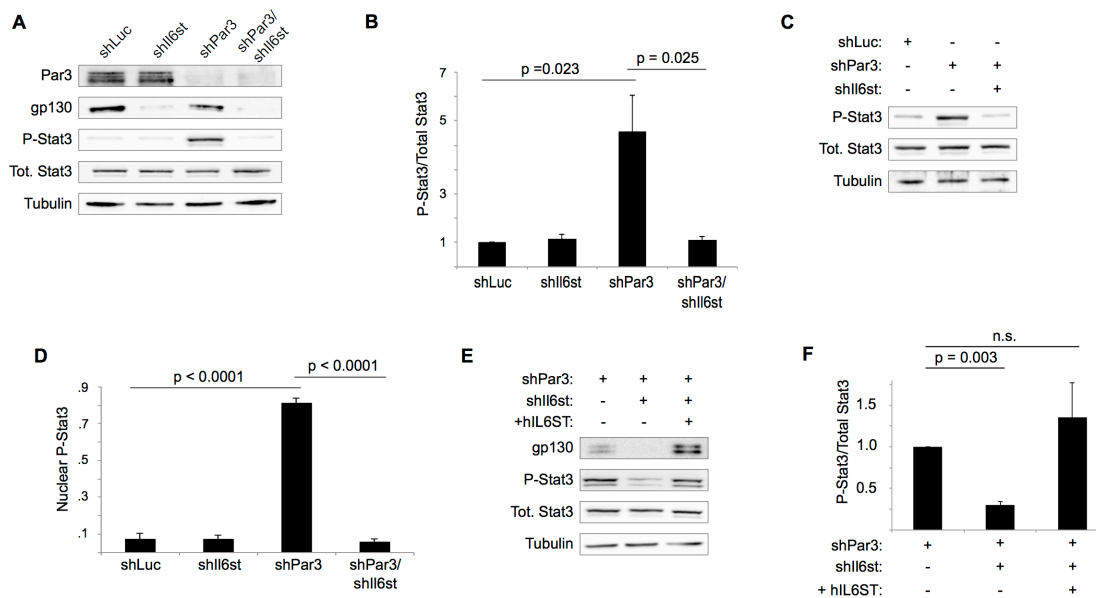
Figure 3. Loss of Par3 triggers Stat3 activation via gp130. (A) NICD1-mMECs, infected with lentivirus to express the indicated shRNAs, were harvested and equal amounts of lysate were immunoblotted for Par3, gp130, phospho-Stat3, total Stat3, and beta tubulin (loading control). (B) Quantitation of phospho-Stat3/total Stat3 from immunoblot experiments shown in (A), n=7. (C) Primary murine mammary cells were harvested and infected with lentivirus to express the constructs indicated, then cultured as mammospheres in non-adherent conditions for 5 days. After culture, the mammospheres were harvested and lysed, and equal amounts of lysate were immunoblotted for phospho-Stat3, total Stat3, and beta tubulin (loading control). (D) Quantitation of the proportion of cells displaying nuclear phospho-Stat3 staining, n=6. (E) NICD1-mMECs infected with lentivirus to express the constructs indicated were harvested and lysates were immunoblotted for gp130, phospho-Stat3, total Stat3, and beta tubulin (loading control). (F) Quantitation of the phospho-Stat3/total Stat3 from immunoblot experiment shown in (E), n=3. Error bars represent SEM.

I next investigated nuclear accumulation of activated Stat3 by immunofluorescence. Following infection with virus encoding a hairpin against the genes for either luciferase or gp130, less than 10% of nuclei displayed staining for phospho-Y705 Stat3 (Fig. 3D and Fig. S2). However, depletion of Par3 led to more than a ten-fold increase in the proportion of nuclei positive for P-Y705 Stat3 (Fig. 3D and Fig. S2). Additional silencing of gp130 caused

nuclear P-Y705 Stat3 accumulation to return to baseline levels (Fig. 3D and Fig.

S2). To determine if the repression of Stat3 activity observed following gp130

knock down was functionally significant, we analyzed the expression of five

genes whose transcripts can be stimulated by Stat3: Socs3, Junb, c-Myc, Stat3,

and Stat1 (Dauer et al., 2005; E. Kim et al., 2013, p. 2). All five genes were

induced when Par3 was knocked down, and three of the five – Junb, Stat3, and

Stat1 – returned towards baseline levels when Par3 and gp130 were knocked

down concurrently (Fig. S3).

Finally, to ensure that the inactivation of Stat3 seen after shRNA knock down of

gp130 was not due to nonspecific effects of the hairpin, I infected cells with

lentivirus encoding human gp130, which can interact with the mouse isoforms of

the IL-6 receptor to activate Stat3 (Hammacher et al., 1998). Expression of

human gp130 successfully restored activation of Stat3 in NICD1/shPar3 cells in

which murine Il6st had been silenced (Fig. 3E, F). These experiments

demonstrate that Stat3 activation following Par3 silencing occurs via a gp130

receptor-mediated signaling pathway.

<u>Aytpical protein kinase C-iota is necessary for Stat3 activation</u>

I next asked how loss of Par3 triggers activation of Stat3 in mammary cells. In

previous studies, it was shown that aPKC becomes activated in transformed

mammary cells following loss of Par3. Moreover, Stat3 activation could be

blocked using pseudosubstrate inhibitors of aPKC (McCaffrey et al., 2012).

However, aPKC pseudosubstrate inhibitors have since been shown to have significant nonspecific activity (Lee et al., 2013; Volk et al., 2013), calling this result into question. I thus used gene silencing to test the hypothesis that signaling via aPKC is necessary for Stat3 activation following Par3 knock down.

I first confirmed that aPKC is activated following loss of Par3, as measured by phosphorylation of threonine 560 (T560). As previously reported, activation of aPKC is significantly induced by Par3 silencing in NICD1-mMECs (Fig. 4A). Silencing of the gene encoding aPKCι/λ, Prkci, reduced phospho-Stat3 to levels seen in shLuc control cells, demonstrating that aPKCι/λ is necessary to induce Stat3 (Fig. 4B, C). To confirm that this effect was due to on-target effects of the shRNA against Prkci, I re-expressed wild-type aPKCι/λ tagged with tRFP and observed rescue of Stat3 phosphorylation (Fig. 4D).

To determine whether aPKCι/λ activation is sufficient to trigger Stat3 activation, I expressed a tRFP-tagged aPKCi-CA in NICD1-mMECs. Stat3 was activated following expression of aPKCi-CA (Fig. 4E, F), demonstrating that activation of aPKCι/λ is sufficient to induce Stat3 in this model. Moreover, silencing of the Il6st gene was sufficient to abrogate the Stat3 activation seen when aPKCi-CA is overexpressed (Fig. 4G, H), showing that aPKCι/λ acts upstream of the gp130 receptor. Finally, to confirm that aPKC activation upon loss of Par3 is not an artifact of NICD1 overexpression, I immunoblotted for phosphorylated aPKCι/λ in freshly isolated, primary mouse MECs grown in 3D suspension culture for 5
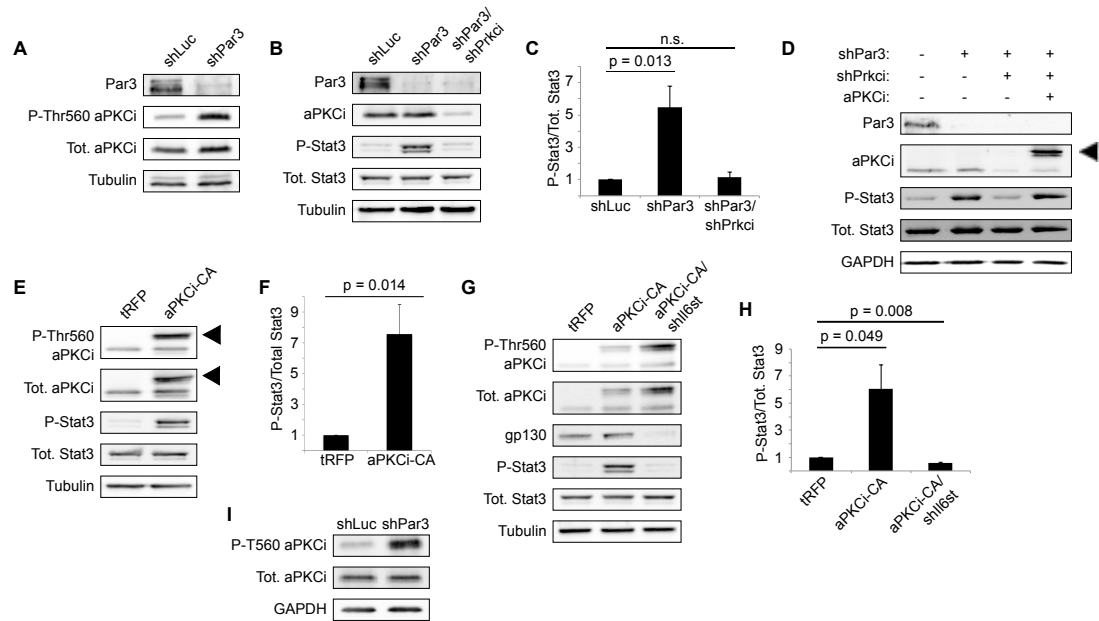
Figure 4. Activation of aPKCι/λ triggers Stat3 activation when Par3 is silenced. (A) NICD1-mMECs infected with lentivirus to express shRNAs indicated were harvested, and equal amounts of lysate were immunoblotted for Par3, phospho-threonine560 aPKC, total aPKCι/λ, and beta tubulin (loading control). (B) NICD1-mMECs infected with lentivirus to express the shRNA constructs indicated were harvested, and equal amounts of lysate were immunoblotted for Par3, aPKCι/λ, phospho-Stat3, total Stat3, and beta tubulin (loading control). (C) Quantitation of phospho-Stat3/total Stat3 in (B), n=4. (D) NICD1-mMECs infected with lentivirus to express the constructs indicated were harvested, and equal amounts of lysate were immunoblotted for Par3, aPKCι/λ, phospho-Stat3, total Stat3, and GAPDH (loading control). Arrowhead indicates expressed tRFP-tagged aPKCι/λ. (E) NICD1-mMECs expressing tRFP tagged aPKCi-CA or tRFP (control) were lysed and immunoblotted for phospho-T560 aPKC, total aPKCι/λ, phospho-Stat3, total Stat3, and beta tubulin (loading control). Arrowheads indicate expressed tRFP-tagged aPKCi-CA. (F) Quantitation of phospho-Stat3/total Stat3 in (E), n=4. (G) NICD1-mMECs infected with lentivirus to express the indicated constructs were harvested and immunoblotted for phospho-T560 aPKC, total aPKCι/λ, gp130, phospho-Stat3, total Stat3, and beta tubulin (loading control). (H) Quantitation of phospho-Stat3/total Stat3 in (G), n=3. (I) Primary murine mammary cells were harvested and infected with lentivirus to express the constructs indicated, then cultured as mammospheres in non-adherent conditions for 5 days. After culture, the mammospheres were harvested and lysed, and equal amounts of lysate were immunoblotted for phospho-T560 aPKC, total aPKCι/λ, and GAPDH (loading control). Error bars represent SEM.

days. Depletion of Par3 was sufficient to activate aPKCι/λ, indicating that

transformation is not necessary for this phenotype, and that the response occurs

in 3D suspension culture as well as in 2D cultures (Fig. 4I).

<u>Increased IL-6 production is triggered by aPKCι/λ activity after Par3 silencing</u>

<u>and induces Stat3 activation</u>

gp130 is the coreceptor for cytokines of the Interleukin-6 (IL-6) family (H. Yu et al., 2009), and IL-6 has been implicated in numerous malignancies including breast cancer (Y. Guo, Xu, Lu, Duan, & Zhang, 2012; Taniguchi & Karin, 2014). I hypothesized, therefore, that loss of Par3 might induce production of IL-6. When I measured IL-6 levels in culture media conditioned with either NICD1/shLuc or NICD1/shPar3 cells for 16 hrs, I observed a near doubling of IL-6 levels in the medium from shPar3 cells (Fig. 5A). To test whether this cytokine can activate Stat3, NICD1-mMECs were treated with increasing concentrations of recombinant murine IL-6. Treatment with IL-6 induced phosphorylation of Stat3 with activation beginning at some point between 10 and 100 pg/mL of IL-6 (Fig. 5B), thus proving that NICD1-transformed mMECs will activate Stat3 in response to IL-6 stimulation.

I next asked whether IL-6 is necessary for Stat3 activation, testing several shRNA vectors against the IL-6 gene. Two of these hairpins, designated shIl6 #1 and shIl6 #3, depleted IL-6 mRNA and IL-6 cytokine levels in conditioned media by roughly 50% when expressed in NICD1/shPar3 cells (Fig. E4). Both hairpins significantly reduced Stat3 activation relative to the Par3 knock down condition, as assessed by Y705 phosphorylation (Fig 3C, D). To prove that the reduction in Stat3 activation was due to reduced IL-6 production, cells were treated with 100 pg/mL of recombinant IL-6 in addition to either hairpin. As would be expected if

the shIl6 hairpins are not acting through off-target mechanisms, treatment with recombinant IL-6 was sufficient to restore Stat3 phosphorylation (Fig. 5E). Increased IL-6 production following Par3 silencing thus triggers Stat3 activation.
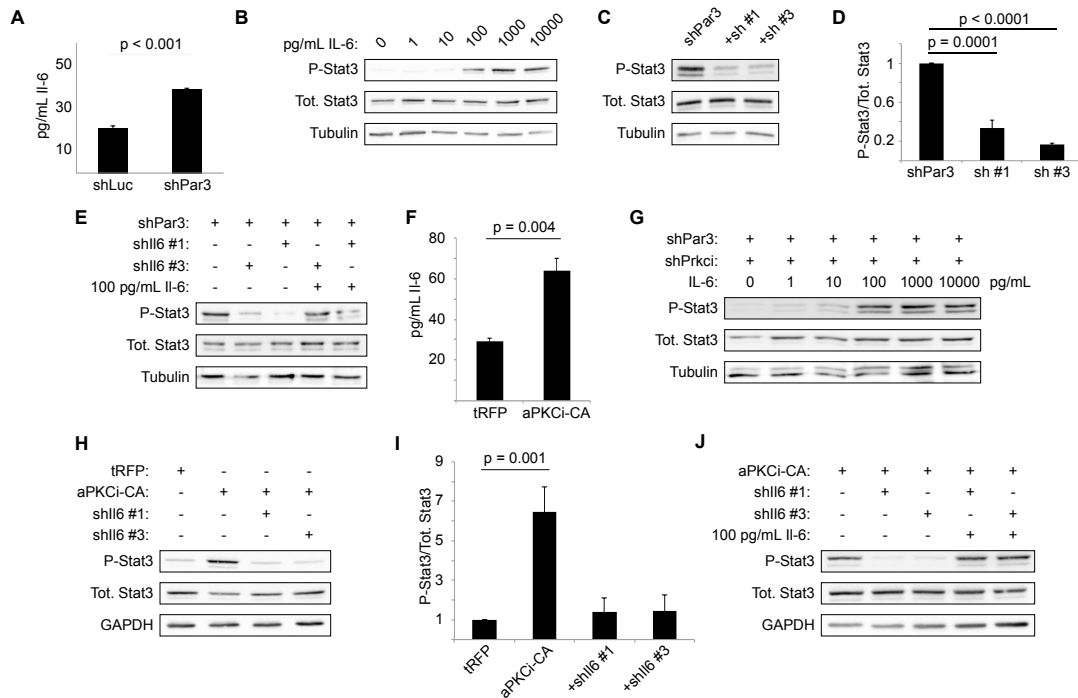
Figure 5. Interleukin-6 production mediated by aPKCι/λ activation triggers Stat3 signaling. (A) Media was collected from cultures of NICD1-mMECs infected with lentivirus to express the shRNAs indicated and analyzed by ELISA for IL-6 levels, n=3. (B) NICD1-mMECs were treated for 20 min with the indicated concentrations of recombinant IL-6, then harvested and equal amounts of lysate were immunoblotted for phospho-Stat3, total Stat3, and beta tubulin (loading control). (C) Cells infected with lentivirus to express the shRNA constructs indicated were harvested, and equal amounts of lysate were immunoblotted for phospho-Stat3, total Stat3, and beta tubulin (loading control). (D) Quantification of immunoblot shown in (C), n=4. (E) NICD1-mMECs expressing shRNAs indicated from lentiviral vectors were treated for 20 minutes with recombinant IL-6 as indicated, and lysates were immunoblotted for phospho-Stat3, total Stat3, and beta tubulin (loading control). (F) Media was collected from cultures of NICD1-mMECs expressing the constructs indicated and analyzed by ELISA for IL-6 levels, n=3. (G) NICD1-mMEC/shPar3/shPrkci cells were treated with the indicated concentrations of recombinant murine IL-6 for 20 minutes, harvested, and then equal amounts of lysates from each were immunoblotted for phospho-Stat3, total Stat3, and beta tubulin (loading control). (H) NICD1-mMECs infected with lentiviruses to express the constructs indicated were harvested, and equal amounts of lysate were immunoblotted for phospho-Stat3, total Stat3, and beta tubulin (loading control) (I) Quantitation of phospho-Stat3/total Stat3 shown in (H), n=5. (J) Cells infected with lentivirus as in (H) were treated with recombinant Il-6 as indicated for 20 minutes prior to harvesting, and equal amounts of lysate were immunoblotted for phospho-Stat3, total Stat3, and GAPDH (loading control). Error bars represent SEM.

I hypothesized that activation of aPKCι/λ induces expression of IL-6, which then

activates Stat3 via the gp130 receptor. As this hypothesis would predict, the

expression of aPKCi-CA induced an approximately 2-fold increase IL-6 cytokine expression in the cell culture medium (Fig. 5F). I observed a dose-dependent activation of Stat3 when recombinant mouse IL-6 was applied to NICD1 /shPar3/shPrkci cells (Fig. 5G). To confirm that aPKC activation triggers Stat3 activity through IL-6, I knocked down the IL-6 gene in cells expressing aPKCi-CA. Silencing of IL-6 eliminated Stat3 activation after aPKCi-CA expression (Fig. 5H, I). Moreover, treatment of NICD1/aPKCi-CA/shIl6 cells with 100 pg/mL of recombinant IL-6 restored Stat3 phosphorylation (Fig. 5J). I conclude that loss of Par3 from murine mammary cells activates aPKCι/λ, leading to production of IL-6 and activation of Stat3 via gp130.

<u>aPKCι/λ-mediated NF-κB activation triggers Stat3 signaling</u>

Multiple signaling pathways can induce IL-6 expression, including activation of NF-κB signaling by aPKCι/λ. aPKCι/λ and aPKCζ interact with and activate multiple components of the NF-κB pathway (Wooten, 1999). Moreover, aPKCι/λ-mediated activation of NF-κB induces IL-6 production in prostate cancer (Ishiguro et al., 2009), and silencing of Par3 can activate NF-κB signaling in human intestinal epithelial cells (Forteza, Wald, Mashukova, Kozhekbaeva, & Salas, 2013). To test whether the activation of Stat3 requires NF-κB signaling, we first treated NICD1-mMEC/shPar3 cells with two chemical inhibitors of NF-κB, JSH-23 and CAPE. Both JSH-23 and CAPE eliminated the Stat3 phosphorylation triggered by loss of Par3 (Fig. 6A, B). In addition, treatment of cells with recombinant IL-6 along with either JSH-23 or CAPE restored Stat3
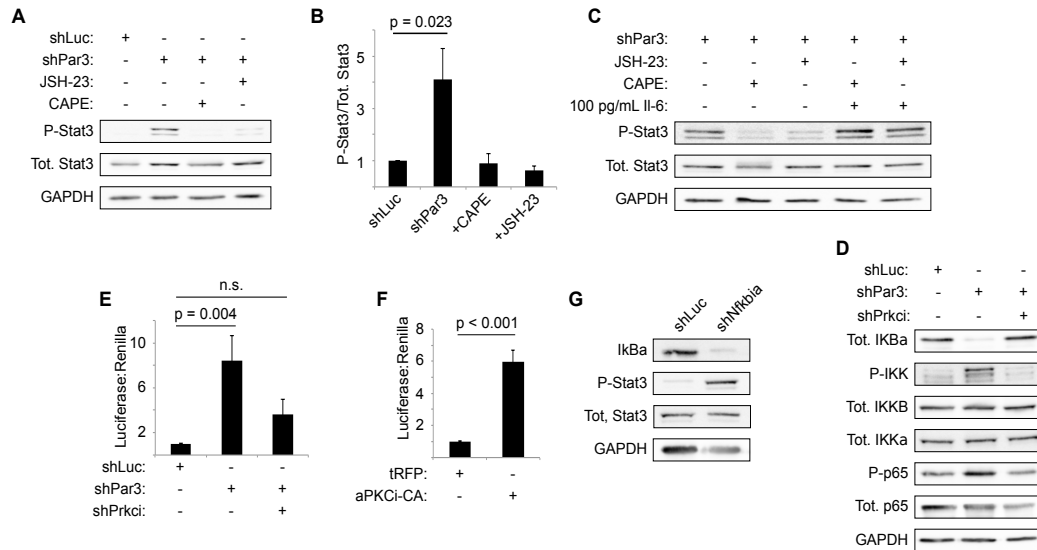
Figure 6. Stat3 activation following aPKCι/λ activation is mediated by NF-κB signaling. (A) NICD1-mMECs infected with lentivirus to express the shRNAs indicated and treated with the NF-κB inhibitors indicated at 30 mg/mL were harvested, and equal amounts of lysate were immunoblotted for phospho-Stat3, total Stat3, and GAPDH (loading control). (B) Quantitation of phospho-Stat3/total Stat3 from (A), n=6. (C) NICD1-mMEC/shPar3 cells were treated with the NF-κB inhibitors indicated at 30 mg/mL and recombinant IL-6 as indicated were harvested, and equal amounts of lysate were immunoblotted for phospho-Stat3, total Stat3, and GAPDH (loading control). (D) NICD1-mMECs infected with lentivirus to express the shRNAs indicated were harvested, and equal amounts of lysate were immunoblotted for IκBα, phospho-IKK (detects both α and β isoforms, β runs slightly higher), total IKKα, total IKKβ, phospho-p65/RelA, total p65/RelA, and GAPDH (loading control). (E) NICD1-mMECs infected with lentiviruses to express the shRNA constructs indicated were calcium phosphate-transfected with an NF-κB reporter plasmid and with constitutively expressed renilla luciferase (normalization control). Cells were lysed 24 hrs after transfection, and luciferase intensity was measured, n=4. (F) NICD1-mMECs infected with lentivirus expressing the constructs indicated were transfected with luciferase plasmids and analyzed as in (E), n=4. (G) NICD1-mMECs infected with lentivirus expressing the shRNAs indicated were harvested, and equal amounts of lysate were immunoblotted for total IκBα, phospho-Stat3, total Stat3, and GAPDH (loading control). Error bars represent SEM.

activation (Fig. 6C), strongly suggesting that NF-κB activity induces IL-6 following loss of Par3.

I next asked directly whether the NF-κB pathway is activated after depletion of Par3. Following loss of Par3, the IκBα subunit is degraded (Fig. 6D),

phosphorylation of both IKKβ and α is increased, and p65 phosphorylation is slightly increased (Fig. 6D). These effects were reversed by concurrent knock down of aPKCι/λ (Fig. 6D). I also observed relocation of the p65 subunit of NF-κB to the nucleus of NICD1-mMECs after Par3 silencing (Fig. S5). To confirm transcriptional activation of NF-κB, I transfected cells with a NF-κB luciferase reporter plasmid (Everhart et al., 2006). Consistent with my prior results, loss of Par3 induced expression of the luciferase gene and concurrent silencing of aPKCι/λ reverted luciferase expression towards baseline (Fig. 6E). In addition, expression of aPKCi-CA induced this luciferase construct (Fig. 6F), demonstrating that aPKCι/λ activation increases NF-κB signaling in these cells. Finally, we silenced the Nfkbia gene, which encodes for the murine IκBα protein. Loss of IκBα will result in the constitutive activation of the NF-κB pathway, and consistent with my previous data, Stat3 in this circumstance was activated (Fig. 6G). I conclude that loss of Par3 triggers NF-κB activity via aPKCι/λ, and that NF-κB induces IL-6 production and activation of Stat3 (Fig. 7), which, as has been shown previously, can in turn promote invasive behavior and metastasis of NICD1-MECs (McCaffrey et al., 2012).

Discussion

In this study, I found that loss of Par3, a protein implicated in a number of human cancers, leads to activation of Stat3 by a mechanism that depends on aPKCι/λ activity in mouse mammary cells. The potential of polarity proteins as tumor suppressors has been recognized for many years, since early studies in *Drosophila* found that polarity pathways restrain tissue proliferation (E. Gateff & Schneiderman, 1974). Loss of polarity genes in *Drosophila* can also synergize with oncogenes to generate tumors that invade and metastasize aggressively (Cordero et al., 2010; Pagliarini & Xu, 2003; M. Wu et al., 2010). However, direct experimental evidence for Par3 as a tumor suppressor in mammalian cancer was lacking until quite recently (Iden et al., 2012; McCaffrey et al., 2012; Xue et al., 2013). Additionally, recent studies have found that Par 3 expression is altered in several cancers, including ESCCs, HNSCCs, and GBMs (Rothenberg et al., 2010; Zen et al., 2009). The mechanisms through which loss of Par3 promotes malignancy remain partially obscure. In the murine skin, Par3 can act either as a tumor suppressor or as an oncogene, depending upon the type of tumor (Iden et al., 2012). In both cases, aPKC is mislocalized from its usual position at the cell cortex, and instead is found diffusely in the cytoplasm. This is similar to the result seen when Par3 is knocked down in 3D cultures of MDCK cells, in which case total levels and activation of aPKC are unchanged but cortical staining is lost (Hao et al., 2010). Similarly, knock down of Par3 in 2D cultures of NICD1-transformed mammary cells is associated with loss of aPKC enrichment at cell-cell contacts (McCaffrey et al., 2012).

Apical

aPKCι   aPKCι

Par3   Par3

IKKβ   IκBα ⊣ p65

Tight Junction

Loss of Par3

Par3   aPKCι   Par3

IKKβ → IκBα   p65

p65 → Il-6
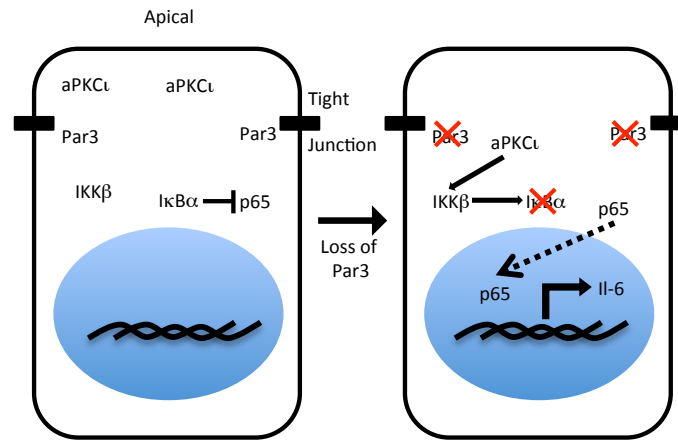
Figure 7. Model for activation of IL-6 production following loss of Par3. When Par3 is normally expressed, aPKCι/λ is restricted to the apical cortex, and IκBα binds to and restrains p65/RelA in the nucleus. When Par3 expression is sufficiently diminished, aPKCι/λ diffuses in the cytoplasm and becomes activated. Activation of aPKCι/λ leads to activation of IKKβ, which phosphorylates IκBα and marks IκBα for degradation. Degradation of IκBα frees p65/RelA to enter the nucleus and induce transcription of target genes, including Il6. Increased Il6 transcription leads to production of the IL-6 cytokine, which activates Stat3 through gp130.

The present work and previous studies from our also lab demonstrate that aPKC can be activated when Par3 is missing (McCaffrey et al., 2012). We believe that the tumor suppressor function of Par3 stems, at least in part, from its role in regulating aPKC's subcellular localization and activity. I propose that a major function of Par3 is to restrict the activity of multiple signaling pathways, of which aPKC is one important example. Loss of Par3 will result in inappropriate activation of these pathways, with sometimes deleterious consequences. Such a model is consistent with aPKC's reported oncogenic role (Linch et al., 2013; Regala, Weems, Jamieson, Copland, et al., 2005; Takagawa et al., 2010). An important limitation of the present study is that while loss of Par3 is shown to

activate aPKCι/λ, and this activation is shown to be necessary and sufficient to induce Stat3, the relative importance of aPKC activation versus mislocalization is not addressed.

I have shown that following loss of Par3, activated aPKCι/λ triggers NF-κB signaling, and NF-κB presumably initiates production of IL-6 (Fig. 7). IL-6 then signals via the canonical gp130-Jak-Stat pathway, leading to activation of networks that promote tumor aggression. These data implicate aPKC isoforms as key mediators of the increased malignancy that occurs after Par3 silencing. In many aspects, this recapitulates what is seen in *Drosophila* models, where the loss of polarity genes in conjunction with activation of oncogenes triggers aggressive, invasive tumors (Brumby & Richardson, 2003). However, the mechanism we propose differs from *Drosophila* biology in several important ways. Although DaPKC may drive tumor growth following disruption of polarity (Leong, Goulding, Amin, Richardson, & Brumby, 2009), it is not known to do so through fly NF-κB homologues. Similarly, loss of Scrib plus activation of Ras induces Upd genes, which are the fly homologues of IL-6 family cytokines (M. Wu et al., 2010). However, this Upd upregulation occurs though JNK signaling, without involvement of DaPKC or NF-κB components. One possible explanation for these discrepancies is that most fly experiments have involved silencing of Scrib, while we have focused our work on Par3. I also speculate that some signaling components that are activated following polarity disruption are

conserved, but the connectivity between these components are different in flies and mammals.

The NF-κB signaling pathway has been implicated in a human cancers (Bassères & Baldwin, 2006). The canonical NF-kB pathway involves activation of IkB kinases (IKKs), which phosphorylate IkBa and mark it for degradation. The degradation of IkBa frees p65/RelA and p50 protein dimers to translocate to the nucleus, where the dimers induce many target genes. The role of aPKC isoforms in NF-κB signaling has been appreciated for some time (Dominguez et al., 1993; S. Ghosh & Baltimore, 1990; Shirakawa & Mizel, 1989). In particular, both aPKCι/λ and aPKCζ have been shown to interact with and activate IKKβ (Lallena et al., 1999; Win & Acevedo-Duncan, 2008). Prior studies have also demonstrated that activated NF-κB induces IL-6 to promote malignancy (Ishiguro et al., 2009; Maeda et al., 2009). However, prior to my current work it has not been obvious that aPKC acts through this path to induce Stat3 following disruption of Par3. This study confirms that loss of Par3 induces preferential phosphorylation of IKKβ (Fig. 6D) and subsequent IL-6 production.

Loss of Par3 was reported to activate NF-κB in the Caco-2 intestinal epithelial cell line (Forteza et al., 2013). However, in contrast with our results, NF-κB activation in Caco-2 cells was not mediated by active aPKCι/λ, but rather inhibited by it. The discrepancy in aPKCι/λ's role may be due to differences in the tissue and species of origin of the cells in each study. Additionally, the report

that loss of Par3 can promote NF-κB activation independently of aPKCι/λ

suggests that the Par complex may impinge upon other pathways that interact

with NF-κB components. Another study recently demonstrated that polarity

proteins including Par3 were required for NF-κB induction in MDCK cells in

response to *Pseudomonas aeruginosa* infection (Tran et al., 2014). When *P.

aeruginosa* contacts the apical surface of these cells, it triggers formation of

membrane protrusions that have characteristics of the basolateral surface, with

Par3 being localized to the boundary of the normal membrane and the basal-like

protrusion. This process is associated with activation of NF-κB signaling in the

host cell. If Par3 is silenced, the NF-κB response is blunted, suggesting that

Par3 organizes signaling molecules that induce NF-κB. In the setting of bacterial

infection, Par3 might act to bring aPKCι/λ to the site of host-microbe interaction,

from where it helps induce NF-κB activity. In contrast, in the absence of Par3,

aPKC localization would be unregulated and aPKCι/λ would be free to interact

with the NF-κB pathway despite the absence of a bacterial cue.

Although Stat3 signaling mediates many of the malignant consequences of Par3

silencing (McCaffrey et al., 2012), loss of Par3 appears to have other effects that

are not yet fully understood. While the Macara lab has observed an increase in

both tumor growth and metastasis upon loss of Par3 (McCaffrey et al., 2012),

another group has only reported increased metastasis, without an impact on

primary tumor latency or size (Xue et al., 2013). This is most likely due to the

differing tumor models used in the studies. Our work has predominantly utilized

primary mouse mammary epithelial cells transformed with NICD1, while the

Muthuswamy lab used ErbB2/Neu-transformed cells transplanted into NOD/SCID

mice. ErbB2/Neu has been reported to activate both the NF-κB and Stat3

signaling networks (DeArmond et al., 2003; Le Page, Koumakpayi, Lessard,

Mes-Masson, & Saad, 2005; Van Laere et al., 2007), which may explain why no

change in primary tumor growth is seen when Par3 is silenced in this model. The

authors demonstrated that increased Tiam1-Rac1 signaling upon loss of Par3

alters cytoskeletal dynamics and weakens E-cadherin-mediated cell adhesions,

thus enabling metastatic spread (Xue et al., 2013). Such effects could also be at

work in my system, potentially representing another pathway by which Par3

restrains the malignant phenotype.

How loss of polarity impacts the inflammatory response to mammalian tumors

remains to be studied, but the activation of NF-κB and IL-6 signaling suggests

that loss of polarity may induce inflammation in mammary tissue. In *Drosophila*

models, deletion of Scrib triggers production of the inflammatory mediator Eiger –

the *Drosophila* homologue of TNF-α – from both tumor cells (Igaki, Pastor-

Pareja, Aonuma, Miura, & Xu, 2009) and circulating hemocytes (Cordero et al.,

2010). This finding raises the tantalizing possibility that loss of Par3 will alter the

inflammatory response to tumors in clinically relevant ways. Indeed, activation of

inflammatory pathways such as NF-κB in tumor cells can recruit immune cells

that subsequently secrete pro-tumorigenic signals in a feed-forward process (H.

Yu et al., 2009). Future studies utilizing immunocompetent mice – such as the

Macara Lab's *in vivo* model of mammary tumors – should address this issue.

In conclusion, the present study has uncovered a mechanism – general to

primary murine mammary epithelial cells, transformed cells, and mammary cell

lines – through which loss of the Par3 polarity protein activates autocrine IL-6

signaling and triggers Stat3 activity. Important events downstream of Par3 are

mediated by aPKC, suggesting that restricting aPKC's activity is a tumor

suppressor function of Par3. Future studies should test the role of NF-κB

signaling following Par3 silencing *in vivo*, and investigate how the distribution of

aPKC within cells impacts tumor growth and invasion.

Methods

Cell Culture, Constructs, and Transfections

Primary mammary epithelial cells were harvest from C3H mice, collagenase

digested, and purified by serial centrifugation as previously described (McCaffrey

& Macara, 2009). Following purification, these cells were infected with lentivirus

expressing the NICD1 at a MOI of 5. These cells were then grown as

mammospheres in ultra low adhesion dishes (Corning) for 5 days, after which

they were transferred to 2D culture. These cells are referred to as NICD1-

mMECs. They were cultured in DMEM:F12 supplemented with 1%

penicillin/streptomycin, 5% fetal bovine serum, 1% insulin-transferrin-selenium, 5

ng/mL EGF, and 2 mg/mL hydrocortisone. All lentiviral overexpressions were

performed at an MOI of 5, and all shRNA infections used an MOI of 10. shRNA against Par3 and Il6st were developed in the Macara lab, by cloning into the ClaI and MluI sites of pLVTHM vector. Sequence for Par3 shRNA is ACAAGCGTGGCATGATCCA, and for Il6st shRNA is GCACAGAGCTGACCGTGAA. shRNA vectors were purchased from Sigma-Aldrich for Il6 (Cat. Nos. TRCN0000067550 and TRCN0000067548), Prkci (Cat. No. TRCN0000278129), and Nfkbia (Cat. No. TRCN0000319455). The overexpression vector for gp130 was generated by cloning human IL6ST cDNA into a multiple cloning site our lab created in the PmeI locus of the pWPI vector. tRFP-tagged aPKCi-CA was cloned into the pLVTHM expression vector. Following knockdown or overexpression, cells were allowed to recover in culture for at least 48 hours prior to further treatment or analysis.

Immunofluoresence

Cells were plated on 8-well chamber slides (Lab-Tek) and grown to approximately 75% confluence, at which point they were fixed with either methanol-acetone (for Stat3 staining) for 4% paraformaldehyde (other stains). Following fixation, cells were permeabilized with 0.25% TX-100, blocked with 3% BSA in PBS for 1 hour at room temperature, stained overnight at 4° C with primary antibodies in 0.3% BSA in PBS, washed three times in 0.3% BSA in PBS for 5 minutes per wash, and stained with Alexa Fluor secondary antibodies in 0.3% BSA in PBS. Antibody dilutions used were: Phospho-Stat3, 1:400 (Cell Signaling), p65/RelA, 1:600 (Cell Signaling), Alexa Fluor secondary antibodies,

1:1000 (Life Technologies). After probing with secondary antibodies, cells were

washed three times in PBS for 5 minutes per wash, then stained with DAPI and

phalloidins as indicated. Images were obtained using a 20x objective on an

Eclipse TI microscope (Nikon) and analyzed in TIFF format using NIS Elements

(Nikon) and ImageJ (NIH) software.

<u>Quantitative (q)-PCR</u>

Total RNA was isolated from cells using RNAEasy kits (Qiagen), treatedwith

RNAse-free DNAse (Qiagen), and reverse transcribed into cDNA with random

hexamers (Invitrogen) and SuperScript II reverse transcriptase (Invitrogen) plus

RNasin (Promega). q-PCR of the reverse transcription products was performed

using a CFX96 Real-Time System (Bio-Rad) and SYBR Green Real Time PCR

Master Mixes (Life Technologies). Primer sequences for Socs3, Myc, Junb,

Stat3, Il6, Stat1, and Gapdh were obtained from the Harvard Medical School

PCR PrimerBank. 18S rRNA primer sequences were previously described

(Bouras et al., 2008).

Immunoblotting

Cells treated as indicated were collected by scraping in ice-cold PBS and centrifugation, followed by direct lysis in 4x Laemmli sample buffer supplemented with 1x protease inhibitors and phosphatase inhibitors (Roche). Lysates were boiled for 5 minutes, briefly sonicated to break chromatin, and either frozen at -20^0 C or immediately run out on 10% acrylamide gels and transferred to nitrocellulose membranes. Blocking was performed with 3% BSA in TBS-T. Primary antibodies used were: anti-Par3 was developed by our lab and described previously (McCaffrey & Macara, 2009), anti-gp130 (Cell Signaling 3732), anti-phospho-Stat3 (Cell Signaling 9145), anti-total Stat3 (Cell Signaling 9139), anti-phospho-aPKCι/λ (Cell Applications CG1453), anti-total aPKCι/λ (Transduction Laboratories 610175), anti-IκBα (Cell Signaling 4814), anti-phospho-IKK (Cell Signaling 2697), anti-total IKKβ (Cell Signaling 8943), anti-total IKKα (Cell Signaling 11930), anti-phospho-p65/RelA (Cell Signaling 3033), anti-total p65/RelA (Cell Signaling 8242), anti-GAPDH (Cell Signaling 2118), and anti-β-tubulin (Santa Cruz 9104). HRP-conjugated secondary antibodies (IgG, Jackson ImmunoResearch Laboratories) were used at a dilution of 1:5,000 in TBS-T with 3% milk. Blots were imaged with an ImageQuant device (General Electric). Band intensities were quantified using ImageJ software (NIH).

ELISA Test

Cells were plated at approximately 75% confluence, and culture media was replaced 16 hours prior to collection. After collecting media, cytokine levels were measured with a Mouse IL-6 Quantikine ELISA Kit (R&D Systems).

NF-κB Luciferase Assay

Cells were grown to approximately 75% confluence in 24-well culture plates and calcium phosphate transfected with 100 ng of 8xNF-κB-GFP-Luciferase reporter plasmid (gift of Fiona Yull, Vanderbilt University; described in (Everhart et al., 2006)) and 50 ng of Renilla luciferase plasmid. 24 hours after transfection, cells were lysed and analyzed for luminosity with a Dual-Glu Luciferase Assay Kit (Promega) and a GloMax luminometer (Promega).

Statistical Analysis

Two-tailed Student's t-tests were used in statistical analyses. All statistical analysis and graphing were done using Excel for Mac version 14.3.9 (Microsoft).
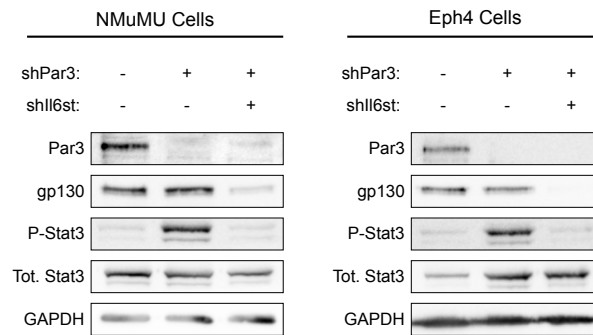
Figure S1. The untransformed mouse mammary epithelial cell lines NMuMG and Eph4 were infected with lentivirus encoding shRNAs as indicated. Cells were lysed, and equal amounts of lysate were immunoblotted for Par3, gp130, phospho-Stat3, total Stat3, and GAPDH (loading control).

shLuc   shII6st

shPar3/
shPar3   shII6st
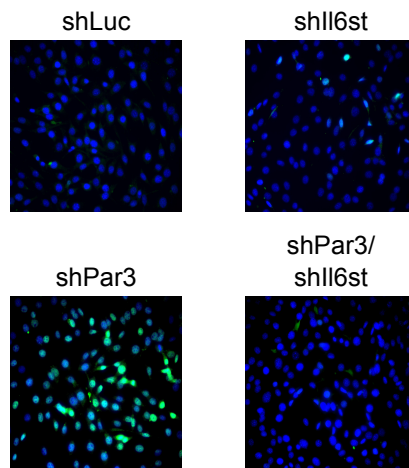
Figure S2. NICD1-mMECs expressing the shRNAs indicated were fixed with

paraformaldehyde and immunostained for phospho-Stat3. DAPI was used to

stain nuclei. Images were taken using a 20x objective.
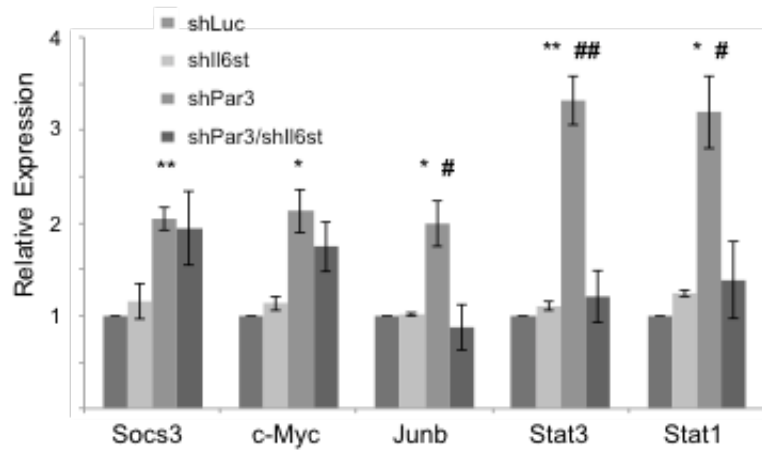
Figure S3. mRNA was isolated from NICD1-mMECs infected with lentivurus expressing the shRNAs indicated, reverse transcribed into cDNA, and expression of the genes indicated was analyzed by qPCR. 18S rRNA was used as a normalization control, n=4. Error bars represent SEM. P-values, shLuc vs shPar3: * < 0.05, ** < 0.01; shPar3 vs shPar3/shIl6st: # < 0.05, ## <0.01.
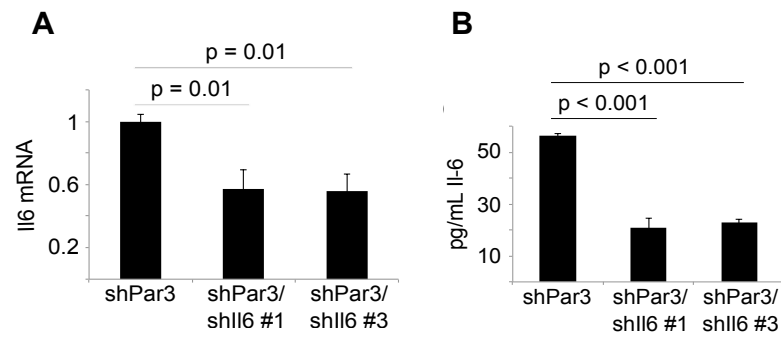
Figure S4. (A) mRNA was isolated from NICD1-mMEC/shPar3 cells infected

with lentivirus to express shRNAs against Il6, reverse transcribed into cDNA, and

expression of the genes indicated was analyzed by qPCR. GAPDH was used as

a normalization control, n=3. (B) Media was collected from cultures of NICD1-

mMEC/shPar3 cells infected with the shIl6 constructs indicated and analyzed by

ELISA for IL-6 levels, n=3. Error bars represent SEM.
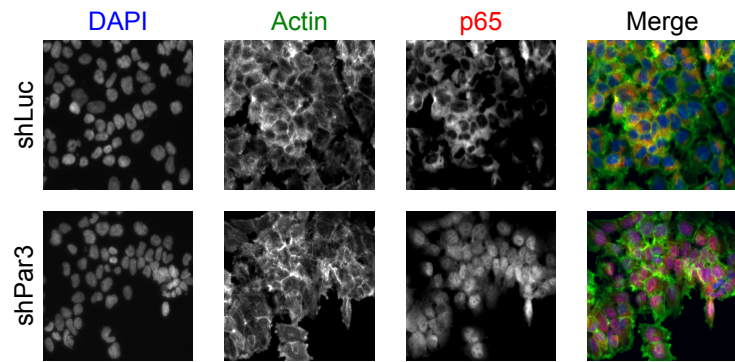
Figure S5. NICD1-mMECs expressing shRNA constructs indicated were fixed and immunostained for anti-p65/RelA, and stained with phalloidin for actin and DAPI for DNA. Images were taken using a 20x objective.

Chapter III

Effects of Par3 in Human Cell Lines and the 4T07 BALB/c Mouse Tumor

Cell Line

Introduction

Par3 is highly-conserved regulator of cell polarity that is necessary for normal

epithelial structure both in culture and *in vivo* (Hao et al., 2010; McCaffrey &

Macara, 2009). Given its importance for tissue structure and function, it has long

been suspected to play a role in cancer. Indeed, it was discovered in a screen

for tumor suppressor genes in Drosophila over a decade ago (Pagliarini & Xu,

2003). Further support for this hypothesis came from the report that Par3 is

frequently mutated or deleted in cell lines derived from human glioblastomas and

squamous cell carcinomas (Rothenberg et al., 2010). However, experimental

proof that Par3 restrains cancer growth and metastasis has only recently been

reported. Loss of the Par3 protein promotes growth and invasion of experimental

mammary and skin tumors (Archibald et al., 2014; Iden et al., 2012; McCaffrey et

al., 2012; Xue et al., 2013).

Prior work has found that loss of Par3 in NICD1-transformed primary mouse

mammary epithelial cells promotes invasion through extracellular matrix in

culture and metastatic dissemination to the lungs *in vivo* (McCaffrey et al., 2012).

In this model, metastasis depends upon Stat3 activation. As described in

Chapter II, I have found that activation of Stat3 in this system depends upon activation of aPKCι/λ, which induces NF-κB activity and Il-6 production. Activation of Stat3 is commonly associated with progression and metastasis of tumors, including mammary tumors (H. Yu et al., 2009). Given this, my discovery that loss of Par3 triggers activation of Stat3 via an aPKC-NF-κB-IL-6 signaling cascade in primary mouse mammary cells suggests that restraint of this pathway could be a general mechanism for Par3's tumor suppressor activity. Immunostaining of sections from human breast tumors has shown that aPKC and Stat3 are preferentially activated in regions of tumor where Par3 staining is reduced (McCaffrey et al., 2012), providing correlative evidence that Par3 may restrain aPKC and Stat3 activity in human tissues.

I undertook the studies in this chapter to determine whether loss of Par3 triggers Stat3 signaling in human mammary cells and human cancer cell lines. In addition to primary human mammary epithelial cells and human breast tumor cell lines, I also sought to determine whether loss of Par3 promotes Stat3 activity in human HNSCC cell lines, since Par3 is frequently mutated or deleted in HNSCC tumors (Rothenberg et al., 2010), and Stat3 signaling has been implicated in HNSCC progression (Squarize, Castilho, Sriuranpong, Pinto, & Gutkind, 2006). To my surprise, loss of Par3 does not appear to induce Stat3 activity in the human cells we examined. However, Par3 does regulate growth of both human tumor cells grown in culture and mouse mammary tumor cells allografted into recipient mice via mechanisms other than Stat3.

Results

Loss of Par3 does not trigger Stat3 signaling in human cells

To test the hypothesis that loss of Par3 triggers Stat3 activation via a mechanism that is conserved among mammals, I obtained HuMEC cells and shRNA against the human PARD3 gene. HuMEC cells are primary human mammary epithelial cells isolated from reduction mammoplasty tissue, and are analogous to untransformed mMEC cells harvested from mice. Although the knockdown of Par3 was very efficient in HuMECs, it did not result in activation of Stat3 (Fig. 8A). To confirm that HuMEC cells will activate Stat3 in response to IL-6, I treated these cells with recombinant human IL-6 and observed robust activation of Stat3 (Fig. 8A).

I next asked whether Par3 is involved in Stat3 signaling in several human tumor cell lines. Given that Par3 is commonly mutated or deleted in HNSCCs (Rothenberg et al., 2010) and that Stat3 signaling has been implicated in these lesions (Squarize et al., 2006), I obtained four HNSCC cell lines. FaDu and SCC-25 cells lack mutations in PARD3 and express the Par3 protein, while BICR78 and BHY cells contain whole-exon deletions in the gene and do not express the protein product (Rothenberg et al., 2010). I hypothesized that BICR78 and BHY cells would have higher levels of Stat3 Y705 phosphorylation than FaDu or SCC-25 cells. To my surprise, Stat3 signaling appears
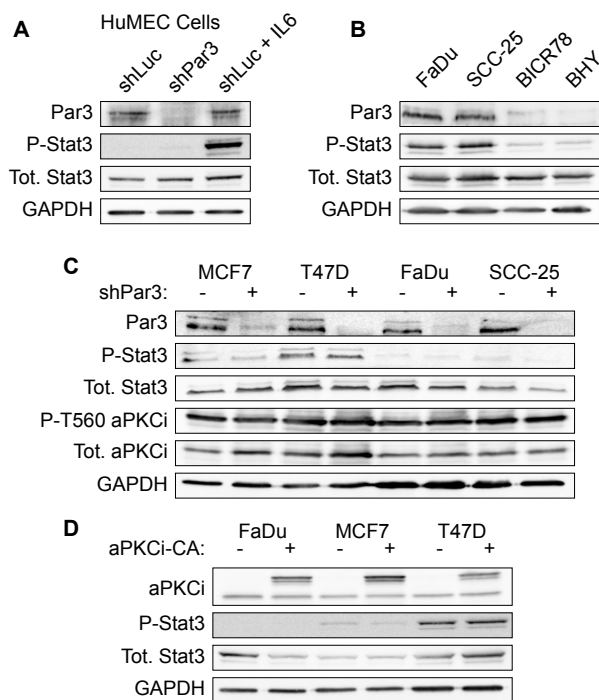
Figure 8. Loss of Par3 does not activate Stat3 or aPKCι/λ in human cells. (A) HuMEC cells, infected with lentivirus to express shRNA against either PARD3 of Luciferase as indicated, were harvested and equal amounts of lysate were immunoblotted for Par3, phospho-Stat ce3, total Stat3, and GAPDH (loading control). shLuc HuMEC cells were treated with 1000 pg/mL of recombinant human IL-6 for 20 minutes before harvesting as a positive control for Stat3 activation. (B) The human HNSCC cell lines indicated were harvested, and equal amounts of lysate were immunoblotted for Par3, phospho-Stat3, total Stat3, and GAPDH (loading control). (C) The human cancer cell lines indicated were infected with lentivirus to expression shRNA against PARD3 as indicated or against Luciferase, then were harvested and equal amounts of lysate were immunoblotted for Par3, phospho-Stat3, total Stat3, phospho-Y560 aPKCι/λ, total aPKCι/λ, and GAPDH (loading control). (D) The human cancer cell lines indicated were infected with lentivirus to express aPKCi-CA as inidicated, then harvested and equal amounts of lysate were immunoblotted for aPKCι/λ, phospho-Stat3, total Stat3, and GAPDH (loading control).

considerably lower in the BICR78 and BHY lines relative to FaDu and SCC-25

cells (Fig. 8B).

To further investigate the role Par3 plays in Stat3 signaling in human tumor cells,

I used lentivirus encoding shRNA against PARD3 to knock down expression of

this gene in 4 cell lines. In addition to FaDu and SCC-25 cells, I also knocked

down PARD3 expression in the MCF7 and T47D mammary tumor lines. In no case did silencing of PARD3 trigger activation of either Stat3 or aPKC (Fig. 8C). Given the lack of aPKC activation above baseline, I theorized that Stat3 was not being activated because aPKC signaling was not induced. However, expression of constitutively active aPKCι/λ in MCF7, T47D, and FaDu cells failed to activate Stat3 (Fig. 8D). This shows that human cells will not necessarily activate Stat3 in response to either loss of Par3 or activation of aPKC.

Expression of Par3 accelerates growth and activates pro-growth signaling mechanisms in human HNSCC cell lines

To further investigate the role of Par3 in human tumors, I used lentivirus to stably express the Par3b splice variant of the human PARD3 gene in BICR78 and BHY cells. This splice variant interacts with aPKC (Gao, Macara, & Joberty, 2002), and has been reported to rescue the effects of Par3 silencing when it is expressed in mammary cells (McCaffrey & Macara, 2009). The BHY and BICR78 cell lines both contain whole-exonic deletions within PARD3, and do not express the Par3 protein (Rothenberg et al., 2010). In prior reports, expression of Par3 in a human esophageal SCC line that lacks the protein slowed growth in culture (Rothenberg et al., 2010). I thus hypothesized that Par3 expression would slow the growth of BHY and BICR78 cells.
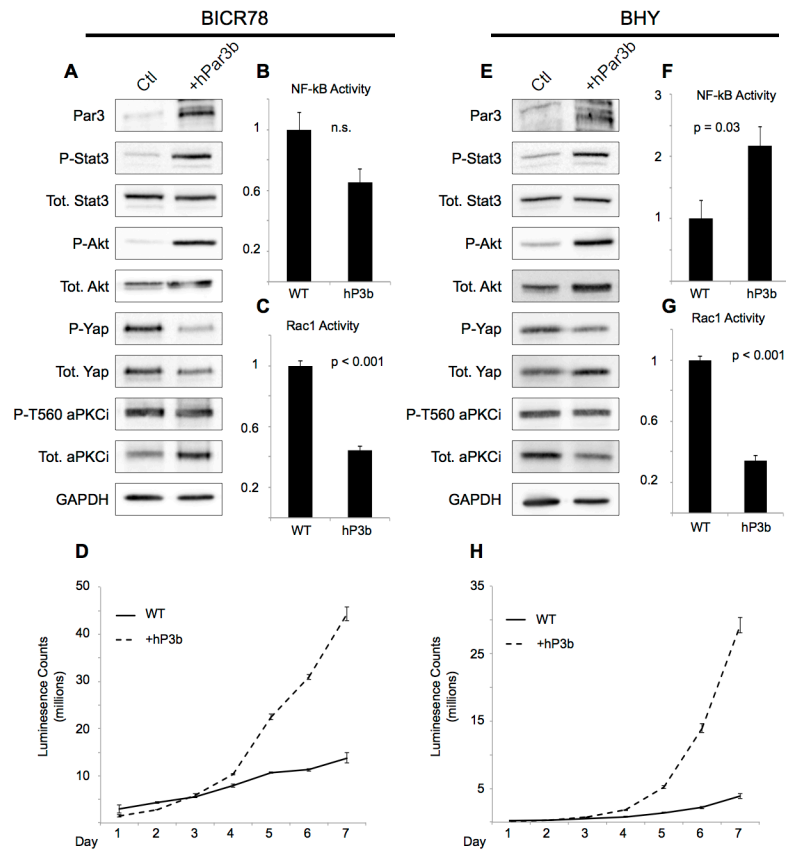
Figure 9. Expression of Par3 in BICR78 and BHY cells activates pro-growth signaling pathways and accelerates growth in culture. (A, E) BICR78 or BHY cells infected with lentivirus to express Par3b as indicated were harvested, and equal amounts of lysate were immunoblotted for Par3, phospho-Stat3, total Stat3, phospho-Akt, total Akt, phospho-Yap, total Yap, phospho-Y560 aPKCι/λ, total aPKCι/λ, and GAPDH (loading control). (B, F) BICR78 or BHY cells infected with lentivirus to express Par3b as indicated were calcium phosphate transfected with an NF-kB reporter plasmid and with constitutively expressed renilla luciferase (normalization control). Cells were lysed 24 hrs after transfection, and luciferase intensity was measured, n=4. (C, G) BICR78 or BHY cells infected with lentivirus to express Par3b as indicated were analyzed for Rac1 activity with a Rac1 G-LISA kit, n=3. (D, H) BICR78 or BHY cells infected with lentivirus to express Par3b as indicated were plated at a density of 10,000 cells/well in 24-well plates and cell counts were estimated by measuring ATP levels at 1-day intervals, n=4.

To my surprise, expression of Par3b in these two lines led to increased activation

of three signaling pathways that are associated with tumor growth and survival –

Stat3, Akt, and Yap (Fig. 9A, D). There was no impact on activation of aPKCι/λ

in these cells when Par3b was expressed (Fig. 9A, D). Consistent with these

signaling alterations, expression of Par3b in either cell line led to a

marked acceleration of growth in 2D culture (Fig. 9C, F). These results contrast

with data previously reported for an esophageal SCC cell line (Rothenberg et al.,

2010), suggesting that the effects of Par3 expression depend on the signaling

context of tumor cells rather than their histological subtype.

Archibald *et al* (2014) recently reported that activation of Rac1 triggers growth of

mammary tumors when Par3 is silenced, presumably via release of Tiam1 from

its normal interaction with Par3. Given that BHY and BICR78 cells grow more

rapidly when Par3b is expressed, I asked whether Rac1 activity would be

increased. In keeping with the data reported by Archibald *et al* (2014),

expression of Par3b in either HNSCC line led to a large decrease in Rac1 activity

(Fig. 9B, E). This decrease suggests that restraint of Rac1 signaling may be a

general role for Par3, but also demonstrates that increased cell proliferation

following Par3 expression in HNSCC cells does not require heightened Rac1

signaling.

I also investigated whether expression of Par3 impacts NF-κB signaling, given

the importance of NF-κB activation following loss of Par3 in NICD1-transformed

murine mammary cells (Chapter 2). In BHY cells, expression of Par3 caused

over a 2-fold increase in NF-κB transcriptional activity (Fig. 9F), while in BICR78

cells a small decrease in NF-κB activity that was not statistically significant was seen (Fig. 9B). The relevance of these changes in NF-κB activity for cell growth and survival is not clear.

<u>Silencing of Par3 promotes *in vivo* growth of a mouse mammary tumor cell line by undefined mechanisms</u>

After determining that loss of Par3 does not activate Stat3 in human mammary cells or in some human tumor cell lines (Fig. 8), I sought to clarify whether activation of Stat3 is a universal effect of Par3 silencing in transformed mouse mammary cells. 4T07 cells are a clonal line are derived from a spontaneously-arising mammary tumor in a BALB/c mouse, and which are tumorigenic when injected into immunocompetent BALB/c recipients (Aslakson & Miller, 1992; Aslakson, Rak, Miller, & Miller, 1991). I used these cells to test the hypothesis that silencing Par3 would activate Stat3 in all mouse mammary cells and mouse mammary tumors. To my surprise, Stat3 is not activated when Par3 is silenced in these cells in culture (Fig. 10A, B). This finding contrasts with primary mouse mammary cells and other mouse mammary cells that we have tested (Chapter 2), but is consistent with results obtained from human breast tumor cell lines and primary human mammary epithelial cells (Fig. 8A, C). Together with data from Chapter 2 and Figure 6 of this chapter, this result shows that activation of Stat3 occurs only in a subset of mouse-derived mammary cells. Whether this response to loss of Par3 occurs in cells from other tissues or other species remains unknown.
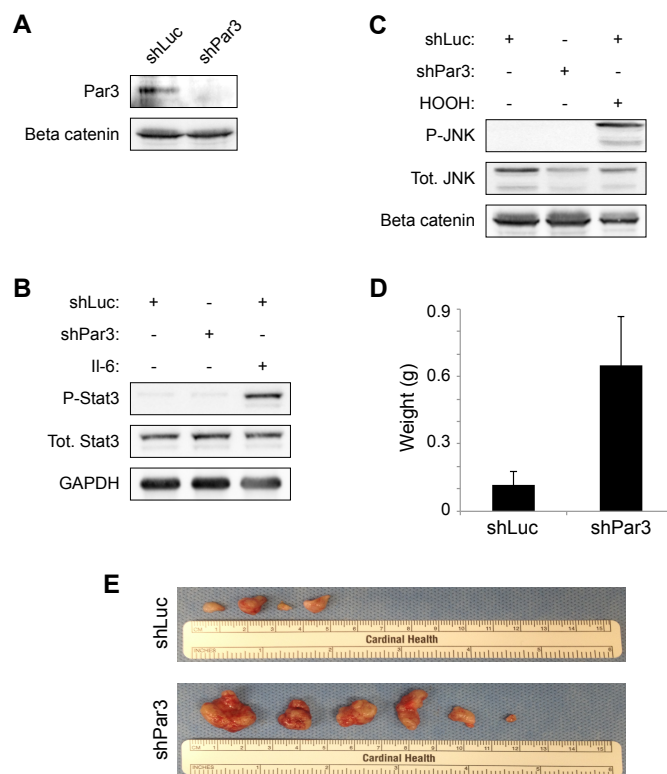
Figure 10. Silencing of Par3 accelerates growth of 4T07 tumor cells *in vivo*, but does not activate Stat3 or JNK. (A) 4T07 cells infected with lentivirus to express the shRNA constructs indicated were harvested, and equal amounts of lysate were immunoblotted for Par3 and beta catenin (loading control). (B) 4T07 cells infected with lentivirus to express the shRNA constructs indicated were harvested, and equal amounts of lysate were immunoblotted for phospho-Stat3, total Stat3, and GAPDH (loading control). As a positive control for Stat3 activity, 4T07/shLuc cells were treated with 1000 pg/mL recombinant mouse Il-6 for 20 minutes prior to harvesting. (C) 4T07 cells infected with lentivirus to express the shRNA constructs indicated were harvested, and equal amounts of lysate were immunoblotted for phospho-JNK, total JNK, and beta catenin (loading control). As a positive control for JNK activity, 4T07/shLuc cells were treated with HOOH prior to harvesting. (D) 4T07 cells infected with lentivirus to express the shRNA constructs indicated were injected into the mammary fat pads of recipient BALB/c mice, and tumors were harvested and weighed 6.5 weeks later. (E) Images of tumors described in (D).

Because activation of JNK following Par3 knock down has been reported to promote tumor growth (Archibald et al., 2014), I also asked whether loss of Par3 activated JNK signaling. However, knock-down of Par3 in these cells does not trigger JNK signaling (Fig. 10C). By treating 4T07/shLuc cells with HOOH prior

to immunoblotting, I confirmed that these cells can activate JNK in response to stress.

Despite failure of Par3 silencing to activate these tumor-promoting signaling networks, 4T07/shPar3 cells form tumors that grow more rapidly than shLuc controls when injected into the mammary fat pads of recipient BALB/c mice (Fig. 10D, E). This suggests that additional signaling mechanisms can promote growth of mammary tumors following Par3 silencing *in vivo*. Signaling via Akt and Yap, both of which are activated in HNSCC cell lines after Par3 is knocked down (Fig. 9A, D), may be worthy of investigation. Notably, these results do not prove that 4T07 cells cannot activate Stat3 or JNK signaling following Par3 silencing *in vivo*. Examining signaling activation in tumor sections may reveal differences between the signaling pathways that are activated in culture and those that are triggered *in vivo*.

Discussion

Contrary to my expectations, loss of Par3 does not trigger activation of Stat3 or aPKC in any of the human cell lines that I tested. Tested lines included HuMEC cells, as well as breast and HNSCC tumor cell lines. Clearly, activation of aPKC and Stat3 is not a universal response to disruption of Par3 in mammalian cells. This lack of universality is also demonstrated by previous work showing that aPKC activity is not affected when Par3 is silenced in MDCK cells (Hao et al., 2010). Immunostaining of human breast tumor sections has revealed that

regions lacking Par3 are enriched for active aPKC and active Stat3 (McCaffrey et al., 2012), but these results are only correlative. To my knowledge, there is currently no experimental evidence that loss of Par3 triggers Stat3 activation in human cells. Further work will be necessary to determine whether Stat3 activation in response to Par3 silencing is unique to the mouse mammary gland.

What can explain the disparate results between mouse models and human tumor cells? I speculate that the length of the human life has provided selective pressure for mechanisms that prevent activation of oncogenic signaling networks. Human cells may have fail-safe strategies to prevent loss of polarity from triggering activation of aPKC. Moreover, human cells may have other programs that prevent oncogenic consequences in the event that aPKC does become active. Such mechanisms may help prevent transformation when one or more signaling network escapes normal regulatory mechanisms. In animals with much shorter life expectancies, such as flies and mice, these mechanisms would confer a smaller competitive advantage. This hypothesis is consistent with papers that report that a greater number of genetic insults are necessary to transform human fibroblasts than their murine counterparts (Hahn et al., 1999; Land, Parada, & Weinberg, 1983).

The results obtained from knocking down Par3 in 4T07 cells suggest that Par3 restrains tumor growth via pathways other than the Jak-Stat axis. Knock down of Par3 in these cells clearly fails to trigger Stat3 activation in culture. Although it is

possible that Stat3 is active when the cells are grown *in vivo*, Stat3 activation

upon Par3 knock down has been seen both in culture and *in vivo* in other mouse

models (McCaffrey et al., 2012). In addition, we do not see an increase in JNK

signaling after Par3 silencing, which contrasts with recent reports of primary

mouse mammary cells (Archibald et al., 2014). However, Archibald *et al* (2014)

utilized a 3D suspension culture system for their study, so our different culture

conditions could account for this disparity. Definitive experiments to rule out JNK

activation in 4T07/shPar3 cells are warranted.

The activation of pro-growth and pro-survival signaling pathways and the

increased growth in culture that I observe when Par3 is expressed in BHY and

BICR78 cells suggests that Par3 may act as an oncogene in some situations.

This increased growth is seen in spite of decreased Rac1 activity, even though

Rac1 activation has been reported to promote proliferation of both untransformed

cells and tumors (Archibald et al., 2014). Together, these data imply that Par3

can impact multiple signaling pathways, and that the effect on cell proliferation

depends on the net impact of changes in each of these pathways. Moreover,

whether Par3 activates or restrains specific pathways may depend on the

signaling context of a cell. This complicated set of effects could be explained by

a scaffolding role for Par3. By acting as a scaffold, Par3 could bring components

of a given network together to promote signaling in some settings, and could

sequester them apart to restrain the same pathway in other contexts.

In conclusion, the data presented in this chapter demonstrate that Par3 impacts tumors through multiple mechanisms. The complement of mechanisms that functions in a given cell type is variable, and likely depends on the signaling context in which the cell resides. Moreover, these results underscore the differences between humans and mice. Although there are remarkable similarities between mouse and human mammary epithelia, significant differences exist. For example, during pubertal development, mouse mammary ducts terminate in hypercellular structures known as terminal end buds that do not exist in humans (Sternlicht, Kouros-Mehr, Lu, & Werb, 2006). Mouse and human mammary organoids grown in 3D culture respond in very different ways to stimulation with growth factors (Pasic et al., 2011), demonstrating that signaling is not perfectly conserved between these species. It is clear that data from mouse models offer valuable insights into vertebrate biology, but such data cannot establish clinical significance for disease mechanisms. Studies utilizing human cells and careful correlation with clinical findings remain essential. With continuing technical improvements, *ex vivo* culture methods that recapitulate human biology more closely than 2D cell cultures may become valuable tools.

Methods

Cell Culture, Constructs, and Transfections

MCF7 cells were cultured in MEM supplemented with 10% FBS and 1% penicillin/streptomycin. T47D cells were cultured in DMEM:F12 supplemented with 10% FBS, 1% penicillin streptomycin, and 0.2 units/mL recombinant human insulin. BICR78 cells were purchased from the European Collection of Cell Cultures (Cat. # 0407211) and cultured in DMEM supplemented with 10% FBS, 0.4 μg/mL hydrocortisone, and 1% penicillin/streptomycin. BHY cells were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Cat. # ACC 404) and cultured in MEM supplemented with 10% FBS and 1% penicillin/streptomycin. FaDu (Cat. # HTB-43) and SCC-25 (Cat. # CRL-1628) were purchased from ATCC and cultured in MEM supplemented with 10% FBS and 1% penicillin/streptomycin or DMEM:F12 supplemented with 10% FBS, 400 ng/mL hydrocortisone, and 1% penicillin/streptomycin, respectively. HuMEC cells were a gift from Deborah Lannigan (Vanderbilt University), and were cultured using MEGM BulletKit media (Lonza, Cat. # CC-3150). The human Par3b overexpression vector has been described previously (McCaffrey & Macara, 2009), and an shRNA vector against human PARD3 was purchased from Sigma-Aldrich (Cat. # TRCN0000118134). Overexpressions were performed at an MOI of 5, and knock-downs were done using an MOI of 10. Following infection with lentiviral vectors, cells were allowed to recover in culture for 48 hours before harvesting or passaging.

Cells treated as indicated were collected by scraping in ice-cold PBS and centrifugation, followed by direct lysis in 4x Laemmli sample buffer supplemented with 1x protease inhibitors and phosphatase inhibitors (Roche). Lysates were boiled for 5 minutes, briefly sonicated to break chromatin, and either frozen at minus-20^0 C or immediately run out on 10% acrylamide gels and transferred to nitrocellulose membranes. Blocking was performed with 3% BSA in TBS-T. Primary antibodies used were: anti-Par3 was developed by our lab and described previously (McCaffrey & Macara, 2009), anti-phospho-Stat3 (Cell Signaling 9145), anti-total Stat3 (Cell Signaling 9139), anti-phospho-aPKCι/λ (Cell Applications CG1453), anti-total aPKCι/λ (Transduction Laboratories 610175), anti-phospho-JNK (Cell Signaling 9251), anti-total JNK (Cell Signaling 9252), anti-phospho-Akt (Cell Signaling 4060), anti-total Akt (Cell Signaling 4691), anti-phospho-Yap (Cell Signaling 4911), anti-total Yap (Santa Cruz 101199) anti-GAPDH (Cell Signaling 2118), and anti-b-catenin (BD Biosciences 610153). HRP-conjugated secondary antibodies (IgG, Jackson ImmunoResearch Laboratories) were used at a dilution of 1:5000 in TBS-T with 3% milk. Blots were imaged with an ImageQuant device (General Electric).

NF-κB Luciferase Assay

Cells were grown to approximately 75% confluence in 24-well culture plates and calcium phosphate transfected with 100 ng of 8xNF-κB-GFP-Luciferase reporter plasmid (gift of Fiona Yull, Vanderbilt University; described in (Everhart et al., 2006)) and 50 ng of Renilla luciferase plasmid. 24 hours after transfection, cells were lysed and analyzed for luminosity with a Dual-Glu Luciferase Assay Kit (Promega) and a GloMax luminometer (Promega).

Rac1 Activity Assay

Cells were plated at approximately 50% confluence, and were harvested approximately 24 hours later. Samples were handled and protein was quantified per the manufacturer's protocol for a Rac1 G-LISA colorimetric assay (Cytoskeleton, Inc.). After equalizing the total protein in each sample, the Rac1 G-LISA assay was performed according to the manufacturer's protocol.

Cell Growth Curves

10,000 cells per well were plated in 24-well culture dishes, with 4 wells seeded per experimental condition. At 24-hour intervals, cells were lysed with TiterGlo reagent (Promega) and 200 μL aliquots were transferred to a 96-well assay plate; 3 technical replicates were taken and averaged for each culture well replicate. Luminosity was measured using a GloMax luminometer (Promega).

In Vivo Tumor Growth

All studies were conducted in accordance with Vanderbilt University IACUC policies. 4T07 cells infected with lentivirus to express either shPar3 or shLuciferase were trypsinized, filtered to ensure a single-cell suspension, and 100,000 cells/mouse were injected into the mammary fat pad of BALB/c mice. Mice were returned to the Vanderbilt University animal housing facility and monitored for tumor growth. After 6.5 weeks, the animals were euthanized and tumors were harvested and weighed. Photographs of the tumors were taken at the time of harvesting.

Chapter IV

The Role of Par3 in Mouse and Human Tumor Biology

Introduction

With the studies presented in this thesis, I have attempted to elucidate

mechanisms through which Par3 impacts tumor biology. There is growing

evidence, both from correlative studies and from direct experimental evidence in

mice, that Par3 is a tumor suppressor gene in mammals (Archibald, Mihai,

Macara, & McCaffrey, 2014; Halaoui & McCaffrey, 2014; Iden et al., 2012;

McCaffrey, Montalbano, Mihai, & Macara, 2012; Rothenberg et al., 2010; Xue,

Krishnamurthy, Allred, & Muthuswamy, 2013). Depending on the biological

context, Par3 can suppress growth of primary tumors (Iden et al., 2012),

metastatic spread (Xue et al., 2013), or both (McCaffrey et al., 2012). In addition,

Par3 appears to promote growth of tumor cells in some situations. Recent

studies have uncovered a number of mechanisms through which these effects

occur, but many aspects of Par3's function in tumors remain obscure. In this

section, I will discuss my findings in light of existing knowledge about the role for

Par3 in normal biology and in cancer.

Future Studies of Par3 and aPKC

The data in this thesis reinforces the notion that an important function of Par3 is to control aPKC activity. It is well established that Par3 is required for localization of aPKC to the apical surface of epithelial cells (Hao et al., 2010; Harris & Peifer, 2005; Morais-de-Sá, Mirouse, & St Johnston, 2010). Previous work from the Macara Lab has suggested that loss of Par3 triggers activation of aPKC in the context of transformed mammary epithelial cells (McCaffrey et al., 2012). My work affirms this result, and also demonstrates that Par3 limits activation of aPKC in untransformed mouse mammary epithelial cells. However, the mechanism for this is unclear and ripe for future studies.

The readout for aPKC activity that I have used is phosphorylation on an activating threonine residue. Increased threonine phosphorylation after loss of Par3 could be due to either increased activity of a kinase that phosphorylates this residue, or decreased activity of phosphatases that dephosphorylate aPKC. A complicating factor is that global activity of phosphatases and kinases may not be affected, but their ability to interact with aPKC may be enhanced or impaired. Several candidate molecules are suggested by the literature. A number of studies have suggested that PP2A regulates aPKC activation (Chabu & Doe, 2009; Nunbhakdi-Craig et al., 2002; Ogawa, Ohta, Moon, & Matsuzaki, 2009; Zhang, Liao, & Dufau, 2008). PP2A dephosphorylates Par6, thus preventing Par6 from activating aPKC (Ogawa et al., 2009). If loss of Par3 impairs the PP2A-Par6 interaction, constitutively phosphorylated Par6 could promote a

higher level of aPKC activity. This model would be consistent with a recent report that Par6 binding to aPKC alters the conformation of aPKC to promote kinase activity (Graybill, Wee, Atwood, & Prehoda, 2012), as well as earlier reports that overexpression of Par6 promotes growth of tumor cells in an aPKC-dependent manner (Nolan et al., 2008). If PP2A's interaction with Par6 and aPKC is hindered following loss of Par3, this mechanism would warrant further investigation.

PDK1 is a kinase that is involved in activating numerous other kinases, including aPKC.   This kinase phosphorylates aPKC on an autoinhibitory domain to induce a conformational change that promotes activation (Balendran, Hare, Kieloch, Williams, & Alessi, 2000; Mashukova, Forteza, Wald, & Salas, 2012; Zabkiewicz et al., 2014). PDK1 targeting of aPKC is important for aPKC activity in polarized epithelial cells, where PDK1 localizes to the apical membrane (Mashukova et al., 2012). Whether this localization of PDK1 is necessary for aPKC activation is unclear, and whether PDK1's subcellular distribution is disrupted when Par3 is silenced is unknown. Studies have suggested that PDK1 is overexpressed in hematological malignancies (Zabkiewicz et al., 2014), and PDK1 activation promotes tumor growth in mouse models (Sundaresan et al., 2011). The hypothesis that PDK1 promotes aPKC activation when Par3 is knocked down merits testing.

In *Drosophila* neuroblasts, Clu interacts with the Baz/DaPKC complex and stabilizes DaPKC levels; this stabilization promotes DaPKC signaling due to increased levels of DaPKC protein (Goh et al., 2013). This mechanism of stabilizing aPKC levels might be conserved in mammalian epithelial tissues, and loss of Par3 might alter the function of Clu. However, neither the data presented in this thesis nor previous studies have found increased levels of aPKC protein when Par3 is silenced (Hao et al., 2010; McCaffrey et al., 2012), suggesting that Clu is not involved in altered aPKC signaling. Another study in *Drosophila* neuroblasts found that Dap160 interacts directly with aPKC and increases the kinase activity of aPKC (Chabu & Doe, 2008). No studies have reported a role for Dap160 in epithelial polarity signaling, but it is conceivable that mislocalized aPKC could interact with either this protein or a similar molecule and have its signaling enhanced.

Another possible mechanism for aPKC activation is perturbation of the cell's phospholipid pool following Par3 silencing. aPKC is known to be activated by PtdIns(3,4,5)P3 signaling (Ivey, Sajan, & Farese, 2014; Standaert, Bandyopadhyay, Kanoh, Sajan, & Farese, 2001), and the importance of PtdIns(4,5)P2 and PtdIns(3,4,5)P3 for polarity signaling is well-established (Claret, Jouette, Benoit, Legent, & Guichet, 2014; Devergne, Tsung, Barcelo, & Schüpbach, 2014; Martin-Belmonte et al., 2007). Since the 3rd PDZ domain of Par3 associates with PTEN (Feng, Wu, Chan, & Zhang, 2008; Tyler, Peterson, & Volkman, 2010; Wu et al., 2007), loss of Par3 could alter the location and activity

of PTEN with possible effects on the pools of PtdIns(4,5)P2 and PtdIns(3,4,5)P3 in the cell. Studies are merited to investigate whether Par3 does, in fact, regulate phospholipid signaling, and to determine the consequences of an altered phospholipid composition.

My results give rise to the question of the relative importance of aPKC activation versus mislocalization. Since aPKC is always mislocalized when Par3 is silenced but has only been reported to be activated in mouse mammary epithelial cells (Hao et al., 2010; Harris & Peifer, 2005; Iden et al., 2012; McCaffrey et al., 2012), aPKC's localization may have broader relevance. The experiments presented here do not address this issue, but future studies that target aPKC to different cell regions may be useful.

Par3 appears to promote tumor growth through mechanisms other than activation of Stat3. One recent paper shows that loss of Par3 promotes tumor growth via Rac1-mediated activation of JNK, without activation of Stat3 (Archibald et al., 2014). Moreover, data I present in Chapter 3 clearly shows that loss of Par3 can promote tumor growth in immunocompetent mice without activation of JNK or Stat3 in the cultured tumor cells. Thus there could be at least three pathways through which loss of Par3 can promote growth of tumor cells: aPKC-mediated activation of Stat3, Rac1-mediated JNK activation, and at least one more mechanism that does not involve Stat3 or JNK activity. Whether aPKC or Rac1 is involved any additional mechanisms remains an open question.

It is possible that mislocalized or activated aPKC can trigger tumorigenic growth via inappropriate interactions with multiple additional signaling pathways. aPKC has been reported to trigger tumor growth by activating Hedgehog signaling (Atwood, Li, Lee, Tang, & Oro, 2013; Justilien et al., 2014), and a recent study demonstrated that activated aPKC can induce resistance to EGFR inhibitors in glioblastomas (Kusne et al., 2014). It is worth studying whether aPKC acts through these or other mechanisms to drive tumor progression following disruption of Par3 even when Stat3 is not activated.

Divergent Roles for Par3 in Different Tumor Types

I have found that expression of Par3 accelerates the growth of two human HNSCC cell lines – BHY and BICR78 – that ordinarily do not express the protein. In addition to growing more rapidly in culture, these cells display activation of numerous signaling pathways that have been implicated in cancer, such as the Akt pathway, Stat3, and Hippo. This suggests that Par3 can be an oncogene in at least some human tumors. The mechanisms through which Par3 activates these signaling networks remain to be uncovered. With respect to Akt, the known interaction between Par3 and PTEN suggests a possible mechanism. Expression of Par3 could lead to binding and sequestration of PTEN away from its substrate PI3K, causing increased PI3K activity. Since PI3K can activate Akt, Par3 expression could maintain active Akt. A similar model is proposed by Iden *et al* (2012), who reported that loss of Par3 attenuates Erk activation in keratinocytes. In addition, they show that Ras is mislocalized in these cells –

both in culture and *in vivo* – when Par3 is deleted. With the backing of this data,

they speculate that loss of Par3 prevents Ras from signaling to its downstream

effectors Erk and Akt. Definitive mapping of the signaling networks implicated by

Iden *et al* (2012) has yet to be performed.

A possible unifying mechanism for the pro-growth effects of Par3 in BHY and

BICR78 cells is increased signaling via EGFR. EGFR activation can trigger Stat3

activation (Zhong Zhong, Wen, & Darnell, 1994), Akt signaling by means of PI3K

(Okano, Gaslightwala, Birnbaum, Rustgi, & Nakagawa, 2000), and has been

reported to activate Yap (Fan, Kim, & Gumbiner, 2013). Par3 both has a known

role in receptor-mediated endocytosis and has been reported to interact with the

exocyst (Balklava, Pant, Fares, & Grant, 2007; Bryant et al., 2010), making it

plausible that Par3 could impact trafficking of EGFR to or from the cell surface.

In principle, Par3 could also impact the secretion of EGFR ligands via its

interaction with the exocyst. Experiments to test this hypothesis are underway.

Interestingly, expression of Par3 in both BICR78 and BHY cells causes a

statistically significant drop in Rac1 activity. This aligns with previous papers that

have reported an increase in active Rac1 when Par3 is knocked down (Archibald

et al., 2014; Xue et al., 2013). These papers have either reported that Rac1

activation promotes tumor growth by triggering JNK activity (Archibald et al.,

2014), or that Rac1 signaling alters E-cadherin dynamics to favor cell motility and

permit metastasis (Xue et al., 2013). The consequences of reduced Rac1

activity in BICR78 and BHY cells that express Par3 is not clear, but it does not prevent faster cell proliferation compared to WT cells. Whether it impacts adhesion, motility, matrix invasion, or *in vivo* invasion and metastasis awaits investigation. It is clear, however, that the effects of Par3 on one signaling pathway cannot be inferred from its effects on another. Additionally, the different outcomes of Par3 status on growth in various cell types may depend upon changes in multiple signaling pathways.

Although the differing results reported for Par3 seem paradoxical, they could be explained by a scaffolding function. In some settings, Par3 may act to bring its binding partners into proximity with their interaction partners, thus facilitating activation of signaling pathways. In this case, loss of Par3 might reduce signaling through these networks. In other situations, Par3 could sequester its binding partners away from inappropriate substrates. In this latter scenario, loss of Par3 would permit aberrant activation of some signaling mechanisms. For example, this could explain how Par3 is necessary for NF-κB activation when *P. aeruginosa* infect MDCK cells (Tran et al., 2014), and yet loss of Par3 triggers NF-κB signaling in NICD1-transformed mouse mammary cells (Chapter 2). This model is speculative, and will require validation utilizing binding deficient mutants of Par3 and its interaction partners.

It is noteworthy that although opposite effects on tumor growth have been found for Par3, loss of Par3 has always been reported to promote tumor invasion and

dissemination. Studies with both mouse and human breast tumor cells have found an increase in metastasis to the lungs upon Par3 knock down (McCaffrey et al., 2012; Xue et al., 2013). Similarly, Iden *et al* (2012) found that while papillomas lacking Par3 arise less often and grow more slowly than controls, they are more likely to invade the local stroma than Par3-WT control lesions. Similarly, tissue invasion was seen exclusively in Par3-KO keratoacanthomas, and never with Par3-WT cells (Iden et al., 2012). It is thus possible that loss of Par3 always impact cell-cell adhesion in ways that promote invasion and metastasis. Rac1 activation in the absence of Par3 may be a unifying mechanism to explain increased motility. A prediction of this model is that expression of Par3 in BHY and BICR78 cells will lead to diminished invasion and metastasis of these cells *in vivo*, even if Par3 expression accelerates growth of the primary tumors grown from these cells. *In vivo* studies to test this hypothesis should be undertaken.

Role of Par3 in Murine versus Human Disease Models

Par3 is often lost is human breast tumors, and this loss correlates spatially with activation of aPCK and Stat3 (McCaffrey et al., 2012). The data I present in this thesis suggests that Par3 can impact human tumors in at least some cases, but the effects are different than those seen in mouse models. Strikingly, knock-down of Par3 does not appear to trigger Stat3 signaling either in cultured human tumor cell lines or in primary human mammary epithelial cells. In addition, there is no evidence that loss of Par3 activates aPKC in these cells, and

overexpression of a constitutively-active aPKCι/λ isoform is not sufficient to activate Stat3. This suggests that human epithelial cells possess mechanisms to protect themselves from at least some of the aPKC-mediated effects of Par3 silencing.

I speculate that the longer lifespan of humans provides a competitive advantage to traits that protect against transformation of epithelial cells. This selective pressure may have fostered mechanisms that limit aPKC's ability to transform cells when polarity is disrupted. Such a model is consistent with early studies of cell transformation, which found that more insults are required to transform human cells than rodent cells (Hahn et al., 1999; Land, Parada, & Weinberg, 1983; O'Brien, Stenman, & Sager, 1986; Stevenson & Volsky, 1986).

These results also demonstrate that regulation of signaling pathways cannot be assumed to be conserved between mice and humans. Previous studies have shown that mouse and human mammary glands respond in markedly different ways to EGF stimulation (Pasic et al., 2011). 3D cultures of human mammary tissue (Pasic et al., 2011) and "humanized" mammary glands grown in mice (D. Proia & Kuperwasser, 2006; T. A. Proia et al., 2011) will be necessary to experimentally validate the role of signaling mechanisms in the human mammary organ. Mice are a valuable model organism for studying both mammary biology and other systems, but translational significance cannot be assumed based on data from murine experiments. This is underscored by the frequency with which

therapeutic strategies developed in mouse models fail to show efficacy in human

trials (Pound, Ebrahim, Sandercock, Bracken, & Roberts, 2004; van der Worp et

al., 2010). Experiments utilizing human cells and careful comparisons between

mouse studies and clinical data are essential to ensure that signaling

mechanisms in mice are conserved in humans.

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