Functional Interactions Between Par-1b Protein Kinase and RNF41 E3 Ubiquitin Ligase

Katherine Terese Vega
Washington University in St. Louis

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WASHINGTON UNIVERSITY IN ST. LOUIS

Division of Biology and Biomedical Sciences
Biochemistry

Dissertation Examination Committee:

Helen Piwnica-Worms, Chair
Phyllis Hanson
Greg Longmore
Linda Pike
Jason Weber
Zhongsheng You

Functional Interactions Between Par-1b Protein Kinase and RNF41 E3 Ubiquitin Ligase

By
Katherine Terese Vega

A dissertation presented to the
Graduate School of Arts and Sciences
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partial fulfillment of the
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ABSTRACT OF THE DISSERTATION

Functional Interactions Between Par-1b Protein Kinase and RNF41 E3 Ubiquitin Ligase

By

Katherine Terese Vega

Doctor of Philosophy in Biology and Biomedical Sciences
(Biochemistry)

Washington University in St. Louis, 2012

Professor Helen Piwnica-Worms

The Par-1 family of serine/threonine protein kinases functions to regulate cell polarity and is conserved from yeast to humans. Par-1 is encoded by one of six Par (partitioning-defective) genes (Par-1-6) originally identified in a genetic screen conducted in Caenorhabditis elegans (C. elegans). The mammalian Par-1 family is comprised of four members (Par-1a, b, c and d). Par-1 kinases are regulated by two arms of the Protein Kinase C (PKC) pathway. Atypical Protein Kinase C (aPKC) phosphorylates Par-1b on a conserved threonine residue (T595) and I participated in studies demonstrating that novel Protein Kinase C (nPKC) activates Protein Kinase D (PKD) to directly phosphorylate Par-1b on serine 400 (S400), a residue that is conserved in all four mammalian Par-1 kinases as well as the fly ortholog. Phosphorylation of Par-1b on T595 and S400 causes Par-1b to relocate from membranes to the cytosol and to bind 14-3-3 proteins. In 2004, Brajenovic, et al. reported the results of a study that used tandem affinity purification (TAP) to isolate human Par-1d along with associated proteins. Nrdp1/RNF41, a RING finger E3 ligase was identified in their screen along with 14-3-3 and aPKC. I found that
Par-1b binds to RNF41 and I identified RNF41 as a novel cell polarity determinant. My work demonstrated that phosphorylation of RNF41 on S254 by Par-1b is necessary for establishing epithelial cell polarity.
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I humbly dedicate this dissertation to my beloved parents, Robert and Teresa,

my brother Mark, and my grandparents

(Roman and Waclawa Franczak and George and Adeline Lewandowski).
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The Par proteins in development and disease

The Par proteins are important for establishing cell polarity in the embryo and are found to play crucial roles in a variety of human diseases. In the *Caenorhabditis elegans* (*C. elegans*) embryo the Par proteins are required for asymmetric cell division of the embryo (Kemphues et al., 1988) and in epithelial cells for the establishment of apical-basal cell polarity (Rodriguez-Boulan and Nelson, 1989). In mice, Par-1b (one of the Par-1 mammalian orthologs) has been show to be important for fertility, immune homeostasis, learning, and memory as well as glucose metabolism (Hurov et al., 2007). It has been suggested that epithelial cell polarity may exert a tumor suppressive function in mammals through its participation in the establishment and maintenance of the 3D organization of epithelial tissues as a whole. Par-1, a serine/threonine protein kinase, is phosphorylated and activated by the Par-4 (LKB1) tumor suppressor (Fig. 1.3) and LKB1 is mutated in Peutz-Jeghers cancer syndrome (PJS) (Baas et al., 2004b). It is important to note that a common theme in establishing and maintaining cell polarity is the antagonistic relationship between the Par proteins through the addition of posttranslational modifications such as phosphorylation. Moreover, phosphorylation of Par proteins also affects protein-protein interactions.

The par genes and Par proteins: Asymmetric localization and fundamental for cell polarization

The *par* genes are required for asymmetric cell division of the *C. elegans* embryo into two daughter cells (Kemphues et al., 1988) and epithelial cells depend on the asymmetric organization of cellular components along their apical-basal axes. It is the activity of asymmetrically localized proteins and protein complexes that regulate this
polarity (Rodriguez-Boulan and Nelson, 1989). Par-1 is one of six par (partitioning-defective) genes (Par-1, Par-2, Par-3/ASIP, Par-4/LKB1, Par-5/14-3-3, and Par-6) identified in a genetic screen conducted in C. elegans (Guo and Kemphues, 1995). Par-1 orthologs have subsequently been identified and studied in a number of organisms, including yeast, Drosophila, and mammals (Drewes et al., 1997; Elbert et al., 2005; La Carbona et al., 2004; Shulman et al., 2000; Trinczek et al., 2004) and have been implicated in regulating cell polarity and other cellular functions, such as mitogenic and Wnt signaling (Bohm et al., 1997; Cox et al., 2001; Muller et al., 2001; Ossipova et al., 2005; Shulman et al., 2000; Sun et al., 2001; Tomancak et al., 2000). In mammals there are four Par-1 family members: mPar-1a (C-TAK1/MARK3), mPar-1b (EMK/MARK2), mPar-1c (MARK1), and mPar-1d (MARKL1, MARK4). Mammalian orthologs have been identified for all of the par genes except for Par-2.

In the C. elegans embryo, all of the Par proteins are enriched at or near the cell cortex, and most of the proteins adopt asymmetric localization patterns as cell polarization is established (Kemphues, 2000). Par-3 and Par-6 become enriched in the anterior cortex during the one-cell stage, and Par-1 and Par-2 become enriched in the posterior cortex (Fig. 1.1). Par-4 (LKB1) and Par-5 remain symmetrically distributed and are both cortical and cytoplasmic. Par proteins drive their own asymmetric localization by regulating motors or by establishing the conditions necessary in the cell cortex for asymmetric movements of cellular components (Goldstein and Macara, 2007). The anterior Par proteins are required to prevent posterior Par proteins from localizing anteriorly, and vice versa (Kemphues, 2000). Par-5 is required for the exclusion of both anterior and posterior Par domains (Cuenca et al., 2003; Morton et al., 2002). Par-1
membrane association at the posterior pole is dependent in part on Par-2 (Boyd et al., 1996).

The *C. elegans* one-cell embryo becomes polarized by the contraction of the actomyosin cortex to one side of the cell (Fig. 1.1) (Munro, 2006). The fertilizing sperm delivers two cues for the contraction: the sperm-derived centrosomes and a RhoGAP called CYK-4. Rho affects myosin organization and activates myosin-based contraction and CYK-4 locally inactivates Rho, thus resulting in an inhibition of myosin and breaking the actomyosin on one side of the cell allowing the cortex to contract away from the site of sperm entry (Goldstein and Macara, 2007). The contraction of the cortex to one side moves the Par-3/Par-6/atyypical protein kinase C (aPKC) complex toward the anterior; however, Par-6 and Par-2 associate with the moving cortex, suggesting that Par proteins may exchange rapidly on and off of the moving cortex rather than be moved (Munro et al., 2004). Once the polarity is established at the anterior of the embryo and anterior Par proteins are in place, posterior Par proteins can then associate with the posterior cortex (Cuenca et al., 2003; Munro et al., 2004).

Once the two Par domains are established, the Par proteins function to maintain polarity on each end of the *C. elegans* embryo. Two distinct domains are maintained in a positive feedback loop using phosphorylation as a means to maintain this polarity. aPKC at the anterior of the embryo and Par-1 at the posterior inhibit specific proteins from associating locally. In the anterior, aPKC phosphorylates Par-2 in order to prevent Par-2 cortical association (Hao et al., 2006). In the posterior, Par-2 prevents reverse cortical flow and blocks anterior Pars from associating by allowing Par-1 to accumulate at the
posterior cortex where it can cause disassembly of the anterior Par complexes (Cuenca et al., 2003).

**Epithelial polarization in *C. elegans, Drosophila, and mammals***

The formation and maintenance of epithelial sheets of cells with apical-basal polarity is essential throughout development and in adult organisms. Junctions between the epithelial cells provide adhesion and control the permeability of the sheets to ions and molecules. The physiological functions of epithelial cells depend on the asymmetric organization of cellular components such as the plasma membrane, organelles, and the cytoskeleton along the apical-basal axis (Rodriguez-Boulan and Nelson, 1989). In the *C. elegans* embryo this organization and establishment of cell polarity has been shown to be dependent on the antagonistic relationship between Par-1 and the Par-3/Par-6/aPKC complex, and the same is likely true in epithelial cells of developed eukaryotic organisms. The fly ortholog of Par-3 functions in *Drosophila* epithelial cells to also drive apical-basal polarization (Kuchinke et al., 1998); furthermore, in mammalian epithelia, Par-3 is localized to tight junctions at the apical/lateral boundary and functions in their assembly (Chen and Macara, 2005). This discussion of polarity proteins extends to Par-1, as the phosphorylation of Par-1 by aPKC and PKD permits binding of Par-5 (14-3-3) and blocks membrane binding (Fig. 1.2) (Hurov et al., 2004; Suzuki et al., 2004; Watkins et al., 2008). Conversely, Par-1 present at the lateral membrane can phosphorylate any Par-3 molecule that is located in its vicinity (Fig. 1.2). Phosphorylated Par-3 binds Par-5 (14-3-3) and is released from the cell membrane, thus preventing the spread of Par-3 into the lateral membrane occupied by Par-1. Therefore, the segregation of the Par proteins into
separate domains is maintained by exclusion which is driven by phosphorylation and Par-5 (14-3-3) binding.

Par-1 and Par-4 also participate in epithelial polarization. In mammalian cells Par-1 determines the organization of microtubules, which establish the position of the luminal surface (Cohen et al., 2004). Hyperactive Par-1 converts columnar epithelial cells with vertical microtubules and an apical luminal surface into a hepatic type of epithelial cells with horizontal microtubules and lumens that form between adjacent cells. Par-1 is phosphorylated and activated by Par-4 (LKB1) (Fig. 1.3). Mammalian Par-4 (LKB1) is mutated in Peutz-Jeghers cancer syndrome (PJS). This disease involves the epithelial cells of the gastrointestinal tract, pancreas, lungs, and reproductive organs (Baas et al., 2004b) even though Par-4 is expressed ubiquitously (Alessi et al., 2006). PJS patients frequently have mutations that truncate the kinase domain or result in loss of expression of LKB1 (Par-4). In mammals, LKB1 (Par-4) functions as a master kinase that can phosphorylate and activate many downstream kinases, including Par-1, and it has been implicated in metabolic control, cell growth, and mitosis, in addition to cell polarization and asymmetric cell division (Alessi et al., 2006). High Par-4/LKB1 activity can drive the polarization of isolated epithelial cells even in the absence of cell-cell contacts; however, the downstream kinase necessary for this is not Par-1, but AMPK, a kinase primarily thought of as a metabolic regulator that responds to energy deprivation (Lee et al., 2007; Mirouse et al., 2007). It is interesting to note that a constitutively active AMPK can rescue many of the polarity defects that occur in Drosophila lkb1 null mutants through phosphorylation of the myosin light chain, and loss of either AMPK or Par-4/LKB1 results in a stress-dependent loss of epithelial cell polarity. On the other
hand, activation of AMPK by energy deprivation can drive epithelial cell polarization. These results reveal a link between energy metabolism and the polarity/proliferation pathways.

**Par-1b and Par-1a knockout mice**

Another link between energy metabolism and the polarity/proliferation pathways is mammalian Par-1b/MARK2/EMK (mPar-1b). Knockout studies of mice performed by our laboratory suggest that polarity kinase Par-1b/MARK2/EMK is required for regulating fertility, immune homeostasis, as well as glucose metabolism *in vivo* (Hurov et al., 2007). Par-1b knockout mice are lean, insulin hypersensitive, resistant to high-fat diet-induced weight gain, and hypermetabolic. Thus, mammalian Par-1b is a regulator of glucose metabolism and adiposity in the whole animal and may be a valuable drug target for the treatment of both type 2 diabetes and obesity.

Similar to Par-1b/MARK2/EMK knockout mice, our lab has also shown that Par-1a/MARK3/C-TAK1 null mice exhibit increased energy expenditure, reduced adiposity with unaltered glucose handling, and normal insulin sensitivity (Lennerz et al., 2010). Par-1a knockout mice are protected against high-fat diet-induced obesity and display attenuated weight gain, complete resistance to hepatic steatosis, and improved glucose handling with decreased insulin secretion (Lennerz et al., 2010). Overnight starvation led to complete depletion of hepatic glycogen supply, associated hypoketotic hypoglycemia, increased hepatocellular autophagy, and increased glycogen synthase levels in Par-1a null but not in control or Par-1b null mice. When Par-1a null mice were intercrossed with Par-1b null mice it was noticed that at least one of the four alleles is necessary for embryonic survival. Interestingly, the loss of one Par-1b allele in Par-1a null mice
conveyed milder phenotypes than the loss of one Par-1a allele in Par-1b null mice. Thus, although Par-1a and Par-1b can compensate for one another during embryogenesis, their individual and specific disruption gives rise to unique metabolic phenotypes in adult mice.

**Par-1b protein domains and known posttranslational modifications**

Par-1 is a serine/threonine protein kinase comprised of an amino-terminal kinase domain, followed by a ubiquitin-associated domain (UBA), a nonconserved spacer region, and ending with a conserved region of about 100 amino acids that terminates with the sequence glutamate-leucine-lysine/asparagine-leucine (Fig. 1.3) (Marx et al., 2006). This region has been referred to as the “ELKL” domain and murine EMK derived its name from ELKL motif kinase. Par-1 is activated by phosphorylation within its activation loop on Threonine (T)208 (Fig. 1.3). Activation loop phosphorylation can be catalyzed by Par-1b itself, by the tumor suppressor LKB1 (Par-4) (Lizcano et al., 2004) or by MARKK (Timm et al., 2003). The presence of a UBA domain adjacent to the catalytic domain suggests potential interactions between Par-1b and proteins involved in the ubiquitin proteasome pathway, DNA repair, and/or cell signaling (Brajenovic et al., 2004; Hofmann and Bucher, 1996; Mueller and Feigon, 2002). However, one study reported that the UBA domain of Par-1b may not bind ubiquitin, but rather serve as an inhibitory domain (Murphy et al., 2007; Panneerselvam et al., 2006).

Downstream of the UBA domain is a region that is not highly conserved among Par-1 orthologs. We identified two phosphorylation sites (Serine (S)400 and T595) within this region that are conserved among many orthologs (Fig. 1.3) (Hurov et al., 2004; Watkins et al., 2008). Furthermore, our work has demonstrated that these sites are regulated by two different arms of the Protein Kinase C (PKC) pathway. T595 is
phosphorylated by aPKC (Hurov et al., 2004) whereas my work has shown that S400 is phosphorylated by PKD through activation of novel PKCs (nPKCs) (Watkins et al., 2008). The nPKC pathway is activated by the PKC activator phorbol-12-myristate-13-acetate (PMA) in vivo. Moreover, both phosphorylation sites contribute to interactions between Par-1b and 14-3-3 proteins (Benton and St Johnston, 2003; Goransson et al., 2006; Suzuki et al., 2004). 14-3-3 proteins, themselves members of the Par family (Par-5), interact with phospho-Ser or phospho-Thr motifs and modulate diverse cellular processes (Fu et al., 2000; Mackintosh, 2004; Muslin and Xing, 2000). In addition to directly binding to 14-3-3 proteins, Par-1 proteins also phosphorylate other proteins to regulate their interactions with 14-3-3 proteins: the kinase suppressor of Raf-1 (KSR1), which functions as a docking platform for components of the Ras-MAPK pathway (Muller et al., 2001); protein-tyrosine phosphatase H1, which regulates cell-cycle progression and attenuates T-cell-receptor signaling (Zhang et al., 1997); plakophilin2, a desmosomal protein (Muller et al., 2003), and PAR-3 (Benton and St Johnston, 2003).

**RNF41 protein domains**

In an effort to identify Par-1 interacting proteins, human Par protein complexes were mapped out using tandem affinity purification (TAP) and the resulting network of proteins identified novel interactors of human Par-1 (hPar-1d/MARK4) such as the ubiquitin ligase NRDP1 (neuregulin receptor degradation protein-1)/RNF41 (Brajenovic et al., 2004). RNF41 is composed of an N-terminal RING domain, two zinc-finger domains collectively called the B-Box, a coiled-coil segment, and a C-terminal receptor binding domain (Abdullah et al., 2001; Bouyain and Leahy, 2007) (Fig. 1.4). Out of the six par genes in *C. elegans*, orthologs of every gene except Par-2 have been identified in
mammals. Par-2 is a RING finger-containing protein and has homology with RNF41 in both the RING domain and a portion of the C-terminus leaving open the interesting possibility that RNF41 is an ortholog of Par-2.

While the N-terminal motif of RNF41 is characteristic of a family of E3 ubiquitin ligases, called TRIM/RBCC (Tripartite Motif/Ring B-Box Coiled-coil) proteins, it is the diversity of the C-terminal ends of these proteins that is thought to be involved in selectively recognizing target proteins. Of interest is the C-terminal end of RNF41 as it has no homology with other proteins of known structure (Diamonti et al., 2002; Qiu and Goldberg, 2002) and binds specifically to its substrates, such as ErbB3, BRUCE, PARKIN, and others via specific residues (Bouyain and Leahy, 2007). Unlike most other membrane receptors, ErbB3 does not undergo degradation by lysosomes (Levkowitz et al., 1999; Qiu and Goldberg, 2002; Waterman et al., 1998), but rather is degraded by the proteasome and this process is catalyzed by the ubiquitin ligase RNF41 (Qiu and Goldberg, 2002). Mutation of cysteine, histidine, and aspartate residues in the RING domain of RNF41 inhibits its E3 ligase activity and thus degradation of ErbB3. Additionally, the RING and B-box motifs at the N-terminus of RNF41 are not required for binding to ErbB3 (Qiu and Goldberg, 2002) yet are required for ERBB3 degradation. Interestingly, expression of an RNF41 mutant containing only the coiled-coil and C-terminal domains caused ErbB3 to accumulate (Diamonti et al., 2002; Qiu and Goldberg, 2002). Therefore, RNF41 binds ErbB3 receptors through its C-terminal end; however, it is its N-terminal RING domain which provides its function as an E3 ubiquitin ligase and signal for ErbB3 degradation.
**RNF41 regulation**

RNF41 is extremely unstable when transfected or transduced in a variety of cell lines, often requiring treatment of cells with the proteasome inhibitor MG132 in order to detect endogenous RNF41 protein by Western blot. Point mutations in the RING finger domain that disrupt zinc ion coordination and thus coupling to E2 ubiquitin conjugating enzymes stabilize RNF41 protein, suggesting that the lability of RNF41 protein may be due in a large part to autoubiquitination (Carraway).

In order to elucidate mechanisms of RNF41 regulation, interacting proteins were identified by affinity chromatography/tandem mass spectrometry. Among the proteins identified was USP8 (Wu et al., 2004), a deubiquitinating enzyme. After characterization of this protein-protein interaction, it was found that the carboxy terminal domain of RNF41 binds to the rhodanese domain of USP8, and that USP8 very efficiently deubiquitinates and stabilizes RNF41 (Wu et al., 2004). Moreover, it was observed that treatment of cells with the growth factor Neuregulin-1β (NRG1β) stabilizes RNF41 through USP8 (Cao et al., 2007). NRG1β stimulation of the ErbB2/ErbB3 heterodimer activates phosphatidylinositol 3-kinase (PI3K), in turn activating AKT, which phosphorylates murine USP8 on threonine 907. USP8 phosphorylation leads to its activation and stabilization, thus stabilizing RNF41. The resulting feedback loop provides a mechanism for ligand-induced degradation of ErbB3, and furthermore it has been demonstrated that RNF41 or USP8 knockdown in MCF7 breast tumor cells suppresses NRG1β-stimulated ErbB3 ubiquitination and degradation(Cao et al., 2007).
ErbB receptor tyrosine kinase signaling and breast cancer

The ErbB family of receptor tyrosine kinases regulates various cellular processes such as proliferation, differentiation, cell survival, migration, and invasion (Yarden and Sliwkowski, 2001; Yen et al., 2006). Members of this family include the epidermal growth factor receptor (EGFR/ErbB1), HER2/c-neu (ErbB2), HER3 (ErbB3), and HER4 (ErbB4). Furthermore, aberrant expression of these receptors is commonly found in human cancers and is associated with aggressive disease (Hynes and Stern, 1994; Salomon et al., 1995; Yarden and Sliwkowski, 2001; Yen et al., 2006).

The ErbB signaling network is complex due to the existence of multiple ligands, each with specificity for distinct members of the ErbB family. The ErbB receptors signal through the formation of ligand-induced homodimers and heterodimers between the different family members (Alroy and Yarden, 1997; Carraway and Cantley, 1994; Riese and Stern, 1998; Yen et al., 2006), which then activate diverse signaling pathways (Alroy and Yarden, 1997; Carraway and Cantley, 1994). Binding of peptides of the EGF-related growth factor family to the extracellular domain of ErbB receptors results in the formation of these homo- and heterodimers. Ligand binding induces the intrinsic receptor kinase activity, leading to stimulation of the intracellular signaling cascades (Yarden and Sliwkowski, 2001). Moreover, the ErbB3 receptor has impaired kinase activity (Guy et al., 1994), whereas the ErbB2 receptor has no known ligand. Thus, both receptors must form receptor heterodimers in order to signal. Neuregulin-1 (NRG1) growth factor binds to its ErbB3 receptor preferentially resulting in ErbB2/ErbB3 heterodimer formation (Citri et al., 2003; Yen et al., 2006) and initiating extracellular-related kinase/mitogen-activated protein kinase (ERK/MAPK) and PI3K pathway
activation (Alimandi et al., 1995; Ben-Levy et al., 1994; Holbro et al., 2003; Peles et al., 1992; Yen et al., 2006).

It has widely been demonstrated that ErbB receptor tyrosine kinases, in particular ErbB1 and ErbB2, have roles in human cancer development, thus making them attractive targets for cancer therapies (Hynes and Stern, 1994; Mendelsohn and Baselga, 2000; Salomon et al., 1995; Shawver et al., 2002). ErbB2 overexpression, which is generally due to gene amplification, occurs in 25-30% of breast cancer and correlates with shorter time to relapse and lower overall survival (Slamon et al., 1987). Overexpressed ErbB2 is constitutively phosphorylated in breast cancer cell lines and in human tumors (Alimandi et al., 1995). It has been observed that targeting overexpressed active ErbB2 results in efficient inhibition of breast cancer cell proliferation (Lane et al., 2000; Munster et al., 2002; Neve et al., 2000; Yakes et al., 2002).

Interestingly, expression of ErbB3 is seen in many tumors that express ErbB2, including breast (Naidu et al., 1998), bladder (Chow et al., 2001), and others. Furthermore, in many ErbB2-overexpressing breast tumors, ErbB3 has elevated levels of phosphotyrosine (Alimandi et al., 1995). Several groups have shown that inactivation of ErbB2 leads to a decrease in ErbB3 tyrosine phosphorylation (Basso et al., 2002; Lane et al., 2000; Motoyama et al., 2002; Neve et al., 2000). ErbB3, which contains six docking sites for the p85 adaptor subunit of PI3K, efficiently couples to this pathway (Fedi et al., 1994; Prigent and Gullick, 1994). Interestingly, it has been observed that a major consequence of targeting overexpressed ErbB2 is decreased PI3K (Basso et al., 2002; Lane et al., 2000; Neve et al., 2000), suggesting a role for ErbB3 in stimulation of this pathway downstream of active ErbB2.
Finally, it has been shown in breast cancer cells that constitutive tyrosine phosphorylation on ErbB3 depends on the activity of overexpressed ErbB2 (Holbro et al., 2003). Activity of the PI3K pathway depends fully on the recruitment of p85 to phospho-ErbB3. Importantly, inactivation of ErbB3 blocks proliferation of breast cancer cells as efficiently as inhibiting ErbB2 signaling (Holbro et al., 2003). Expression of constitutively active PI3K rescues the proliferative block induced due to the loss of ErbB2 or ErbB3 signaling. Taken together, these results suggest that the ErbB2/ErbB3 dimer functions as an oncogenic unit to drive tumor cell proliferation.

**RNF41 involvement in ErbB3 overexpression in mammary tumors**

Several mechanisms account for ErbB3 overexpression in tumors. Amplification of the erbB3 gene has been reported, but PCR and FISH results suggest that this is not as prevalent as erbB2 amplification (Sassen et al., 2008; Zaczek et al., 2008) and does not account for the frequency of elevated ErbB3 protein observed in tumors. Although ErbB3 overexpression is a common occurrence in breast tumors, and ErbB3 levels may be regulated post-transcriptionally in mouse mammary tumors (Miller et al., 2008; Siegel and Muller, 1996; Yen et al., 2006), the question remains whether the RNF41 pathway is deregulated in tumors, and if so, whether deregulation contributes to tumor initiation or progression. It has previously been observed that RNF41 protein is lost in ErbB2-driven mammary tumors from transgenic mice and this loss cannot be attributed to a reduction in message levels (Carraway). Furthermore, RNF41 protein is suppressed in 57% of tumors when comparing patient-matched normal and tumor breast tissues, and there is a strong inverse correlation between RNF41 and ErbB3 protein levels in patient tumors (Yen et al.,
All of this data is suggestive that loss of RNF41 contributes to ErbB3 protein overexpression in both human and mouse tissue samples.

Surprisingly, crossing MMTV-RNF41 mice into an ErbB2 overexpression model had little effect on tumor latency or burden, or on ErbB3 protein overexpression (Ingalla et al.), even though RNF41 transcript levels were 10-fold higher in bigenic animals than in MMTV-ErbB2 mice. Immunoblotting studies revealed that the ErbB2-induced mouse mammary tumors lacked both endogenous and overexpressed RNF41 protein. Consistent with this, cultured ErbB2-positive breast tumor cells are resistant to the expression of exogenous RNF41, while non-transformed cells efficiently support RNF41 expression (Ingalla et al.). These observations indicate that like ErbB2 and ErbB3, RNF41 levels are deregulated in tumor cells by posttranscriptional mechanisms.

3D models of glandular epithelium

Under 3D culture conditions, normal epithelial cells proliferate and organize into spheroids (acini), which are known to have a centrally-localized, hollow lumen with polarized cells surrounding this lumen (Fig. 1.5) (Debnath et al., 2003; Petersen et al., 1992; Streuli and Bissell, 1990). These 3D culture systems address some of the limitations of monolayer (2D cell culture) cell cultures, mouse models, or human tissues. 3D cell cultures are easily manipulated experimentally and can be analyzed biochemically and using microscopy. Furthermore, 3D cell cultures contain essential structural features of glandular epithelium in vivo. Early studies revealed that human breast tumor cell lines did not form acini when grown in 3D culture; rather, they develop into nonpolarized clusters (Petersen et al., 1992). These experiments illustrated the
difference in behavior between normal and tumor cells in 3D culture, even though subtle
difference were noted when the same cells were grown as monolayers.

The cells comprising glandular epithelial tissues exhibit a characteristic polarity,
such that their apical poles point towards the central lumen. This polarity is often
disrupted in carcinomas, which is generally considered a poor prognostic sign. MDCK
cyst models have demonstrated that epithelial cells possess hard-wired mechanisms for
generating polarity within the context of a 3D tissue structure. Furthermore, it has been
demonstrated that the ECM plays a fundamental role in directing these mechanisms
(O'Brien et al., 2002).

**Epithelial cell polarity and cancer**

The majority of human cancers are derived from epithelial tissues, and display
loss of cell polarity and often, as a consequence, tissue disorganization. Although the
tumor suppressive function of polarity complexes is well established in *Drosophila*, it
remains unclear whether a loss of cell polarity is a consequence or cause of human
cancers. However, it is now suggested that epithelial cell polarity may exert a tumor
suppressive function in mammals through its participation in the establishment and
maintenance of the 3D organization of epithelial tissues as a whole. This is supported by
the findings that polarity proteins are cellular targets of oncogenes and tumor suppressors
(Royer and Lu).

Apical-basal polarity has two fundamental roles in epithelial cells that are linked
to tumor suppression: the regulation of asymmetric cell division and the maintenance of
the apical junctional complex (AJC). In epithelial stem cells, polarity proteins control
asymmetric cell divisions by regulating the localization of cell fate determinants and the
correct orientation of mitotic spindles. Thus, asymmetric cell division has a role in the control of progenitor or stem cell numbers and differentiation. This is of interest in the context of the cancer stem cell theory, as shifting from asymmetric division of epithelial stem cells or cancer-initiating cells towards symmetric divisions would result in dedifferentiation and an increase in cancer-initiating cells. Thus, a defect in asymmetric division could contribute to the development of tumors.

In addition to their role in the prevention of tumor initiation, core epithelial cell polarity mechanisms may also behave as a barrier to tumor metastasis and malignancy through their close connection to the AJC. The AJC contains both tight and adherens junction complexes, and its structure is dependent on the integrity of the apical and basolateral polarity complexes. The loss of one of the key components of adherens junctions, E-cadherin, often occurs in later stages of tumorigenesis and is thought to contribute to epithelial mesenchymal transition (EMT), which represents a crucial step in metastasis. The importance of the AJC in supporting cancer malignancy is supported by cancer genome sequencing data which show that a large number of AJC components are frequently mutated in human cancers.

Evidence indicates that loss of cell polarity is not merely a byproduct of abnormal cell proliferation, but rather is caused by the direct disruption of cell-polarity mechanisms by oncogenic signaling (Fig. 1.6). One example, E6 oncogenes, found in human papilloma virus (HPV), target cell polarity proteins Dislarge (Dlg) and Scribble (Scrib) for proteolytic degradation (Fig. 1.6) (Lee and Vasioukhin, 2008). The ability to degrade these cell polarity proteins correlates with the malignant potential of E6 oncogenes. In addition to viral oncogenes, malignant transformations can be induced by the abnormal
activation of various growth factor signaling pathways such as transforming growth factor β (TGFβ), resulting in the stimulation of cell proliferation and disruption of apical-basal polarity, cell-cell adhesion, and sometimes a complete epithelial-mesenchymal transition (EMT). AJC-localized Par6 directly interacts with TGFβ receptors and is a substrate for TGFβRII-mediated phosphorylation (Ozdamar et al., 2005). Activation of TGFβ signaling results in phosphorylation of Par6, which promotes its interaction with the E3 ubiquitin ligase Smurf1. Localized to the AJCs, Smurf1 targets junctional RhoA for degradation. RhoA is crucial for the maintenance of the actin cytoskeleton and stabilization of AJCs; thus, activation of TGFβ signaling results in the destabilization and loss of AJCs and the initiation of EMT (Fig. 1.6).

Abnormal activation of the receptor tyrosine kinase ErbB2 is implicated in human breast, ovarian, gastric, esophageal, and endometrial cancers (Hynes and Lane, 2005; Linggi and Carpenter, 2006; Moasser, 2007). Whereas ErbB2 stimulates cell proliferation by activating the PI3K pathway, it can also directly disrupt cell polarity and provide protection from apoptosis through its interaction with the Par6-aPKC protein complex (Aranda et al., 2006). Activation of ErbB2 results in the dissociation of Par3 from the Par6-aPKC complex (Fig 1.6). Inhibition of this dissociation restores correct cell polarity and abrogates the anti-apoptotic effects of ErbB2. These findings indicate that growth-factor receptors can use independent mechanisms to regulate proliferation and polarity, and that it is possible to activate cell proliferation without inducing cell-polarity defects.

In addition to oncogenes and proto-oncogenes, tumor suppressors are also involved in the regulation of apical-basal cell polarity. Mutations in the von Hippel-
Lindau (VHL) tumor suppressor are responsible for von Hippel-Lindau disease, which is characterized by the development of hemangioblastoma, clear-cell renal carcinoma and pheochromocytomas (Kaelin, 2005; Kaelin, 2007). VHL polyubiquitinates and targets the transcription factor hypoxia-inducible factor 1 (HIF1) for degradation. VHL directly impacts cell polarity pathways by ubiquitin-mediated degradation of activated aPKC (Fig 1.6) (Okuda et al., 2001). Furthermore, interaction between VHL and the Par3-Par6-aPKC complex is involved in VHL-mediated regulation of polarized microtubule growth and formation of primary cilia (Schermer et al., 2006). This function makes VHL an important regulator of cell polarity because polarized growth of microtubules is crucial for cell polarization.

Phosphatase and tensin homolog (PTEN) is another tumor suppressor protein that is implicated in the regulation of cell polarity. PTEN negatively regulates the PI3K pathway (Rossi and Weissman, 2006). Spatial membrane segregation of PI3K pathway regulators is important for apical-basal cell polarity (Fig.1.6)(Martin-Belmonte et al., 2007). Thus, PTEN is necessary for the establishment and maintenance of apical-basal cell polarity.

Finally, in addition to PTEN, LKB1 is another dual-function protein that is directly involved in both cell polarity and tumor suppression. As previously described, mutations in LKB1 are responsible for Peutz-Jeghers syndrome. LKB1 is a homologue of the Drosophila and C. elegans cell polarity protein Par4 and its function is also important for establishing polarity in mammalian cells (Baas et al., 2004a). LKB1 phosphorylates and activates several other serine/threonine protein kinases including AMP-activated protein kinase (AMPK) and Par1 (Fig 1.6) (Lizcano et al., 2004). AMPK
controls cell polarity by regulating the acto-myosin cytoskeleton via phosphorylation of the non-muscle myosin regulatory light chain (MRLC) protein (Lee 2007). The discovery of AMPK as a regulator of cell polarity indicates that LKB1-mediated control of cell growth and polarity are connected to each other.

**Summary**

The major goal of my thesis work was to investigate functional interactions between the Par-1b protein kinase and the RNF41 E3 ubiquitin ligase. Atypical Protein Kinase C (aPKC) phosphorylates Par-1b on a conserved threonine residue (T595) and I participated in studies demonstrating that novel Protein Kinase C (nPKC) activates Protein Kinase D (PKD) to directly phosphorylate Par-1b on serine 400 (S400), a residue that is conserved in all four mammalian Par-1 kinases as well as the fly ortholog. Phosphorylation of Par-1b on T595 and S400 causes Par-1b to relocate from membranes to the cytosol and to bind 14-3-3 proteins. In 2004, Brajenovic, et al. reported the results of a study that used tandem affinity purification (TAP) to isolate human Par-1d along with associated proteins. Nrdp1/RNF41, a ring finger E3 ligase was identified in their screen along with 14-3-3 and aPKC. I found that Par-1b also binds to RNF41 and identified RNF41 as a novel cell polarity determinant. My work demonstrated that phosphorylation of RNF41 on S254 by Par-1b is necessary for establishing epithelial cell polarity.
Fig. 1.1 Mechanisms that localize Par proteins.
Sperm-contributed nucleus and centrosome-nucleated microtubules are on the right side of the *C. elegans* embryo. Arrows indicate spread of the cortex to which Par-2 associates as actomyosin contraction occurs. [Adapted from (Goldstein and Macara, 2007)]
Fig. 1.2 Mechanisms of establishing epithelial cell polarity. Par-1 that has diffused onto the apical domain is phosphorylated by aPKC, thus inhibiting the Par-1 kinase activity and inducing binding of Par-5/14-3-3, which in turn triggers release of Par-1 into the cytoplasm.
Fig. 1.3 Diagram of the domains of Par-1b protein and upstream signaling. [(*) indicates autophosphorylation] LKB1 phosphorylates Par-1b to activate Par-1b signaling. Additionally, Par-1b is phosphorylated by two arms of the PKC pathway on two sites (S400 and T595).
Fig. 1.4 Diagram of the domains of RNF41 protein. Indicated are amino acids known to be of importance for substrate binding and/or E3 ligase activity. Serine 254, a site phosphorylated by Par-1b, is indicated in bold.
Fig. 1.5 Events in MCF10A morphogenesis in 3D culture.
During the early stages of 3D culture, apical-basal polarization becomes evident within cell clusters before evidence of lumen formation. At day 5-8 in culture, two distinct populations of cells become evident within each acinus: an outer layer of cells in direct contact with the matrix (marked by laminin 5 staining on day 4) and inner cells lacking matrix contact. Throughout morphogenesis, this outer cell layer remains polarized with respect to the acinus center (indicated by the apical orientation of the golgi protein, GM130). Starting at day 8, the centrally located cells undergo cell death, which involves apoptosis, characterized by the expression of active caspase 3. This cell death contributes to the formation of a hollow lumen. The acinus remains hollow thereafter.
[Adapted from (Debnath and Brugge, 2005)]
Fig. 1.6 Oncogenic and tumor suppressor signaling pathways target cell-polarity mechanisms. (A) Viral E6 oncogene from human HPV targets Dlg and Scrib proteins for ubiquitin-mediated degradation. (B) Activation of TGFβ signaling results in phosphorylation of Par6, and targeting of the E3 ubiquitin ligase Smurf1 to the AJCs, where Smurf1 destroys RhoA, disrupts the integrity of AJCs and causes EMT. (C) Activation of ErbB2 signaling results in disruption of the apical Par6-Par3-aPKC polarity complex by promoting dissociation between Par3 and Par6-aPKC. (D) The VHL tumor suppressor ubiquitinates active aPKC and targets it for proteasome-mediated degradation. In addition, interaction between VHL and the Par3-Par6-aPKC polarity complex is necessary for correct orientation of the microtubules and for primary cilia formation. (E) During polarization of epithelial cells PTEN is targeted to the future apical membrane domain. (F) The tumor suppressor LKB1 controls cell polarity, growth and proliferation by regulating the activities of AMPK and Par1 protein kinases. [Adapted from (Lee and Vasioukhin, 2008)]
References


CHAPTER 2
Phosphorylation of the Par-1 Polarity Kinase by Protein Kinase D Regulates 14-3-3 Binding and Membrane Association.

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Phosphorylation of the Par-1 Polarity Kinase by Protein Kinase D Regulates 14-3-3 Binding and Membrane Association.

Janis L. Watkins¹,#, Katherine T. Lewandowski¹,#, Sarah E. M. Meek¹,³,§, Peter Storz⁴, Alex Toker⁵ and Helen Piwnica-Worms¹,²,³ *

¹Department of Cell Biology and Physiology, ²Department of Internal Medicine, Washington University School of Medicine, 660 South Euclid Avenue St. Louis, MO 63110, USA, ³Howard Hughes Medical Institute, ⁴Department of Cancer Biology, Mayo Clinic Comprehensive Cancer Center, 4500 San Pablo Road, Jacksonville, FL 32224, USA, ⁵Department of Pathology, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, Boston MA 02215, USA

Running Title: Regulation of Par-1b by nPKC/PKD pathway

#equal contribution

*Corresponding author: Helen Piwnica-Worms, Department of Cell Biology and Physiology Washington University School of Medicine, Box 8228, 660 South Euclid Ave. St. Louis, MO 63110. Tele: (314) 362-6812, FAX: (314) 362-3709, email: hpiwnica@cellbio.wustl.edu

§Current address: Department of Oncology, University of Edinburgh, CR-UK Building, Western General Hospital, Crewe Road South, Edinburgh, EH4 2XU, UK.
Abstract

The Par-1 protein kinases are conserved from yeast to humans where they function as key polarity determinants. The mammalian Par-1 family is comprised of four members (Par-1a, b, c and d). Previously, we demonstrated that atypical protein kinase C (aPKC) phosphorylates the Par-1 kinases on a conserved threonine residue (T595) to regulate localization and kinase activity. Here we demonstrate that Par-1b is also regulated by another arm of the PKC pathway, one that involves novel PKCs (nPKC) and Protein Kinase D (PKD). Treatment of cells with the PKC activator phorbol-12-myristate-13-acetate (PMA) potently stimulated phosphorylation of Par-1b on serine 400 (S400), a residue that is conserved in all four mammalian Par-1 kinases as well as the fly ortholog. We demonstrate that PMA stimulates nPKC to activate PKD, which in turn directly phosphorylates Par-1b on S400 to positively regulate 14-3-3 binding and to negatively regulate membrane association. Thus, two arms of the PKC pathway regulate interactions between Par-1b and 14-3-3 proteins: one involving aPKC and the other nPKC/PKD.
Introduction

Establishing and maintaining cellular polarity is critical for the homeostasis of unicellular and multicellular organisms alike. The PAR (partitioning-defective) genes (PAR 1-6) were identified in Caenorhabditis elegans as essential determinants of asymmetric cell division and polarized cell growth (Kemphues, 2000; Kemphues et al., 1988). Par-1 is a serine/threonine protein kinase and Par-1 homologues have been identified and studied in a number of organisms, including yeast, fruitflies, frogs and mammals (Goldstein and Macara, 2007; Tassan et al., 1994). These studies have revealed disparate roles for Par-1 not only as a regulator of cell polarity but also as a component of mitogenic and Wnt signaling (Hurov and Piwnica-Worms, 2007; Tassan et al., 1994). In mammals there are four Par-1 family members named Par-1a (C-TAK1/MARK3), Par-1b (EMK/MARK2), Par-1c (MARK1), and Par-1d (MARKL1, MARK4).

Several Par-1 substrates have been identified, including Par-3 (Benton and St Johnston, 2003; Hurd et al., 2003). An antagonistic relationship between Par-1 and the Par-3/Par-6/aPKC complex has been revealed. In C. elegans embryos Par-1 is located at the posterior cortex whereas the Par3/Par6/aPKC complex is located at the anterior cortex. In epithelial cells, the Par3/Par6/aPKC complex is found at tight junctions whereas Par1 is located laterally beneath tight junctions. Par-1 phosphorylates Par-3 to exclude it from lateral membranes of epithelial cells (Benton and St Johnston, 2003; Hurd et al., 2003), whereas aPKC in complex with Par-3/Par-6 phosphorylates Par-1 to dislodge it from plasma membranes (Hurov et al., 2004a; Suzuki et al., 2004). Thus, the establishment and/or maintenance of cell polarity likely require that Par-1 be physically sequestered from the Par-3/Par-6/aPKC complex and phosphorylation of Par-1 by aPKC may enforce
the mutual exclusion of Par-1 and Par-3/Par-6/aPKC. Negative regulation of Par-1b by the Par-3/Par-6/aPKC complex is also observed in hippocampal neurons (Chen et al., 2006). Here, we identify another protein kinase pathway that regulates Par-1 localization. We demonstrate that treatment of cells with PMA activates nPKCs to activate PKD and that PKD directly phosphorylates Par-1b on S400. Phosphorylation of S400, like phosphorylation of T595, regulates Par-1b/14-3-3 interactions and the ability of Par-1b to associate with cellular membranes.
Results

Using a combination of site-directed mutagenesis and tryptic phosphopeptide mapping, we identified serine 400 (S400) as a potential site of Par-1b phosphorylation in vivo (data not shown). To verify that Par-1b is indeed phosphorylated on S400 in vivo, a phosphospecific antibody was generated and used in Western blotting experiments (Fig. 2.1A, upper panel). Whereas the pS400 antibody recognized Par-1b (lane 2), mutation of S400 eliminated its recognition by the antibody (lane 4). Thus, the antibody is specific for Par-1b when it is phosphorylated on S400 and ectopically expressed Par-1b is phosphorylated on S400 in cultured cells. In addition, phosphorylation of S400 did not require the kinase activity of Par-1b as a kinase-inactive mutant of Par-1b was also phosphorylated on S400 in vivo (lane 3). The status of endogenous Par-1b phosphorylation was interrogated in several mammalian cell lines using the pS400 antibody (Fig. 2.1B). Two electrophoretic forms of Par-1b that arise by alternative splicing (Hurov et al., 2001; Hurov et al., 2004b) were detected in each cell line and both splice variants reacted with the phosphospecific antibody. Thus, endogenous Par-1b is phosphorylated on S400 in vivo.

Members of the Par-1 family share a conserved amino-terminal kinase domain, followed by a UBA domain, a divergent region of unknown function and a conserved C-terminal region of approximately 100 amino acids (Fig. 2.S1). Serine 400 resides within the divergent region and is conserved in all four human Par-1 kinases as well as the fly, but not worm ortholog (Fig. 2.1C). Par-1a also reacted with the pS400 antibody demonstrating that Par-1a is also phosphorylated on S410 (S400 equivalent) in vivo (Fig. 2.S1B). The pS400-specific antibody did not recognize Par-1c and Par-1d and this is
likely due to the fact that the phosphopeptide used to generate the pS400-specific antibody varies significantly in sequence from residues surrounding the equivalent phosphorylation sites in Par-1c and Par-1d (Fig. 2.1C).

Interestingly, sequences inclusive of and surrounding S400 resemble a mode I 14-3-3 binding motif (Muslin et al., 1996; Yaffe et al., 1997) and Par-1b binds 14-3-3 proteins (Benton and St Johnston, 2003; Goransson et al., 2006; Meek et al., 2004a; Suzuki et al., 2004). We tested whether S400 regulated interactions between Par-1b and 14-3-3 proteins in two ways. First, co-precipitation of 14-3-3 proteins with wild type and mutant forms of Par-1b were examined and secondly Far Western analysis with purified 14-3-3 proteins was employed. As seen in Fig. 2.2A, co-precipitation of 14-3-3 proteins with wild-type (WT, lane 2) and kinase-inactive Par-1b (lane 3) was observed. Substitution of S400 with alanine diminished, but did not eliminate, 14-3-3 binding to Par-1b (lane 4). Far Western analysis confirmed that mutation of S400 reduced interactions between Par-1b and 14-3-3 proteins (Fig. 2.2B, lane 5). A truncation mutant of Par-1b consisting of amino acids 1-470 but lacking S400 failed to bind 14-3-3 proteins (lane 6) indicating that phosphorylation of S400 regulated interactions between 14-3-3 proteins and the amino terminus of Par-1b. Additional 14-3-3 binding site(s) must reside within the C-terminus of Par-1b given that mutation of S400 diminished but did not eliminate interactions between 14-3-3 proteins with full length Par-1b (Fig. 2.2A). A previous study reported that 14-3-3 binding is facilitated by T595 phosphorylation (Suzuki et al., 2004). We monitored WT and mutant forms of Par-1b for their ability to bind to 14-3-3 proteins and to be phosphorylated on S400 and T595 in vivo (Fig. 2.2C, 2.S1C). We observed that substitution of T595 with alanine reduced interactions between
Par-1b and 14-3-3 proteins as did substitution of S400 with alanine. Simultaneous mutation of both phosphorylation sites severely compromised binding of 14-3-3 proteins to Par-1b.

In our search to identify signaling pathways that regulate Par-1b in vivo, we observed that treatment of cells with the protein kinase C (PKC) activator PMA potently stimulated phosphorylation of endogenous (Fig. 2.2D) and ectopically-produced (Fig. 2.2E, lane 2) Par-1b on S400 in vivo. In addition, Far Western analysis demonstrated that PMA-treatment enhanced the binding of 14-3-3 proteins to Par-1b (Fig. 2.2E, lane 4). Although PMA enhanced the binding of 14-3-3 proteins to Par-1b, its relative binding to WT and mutant forms of Par-1b was not significantly altered in PMA-treated cells (Fig. 2.S1D). Enhanced Par-1b phosphorylation on S400 by PMA implicated members of the PKC family as upstream regulators of Par-1b. In particular, both the conventional PKC (cPKCs) and novel PKCs (nPKC) are activated by DAG or phorbol esters (Newton, 1997). Sequences surrounding and inclusive of S400 (KVQRSVpSA) do not form a consensus PKC phosphorylation site but rather more closely resemble that of a PKD consensus site (Fig. 2.1C) (Nishikawa et al., 1997). A major pathway for the activation of PKD is translocation to membrane compartments via binding to DAG or phorbol esters followed by phosphorylation of activation loop residues by novel PKCs (δ, ε, η, θ), which are directly activated by PMA (Rozengurt et al., 2005). As seen in Fig. 2.S1E, PMA treatment resulted in activation loop phosphorylation of both PKD1 (lane 2) and PKD2 (lane 4) indicative of PKD activation.

Several experiments were performed to determine if Par-1b is directly regulated by PKD. First, the phosphorylation status of Par-1b was monitored after co-production
with wild type and mutant forms of PKD1 and PKCε (Fig. 2.3A). Enhanced S400-phosphorylation was observed when Par-1b was co-produced with either WT PKD1 (lane 3), WT PKCε (lane 4) or both PKD1 and PKCε (lane 5). In addition, the stimulatory effects of PKD1 and PKCε on Par-1b S400-phosphorylation were blocked by kinase-inactive PKCε (lane 6) and kinase-inactive PKD1 (lane 7), respectively. Importantly, kinase–inactive PKCε was unable to block the stimulatory effects of constitutively active PKD1 on Par-1b S400 phosphorylation (lane 8). These findings suggest that PKCε functions upstream of PKD to regulate phosphorylation of Par-1b on S400. Next, kinase assays were performed in vitro to determine if Par-1b was a direct substrate of either PKCε or PKD1 (Fig. 2.3B). PKD1 phosphorylated a kinase-inactive mutant of Par-1b on S400 in vitro (lane 2, bottom panel). Although PKCε phosphorylated activation loop residues of PKD1 (lane 6, bottom panel), it was incapable of phosphorylating Par-1b on S400 in vitro (lane 1, bottom panel). In addition, PKD1 did not phosphorylate Par-1b on T595, the aPKC site, in vitro (Fig. 2.4A, bottom panels). Interestingly, S400 (Fig. 2.4A, upper panels) but not T595 (Fig. 2.4A, lower panels) was a site of Par-1b autophosphorylation (lane 1). To determine if auto/trans phosphorylation contributed significantly to S400 phosphorylation in vivo, WT and mutant forms of Par-1b were expressed in Par-1b null MEFs (Hurov et al., 2001). Note that kinase-active (Fig. 2.4B, lane 2) and kinase-inactive (lane 3) forms of Par-1b were similarly phosphorylated on S400. Thus, although Par-1b is capable of phosphorylating itself on S400, additional cellular kinase(s) also serve this function in vivo.

Next, siRNA was used to knockdown expression of PKD1 and PKD2 in Hela cells. As seen in Fig. 2.4C, stimulation of S400 phosphorylation was not observed in
PMA-treated cells deficient for PKD1 and PKD2 (lane 4) whereas control cells (lane 2) or cells incubated with scrambled siRNA (lane 3) showed a robust stimulation of S400 in response to PMA-treatment. Taken together, these results suggest that PMA stimulates a signaling pathway from nPKCs to PKD to Par-1b to regulate S400-phosphorylation and 14-3-3 binding.

Phosphorylation of Par-1b by aPKC induces dissociation of Par-1b from the plasma membrane into soluble fractions in a T595-dependent manner (Suzuki et al., 2004). To test the consequences of S400 phosphorylation, PMA-treated HeLa cells were fractionated by sequential centrifugation and membrane (P) and soluble (S) fractions were analyzed for endogenous Par-1b by Western blotting (Fig. 2.4D). As expected, phosphorylated PKD was found in membrane fractions whereas 14-3-3 proteins were observed in soluble fractions. Importantly, endogenous Par-1b phosphorylated on S400 was present predominantly in soluble fractions.
Discussion

In this study, we identified S400 as a novel Par-1b phosphorylation site. Phosphorylation of S400 was shown to regulate interactions between Par-1b and 14-3-3 proteins and to be mediated by PKD. Par-1b is also phosphorylated on T595 and this is catalyzed by atypical PKC (Hurov et al., 2004b; Suzuki et al., 2004). Thus, Par-1b is regulated by two arms of the PKC pathway: one arm is indirect involving activation of nPKCs which, in turn, activate PKD to phosphorylate Par-1b on S400, the second arm is direct and involves phosphorylation of T595 by aPKCs.

Previous studies reported that 14-3-3 binding to Par-1 either does not involve Par-1 phosphorylation (Benton et al., 2002) or is facilitated by T595 phosphorylation (Suzuki et al., 2004). Another study reported 17 new Par-1b phosphorylation sites and mutation of all 17 of these residues eliminated 14-3-3 binding. Surprisingly S400 was not identified as a site of phosphorylation in this study (Goransson et al., 2006). Here, we report that mutation of the two PKC-regulated sites (S400 and T595) is sufficient to ablate 14-3-3 binding to Par-1b. It may be that mutation of 17 residues of Par-1b indirectly effected phosphorylation at S400 and this, in turn, explains the loss of 14-3-3 binding in the Goransson et al. study (Goransson et al., 2006).

The PKC family of protein kinases are subdivided into conventional PKCs that are activated by calcium, acidic phospholipids and diacylglycerol (DAG); novel PKCs activated by DAG and acidic phospholipids but insensitive to calcium and atypical PKCs that are activated, in part, by PKD1 (Newton, 1997). The PKD family consists of three members. PKD1 was originally reported to be a novel PKC and was given the name PKC\(\mu\). However, it was later appreciated that the PKD family distinguished itself from
the PKC family in amino acid composition, domain structure, regulation, and substrate specificity (Rykx et al., 2003). A major pathway for the activation of PKD is translocation to membrane compartments via binding to DAG or phorbol esters followed by phosphorylation of activation loop residues by the nPKCs, which are directly activated by PMA (Rozengurt et al., 2005). Stimulation of S400 phosphorylation by PMA implicated members of the PKC family as regulators of Par-1b. However, sequences inclusive of and surrounding S400 do not conform to a typical PKC consensus sequence but rather more closely resembles that of a PKD phosphorylation site (Nishikawa et al., 1997). The experimental evidence leading to the conclusion that PKD directly phosphorylates Par-1b on S400 in a nPKC-dependent manner is as follows: enhanced S400-phosphorylation was observed when Par-1b was co-produced with PKD1 and/or PKCε (Fig. 2.3A); the stimulatory effects of PKD1 and PKCε on Par-1b S400-phosphorylation were blocked by kinase-inactive forms of each kinase (Fig. 2.3A); importantly kinase–inactive PKCε was unable to block the stimulatory effects of constitutively-active PKD1 on Par-1b S400 phosphorylation (Fig. 2.3A); PKD, but not nPKC, directly phosphorylated Par-1b on S400 in vitro (Fig. 2.3B) and finally, stimulation of S400 phosphorylation by PMA was not observed in cells deficient for PKD1 and PKD2 (Fig. 2.4C). Taken together this data argues that PMA stimulates nPKCs to activate PKD and PKD, in turn, directly phosphorylates Par-1b on S400.

PKD functions downstream of activated G-protein coupled receptors and receptor tyrosine kinases that signal through phospholipase C. PKD is activated by any agent that activates nPKCs and, as such PKD functions to regulate a diverse set of cellular processes including proliferation, apoptosis, stress responses, vesicle trafficking from
golgi, neuronal development, and immune cell signaling (Toker, 2005; Van Lint et al., 2002; Wang, 2006). If Par-1b is an important downstream target of PKD in each of these pathways, it may explain the diverse phenotypes observed in mice disrupted for Par-1b (Hurov and Piwnica-Worms, 2007). It has been demonstrated that the regulated cycling of Par-b on and off lateral membranes is critical for maintenance of apicobasal polarity in mammalian cells (Suzuki et al., 2004). Our study provides mechanistic insight into how Par-1b shuttling is regulated. Phosphorylation of Par-1b on S400 by PKD, like phosphorylation of T595 by aPKC, not only promotes the binding of 14-3-3 proteins to Par-1b but also promotes the release of Par-1b off of cellular membranes. Furthermore, these results implicate a role for PKD in regulating cellular polarity through phosphorylation of Par-1b.
Materials and Methods

Cell Culture

HeLa, HEK-293 and HCT116 cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) supplemented with 10% bovine growth serum (Hyclone, Logan, UT), 100 units/ml penicillin and streptomycin, and 1mM L-glutamine. MCF7 cells were grown in the presence of 10% FBS (Hyclone). Par-1b null MEFs (Hurov et al., 2001) were cultured in DMEM, 10% FBS (Hyclone) and 1 mM L-glutamine, 0.2 mM nonessential amino acids, 140 mM 2-mercaptoethanol, 100 U/ml penicillin G and 100 µg/ml streptomycin.

Plasmids and reagents

PMA was purchased from Sigma Chemical Company (St. Louis, MO). Purified PKCε and the PKC lipid activator were purchased from Upstate Cell Signaling (Lake Placid, NY). Sf9 cell purified HA-PKD and HA-PKD (K612W) as well as plasmids pcDNA3.HA-PKD1, pcDNA3.HA-PKD1(K612W), pcDNA3.HA-PKD1 (S738ES742E) have been described (Storz et al., 2003; Storz et al., 2004). Plasmids encoding PKCε(WT) and PKCε(KD) and Flag tagged Par-1b, Par-1b(D193N) and Par-1b(T595A) have been described (Cenni et al., 2002; Hurov et al., 2004a). Substitution of alanine for serine at position 400 and/or threonine at position 595 was accomplished using the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene, LaJolla. CA). DNA sequencing was performed to verify the mutant sequence. siRNAs specific for PKD1 and PKD2 were purchased from Dhharmacon Inc. (Lafayette, CO) as well as SiGenome Smart
Pool reagent to Human PRKCM (PKD1) and Smart Pool reagent to Human PRKD2 (PKD2). The control siRNA sequence was: 5’UAAGGCUAUGAAGAGAUACUU.

Transient transfections were performed using TransIT HeLa MONSTER reagent (Mirus Bio Corporation, Madison, WI), with Superfect reagent (Qiagen, Valencia, CA) or with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). Par-1b null MEFs have been described (Hurov et al., 2001)

**Antibodies and Western Blotting**

Antibodies against 14-3-3 (K19) and glutathione S-transferase (GST) were purchased from Santa Cruz (Santa Cruz, CA). Other antibodies used in this study were specific for PKD1 (Abcam or Cell Signaling Technology), PKD2 (Abcam, Cambridge, MA), phosphoPKD (Cell Signaling Technology, Boston, MA) and PKCe (Upstate Cell Signaling). Antibodies used for experiments shown in Fig. S1E include a rabbit polyclonal antibody specific for PKD1 raised against a NH$_2$-MAECQNDSGEMQDP-amide peptide (amino acids 372-385 in human PKD1), a rabbit polyclonal antibody specific for PKD2 was from Upstate Cell Signaling (Charlottesville, VA), and a rabbit polyclonal antibody specific for PKD3 from Bethyl Laboratories (Montgomery, TX). These antibodies are specific for the respective PKD isoenzyme and do not cross react with other PKD family members. Antibodies specific for Par-1b have been described (Hurov et al., 2001). Antibodies specific for Par-1b phosphorylated on serine 400 were generated by immunizing rabbits with the phosphopeptide CQRSV-pS-ANPKQ coupled to keyhole limpet hemocyanin (KLH). Antibodies specific for Par-1b phosphorylated on T595 have been described (Hurov et al., 2004b). Flag-fusion proteins were precipitated
with anti-Flag M2 antibody-agarose affinity gel (Sigma Chemical Co) and detected by Western blotting with anti-Flag M2 monoclonal antibody (Sigma Chemical Co.). For Western blotting, antibodies were diluted in 5 % milk in TBST (50 mM Tris-HCl pH 8.0, 0.15 M NaCl, 0.02 % Tween-20), and membranes were washed 4 times in TBST after application of both primary and secondary antibody. Bound primary antibodies were detected with horseradish peroxidase-conjugated goat anti-mouse (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), goat anti-rabbit (Invitrogen, Carlsbad, CA), or donkey anti-goat (Santa Cruz) secondary antibodies, and visualized using the ECL reagent (GE Healthcare, Piscataway, NJ) or Super Signal West Femto reagent (Pierce Biotechnology, Rockford, IL).

**Immunoprecipitations**

Cells were lysed in MCLB (50 mM Tris-HCl pH 8.0, 0.1 M NaCl, 5 mM EDTA, 0.5 % NP-40, 1 mM DTT) supplemented with 1 µM microcystin-LR and protease inhibitors (2 mM PMSF, 0.15 U/ml aprotinin, 20 µM leupeptin, and 20 µM pepstatin.). Lysates were clarified by centrifugation at 16, 000 x g. Cell lysates were pre-cleared with protein A agarose for 1 hour at 4 °C, then incubated with anti-FLAG agarose (Sigma Chemical Co.) for 1 h at 4°C. Beads were washed four times in MCLB, and bound proteins eluted by boiling in SDS–PAGE sample buffer.

**14-3-3 Far Western Analysis**

Samples were subjected to SDS-PAGE and transferred to nitrocellulose membranes as for a Western blot. Proteins bound to the membrane were denatured by incubating for at
least 1 h in denaturation buffer (50 mM Tris-HCl pH 8.0, 6 M guanidine-HCl, 6.25 mM EDTA, 1 mM DTT, 10 % glycerol, 0.05 % Tween-20), then renatured by incubating for at least 1 h in renaturation buffer (denaturation buffer without guanidine-HCl or DTT).

Membranes were blocked for 1 h in 5 % milk in TBST, rinsed in TBST, then incubated for 2 h at room temperature or overnight at 4°C in TBST containing 0.1 µg/ml GST-14-3-3ζ and σ, and 1 mg/ml bovine serum albumin. GST-14-3-3 proteins were purified as described (Meek et al., 2004b). Membranes were washed 4 times in TBST, then incubated for 1 h with anti-GST primary antibody in 5% milk / TBST. Bound primary antibody was detected with horseradish peroxidase-conjugated secondary antibody, and visualized using ECL reagent, as for Western blotting.

**Co-precipitation of 14-3-3 proteins with WT and mutant forms of Par-1b**

HeLa cells at 75% confluency were transfected using Lipofectamine 2000 reagent for 24 h. Cells were lysed in MCLB supplemented with 1µM microcystin and protease inhibitors. Lysates were clarified by centrifugation at 16,000 x g. Cell lysates were pre-incubated with protein A agarose for 1 hour at 4 °C, then incubated with anti-FLAG agarose for 1 h at 4°C. Bound proteins were eluted by incubating 1 h with a 3X flag peptide (Sigma Chemical Co.).

**Kinase Assays**

Kinase reactions were carried out in the presence of 0.5 µg bacterially-purified GST-Par-1b, GST-Par-1b (S400A), or GST-Par-1b (D160N) with 250 ng Sf9 cell purified HA-
PKD or HA-PKD (K612W) or with 5 ng active PKCε (Upstate Cell Signaling) and a buffer consisting of 12 µM ATP, 2 mM DTT, 10 mM MgCl₂, and 50 mM Tris pH 7.5 supplemented with sonicated PKC lipid activator containing phosphatidyl serine and diacylglycerol (Upstate Cell Signaling). Samples were incubated at room temperature for 30 min. Reactions were terminated by the addition of SDS sample buffer followed by incubation for 10 min at 37°C.

**RNAi Experiments**

RNA interference (RNAi) was performed using protocols supplied by Dharmacon, Inc. siRNA duplexes were transfected into HEK293 cells using Dharmafect #2 at a final concentration of 10 nM. After ~30 h cells were transfected a second time with siRNA at 10 nM final concentration. Cells were incubated an additional 48 h and then incubated in the absence or presence of 200 ng/ml PMA for 1 min. Cells were lysed in MCLB and analyzed by Western blotting.

**Cell Fractionation**

HeLa cells were grown to near confluency on 10 cm tissue culture dishes. Cells were treated with 200 ng/ml PMA for 1 min prior to harvest. Cells were trypsinized and washed in ice cold PBS. Cells were suspended in 800 µl of hypotonic buffer (HB: 12.5 mM Tris pH 7.5, 1 mM EDTA, 1 mM DTT) supplemented with 5 mM Na₃PO₄, 1 µM microcystin, 1 mM sodium orthovanadate, 2 mM PMSF, 0.15 U/ml aprotinin, 20 µM leupeptin, and 20 µM pepstatin for ~40 min. Cells were dounce homogenized and when the majority of cells were lysed, cell lysates were centrifuged at 1000 x g for 5 min. The
resulting supernatant was transferred to a fresh tube and the pellet was washed once with 200 µl of HB. The washed supernatant was added to the first collected supernatant (= total (T)). A portion of this was reserved for Western blotting and the remainder was centrifuged at 100,000 x g for 30 min. The supernatant was transferred to a fresh tube (= soluble (S) fraction) and the pellet was vortexed in 100 µl HB and recentrifuged at 100,000 x g for 15 min. The supernatant was added to the soluble fraction and the pellet (= membrane (M) fraction) was resuspended in 300 µl of MCLB supplemented with 1 µM microcystin, 1 mM sodium orthovanadate and protease inhibitors. Fractions were resolved by SDS-PAGE and proteins visualized by Western blotting using Super Signal West Femto reagent.
Acknowledgments

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\begin{array}{cccccc}
-5 & -3 & -1 & & & \\
V & Q & R & S & I & S \ A \ N - & Q & K & Q & \text{HaPar-1c/MARK1} \\
V & Q & R & S & V & S & A & N - & P & K & Q & \text{HaPar-1b/Emk/MARK2} \\
V & Q & R & S & V & S & S & S - & Q & K & Q & \text{HaPar-1a/C-TAK1/MARK3} \\
G & Q & R & S & S & S & S & T & Y & H & R & Q & \text{HaPar-1d/MARK4/MARKL1} \\
V & H & R & S & I & S & A & S & S & T & K & P & \text{DmPar-1} \\
V & V & R & T & L & S & V & & & & & & \text{PKD(PKC}_\mu\text{) consensus} \\
R & S & X & S & A & P & G & & & & & & 14-3-3 \text{ consensus I} \\
Q & R & S & V_pS & A & N - & P & K & Q & \text{pS400-Specific Antibody} \\
\end{array}
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Fig. 2.1 Par-1b is phosphorylated on serine 400 in vivo.

(A) HeLa cells were transfected with plasmids encoding the indicated proteins using HeLa MONSTER reagent for 48 h. Flag-tagged Par-1b proteins were resolved by SDS-PAGE and Western blotting was performed with an antibody specific for Par-1b phosphorylated on S400 (top panel). Blots were stripped and re-probed with an antibody specific for the flag epitope (bottom panel). (B) Lysates prepared from the indicated cell lines were resolved by SDS-PAGE and Western blotting was performed with an antibody specific for Par-1b phosphorylated on S400 (top panel). Blots were stripped and re-probed with an antibody specific for Par-1b (bottom panel). (C) Sequence alignment of Par-1 orthologs illustrates conservation of the S400 phosphorylation site. The mode I 14-3-3 binding motif, the PKD phosphorylation motif and the phosphopeptide used to generate the pS400-specific Par-1b antibody are also indicated.
Fig. 2.2 Phosphorylation of Par-1b on S400 regulates 14-3-3 binding and is stimulated by PMA.

(A) HEK293 cells were transfected with plasmids encoding WT Par-1b (WT), kinase-inactive Par-1b (D193N) or a phosphorylation-site mutant of Par-1b (S400A) using Superfect reagent for 48 h. Lysates were resolved directly by SDS-PAGE (lanes 5-8) or were incubated with anti-flag agarose prior to SDS-PAGE (lanes 1-4). Western blotting was performed with an antibody specific for the flag epitope to detect Par-1b (top panel) or with an antibody specific for 14-3-3 proteins (bottom panel). Relative levels of 14-3-3 in each precipitate were determined from Western blots using the ImageJ program and are indicated below the blot. (B) HEK293 cells were transfected with plasmids encoding the indicated fusion proteins using Superfect reagent for 48 h. Lysates were incubated with anti-flag agarose and precipitates were resolved by SDS-PAGE. Precipitates were subjected to Western blotting to monitor Par-1b levels (lanes 1-3) or to Far Western analysis to monitor 14-3-3 binding (lanes 4-6). (C) HeLa cells were transfected with plasmids encoding the indicated flag-tagged proteins using Lipofectamine 2000 for 24 h. Lysates were incubated with flag agarose. Precipitates were resolved by SDS-PAGE and analyzed by Western blotting using the indicated antibodies. Relative levels of 14-3-3 in each precipitate were determined from Western blots using the ImageJ program and are indicated below the blot (Fig. S1C). A representative experiment is shown in panel C. (D) Lysates prepared from HeLa cells that had been cultured in the absence of serum for 16 h and then treated with vehicle (lane 1) or with 400 ng/ml PMA for 10 min. (lane 2) were resolved by SDS-PAGE and Western blotting was performed with pS400 antibody (top panel). Blots were stripped and re-probed with a Par-1b specific antibody (bottom panel).
HEK293 cells were transfected with plasmid encoding Flag-Par-1b using Superfect reagent for 48 h. Cells were cultured in the absence of serum for 3 h and then treated with vehicle (lanes 1, 3) or with 400 ng/ml PMA for 20 min (lanes 2, 4). Flag-tagged Par-1b was precipitated using anti-flag agarose and resolved by SDS-PAGE. Flag-Par-1b was subjected to Western blotting to monitor S400 phosphorylation (lanes 1, 2) or to Far Western analysis to monitor 14-3-3 binding (lanes 3, 4). The pS400 blot was stripped and probed with antibody specific to the flag epitope (lanes 5, 6).
Fig. 2.3 Phosphorylation of Par-1b on S400 is regulated by nPKC/PKD pathway

(A) HEK293 cells were transfected with the indicated plasmids using Superfect reagent for 24 h. Lysates were resolved by SDS-PAGE and subjected to Western blotting. (B) Kinase assays were performed in vitro with a kinase-inactive mutant of Par-1b and the indicated purified protein kinases. Western blotting was performed to monitor levels of each protein in the assay (top 3 panels) and to monitor the phosphorylation status of Par-1b (bottom panel, lanes 1-5) and PKD1 (bottom panel, lane 6).
**Fig. 2.4 Phosphorylation and localization of Par-1b regulated by PKD *in vivo.***

(A) Kinase assays were performed *in vitro* with kinase-active Par-1b alone (lane 1) or in the presence of kinase-active (lane 2) or kinase-inactive (K612W, lane 3) PKD1. Reactions were resolved by SDS-PAGE and subjected to Western Blotting. The phosphorylation status of Par-1b was monitored using the indicated phospho-specific antibodies. (B) Par-1b null MEFs (Hurov et al., 2001) were transfected with plasmids expressing the indicated proteins using Lipofectamine 2000 for 24 h. Lysates were incubated with anti-flag agarose and precipitates were resolved by SDS-PAGE. Precipitates were subjected to Western blotting with antibodies specific for Par-1b phosphorylated on S400 (top panel). Blots were stripped and re-probed with Flag-specific antibodies (bottom panel). (C) HeLa cells were untreated, incubated with control siRNAs or with siRNAs specific for PKD1 and PKD2 as described in the methods section. Cells were incubated for 1 min with 200 ng/ml PMA, lysed and subjected to Western blotting with the indicated antibodies. Relative levels of S400 phosphorylation and Par-1b protein are indicated above each blot. (D) Lysates from PMA-treated HeLa cells (T) were fractionated into soluble (S) and membrane (M) compartments. Fractions were probed for the indicated proteins by Western blotting. Arrows denote two isoforms of Par-1b.
References


-66-


CHAPTER 2. Supplemental Data

SUPPLEMENTARY FIGURE 1

A

B

C

D

E

Relative 14-3-3 levels:

-68-
Fig. 2.S1 Par-1a is phosphorylated on serine 400 in vivo.

(A) The kinase-, UBA- and ELKL-domains of Par-1b are indicated. Black boxes represent regions that are absent in spliced variants of human Par-1b. The signaling pathways regulating phosphorylation of S400 and T595 are indicated. (B) HeLa cells were transfected with plasmid encoding flag-tagged Par-1a using Lipofectamine 2000 for 24 h. Lysates from control cells (lane 1) or cells expressing flag-tagged Par-1a (lane 2) were resolved directly by SDS-PAGE. Western blotting was performed with indicated antibodies. (C) HeLa cells were transfected with plasmids encoding the indicated flag-tagged proteins using Lipofectamine 2000 for 24 h. Lysates were incubated with flag agarose. Precipitates were resolved by SDS-PAGE and analyzed by Western blotting using the indicated antibodies. Relative levels of 14-3-3 in each precipitate were determined from Western blots using the ImageJ program and are indicated below the blot. The mean +/- SEM for five independent experiments is shown. Student’s t test was performed for comparisons between groups. p value designations are as follows: * (p<0.005), **(p<0.001). A representative Western blot is shown in Figure 2C. (D) HeLa cells were transfected with plasmids encoding the indicated flag-tagged proteins using Lipofectamine 2000 for 24 h and then incubated with 200 ng/ml PMA for 1 min. Lysates were incubated with flag agarose and precipitates were resolved by SDS-PAGE and analyzed by Western blotting for the indicated proteins. Relative levels of 14-3-3 in each precipitate were determined from Western blots using the ImageJ program and are indicated below the blot. (E) HeLa cells were cultured in the absence of serum for 16 h and treated with vehicle (lanes 1, 3, 5) or with 400 ng/ml PMA for 10 min (lanes 2, 4, 6). PKD1, PKD2 and PKD3 were immunoprecipitated and samples were resolved by SDS-
PAGE. Immunoblotting was performed with a phospho-specific antibody that recognizes activated PKD1. Blots were stripped and re-probed with PKD1-, PKD2- or PKD3-specific antibodies (bottom panel).
CHAPTER 3

Phosphorylation of RNF41 E3 Ubiquitin Ligase by Par-1 Protein Kinase

Required for Epithelial Cell Polarity

(Unpublished Manuscript)
Phosphorylation of RNF41 E3 Ubiquitin Ligase by Par-1 Protein Kinase
Required for Epithelial Cell Polarity

Katherine T. Vega¹, ² and Helen Piwnica-Worms¹, ², ³*

¹Department of Cell Biology and Physiology
²BRIGHT Institute
³Department of Internal Medicine
Washington University School of Medicine
Campus Box 8228, 660 S. Euclid Ave.
St. Louis, MO 63110-1093
USA

Running Title: Functional interactions between Par-1 and RNF41

*Corresponding author. Mailing address: Helen Piwnica-Worms, Ph.D., Department of Cell Biology and Physiology, Washington University School of Medicine, Box 8228, 660 South Euclid Ave., St. Louis, MO 63110. Phone: (314) 362-6812. Fax: (314) 362-3709. E-mail: hpiwnica@wustl.edu
Abstract

Establishing and maintaining cellular polarity is critical for the homeostasis of unicellular and multicellular organisms alike. The *PAR* (partitioning-defective) genes (*PAR*-1–6) were identified in *Caenorhabditis elegans* (*C. elegans*) as essential determinants of asymmetric cell division and polarized cell growth. Orthologs of *PAR*-1, 3, 4, 5, and 6 but not *PAR*-2 have been identified in mammals. The mammalian Par-1 family is comprised of four members (Par-1a, b, c and d). We have shown that Par-1b and the RING finger containing protein RNF41 bind one another. RNF41 and Par-2 share homology in their RING finger domain and part of their C-terminal domain suggesting that RNF41 may be an ortholog of Par-2. RNF41 binds the N-terminal kinase and ubiquitin associated (UBA) domains of Par-1b and Par-1b phosphorylates RNF41 on serine 254. MCF-10A cells knocked down for RNF41 failed to form polarized acini-like spheroids when grown in either collagen I matrix or in Matrigel. Polarity was rescued when knockdown cells were engineered to express wild-type RNF41 but not the phosphorylation-site mutant of RNF41 encoding alanine for serine at position 254. This study identifies RNF41 as a novel substrate of Par-1 and demonstrates that phosphorylation of RNF41 by Par-1 is required for mammary epithelial cells to polarize when grown in three-dimensional cultures.
Introduction

Epithelial cells depend on the asymmetric organization of cellular components along their apical-basal axis and it is the activity of these asymmetrically localized proteins and protein complexes that regulate polarity (Rodriguez-Boulan and Nelson, 1989). Par-1 encodes a serine/threonine protein kinase and is one of six Par (partitioning-defective) genes (Par-1, Par-2, Par-3/ASIP, Par-4/LKB1, Par-5/14-3-3, and Par-6) identified in a genetic screen conducted in Caenorhabditis elegans (C. elegans) (Kemphues et al., 1988). The Par genes are required for the asymmetric cell division of the C. elegans zygote (Kemphues et al., 1988). Orthologs of each of the C. elegans Par genes have been identified except for Par-2. Par-2 is a RING finger containing protein that co-localizes with Par-1 to the posterior pole of the C. elegans zygote and cooperates with other Par gene products to establish polarity during early embryonic development.

In epithelial cells, the organization and establishment of cell polarity is dependent on the antagonistic relationship between Par-1 and the Par-3/Par-6/atypical protein kinase C (aPKC) complex. The Par-3/Par-6/aPKC complex localizes to tight junctions and the integrity of this complex is required for maintaining polarity (Etienne-Manneville and Hall, 2003; Izumi et al., 1998; Joberty et al., 2000). Mammalian Par-1 kinase is excluded from tight junctions and instead localizes to basolateral membranes (Bohm et al., 1997). Par-1 phosphorylates Par-3 to exclude it from tight junctions (Benton and St Johnston, 2003; Hurd et al., 2003) whereas aPKC in complex with Par-3/Par-6 phosphorylates Par-1 on T595 to displace it from membranes (Chen et al., 2006; Hurov et al., 2004; Suzuki et al., 2004). In mammals there are four Par-1 family members: Par-1a (C-TAK1/MARK3), Par-1b (EMK/MARK2), Par-1c (MARK1) and Par-1d (MARKL1, MARK 4) (Drewes et
al., 1997; Espinosa and Navarro, 1998; Hurov et al., 2001; Inglis et al., 1993; Kato et al., 2001; Muller et al., 2001; Peng et al., 1998).

A proteomic screen employing tandem affinity purification (TAP) followed by tandem mass spectrometry identified a complex of proteins that associate with one of the human Par-1 orthologs (Par-1d/MARK4) (Brajenovic et al., 2004). This screen identified both known (aPKC and 14-3-3) and novel Par-1 interacting proteins (Brajenovic et al., 2004). Of particular interest was the identification of the RING finger E3 ubiquitin ligase RNF41 as a Par-1d interacting protein. RNF41 is also known as NRDP1 (neuregulin receptor degradation protein-1) based on its role in facilitating the ubiquitin-mediated proteolysis of ErbB3 (Diamonti et al., 2002). RNF41 contains an N-terminal RING domain, two zinc-finger domains (B-Boxes), a coiled-coil domain, and a C-terminal domain that binds to various substrates (Abdullah et al., 2001; Bouyain and Leahy, 2007; Diamonti et al., 2002; Qiu and Goldberg, 2002).

RNF41 has been shown to regulate the steady-state levels of ErbB3 and ErbB4 but not epidermal growth factor receptor (EGFR, ErbB1) or ErbB2 (Her2/neu). RNF41 associates with ErbB3 and ErbB4 independently of receptor stimulation (Diamonti et al., 2002; Qiu and Goldberg, 2002) and elicits ligand-independent ErbB3 ubiquitination and degradation (Qiu and Goldberg, 2002). Importantly a concomitant reduction in ErbB3 levels and neuregulin-1 (NRG1) growth factor signaling are observed when RNF41 is overproduced whereas ErbB3 accumulates and NRG1 growth factor signaling is enhanced when RNF41 is knocked down or a ligase-deficient (dominant-negative) mutant is expressed (Yen et al., 2006). Finally, decreased RNF41 levels in tumors correlates with higher levels of ErbB3 in both an in vivo transgenic mouse model of
ErbB2-induced mammary tumorigenesis and in a panel of primary breast tumors (Yen et al., 2006).

In addition to its role in regulating NRG1 growth factor signaling by modulating ErbB3 levels, RNF41 also regulates ligand-independent expression of the interleukin-3 and erythropoietin cytokine receptors (Sausville et al., 2001) by controlling receptor degradation and shedding (Wauman et al.) and RNF41 regulates Toll-like receptor (TLR)-mediated responses through ubiquitination of MyD88 and TBK1 (Singh et al., 2009). Finally, RNF41 has been implicated in the ubiquitin-mediated proteolysis of two ubiquitin ligases. These include the inhibitor of apoptosis BRUCE and PARKIN, a protein involved in the onset of Parkinson's disease (Qiu et al., 2004; Zhong et al., 2005).

Although several targets of RNF41 have been identified, very little is known regarding how RNF41 itself is regulated. RNF41 is subject to auto-ubiquitination and proteolysis and this is counterbalanced by the activity of USP8, a deubiquinating enzyme (Cao et al., 2007; Wu et al., 2004). In this study, we demonstrate that RNF41 is regulated by the Par-1 polarity kinase and identify a novel role for RNF41 in regulating epithelial cell polarity.
Results

RNF41 co-precipitates with Par-1b.

A proteomic screen employing tandem affinity purification (TAP) followed by tandem mass spectrometry identified RNF41 in a complex of proteins that associate with Par-1d (MARK4) (Brajenovic et al., 2004). To determine if RNF41 interacted with a second human Par-1 ortholog, HeLa and HEK 293T cells were transfected with control plasmid (-), plasmid encoding human Flag-tagged CHK2 as a negative control or plasmid encoding Flag-tagged Par-1b. Lysates were prepared and incubated with Flag agarose followed by Western blotting. As seen in Fig. 3.1A, endogenous RNF41 was detected in Par-1b (lanes 3 and 6) but not in control (lanes 1, 2, 4, 5) precipitates. Two electrophoretic forms of RNF41 are present in HeLa and HEK 293T cells.

Members of the Par-1 family are characterized by having a conserved amino-terminal kinase domain, followed by a ubiquitin-associated (UBA) domain, a divergent region of unknown function (spacer) and ending with a conserved region of about 100 amino acids known as the ELKL domain because it terminates with the sequence glutamate-leucine-lysine/asparagine-leucine (Fig. 3.1B) (Inglis et al., 1993). Par-1b deletion mutants that express only the amino-terminal domain or C-terminal domain were expressed in cells to identify regions of Par-1b important for interactions with RNF41. As seen in Fig. 3.1C, endogenous RNF41 co-precipitated with full length (FL) Par-1b (lane 2) as well as the Par-1b mutant encoding the amino terminus and UBA domain (N-UBA, lane 3). Removal of the UBA domain significantly reduced RNF41 binding (lane 5) and RNF41 did not associate with the C-terminal domain of Par-1b (lanes 4 and 6).
Par-1b phosphorylates RNF41 on S254 \textit{in vitro} and RNF41 is phosphorylated on S254 \textit{in vivo}.

To determine if RNF41 is a substrate of Par-1b, kinase assays were performed \textit{in vitro} in conjunction with two-dimensional tryptic phosphopeptide mapping. RNF41 was phosphorylated by Par-1b \textit{in vitro} (Fig. 3.2A) and this occurred on one or more serine residues (Fig. 3.2B). Mapping studies revealed several phosphopeptides and substitution of alanine for threonine at position 254 resulted in the disappearance of a single phosphopeptide (Fig. 3.2C) suggesting that Par-1b phosphorylates RNF41 on S254 \textit{in vitro}. To confirm that Par-1b phosphorylates RNF41 on S254 \textit{in vitro} and that RNF41 is phosphorylated on S254 \textit{in vivo}, a phosphospecific antibody recognizing RNF41 only when it is phosphorylated on S254 was generated (Fig. 3.2D). RNF41 consists of an amino terminal RING domain followed by a B box, a coiled-coil domain and a C-terminal region that mediates interactions between RNF41 and ErbB3 (Receptor binding domain) (Fig. 3.1B). Mutation of two residues within the RING domain (cysteine (C) 34 and histidine (H) 36) ablates the E3 ubiquitin ligase activity of RNF41 (Qiu and Goldberg, 2002). Due to the ability of RNF41 to auto-ubiquitinate and promote its own proteosomal degradation, we generated a ligase deficient mutant of RNF41 encoding serine for C34 and glutamine for H36 (denoted ΔCH) in order to produce adequate levels of RNF41 \textit{in vivo}. As seen in Fig. 3.2E, the pSer254 antibody recognized ectopic RNF41 (lane 2) and phosphorylation of S254 increased when RNF41 was co-produced with wild-type Par-1b \textit{in vivo} (lane 3). In contrast, phosphorylation of RNF41 on S254 was reduced in cells expressing kinase-inactive Par-1b (lane 4). Mutation of serine 254 eliminated recognition of RNF41 by the phosphospecific antibody under similar conditions (lanes 5-
7). These results demonstrate that the antibody is specific for RNF41 when it is phosphorylated on S254 and that ectopically expressed RNF41 is phosphorylated on S254 in vivo. Furthermore, the ability of kinase-active Par-1b to enhance S254 phosphorylation and kinase-inactive Par-1b to decrease S254 phosphorylation suggests that phosphorylation of RNF41 on S254 is regulated in vivo by Par-1b.

**Phosphorylation of endogenous RNF41 on S254 increases as MCF-10A cells polarize in 3D culture.**

We were unable to detect phosphorylation of endogenous RNF41 on S254 when cells were grown in two-dimensional (2D) cultures (Fig. 3.3D, lane 1 and data not shown). Given the critical role played by Par proteins in establishing and maintaining apical-basal polarity, we investigated the phosphorylation status of both RNF41 and Par-1b in mammary epithelial cells (MCF-10A) grown in 3D cultures. MCF-10A is a spontaneously immortalized but untransformed mammary epithelial cell line. When grown in 3D cultures, MCF-10A cells form polarized acini-like spheroids (Debnath et al., 2003). Parental MCF-10A cells formed normal acinar architecture along with a hollow lumen over a 20 day period when cultured in collagen I matrix and Matrigel (Fig. 3.3A). Serial confocal cross sections of an acinus were stained with To-Pro-3 to detect nuclei and antibody against either E-cadherin to detect adherins junctions; Alpha 6- Integrin to detect basolateral membranes; GM130 to detect golgi at the apical surface; Laminin V to detect basement membrane; and Flag epitope to detect ectopically expressed RNF41. The results demonstrate that normal acinar architecture is formed under our culture conditions and that cells are able to secrete Laminin V to form a basement membrane (Fig 3.3B,C).
Spheroids were also isolated at various times during the 3D culturing period and subjected to Western blotting. As seen in Fig. 3.3D, enhanced phosphorylation of Par-1b on both S400 and T595 was observed in 3D cultures (lanes 2-5) relative to the 2D cultures (lane 1). Two electrophoretic forms of RNF41 were detected in MCF-10A cells and the faster migrating form was phosphorylated on S254 in cells grown in 3D (lanes 2-5) but not 2D (lane 1) cultures.

**RNF41 is required for polarization of MCF-10A cells in 3D cultures.**

Knockdown experiments were conducted to determine if RNF41 is required for MCF-10A cells to polarize into acini-like spheroids in 3D cultures. We employed a lentiviral vector that concurrently knocks down and rescues expression of a target gene in the same cell (Feng et al., 2010). Lentiviral vectors encoding shRNAs specific for luciferase (shLuc) as a control or shRNA specific for sequences within the 3’UTR of RNF41 (shRNF41) were generated. In addition, vectors simultaneously expressing shRNF41 with the coding regions of either RNF41 (WT) or RNF41 (S254A) with a C-terminal flag epitope followed by six histidines (FlagHis₆) were generated. In this way we could determine whether phenotypes observed upon knockdown of RNF41 could be rescued by expression of either wild-type RNF41 or the S254A mutant of RNF41.

The maximum knockdown of RNF41 we were able to achieve in MCF-10A cells was ~ 50%. In the experiment shown in Fig. 3.4B, shRNA treatment resulted in a 48% knockdown of RNF41 (lane 2). The ectopically produced tagged form (FlagHis₆) of RNF41 co-migrated with the slower electrophoretic form of endogenous RNF41 and as
seen in Fig. 3.4B expression of WT and mutant forms of RNF41 (lanes 3-5) restored RNF41 levels in knockdown cells to approximately those detected for endogenous RNF41 in control cells (lane 1). Despite only achieving a 50% knockdown of RNF41, dramatic effects were observed when MCF-10A were cultured in 3D. As seen in Fig. 3.4A, cells expressing RNF41 shRNA formed abnormal architecture after growing for 20 days in collagen matrix or Matrigel. Cells expressing RNF41 shRNA and grown in collagen matrix were not able to establish apical-basal polarity or adherins junctions (Fig. 3.4A-E). Furthermore, the polarity defect was rescued when wild-type RNF41 but not the S254A mutant of RNF41 was expressed in MCF-10A cells knocked down for RNF41 (Fig. 3.4A-E) suggesting that phosphorylation of RNF41 on S254 is essential for establishing apical-basal cell polarity.
Discussion

In this study, we identified RNF41 as a novel substrate of the Par-1 polarity kinase and discovered a role for RNF41 in regulating epithelial cell polarity. The Par-1 protein kinase regulates cell polarity in worms, flies, frogs and mammals (Bohm et al., 1997; Cohen et al., 2004; Cohen and Musch, 2003; Cox et al., 2001; Muller et al., 2001; Ossipova et al., 2005; Shulman et al., 2000; Sun et al., 2001; Tomancak et al., 2000). In order to identify Par-1 binding proteins, mammalian Par-1 orthologs were used as bait in a Tandem Affinity Purification (TAP) screen (Brajenovic et al., 2004). The RING finger containing E3 ubiquitin ligase RNF41/NRDP1 was found in Par-1d complexes. Mammalian orthologs of 5 out of 6 C. elegans Par genes have been identified. The exception is Par 2, which encodes a RING finger containing protein that co-localizes with Par-1 in the C. elegans zygote. Par-2 and RNF41 share homology in their RING finger and C-terminal domains leaving open the interesting possibility that RNF41 is a Par-2 ortholog. Here we show that human RNF41 binds to the amino terminal domain of human Par-1b. This region contains both the kinase and UBA domains of Par-1b and deletion of the UBA negatively impacted binding of RNF41 to Par-1b. Since RNF41 undergoes ubiquitination, this may indicate that a ubiquitinated form of RNF41 binds to the UBA domain of Par-1b.

In the early C. elegans embryo, Par-1 is among several partitioning-defective mutants that disrupt asymmetric cell division, blastomere cell fate, localization of P granules, and mitotic spindle orientation (Kemphues et al., 1988; Kirby et al., 1990). Some of the mutations are within the kinase domain of Par-1, indicating kinase activity is necessary for its polarity function(Guo and Kemphues, 1995). Of the reported Par-1
substrates, only Par-3 is known to play a role in regulating polarity. Par-3 in complex with Par-6 and aPKC localizes to tight junctions and the integrity of this complex is required for maintaining polarity (Etienne-Manneville and Hall, 2003; Izumi et al., 1998; Joberty et al., 2000). Par-1 phosphorylates Par-3 to exclude it from tight junctions and this, in turn disrupts epithelial cell polarity (Benton and St Johnston, 2003; Hurd et al., 2003).

Here we identify a second substrate of Par-1 (RNF41) that also plays a role in regulating cell polarity. Par-1 phosphorylates RNF41 on S254, a site that is conserved in mammals and frogs but not in flies or the C. elegans Par-2 protein. MCF-10A cells knocked down for RNF41 failed to polarize when grown in either collagen I matrix or Matrigel. Interestingly, 50% knockdown of RNF41 was sufficient to prevent MCF-10A cells from forming polarized acini-like spheroids in 3D culture. Polarity could be rescued if WT RNF41 was expressed in knockdown cells. Phosphorylation of RNF41 on S254 was observed to increase as MCF-10A cells polarized in 3D cultures and expression of the S254A mutant of RNF41 failed to rescue the polarity defect of RNF41-deficient MCF-10A cells. These results indicate that phosphorylation of S254 is important for the polarity function of RNF41.

Serine 254 resides within the C-terminus of RNF41, and this region of RNF41 has been shown to bind to both targets of RNF41 (ErbB3 and BRUCE) (Diamonti et al., 2002; Qiu et al., 2004) and regulators of RNF41 (USP8) (Wu et al., 2004). USP8 is a deubiquitinating enzyme that binds to RNF41 and stabilizes it through deubiquitination. Serine 254 lies within the USP8 binding domain and also neighbors residues involved in ErbB3 binding (Avvakumov et al., 2006; Bouyain and Leahy, 2007). Future experiments
will be directed at determining if phosphorylation of RNF41 on S254 regulates its interactions with USP8 and ErbB3.

Preliminary studies indicate that ectopic RNF41 localizes to basolateral membranes in polarized MCF-10A cells grown in 3D cultures whereas a significant fraction of the S254A mutant protein appears to reside in the cytoplasm of the same cells. Future experiments will also be directed at determining if S254 phosphorylation regulates the intracellular localization of RNF41. The failure of the S254A mutant of RNF41 to localize properly or to bind to key substrates would account for its failure to rescue polarity in RNF41 deficient MCF-10A cells.

The establishment and maintenance of cell polarity is an essential property governing organismal homeostasis and loss of polarity is a common feature of transformed cells. For example, the polarity proteins Scribble, lethal giant larvae (LGL) and discs large (DLG) were isolated as tumour-suppressor mutations in Drosophila melanogaster, based on the distinctive ‘giant larvae’ phenotype of zygotic mutant animals (Bilder, 2004). Little is known about their roles in human disease, but the loss or decreased expression of human homologues of Scribble and LGL has been found in colorectal cancers and malignant melanoma (Gadgil et al., 2006). RNF41 is an E3 ubiquitin ligase that regulates steady state levels of ErbB3 (Diamonti et al., 2002). ErbB3 is overexpressed in a subset of breast cancers. There is a correlation between decreased levels of RNF41 and increased levels of ErbB3 in a transgenic mouse model of ErbB2-induced mammary tumorigenesis and in a panel of primary breast tumors (Yen et al., 2006). It has been assumed that higher levels of active ErbB3 signaling is the major contributing factor driving tumorigenesis under these conditions. Our study suggests that
disruption of polarity due to RNF41 loss may also be a contributing factor driving 
tumorigenesis.
Materials and Methods

Cell culture. HeLa cells were routinely maintained in DMEM (Gibco-BRL) supplemented with 10% Bovine Growth Serum (HyClone), 100 units per mL of penicillin and streptomycin, and 1 mM L-glutamine. HEK293T cells were grown in the presence of 10% FBS (HyClone). MCF-10A cells were maintained in DMEM/F12 (Invitrogen) supplemented with 5% Horse Serum (Invitrogen), 20 ng/ml Epidermal Growth Factor (EGF), 0.5 mg/ml Hydrocortisone, 100 ng/ml Cholera Toxin, 10µg/ml Insulin, and 100 units per mL of penicillin and streptomycin.

Cloning and mutagenesis. The Flag3-Par-1b N-Term, C-Term, N-UBA, and C-UBA deletion mutants were cloned by PCR from the pFLAG3-CMV-10-Par-1b plasmid (Watkins et al., 2008) and ligation into pFLAG3-CMV-10 plasmid (Sigma Chemical Co). GST-RNF41 was cloned by PCR from the pCDNA3.1-RNF41-Flag plasmid (Diamonti et al., 2002) and ligation into the pGex2T vector (GE Healthcare). The pFLRU6RF41shRNA-cmv-MCS-FH plasmid was generated by PCR. First, PCR of the U6 promoter from the pFLRU6Luc-cmv-MCS-FH plasmid was performed followed by PCR of the RNF41shRNA sequence targeted against the 3’UTR of RNF41 (5’GCCACATTGTGGCAATTTAA 3’). The final PCR was performed with the U6 promoter and RNF41shRNA template generated in the previous step in order to create a U6-RNF41shRNA fusion. The fusion PCR product was ligated into the Xba I and Xho I restriction sites of the pFLRcmv-MCS-FH plasmid. The pFLRU6RF41shRNA-cmRVRF41-FlagHis6-FH (WT and S254A) plasmids were generated by PCR of RNF41
(WT and S254A) from the appropriate pCDNA3.1-RNF41-Flag plasmid and inserted into the pFLRU6RNF41shRNA-cmv-MCS-FH plasmid. The RNF41-Flag C34S/H36Q (ΔCH), RNF41-Flag C34S/H36Q/S254A, and GST-RNF41 S254A mutants were generated by using the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene). The sequences of all constructs were verified by sequencing.

**Plasmids.** Plasmids encoding Flag3-Par-1b, Flag3-Par-1b (D193N) (KD), GST-Par-1b (Hurov et al., 2004) and pCDNA3.1-RNF41-Flag have been described (Diamonti et al., 2002). The pFLRU6Luc-CMV-MCS-FH and pFLRCMV-MCS-FH plasmids were a generous gift from Dr. Greg Longmore (Washington University in St. Louis).

**Transient transfections.** Transient transfections were performed using Lipofectamine 2000 reagent according to the recommendations of the manufacturer (Invitrogen). Cells were harvested 24 h post transfection.

**MCF-10A-derived cell lines.** HEK293T cells were plated at $8 \times 10^5$ cells/6 cm dish the day before transfection. The next day, cells were transfected with a mixture of 1 µg viral DNA encoding the gene of interest and 1 µg pHR’8.2deltaR packaging plasmid at a ratio of 8:1 with pCMV-VSV-G envelope plasmid using Mirus LTR1 transfection reagent (Mirus). MCF-10A cells were plated at $1 \times 10^6$ cells/10 cm dish the day before infection with the lentivirus. After HEK293T cells were transfected with viral DNA for 48 h, target MCF-10A cells were infected in the presence of 10 µg/ml protamine sulfate for 4 h and then the virus was removed and media replaced. Infected cells were allowed to
recover for 24 h and then were re-infected with lentivirus. Infected cells were trypsinized and plated in media containing 1 µg/ml puromycin 48 h after the first infection with lentivirus. RNF41 protein expression of selected cells was measured by Western blot.

**Antibodies and Western blotting.** Antibodies specific for RNF41 (Bethyl), Flag M2 (Sigma Chemical Co.), Alpha Tubulin (Sigma Chemical Co.), and GAPDH (IMGENEX) were purchased. Par-1b was detected with ascites made from a monoclonal antibody that has been previously described (Hurov et al., 2001). Par-1b protein phosphorylated on S400 or T595 was detected using previously described phospho-specific antibodies (Hurov et al., 2004; Watkins et al., 2008). The antibody specific for RNF41 phosphorylated on serine 254 was generated by immunizing rabbits with the phosphopeptide ENAHER-pS-WPQGLATC, coupled to keyhole limpet hemocyanin (KLH). Par-1b fusion proteins were precipitated with anti-Flag M2 antibody-agarose affinity gel (Sigma Chemical Co.) and detected by Western blotting with anti-Par-1b ascites. RNF41-FlagHis6 fusion protein was precipitated with anti-Flag M2 antibody-agarose affinity gel (Sigma Chemical Co.) and detected by Western blotting with anti-Flag M2 monoclonal antibody (Sigma Chemical Co.). Indirect immunofluorescence was performed with FlagM2 (Sigma Chemical Co.), GM130 (BD Biosciences), Alpha6-Integrin (Millipore), E-cadherin (BD Biosciences), and Laminin V (Millipore) antibodies.

**Immunoprecipitation.** HeLa and HEK293T cells were transfected with the indicated plasmids using Lipofectamine 2000 reagent (Invitrogen) at 75% confluence. Cells were grown for 24 h before washing in ice-cold phosphate-buffered saline (PBS) once and
harvesting in ice-cold mammalian cell lysis buffer (MCLB: 50 mM Tris HCl pH 8.0, 100 mM NaCl, 2 mM DTT, 5 mM EDTA, 0.5% NP-40, 1 μm microcystin, 1 mM sodium orthovanadate, 2 mM PMSF, 0.15U/ml aprotinin, 20 μm leupeptin, and 20 μm pepstatin). One mg of total cell lysate was used for the indicated immunoprecipitations (IP). M2 Flag-agarose (Sigma Chemical Co.) (1:1 slurry in MCLB, 10 μl) was used to immunoprecipitate Flag3-Par-1b and RNF41-FlagHis6 proteins for 1 h at 4°C. Immunoprecipitations were washed 6 times with ice-cold MCLB before boiling in SDS-PAGE sample buffer and loading on an SDS gel.

**Expression and purification of proteins in bacteria.** JM109 cells were transformed with a plasmid encoding GST-RNF41. Cultures were grown at 37°C to an A₆₀₀ of 0.6, and isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.5 mM. After growing for an additional 4 h at 30°C, cells were pelleted by centrifugation. Cell pellets were washed with PBS buffer and resuspended in STE (100 mM NaCl, 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA) supplemented with 2 mM phenylmethylsulfonyl fluoride (PMSF), 0.15 unit/ml aprotinin, 20 μM leupeptin, 20 μM pepstatin (referred to as 1 x protease inhibitors), and 1.0 mg/ml lysozyme. After rocking at 4°C for 20 min, Sarkosyl was added to a final concentration of 1.5%, and lysis was accomplished by sonication. Lysates were clarified by centrifugation, and Triton X-100 was added to a final concentration of 2%. Proteins were precipitated with GSH agarose and washed twice in STE, twice in LiCl buffer (0.5 M LiCl, 50 mM Tris-HCl, pH 8.0), and twice in 50 mM Tris-HCl, pH 7.4. GST-fusion proteins were eluted with 20 mM glutathione in 50 mM Tris-HCl (pH 7.4), and the concentration of each GST-fusion
protein was estimated by comparison to known Bovine Serum Albumin (BSA) standards after SDS- PAGE and Coomassie Blue staining.

To express GST-Par-1b in bacteria, the *E. coli* strain JM109 was transformed with GST-Par-1b and cultures were grown at 37°C until reaching an $A_{600}$ of ~0.6. Cells were induced with 100 µM IPTG, grown at 28°C for 11 h, collected by centrifugation and resuspended in STE buffer supplemented with 1x protease inhibitors and 1.0 mg/ml lysozyme. After rocking at 4°C for 20 min, cells were lysed by sonication and centrifuged at 3,000 x g for 10 min. Proteins were precipitated with GSH agarose and washed as described above. Finally, precipitated proteins were washed with incomplete kinase buffer (50 mM Tris-HCl, pH 7.5, 12.5 mM MgCl$_2$, 1 mM DTT).

**Kinase assays.** Recombinant GST-Par-1b bound to GSH agarose was incubated with 5 µg of recombinant GST-RNF41 in complete kinase buffer (50 mM Tris-HCl, pH 7.5, 12.5 mM MgCl$_2$, 1 mM DTT, 440 µM ATP, and 7 µCi $\gamma^{32}$P ATP [>4000 Ci/mmol]) at 37°C for 30 min. Samples were boiled, resolved by SDS-PAGE, and transferred to nitrocellulose.

**Mapping studies.** The nitrocellulose containing radiolabeled GST-RNF41 was blocked with 0.5% polyvinylpyrrolidone (PVP-40) in 100 mM acetic acid for 30 min at 37°C, followed by washing 6 times with water. Radiolabeled GST-RNF41 was digested in a solution containing 0.2 mg of trypsin (Worthington) per ml in 50 mM ammonium bicarbonate. Tryptic phosphopeptides were separated in the first dimension by thin layer
chromatography at pH 1.9 and in the second dimension by ascending chromatography in a buffer consisting of n-butanol-pyridine-acetic acid-water in a ratio of 75/50/15/60. For PAA analysis, washed tryptic labeled peptides were boiled in 6N hydrochloric acid for 90 min, followed by washing six times with water (van der Geer and Hunter, 1994).

**Three-dimensional (3D) cultures.** Each well of a 6-well plate was coated with 500 µl of a mixture of collagen type 1 (BD Biosciences), 1N Sodium Hydroxide, Horse Serum (Invitrogen), 5x DMEM (Sigma Chemical Co.) and sterile water at a 1.0/0.02/0.08/0.3/0.3 ratio. The collagen coating was allowed to polymerize in a tissue culture incubator for 30 min at 37°C. MCF-10A cells or transduced MCF-10A cells selected with puromycin were trypsinated and 7.49 x 10⁴ cells were added to mixture consisting of collagen type 1, 1N Sodium Hydroxide, Horse Serum, 5x DMEM and sterile water mixture (1.0/0.02/0.08/0.3/0.06 ratio) on ice. The cell/collagen mixture was gently mixed so that cells were in suspension and 1 ml of the mixture was added to each of 3 wells of the 6-well plate. The cell/collagen mixture was allowed to polymerize in a tissue culture incubator for 1 h at 37°C and then 2 ml of media with or without 1 µg/ml puromycin was added to each well. Cells were harvested after 8, 10, 14 or 20 days in culture. Cells were harvested by pooling each piece of polymerized collagen and cells from each of 3 wells and incubating them in 20 mg/ml Collagenase A (Roche) in PBS for 5 min at 37°C. The cells were washed three times with ice-cold PBS and either spotted on a glass slide for analysis by indirect immunofluorescence microscopy or lysed in ice-cold MCLB followed by Western blotting.
For cells grown in Matrigel (BD Biosciences), each well of a 6-well plate was coated with 300 µl of Matrigel and the coating was allowed to polymerize in a tissue culture incubator for 30 min at 37°C. MCF-10A cells or transduced MCF-10A cells selected with puromycin were trypsinized and 6 x 10^5 cells were suspended in 1.2 ml matrigel per well and plated. The cell/Matrigel mixture was allowed to polymerize in a tissue culture incubator for 1 h at 37°C and then 2 ml of media with or without 1 µg/ml puromycin was added to each well. Cells were harvested after 8, 10, 14 or 20 days in culture. Cells were harvested by washing each well twice with 2 ml ice-cold PBS and then adding 6 ml of ice-cold 5mM Ethylenediaminetetraacetic acid (EDTA) in 1X PBS. Cells were rocked in buffer containing EDTA/PBS at 4°C until all of the Matrigel was dissolved. The cells were washed three times with ice-cold PBS and either spotted on a glass slide for analysis by indirect immunofluorescence microscopy or lysed in ice-cold MCLB and analyzed by Western blotting.

**Indirect immunofluorescence microscopy.** MCF-10A cells grown in 3D culture were spotted onto microscope slides and fixed with 4% Paraformaldehyde (PFA) in PBS for 20 min at room temperature. Slides were washed two times with room temperature PBS and three times (10 min each wash) at room temperature in 100 mM glycine in PBS. Fixed cells were blocked in IF blocking solution (10% normal goat or donkey serum, 0.2% Triton X-100, 0.1% BSA (radioimmunoassay grade, Sigma Chemical Co.) and 0.05% Tween 20 in PBS, pH 7.4) for 1.5 h at room temperature in a humidified chamber. Fixed cells were stained with primary antibody diluted in IF buffer (IF blocking solution without 10% normal goat or donkey serum) for 2 h at room temperature in a humidified
chamber. Slides were washed three times for 20 min in IF buffer in a Coplin jar at room temperature with gentle shaking. Cells were then incubated with a secondary antibody (Alexa488 or Alexa594) diluted in IF buffer for 45 min at room temperature in a humidified chamber. Slides were washed once with IF buffer for 20 min and then two times with PBS for 10 min at room temperature with gentle shaking. Finally, cells were stained with 1 µM TO-PRO-3 Iodide (Invitrogen) for 10 min at room temperature in a humidified chamber, slides were washed once with PBS for 10 min with gentle shaking, and slides were mounted with Prolong Antifade mounting media (Molecular Probes). Images were obtained using an Olympus FV-500 confocal microscope with a 60x water objective. Images were processed using the Olympus FLUOVIEW Ver.2.1a Viewer software.
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**Fig. 3.1 RNF41 binds to the amino terminus of Par-1b.**

(A) HeLa (lanes 1-3) and HEK293T (lanes 4-6) cells were transfected with plasmids encoding Flag3-Chk2 (lanes 2 and 5) or Flag3-Par-1b (lanes 3 and 6) for 24 h. Cells were harvested and lysates resolved by SDS-PAGE or incubated with anti-Flag agarose before SDS-PAGE. Total lysates and immunoprecipitations were analyzed by Western blotting using the indicated antibodies. (B) Schematic representation of Par-1b and RNF41 proteins. Protein domains, point mutations and deletion mutants are indicated. (C) HEK293T cells were transfected with plasmids encoding Flag3-Par-1b full length (FL) (lane 2), N-UBA (lane 3), C-UBA (lane 4), N-Term (lane 5), or C-Term (lane 6) deletion mutants. Cells were harvested 24 h later and lysates were resolved by SDS-PAGE or were incubated with anti-Flag agarose prior to SDS-PAGE. Total lysates and immunoprecipitations were analyzed by Western blotting using the indicated antibodies.
Fig. 3.2 Phosphorylation of RNF41 by Par-1b.

(A, B, C) Bacterially produced GST-Par-1b was incubated in the absence or presence of bacterially produced GST-RNF41 and kinase assays were performed in vitro. Radiolabeled reaction products were resolved by SDS-PAGE (panel A) and phosphotryptic peptides were subjected to phosphoamino acid analysis (PAA analysis) (panel B). A kinase assay was also performed with GST-Par-1b and GST-RNF41 (WT or S254A) and two-dimensional phosphopeptide mapping (panel C) was performed. Arrows indicate the direction of electrophoresis (x axis) and chromatography (y-axis).

(D) Kinase assays were performed in vitro using GST-RNF41 WT (lanes 1 and 3) or GST-S254A (lanes 2 and 4) with (lanes 3 and 4) or without GST-Par-1b (lanes 1 and 2). Proteins were resolved by SDS-PAGE and detected by Western blotting using the indicated antibodies.

(E) Lysates prepared from mock-transfected HEK293T cells (lane 1) or from HEK293T cells expressing RNF41-FlagΔCH (lanes 2-4) or RNF41-FlagS254AΔCH (lanes 5-7) with Flag3-Par-1b WT (lanes 3 and 6) or Flag3-Par-1b KD (lane 4 and 7) were resolved by SDS-PAGE and immunoblotted with the indicated antibodies.
Fig. 3.3 RNF41 is phosphorylated on S254 as MCF-10A cells polarize in 3D cultures.

(A) MCF-10A cells were grown in collagen I matrix or in Matrigel and harvested at the indicated times. (B,C) Cells described in panel A were fixed and proteins were detected using indirect immunofluorescent staining and confocal microscopy. (D) MCF-10A cells were grown in 2D or 3D culture. Cells grown in 3D culture were harvested at the indicated times. Cell lysates were resolved by SDS-PAGE and immunoblotted with the indicated antibodies.
A. Luc shRNA, RNF41 shRNA, RNF41 shRNA + WT-RNF41, RNF41 shRNA + S254A

Collagen I

Matrigel

B. shRNF41

shLuc shRNF41 + WT + A CH + S254A

RNF41-FlagHis

Endog.RNF41

Par-1b

TUBULIN

C. GM130, Alpha6-Integrin, TO-PRO-3, Merge

Luc shRNA

RNF41shRNA

RNF41shRNA + WT RNF41

RNF41shRNA + S254A RNF41

D. E-cadherin, TO-PRO-3, Merge

Luc shRNA

RNF41shRNA

RNF41shRNA + WT RNF41

RNF41shRNA + S254A RNF41

E. % cyst structure +/- SEM

Luc shRNA

WT

S254A

RNF41 shRNA

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Fig. 3.4 RNF41 is required for MCF-10A cells to polarize in 3D cultures.

(A) MCF-10A cells expressing either lucshRNA, RNF41shRNA, RNF41shRNA + RNF41WT-Flag His6, or RNF41shRNA + RNF41S254A-Flag His6 were grown in plastic culture dishes (2D), in collagen I matrix, or in Matrigel. Cells were harvested and lysates were resolved by SDS-PAGE (panel C lanes 1-5) or incubated with anti-Flag agarose prior to SDS-PAGE (panel C, subpanel *). Total lysates and immunoprecipitates were analyzed by Western blotting using the indicated antibodies. (D, E) Cells described in panel A were grown in collagen I matrix, and harvested after 20 days. Cells were fixed and proteins were detected using indirect immunofluorescent staining and confocal microscopy. (F) The number of polarized acini-like spheroids were counted in 3 separate experiments (n = 3). A one way ANOVA was performed and p<0.0001 (****). A Tukey’s multiple comparison posttest showed p values as described on the graph (*** = p<0.001, ** = p<0.01).
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CHAPTER 4

Loss of Par-1a/MARK3/C-TAK1 kinase leads to reduced adiposity, resistance to hepatic steatosis, and defective gluconeogenesis.

**Contributions:**

Results of my study of the protein levels of glycogen synthase (GS) and phosphorylated glycogen synthase (pGS) in the liver of fasted Par-1a -/- or Par-1b -/- mice were published in Figure 4.5 of this paper (Lennerz et al., 2010). I analyzed protein lysate from Par-1a or Par-1b null mouse livers and showed that upon short-term starvation, Par-1a null mice had increased levels of GS and pGS protein in the liver whereas Par-1b null mice do not (Fig. 4.5F). Since Par-1a null mice demonstrate resistance to steatosis (accumulation of lipids, leading to fatty liver) as well as defective gluconeogenesis, I probed for UCP2 (an uncoupling protein) in fasted and nonfasted liver samples but found similar protein levels (Fig. 4.S1A). Most recently, autophagy has been linked to lipid (Singh et al., 2009) as well as glycogen/glucose homeostasis (Kotoulas et al., 2004; Kotoulas et al., 2006). Therefore, I detected LC-3 protein levels by Western blot as a marker for membrane formation and early stages of autophagy, but observed no differences (Fig. 4.S1B).

In summary, livers from Par-1a null mice are depleted of lipid and glycogen stores, upregulate glycogen synthase, and activate autophagy. The increase in total glycogen synthase protein might be to compensate for the decreased levels of stored glycogen. However, we also observe an increase in the inhibited (phosphorylated) form of glycogen synthase (pGS). Thus, it is unclear whether glycogen synthase activity is altered in the livers of Par-1a null mice even though an increase in total glycogen synthase protein is observed.
Loss of Par-1a/MARK3/C-TAK1 kinase leads to reduced adiposity, resistance to hepatic steatosis and defective gluconeogenesis

Jochen K. Lennerz1, 2, †, *, Jonathan B. Hurov2, 3, †, *, Lynn S. White2, 8, 9, Katherine T. Lewandowski2, 8, Julie L. Prior3, 8, G. James Planer4, Robert W. Gereau IV5, David Piwnica-Worms3, 6, 8, Robert E. Schmidt1 and Helen Piwnica-Worms2, 7, 8, 9

1Department of Pathology and Immunology, 2Department of Cell Biology and Physiology, 3Molecular Imaging Center, Mallinckrodt Institute of Radiology, 4Department of Neurology, Neuromuscular Laboratory, 5Washington University Pain Center and Department of Anesthesiology, 6Department of Developmental Biology, 7Department of Internal Medicine, 8BRIGHT Institute, Washington University School of Medicine, St. Louis, MO 63110; 9Howard Hughes Medical Institute

*These authors contributed equally to this study.
† Present Address: Department of Pathology, Massachusetts General Hospital/Harvard Medical School, Boston, MA 02114-2621
‡ Present Address: Agios Pharmaceuticals, Cambridge, MA 02139-4169

Corresponding author: Helen Piwnica-Worms, Ph.D., Department of Cell Biology and Physiology Washington University School of Medicine, Box 8228, 660 South Euclid Ave. St. Louis, MO 63110. TEL: (314) 362-6812, FAX: (314) 362-3709, email: hpiwnica@wustl.edu

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Abstract

Par-1 is an evolutionarily conserved protein kinase required for polarity in worms, flies, frogs and mammals. The mammalian Par-1 family consists of four members. Knockout studies in mice implicate Par-1b/MARK2/EMK in regulating fertility, immune homeostasis, learning and memory as well as adiposity, insulin hypersensitivity and glucose metabolism. Here we report phenotypes of mice null for a second family member (Par-1a/MARK3/C-TAK1), which exhibit increased energy expenditure, reduced adiposity with unaltered glucose handling and normal insulin sensitivity. Knockout mice were protected against high-fat diet-induced obesity, displayed attenuated weight gain, complete resistance to hepatic steatosis and improved glucose handling with decreased insulin secretion. Overnight starvation led to complete hepatic glycogen depletion, associated hypoketotic hypoglycemia, increased hepatocellular autophagy and increased glycogen synthase levels in Par-1a<sup>−/−</sup>, but not in control or Par-1b<sup>−/−</sup> mice. Intercrossing of Par-1a<sup>−/−</sup> with Par-1b<sup>−/−</sup> mice revealed that at least one of the four alleles is necessary for embryonic survival. Severity of phenotypes followed a rank-order whereby loss of one Par-1b allele in Par-1a<sup>−/−</sup> mice conveyed milder phenotypes than loss of one Par-1a allele in Par-1b<sup>−/−</sup> mice. Thus, although Par-1a and Par-1b can compensate for one another during embryogenesis, their individual disruption gives rise to distinct metabolic phenotypes in adult mice.
Introduction

Cellular polarity is a fundamental principle in biology (Banks and Humbert, 2008; Geldner, 2009; Lindsey, 2004). The prototypical protein kinase originally identified as a regulator of polarity was termed partitioning defective (Par-1) due to early embryonic defects in C. elegans (Kemphues et al., 1988). Subsequent studies revealed that Par-1 is required for cellular polarity in worms, flies, frogs and mammals (Arimura and Kaibuchi, 2007; Krummel and Macara, 2006; Lizcano et al., 2004; Martin and St Johnston, 2003; Nance, 2005; Shin et al., 2006; Sodha et al., 2002). An integral role for Par-1 kinases in multiple signaling pathways has also been established and although not formally addressed, multi-functionality for individual Par-1 family members is implied when reviewing the list of recognized upstream regulators (Table 4.1A) and downstream substrates (Table 4.1B). Interestingly, for many Par-1 substrates the phosphorylated residues generate 14-3-3 binding sites (Dalal et al., 1999; Dequiedt et al., 2006; Goransson et al., 2006; Kao et al., 2001; Kusakabe and Nishida, 2004; Lin et al., 2009; Muller et al., 2001; Muller et al., 2003; Peng et al., 1998; Suzuki et al., 2004; Watkins et al., 2008; Zhang et al., 1997). 14-3-3 binding, in turn, modulates both nuclear/cytoplasmic as well as cytoplasmic/membrane shuttling of target proteins thus allowing Par-1 activity to establish intracellular spatial organization (Bronisz et al., 2006; Watkins et al., 2008). Phosphorylation of Par-1 itself promotes 14-3-3 binding thereby regulating its subcellular localization (Goransson et al., 2006; Kusakabe and Nishida, 2004; Watkins et al., 2008).

The mammalian Par-1 family contains four members (Table 4.2). Physiological functions of the Par-1b kinase have been studied using targeted gene knockout
approaches in mice (Bessone et al., 1999; Hurov et al., 2001). Two independently derived mouse lines null for Par-1b, have implicated this protein kinase in diverse physiological processes, including fertility (Bessone et al., 1999), immune system homeostasis (Hurov et al., 2001), learning & memory (Segu et al., 2006), positioning of nuclei in pancreatic beta cells (Fu et al., 2009; Granot et al., 2009) as well as growth and metabolism (Hurov et al., 2007).

Beyond Par-1b, most information regarding the cell biological functions of the Par-1 kinases comes from studies on Par-1a. Specifically, Par-1a has been implicated in pancreatic- (Parsa, 1988), and hepato-carcinogenesis (Kato et al., 2001), as well as colorectal tumors (Parsons et al., 2005), hippocampal function (Wang et al., 2007), CagA (Helicobacter pylori) associated epithelial cell polarity disruption (Saadat et al., 2007) and Peutz-Jeghers-Syndrome (Jansen et al., 2006), although the later association has recently been excluded (Jansen et al., 2006). As a first step towards determining unique and redundant functions of Par-1 family members, mice disrupted for a second member of the family (Par-1a/MARK3/C-TAK) were generated. We report that Par-1a−/− mice are viable and develop normally; adult mice are hypermetabolic, have decreased white and brown adipose tissue mass, and unaltered glucose/insulin handling. However, when challenged by high-fat diet, Par-1a−/− mice exhibit resistance to hepatic steatosis, resistance to glucose intolerance and delayed onset of obesity relative to control littermates. Strikingly, overnight starvation results in a complete depletion of glycogen and lipid stores along with an increase in autophagic vacuoles in the liver of Par-1a−/− but not Par-1b−/− mice. Correspondingly, Par-1a−/− mice develop hypoketotic hypoglycemia. These findings reveal unique metabolic functions of two Par-1 family members.
Results

Gene targeting was employed to determine the consequences of Par-1a loss in mice (Fig. 4.1A). The targeted Par-1a allele was introduced by electroporation into RW4 embryonic stem (ES) cells, derived from the mouse strain 129X1/SvJ. Southern blot analysis identified correct targeting events (Fig. 4.1B). Southern blotting and PCR analysis demonstrated that the agouti offspring produced by the chimeric males were heterozygous for the Par-1a locus (data not shown). F1 heterozygous offspring were inter-crossed and F2 offspring were genotyped by PCR (Fig. 4.1C). Western blotting demonstrated that the targeted disruption of Par-1a produced a null allele of the locus (Fig. 4.1D) and multi-tissue Western blotting confirmed ablation of Par-1a/b in all tissues of the corresponding null-mice (Fig. 4.1E).

Higher levels of Par-1a were noted in several tissues of Par-1b null mice including brain, BAT, thymus and muscle (Fig. 4.1 E, lanes 2, 5, 17, 26), which may provide an explanation for Par-1a compensation of Par-1b loss (see below). However, increased Par-1b levels were not observed in Par-1a null tissues. Also observed in Fig. 4.1E is the differential expression of Par-1a splice variants in various mouse tissues. The NCBI database contains 5 Par-1a splice variants with their respective protein products ranging from 659 to 753 amino acids. It is not known whether functions differ among the various splice variants.

**Par-1a<sup>−/−</sup>-mice are hypermetabolic and show 9% reduction in body weight**

When Par-1a heterozygotes were intercrossed, the offspring contained WT, HET and KO pups, although genotype proportions deviated significantly from expected Mendelian ratios with loss of homozygous KO mice (n=750; WT=30%, HET=52%,
KO=17%; $\chi^2=27.864$, $P<0.0001$). All pups developed without any visible dysmorphisms or obvious external pathology; and animals survived to adulthood. *Par-1a* null mice exhibited significant weight differences compared with their wild-type littermates (e.g. at 32 weeks: WT (n=6) 33.4±0.94g; vs. *Par-1a*−/− (n=6) 25.5±0.42g; $P<0.0001$, t-test). In contrast to *Par-1b* null mice that show reduced weights at 6 weeks of age (Bessone et al., 1999; Hurov and Piwnica-Worms, 2007), in *Par-1a* null mice the difference was not discernable this early (Fig. 4.2A, 4.2B).

Body weight represents a net balance of food intake and energy expenditure. Based on the decreased weight in *Par-1a* null mice we measured total energy expenditure (EE), total metabolic rate (MR) and respiratory quotients of male mice on a regular chow diet. *Par-1a* null mice exhibited higher O$_2$ consumption (MR: ~9% increase; Fig. 4.2C) and higher EE (10% increase; Fig. 4.2D). We also compared the respiratory quotient (RQ=VCO$_2$/VO$_2$) as a measure of fuel-partitioning patterns and RQ ranged from 0.76-0.78 (Fig. 4.2E; $P=0.32$, t-test). Food intake in *Par-1a* null mice was slightly lower than WT littermates, although this difference was not statistically significant (WT 3.15±0.09 vs. *Par-1a*−/− 2.94±0.04; $P=0.16$, t-test; Fig. 4.2F). Thus, unlike *Par-1b* null mice, *Par-1a* null mice are not hyperphagic (Hurov et al., 2007). The lack of effect on RQ suggests that there was no difference in fuel selection between carbohydrates and lipids, leading us to explore whether the increase in energy expenditure might occur secondarily to increased thermogenesis. However, body temperatures of *Par-1a* null mice were similar to WT littermates (WT 34.3±0.5 vs. *Par-1a*−/− 34.1±0.8; $P=0.8$, t-test) and expression of uncoupling proteins in selected tissues showed no significant difference (Fig. 4.5S1). Lastly, *Par-1a* null mice showed similar levels of activity (comparison to WT; $P=0.96$)
and basic sensorimotor testing as well as open field tests showed no significant difference

(P=0.64; not shown).

**Par-1a** -mice are normoglycemic, normoinsulinemic and show no insulin sensitivity

Serum levels of insulin, glucagon, leptin, adiponectin, triglycerides, cholesterol and free fatty acids in Par-1a null mice were measured and compared to values from WT and Par-1b null mice (20-week-old on regular chow diet; Table 4.3). Male Par-1a null mice exhibited 1.6-fold higher levels of adiponectin when compared to WT littermates. Par-1b null mice showed a significant decrease in female adiponectin values. In contrast to Par-1b null mice, Par-1a null mice showed no difference in serum insulin levels (Table 4.3) and blood glucose was normal (WT 152±18mg/dl vs. Par-1a+/− 147±5 mg/dl; P=0.216, t-test). Par-1a null mice on CD exhibited ITT curves identical to that of WT littermates (Fig. 4.2G; P=0.87; 1-way ANOVA). On CD, Par-1a null mice showed no significant difference in GTT relative to WT littermates (Fig. 4.3C) (P=0.15; t-test and P=0.181, 2-way ANOVA for entire time course; Par-1b+/− not shown).

**Par-1a** - mice show delayed weight gain on high-fat diet

The striking resistance to high fat diet (HFD)-induced weight gain in Par-1b null mice (Hurov et al., 2007), prompted us to challenge Par-1a null mice with a similar HFD for 16 weeks (Fig. 4.3A, 4.3B). The main finding was reduced weight gain of Par-1a null mice compared to WT littermates on HFD (Fig. 4.3A). Specifically, while Par-1b null mice on HFD showed statistically significant lack of weight gain after 7 weeks [t(7w)=3.297 P<0.05 to t(17w)=7.234; P<0.001, 2 way ANOVA with Bonferroni posttests; weight gain: +5%; CD: 19±0.75g vs. HFD: 20.3±1.34, P=0.4097, t-test], Par-1a null mice on HFD continued to gain weight during the entire experiment (Par-1a+/−:
+39%, CD: 23.47±1.6g vs. HFD: 32.4±1.47, \( P=0.004 \), \( t \)-test), albeit at a reduced rate and significantly different from WT littermates (WT: +70%, CD: 27.12±0.5g vs. HFD: 46±0.9, \( P=0.001 \); \( t \)-test). HFD food intake of \textit{Par-1a} null mice was similar to that of WT littermates (\( P=0.39 \), \( t \)-test). While the \textit{Par-1a} phenotype with reduced rate of weight gain is at first glance less dramatic than that observed in \textit{Par-1b} null mice [\textit{Par-1a} \^/- vs. WT: \( t\)(all)=2.779, \( P<0.05 \), 1 way ANOVA with Bonferroni’s Multiple Comparison test], it is a highly significant finding (Fig. 4.3B, \( P=0.001 \)). \textit{Par-1a} null mice required 7 additional weeks of HFD to reach the original weight of their WT littermates.

\textbf{\textit{Par-1a} \^/- mice show resistance to glucose intolerance on high-fat diet}

Next, \textit{Par-1a} null mice fed a HFD were subjected to glucose tolerance testing (Fig. 4.3C). As expected, WT mice on HFD exhibited glucose intolerance relative to WT mice on CD with prolonged elevation of blood glucose of \( \sim \)400% from 15 min onwards (\( t=3.645, \ P<0.01 \); 2-way ANOVA). Strikingly, \textit{Par-1a} null mice exhibited improved glucose tolerance on HFD relative to WT-controls on the same diet [Fig. 4.3D; \( P=0.041 \), \( t \)-test (two genotypes); \( t=3.38; \ P<0.01 \); 1-way ANOVA (all genotypes)]. GTT response in \textit{Par-1a} null mice under HFD-conditions was essentially identical to WT and \textit{Par-1a} null mice on CD [Fig. 4.3D; \( P=0.4781 \), \( t \)-test (two genotypes); \( t=0.346; \ P>0.05 \) 1-way ANOVA (all genotypes); not shown]. We conclude that \textit{Par-1a}-disruption leads to maintenance of normal glucose tolerance even under HFD-conditions; accordingly \textit{Par-1a} null mice on HFD were normoglycemic (\( P=0.3 \), \( t \)-test). Next, we examined serum insulin levels in HFD-fed mice during the first 60 min of the GTT (Fig. 4.3D) and found significantly lower levels in \textit{Par-1a} null mice (\( t=3.16, \ P=0.0194 \), \( t \)-test (two genotypes); \( F=6.454, \ P=0.018 \), 1-way ANOVA). Although we did not formally assess insulin
tolerance in Par-1a null mice on HFD, the combination of improved glucose tolerance and reduced insulin secretion during the GTT is indicative of peripheral insulin hypersensitivity. Thus, Par-1a null mice demonstrate normal insulin and glucose sensitivity, unless challenged by HFD. In contrast Par-1b null mice exhibited insulin hypersensitivity even on a CD (Hurov et al., 2007). Direct assessment of pancreatic islets has proven difficult in Par-1 mice (Granot et al., 2009), however, morphological and morphometric differences were not observed in the islet organ of Par-1a null mice (not shown).

**Par-1a-/- mice show diet-dependent reduced adiposity**

Next, we determined whether the reduced body weight of Par-1a null mice was reflected disproportionately in different tissues (Fig. 4.4A). Measurements of tissue weights in Par-1a null mice indicated that decreased weights of most organs were proportional to total body weight and consistent with a decrease in overall body mass, rather than in specific organs. However, two tissues did not follow this rule and were disproportionately smaller: white-adipose tissue (WAT from gonadal fat pads) and brown-adipose tissue (BAT). We also performed dissection of tissues in the mice on HFD and, as expected, the energy excess in WT-mice led to a disproportionate increase in overall adiposity (Fig. 4.4A; BAT-CD vs. BAT-HFD \( P=0.001 \); WAT-CD vs. WAT-HFD \( P=2.5e^{-5} \); \( t \)-tests). Par-1a null mice showed a similar disproportionate increase in overall adiposity (BAT-CD vs. BAT-HFD \( P=0.007 \); WAT-CD vs. WAT-HFD \( P=1.4e^{-9} \); \( t \)-tests) with a weight increase in BAT and gonadal WAT that eliminated the reduced adiposity observed under chow diet (Fig. 4.4A columns a vs. b and c vs. d, both \( P=0.08 \); \( t \)-tests).
Dissection of the gonadal fat pads (Fig. 4.4B), representative of the WAT showed a disproportionate decrease in fat mass of Par-1a null mice relative to WT on CD (Fig. 4.4C; raw weight values provided). The weight difference corresponds to ~48% (Par-1a−/−) and ~36% (Par-1b−/−) of the WT-WAT weight, respectively (Fig. 4.4C). Although we did not quantitatively assess abdominal fat, during dissection the dimensions of mesenteric root, omental-, and retroperitoneal fat content were strikingly lower in Par-1a null mice and this difference is not accounted for in the displayed values of gonadal WAT (Fig. 4.4C).

Dissection of the interscapular fat depot, representative of BAT, is shown in Fig. 4.4E. The weight difference corresponds to ~52% (Par-1a−/−) and ~38% (Par-1b−/−) of the WT-BAT weight, respectively (Fig. 4.4F). For both adipose tissues, the difference significantly exceeded the overall ~9% weight difference observed in Par-1a null mice. Adipocyte counts from six independent samples of WAT and BAT indicated no differences in adipocyte size from WT and Par-1a null mice: WAT (WT 436±100 adipocytes per mm² vs. Par-1a−/− 419±74; P=0.137, t-test) and BAT (WT 1894±349 vs. Par-1a−/− 1385±434; P=0.382, t-test). Thus, we conclude that the observed decrease in adipose tissue of Par-1a null mice is due to decreases in total adipocyte cell number, not cell size; findings similar to those made in Par-1b null mice (Hurov et al., 2007).

We also determined the weight of muscle samples (tibialis anterior, gastrocnemius, plantaris, diaphragm, extensor digitorum longus), typically used for assessment of myogenic phenotypes and fiber-type composition (Corbett et al., 2001; Sanoudou et al., 2006), which, under HFD conditions showed significantly decreased weights (Fig. 4.4A, subheading e; WT: P=3.7e−6; Par-1−/−: P=0.006, both t-tests). Skeletal
muscle plays an integral role in the coordination of fuel homeostasis (Hegarty et al., 2009) and is the most important site of insulin-stimulated glucose disposal (DeFronzo et al., 1981). We therefore performed functional- (motor, biodistribution and microPET analysis), biochemical- (glycogen quantification) and morphologic- (routine, PAS, fiber-type analysis and ultrastructure) screening of skeletal muscle but observed no differences between wild-type and Par-1a null mice (not shown). Thus, the reduced body weight of Par-1a mice on HFD is likely accounted for by a combination of reduced muscle mass (Argiles et al., 2007; Sitnick et al., 2009) and reduced adiposity; although the latter is not reflected in the gonadal WAT measurements (Fig. 4.4Ac,d).

**Par-1a loss causes decreased WAT glucose uptake in the absence of BAT-phenotype**

MicroPET imaging of WAT was performed (not shown); however the reduced size of Par-1 null mice and high tracer concentrations in kidneys, bladder and skeletal muscle precluded meaningful quantification of uptake in retroperitoneal, gonadal, and calf-fat deposits, respectively. We therefore applied invasive biodistribution analysis and in contrast to Par-1b null mice, WAT of Par-1a null mice showed significantly decreased glucose uptake under normal dietary conditions [Fig. 4.4D; WT: 0.52±0.08%ID/g (n=9) vs. Par-1a^-/-: 0.32±0.05%ID/g (n=8); U=14, P=0.036, Mann-Whitney U test; P=0.05, t-test]. When challenged with HFD, Par-1a null mice showed a statistically significant increase in ^18^F-FDG-uptake compared to Par-1a null mice on chow diet that eliminated the difference of glucose uptake in comparison to WT on HFD (Fig. 4.4D).

We next examined glucose uptake in BAT of chow fed Par-1a null mice by microPET analyses of the interscapular BAT (Fig. 4.4G). We evaluated 4 different conditions at 1h post ^18^F-FDG injection: the fed and fasted state using acute insulin
challenge vs. saline. As part of the HFD-trial, we also examined BAT glucose uptake via biodistribution analysis in all genotypes. We found that loss of Par-1a did not significantly alter BAT glucose uptake in any of these 6 tested conditions (not shown).

Although Par-1a null mice exhibited reduced fat mass on chow diet, and delayed weight gain relative to WT on HFD, sampled gonadal adipose mass in Par-1a null mice was not significantly different compared to WT after 16 weeks of HFD conditions [Fig. 4.4C, F; \( P=0.12/P=0.13 \), both t-tests; WAT/BAT; n=8 Par-1a\(^{-/-} \) vs. n=17 WT]. In contrast, the BAT/WAT mass in Par-1b null mice remained reduced under HFD conditions when compared to WT-mice on the same diet [Fig. 4.4C, F; \( P=0.002/P=0.04 \), t-tests (WAT/BAT); n=6 Par-1b\(^{-/-} \) vs. 17 WT].

**Par-1a\(^{-/-} \)-mice show resistance to hepatic steatosis**

Chronic exposure of mice to HFD causes liver injury via accumulation of lipids, leading to fatty liver (steatosis) (Bradbury, 2006; Postic et al., 1999). The absence of steatosis in Par-1a null mice was apparent from gross examination of the liver, which was notably darker than that of the WT littermates (not shown) and histologic examination that showed abundant panlobular, macrovesicular steatosis in WT (Fig. 4.5Ab, inset) but not Par-1a null livers (Fig. 4.5Ad). Remarkably, with respect to hepatic steatosis, Par-1a null mice on HFD were no different than WT mice on CD (compare Fig. 4.5Ad with 4.5Aa). Based on previous findings (Hurov et al., 2007), we quantified hepatic lipid content in fresh-frozen sections stained with Oil-red O and found 14.6-25% in WT vs. 6.4-7.7% in Par-1a null mice, when expressed as percent fat per high-power field (Fig. 4.5B). The quantitative difference in the WT on chow diet (Fig. 4.5B) corresponds to an absence of centrilocublar (Zone III) lipid accentuation in Par-1a null
mice (compare WT Fig. 4.5Aa to Fig. 4.5Ac). In addition, biodistribution studies demonstrated identical $^{18}$F-FDG-uptake in livers of Par-1a null- and WT-mice on chow standard CD and HFD (not shown). Similarly, microPET examination of the liver using four different metabolic conditions (see methods) did not show significant differences ($P$-value range: 0.12-0.71, $t$-tests). These findings indicate that Par-1a null mice show resistance to steatosis in the absence of altered hepatic glucose uptake under both normal and HFD-conditions.

**Decreased glycogen deposition in Par-1a$^{-/-}$-livers leads to hypoketotic hypoglycemia**

Storage and utilization of glucose is one of the main contributors to the pathophysiologic changes in diabetes (Cline et al., 1994; Shulman et al., 1990). Periodic acid Schiff (PAS) staining was used to detect glycogen content (Fig. 4.5C). Fasted WT liver showed partially depleted liver glycogen stores (Fig. 4.5Ca), whereas liver sections from fasted Par-1a null mice exhibited complete depletion of hepatic glycogen content (Fig. 4.5Cc; PAS-quantification (fasted): WT 23.6±8.5 vs. Par-1a$^{-/-}$ 0.17±0.13, $P=0.01$, $t$-test). This depletion was not observed in Par-1b null mice (Fig. 4.5Ce; Par-1b$^{-/-}$ 26.68±2.8, $P=0.73$, $t$-test). Two hours after glucose administration, glycogen levels were substantially elevated and visible as confluent PAS-positive in livers of WT mice (Fig. 4.5Cb). In contrast Par-1a null mice showed absence of restoration of hepatic glycogen content with only spotty PAS-positive islands (Fig. 4.5Cd). Quantitative enzymatic analysis of glycogen was consistent with the PAS staining such that fasted and glucose-stimulated liver glycogen was significantly decreased in Par-1a null animals (Fig. 4.5D) with no significant difference in Par-1b null mice. After feeding glycogen levels were
normal and no significant histological differences were observed between groups (not shown).

To test the physiologic significance of hepatic glycogen depletion in Par-1a null mice we performed an extended fasting experiment and observed significantly decreased glucose levels in Par-1a null mice after overnight fasting (20 h) when compared to WT littermates (Fig. 4.5E). After 4 additional hours without external energy sources, Par-1a null animals blood glucose levels further declined ($P=0.04$ in comparison to WT, $t$-test) and the mice developed hypoketotic hypoglycemia (Fig. 4.5E). In contrast, Par-1b null mice showed an absence of physiologic decline of blood glucose levels with appropriately low ketone levels (Fig. 4.5E).

To test whether the tissue differences in glycogen levels relate to glycogen synthesis we determined total and phospho-glycogen synthase (GS) levels (pGS; Fig. 4.5F) and found increased levels of GS and pGS in the livers of Par-1a null mice. Since Par-1a null mice demonstrate resistance to steatosis as well as defective gluconeogenesis we probed for UCP2 in fasted and non-fasted liver samples but found similar protein levels (Fig 4.S1). Most recently autophagy has been linked to lipid (Singh et al.) as well as glycogen/glucose homeostasis (Kotoulas et al., 2004; Kotoulas et al., 2006). Therefore, we monitored LC-3 as a marker for membrane formation and early stages of autophagy but observed no differences (Fig. 4.S1). Electron microscopic examination of livers from fasted WT mice demonstrated irregularly shaped glycogen islands (Fig. 4.5Ga, 4.5Gb) and variably sized lipid vacuoles (Fig. 4.5Gb) in close proximity to mitochondria with normal morphology (Fig. 4.5Gc). In striking contrast, livers from fasted Par-1a null mice did not have lipid vacuoles (Fig. 4.5Gd, 4.5Ge) were completely depleted for glycogen
(Fig. 4.5Gd, 4.5Ge) and had abundant autophagocytic vacuoles throughout the hepatocytes (Fig. 4.5Ge, 4.5Gf). These autophagic vacuoles were normally configured and frequently associated with mitochondria (Fig. 4.5Gf). Our ultrastructural findings with WT mice indicate a catabolic state whereas the findings in Par-1a null liver are indicative of more extreme energy deprivation with self-digestion of organelles (Fig. 4.5Gf).

**Par-1b/Par-1a-double mutants are not viable and at least one allele is necessary for embryonic survival**

Next, crossbreeding experiments were performed to obtain mice disrupted for both Par-1a and Par-1b. After an initial experiment where 24 intercrosses were performed and only 6 of the 9 possible genotypes were obtained, genotype frequencies in ongoing HH x HH crosses were monitored. Eventually 2 of the 3 missing genotypes were obtained (Fig. 4.6A) but at a substantial deviation from the expected Mendelian ratio [Chi-squared (8)>944= 2.18e^{-198}] and with a complete absence of mice lacking both Par-1a and Par-1b (KK). In addition, there was a drastic reduction in numbers of KH and HK mice (0.5% and 2.7%, respectively). Instead of the expected 12.5% (corresponding to an estimated n of 308 mice; grey background in Fig. 4.6A), we obtained a total of 14 KH and 69 HK mice (out of n=2466). Next, pregnant females that had been superovulated from HH intercrosses were sacrificed and fetuses at day 10.5 (n=4) or day 8.5 (n=4); post coitum were isolated. None of the obtained sets of embryos (total n=31) included the KK genotype (expected frequency: ~2 mice). All other genotypes were obtained (not shown). These results illustrate Par-1b/Par-1a double mutants are not viable and that at least one allele of either Par-1a or Par-1b is necessary for viability and likely embryonic survival.
When normalized to age-matched WT-controls, Par-1b null mice are ~20% reduced in body weight (Hurov et al., 2007) and Par-1a null mice are ~9% reduced in body weight (this study). Loss of one Par-1a allele in Par-1b null mice (KH) led to a more pronounced reduction in body weight (30%) than that observed in Par-1a null mice containing a single allele of Par-1b (HK, 23%; Fig. 4.6B). HK and KH mice were not healthy and a high proportion of these animals died shortly after birth (not shown). The severity of phenotypes observed in each genotype followed the order: KK>KH>HK>KW>WK>WW and suggests an intriguing gene-dosage effect at the organism level.
Discussion

This study describes metabolic phenotypes arising in mice disrupted for Par-1a and the consequences of intercrossing of Par-1a with Par-1b deficient mice. Par-1a null mice are hypermetabolic, show reduced body weight, decreased adiposity, resistance to hepatic steatosis and hypofertility; features reminiscent of the Par-1b null phenotype (Hurov et al., 2007). Metabolic phenotypes of Par-1a null mice not shared by Par-1b null mice include 1) hepatic glycogen depletion after starvation associated with hypoketotic hypoglycemia and increased autophagic vacuoles in the liver; and 2) a modestly reduced glucose uptake by WAT when mice were fed a standard chow diet. Features observed in Par-1b null mice but not in Par-1a mice include 1) hyperphagy (food intake of Par-1a null mice was equivalent to controls); 2) reduced serum insulin levels under both fed and fasting conditions on a standard CD (serum insulin levels in Par-1a null mice were lower than those measured in WT littermates fed a HFD but not standard CD); 3) increased insulin sensitivity on standard CD (Par-1a null mice were insulin sensitive on a HFD but not standard CD); 4) resistance to HFD-induced weight gain (Par-1a null mice showed a reduced rate of weight gain relative to control littermates when fed a HFD); 5) enhanced glucose uptake in BAT (loss of Par-1a did not affect glucose uptake in BAT under any condition); and 6) resistance to hepatic steatosis accompanied by enhanced glucose uptake in liver (Par-1a null mice, resistance to hepatic steatosis was not accompanied by enhanced hepatic glucose uptake).

Strikingly, Par-1a null mice on HFD exhibited profound resistance to the development of glucose intolerance (Fig. 4.3D) as well as delayed weight gain on HFD (Fig. 4.2A). These findings are most likely related to peripheral insulin hypersensitivity.
as illustrated by resistance to glucose intolerance and decreased insulin secretion during GTT (on HFD). Par-1a null livers maintained resistance to steatosis despite HFD challenge (Fig. 4.5A, 5B). However, the adiposity and WAT glucose uptake measured in Par-1a null mice fed a HFD were similar to those of WT littermates fed the same diet. Although this study does not provide conclusive evidence for a hepatocyte-autonomous role for either Par-1a or Par-1b in lipogenesis, the striking resistance to hepatic steatosis seen in both models may suggest such a function.

Upon short-term starvation, the livers of Par-1a null mice exhibited profound defects in both glycogen storage and glucose mobilization (gluconeogenesis), phenotypes not observed in Par-1b null mice. Depletion of both lipid and glycogen stores, upregulation of glycogen synthase as well as activation of autophagy are observed in the livers of Par-1a null mice. The increase in total glycogen synthase might be a compensatory response in light of the decreased glycogen storage. However, we also observe an increase in the inhibited (phosphorylated form) of glycogen synthase. Thus, it is unclear whether the net glycogen synthase activity is altered in the livers of Par-1a-/- mice. Our ultrastructural findings of increased autophagy in the absence of glycogen or lipid vacuoles in the livers of Par-1a null mice, suggests substantial energy deprivation with self-digestion of organelles. The absence of both hepatic glycogen mobilization and gluconeogenesis required to maintain normal blood glucose levels during starvation, in turn, leads to hypoglycemia and hypoketosis in Par-1a null mice.

The underlying molecular mechanisms driving the hypermetabolic phenotype and resistance to hepatic steatosis in Par-1a null mice and the inability of these mice to maintain metabolic homeostasis during periods of starvation remain unclear. The
regulation of lipogenesis, glucose metabolism and autophagy are coordinated, at least in part, through the Par-1 related kinase AMPK. It is tempting to speculate that Par-1 may regulate these pathways via a common mechanism. To test this, we monitored the activation state of the mTOR pathway by assessing levels of phospho- and total AKT, phospho- and total AMPK, phospho- and total p70S6K, phospho- and total pS6 ribosomal protein, and LC3 in the livers of Par-1 deficient mice during starvation and upon re-feeding. We did not observe any consistent alterations in the levels or activation state of these proteins. Curiously, although both AMPK and SIK kinases negatively regulate lipogenesis via SREBP1c, the phenotypes of Par-1 null mice suggests Par-1 is a positive regulator for lipogenesis in adipocytes and hepatocytes. The molecular mechanism underlying the phenotypes of Par-1a null mice will require tissue specific deletion and identification of specific downstream targets of Par-1a mediating these effects.

Another major finding of this study is that Par-1b compensates more effectively for loss of Par-1a than the reverse and that embryonic survival requires the presence of at least one allele of Par-1a/b. Moreover, the presence of only one Par-1a allele in the complete absence of Par-1b conveys the most severe phenotype (growth restriction); supporting the notion of ranked redundancy with the most severe phenotype observed in the very rare KH (Par-1b⁻/⁻/Par-1a⁺/+ ) mutant.
Materials and Methods

Animal Procedures. The Washington University School of Medicine Animal Studies Committee approved all animal procedures. Unless stated otherwise, animals were fed a standard chow diet (Purina Mills, St. Louis, MO, Lab Diet 5063 containing 4.5% fat, 55% carbohydrate, 20% protein and 4.7% fiber) and were housed with free access to food and water under a 12h light/dark cycle. Unless indicated, only male mice were analyzed throughout this study.

Construction of the Par-1a targeting construct. A Par-1a genomic clone was isolated from a 129X1/SvJ mouse embryonic (ES) cell genomic library by hybridization with an isogenic 0.9 kb Par-1a-specific cDNA probe. The genomic organization of the mouse Par-1a gene was disrupted by replacing a 1.9 kb Sac I/Hpa I fragment with a 1.2 kb neomycin phosphotransferase cassette (Fig. 4.1A). The replacement disrupts exon 2 encoding the ATP binding domain of Par-1a and also destroys the 5’ splice site between introns 2 and 3. The insertion of the neomycin selection cassette alters the Pst I fragment from a single 13.8 kb fragment to two fragments of 7.5 and 5.9 kb, and this change was used to identify targeted ES cell clones and knockout animals by Southern blotting (Fig. 4.1B) using the 5’ and 3’ probes indicated in Fig. 4.1A. Using mouse genomic DNA as template, the 425 bp 5’-probe was amplified by PCR with primers of 5’-TGGTGGTACACGCCTGTATTC and 5’-CTGATCTACACAATTCCAGGAC. The 204 bp of 3’-probe was amplified by PCR with primers of 5’-GCATCTTGGGCTGTAAGTGATG and 5’-GGCTTACAACCATCTGTACAG.

PCR analysis. PCR of tail DNA/tissues was achieved using a three-primer PCR reaction with one 5’ primer from exon 2 (5’-GGAGACGGGAGGCAAGAAGTCA), a 3’
primer from the intronic sequence between exons 2 and 3 (5'-
GGCTCATTCCCTTGTTGTTACCTT), and a 3’ primer from the neomycin cassette
(5’GAGCAGCCGATTGTCTGGTGTT), resulting in 400 bp (KO) or 300 bp (WT) bands,
respectively (Fig. 4.1C).

Generation of mice harboring the Par-1a mutation. RW4 ES cells were
electroporated with linearized targeting vector and selected with Geneticin (G418;
Invitrogen) using established protocols developed in the Murine Embryonic Stem Cell
Core of the Alvin J. Siteman Cancer Center at Washington University School of
Medicine and Barnes-Jewish Hospital; details available online http://escore.im.wustl.edu.
A total of 96 G418-resistant ES cell clones were analyzed for homologous recombination
by Southern blotting and 3 clones were found to be positive. Positive ES clones were
karyotyped and microinjected into 3.5 dpc C57BL/6 blastocysts, which were
subsequently implanted into the uteri of pseudopregnant C57BL/6 X C3HF1 foster
mothers. Male chimeras selected by percentage of agouti color were mated to C57BL/6
females. Germ line transmission was determined by agouti coat color. F1 animals were
tested for the targeted Par-1a allele by Southern blotting and PCR analysis of tail DNA.
Heterozygous F1 males/females were interbred to generate F2 littermates used for
subsequent breeding and analysis; referred to as wild type (WT), heterozygotes (HET)
and null (KO) pups/mice.

Western blotting. Tissues from wild-type (WT) and knock-out animals were
homogenized in 800 µl of mammalian cell lysis buffer (MCLB: 50 mM Tris-HCl pH 8.0,
5 mM EDTA, 0.1 M NaCl, 0.5% NP40, 2 mM DTT) containing 1 µM microcystin, 1 mM
sodium orthovanadate, 10 mM β-glycerol phosphate, 1 mM sodium fluoride, 2µM PMSF,
plus protease- (Sigma, St. Louis, MO) and phosphatase-inhibitor cocktails (Calbiochem, Gibbstown, NJ). Homogenates were rocked for 15 min at 4°C and then clarified twice by centrifugation. Clarified lysates were resolved by SDS-PAGE on a 10% SDS gel followed by Western blotting was carried out with antibodies specific to actin (1:4,000, Sigma Chemical Co.), tubulin (Sigma, 1:1000), β-catenin (1:10,000, BD Biosciences/Pharmagen) and Par-1a ascites (1:10,000) (Hurov et al., 2004), Glycogen Synthase (1:1000; Cell Signaling), phospho-Glycogen Synthase (1:1000; Cell Signaling) and LC-3 (1:1000; Cell Signaling). Par-1b was detected with ascites generated using a monoclonal antibody produced against KLH-conjugated peptide corresponding to amino acids 288-307 (PDYKDPRRTLMVSMGYTRE) of human Par-1b. Bound primary antibodies were detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Jackson, West Grove, PA) and proteins were visualized by chemiluminescence.

**Tissue distribution.** Distribution of Par-1a and Par-1b in various tissues was examined using a modified Western blot protocol. In brief, mouse tissues harvested from ~6-month-old mice were homogenizing in 0.8 ml of Mammalian Cell Lysis Buffer (see above). Lysates were rocked for 15 min at 4°C followed by centrifugation for 10 min at 20,800g. Clarified supernatants were transferred to pre-chilled tubes. Proteins were resolved by SDS-PAGE on 7.5% gels until the 50 kDa molecular weight marker was at the bottom of the gel. Proteins were transferred to nitrocellulose membranes in CAPS buffer (Sigma C2632, St. Louis, MO) 0.1M 3-(cyclohexylamino)-1-propanesulfonic acid and 10% methanol at 1.2 mA for 1.5 h. Membranes were blocked in 1xTBST (100 mM Tris pH 8.0, 1.5 M Sodium Chloride, 2% Tween-20) containing 5% milk for 1h. Membranes were probed with Par-1a ascites (1:10,000), Par-1b ascites (1:1,000), actin
(1:4,000, Sigma Chemical Co.), or β-catenin antibody (1:30,000) by rocking overnight at 4°C. Membranes were washed 4 times for 13 min each in 1x TBST. Membranes were then incubated with a goat anti-mouse antibody (dilution 1:30,000; Jackson West Grove, PA) for 1 h at room temperature. Membranes were then washed 4 times in 1x TBST for 13 min each. Blots were developed using ECL detection reagent (GE Healthcare, Piscataway, NJ).

Breeding, animal weights and growth measurements. Embryos and pups generated from heterozygous crosses were weighed and genotyped by PCR analysis. Plug observation was recorded as 0.5 days post coitus (dpc); embryos were harvested, rinsed and weighed. Mean body weights at each time point were compiled from at least 6 mice per genotype. Breeding schemes followed standard protocols and for double-knock-out (DKO) experiments genotypes are abbreviated by a two-letter code (first letter: Par-1b; second letter: Par-1a; W=+/+; H=+/−; K=−/−; e.g. HK). Binning and comparison within and between genotypes via uni- and multivariate analysis tested trends over time. We assessed number and genotype of litters in 10 superovulated timed pregnancies at 8.5 dpc and 10.5 dpc. Offspring ratios for Par-1a−/−, Par-1b−/− as well as all genotypes in DKO-experiments were recorded as pups/litter and total number per genotype. Significance of deviations from expected Mendelian ratios were tested using Chi-square statistics with 2-degrees (WT, HET, KO) or 8-degrees of freedom (KK, WW, WK, KW, WH, HW, KH, HK, HH) in Par-1a null and DKO experiments, respectively (two-tailed P values are provide).

Metabolic measurements (indirect calorimetry). Metabolic measurements followed previously established protocols (Bernal-Mizrachi et al., 2002; Hurov et al.,
Briefly, metabolic characterization included metabolic rate, energy expenditure, activity, food consumption (in g/day), and body temperature. During data analysis, energy expenditure and metabolic rate were normalized with respect to body weight.

Energy expenditure and respiratory quotient (RQ) were calculated from the gas exchange data: RQ as the ratio of VCO$_2$ to VO$_2$, \[ \text{Heat} = (3.815 + 1.232 \times \text{RQ}) \times \text{VO}_2 \]. Activity was measured on an x- and z-axis using infrared photobeams to count the amount of beam breaks.

**High-fat diet (HFD) studies.** HFD studies followed previously published protocols (Hurov et al., 2007) and consisted of a total of 55 mice. In thirty-five 3-week-old mice (WT, Par-1a$^{-/-}$, Par-1a$^{+/+}$) the regular rodent chow diet (CD) was replaced with *Adjusted Calories Diet* (42% fat) from Harlan Teklad (88137 ‘Western’ diet; Madison, WI). The weight of the HFD chow was recorded and food consumption as well as mouse weight was determined for individual mice on a weekly basis. Although resistance to weight gain on HFD has been previously reported for Par-1b null mice (Hurov et al., 2007), for direct comparison and determination of specific organ uptake values, the HFD experiment included a new group of 9 Par-1b null mice and therefore consisted of six different trial-groups: WT-CD, WT-HFD, Par-1a$^{-/-}$-CD, Par-1a$^{+/+}$-HFD, Par-1b$^{+/+}$-CD, Par-1b$^{-/-}$-HFD. Metabolic profiling after a total diet time of 16 weeks consisted of a) fasting- (n=18CD, 35HFD) and fed blood glucose levels (n=6 per genotype, randomly selected); b) glucose tolerance testing (GTT) on 18 CD-mice (6 per genotype, randomly selected) and all HFD-mice; c) $^{18}$F-FDG-biodistribution experiments (n=19CD, 31HFD), d) organ weights and e) histologic examination. The HFD-trial was designed to allow at least 24h
between GTT and $^{18}$F-FDG-biodistribution studies, during which animals were kept on HFD.

**Serum Factor Quantification.** Serum levels of insulin, triglycerides, adiponectin, leptin, and cholesterol were determined by Ani Lytics Incorporated (Gaithersburg, MD; n=11 animals per genotype and sex); glucagon and free fatty acids were determined according to previously established protocols (Chakravarthy et al., 2005).

**Glucose and insulin measurements and tolerance tests.** Randomly fed or fasted mice were analyzed and blood glucose levels were determined using a $\beta$-glucose photometer and $\beta$-glucose cuvettes (HemoCue AB, Angelholm, Sweden) or Accucheck Advantage glucometer (Roche Diagnostics Corp., Indianapolis, IN). Normoglycemia was defined as blood glucose values between 90-130 mg/dl. Intraperitoneal (i.p.) glucose tolerance tests (GTT) were performed on fasted (12 h) animals on CD and all HFD-trial mice. Mice were injected i.p. with D-glucose (20% solution; 1 g/kg of body weight) and blood glucose levels were determined at 15, 30, 60, 90 120 min and >24h post injection. In addition, insulin levels were determined during GTT immediately before, at 15, 30 and 60 min after glucose injection. Therefore, ~20-30 µl of blood were collected using the Microvette 200 capillary blood collection system (Sarstedt, Newton, NC) and centrifuged at 30,000 g for 5 min. Fasting serum insulin levels were determined for 50 male mice on CD (n=24WT, 8Par-1a$^{+/+}$, 18Par-1b$^{-/-}$) and 18 randomly selected mice on HFD (n=6 per genotype) using rat insulin as a standard and ELISA as specified by manufacturer (Crystal Chem, Inc, IL). Insulin tolerance tests (ITT) were performed on 6-h-fasted male mice (n=9Par-1a$^{+/+}$, 9Par-1a$^{-/-}$). Insulin sensitivity in Par-1b null mice has been reported
Blood glucose values were measured immediately before and at 15 min intervals for a total of 90 min after i.p. injection of insulin (0.30 IU/kg HumulinR, Eli Lilly and Company, Indianapolis, USA).

MicroPET studies. MicroPET studies entailed imaging of cohorts of male WT and Par-1a null mice that were repetitively imaged once a week for four consecutive weeks, each time under a different metabolic condition. Brown adipose tissue (BAT), skeletal muscle (SM), heart, liver and brain were examined under the following 4 conditions: mice were either fasted overnight or allowed access to CD (non-fasted). The next morning, either 0.5 unit/kg insulin (Humulin N; Eli Lilly, Indianapolis, IN) or saline was administered to mice by i.p. injection. Thirty minutes after the injection, mice were lightly anesthetized with isoflurane followed by a tail vein injection of $^{18}$F-FDG (~200-500 µCi in saline). Immediately after injection of radiotracer, mice were placed supine in the microPET scanner (Focus120 or 220 scanner, Concorde MicroSystems, Knoxville, TN) and imaged (acquisition time: ~10 min; 1 bed position; OSEM reconstruction). Mice were allowed to recover, then anesthetized and imaged again at 1 h and 2 h post injection of radiotracer. The Par-1b null and WT mice used for comparison were age matched males and treated as above except that they were imaged on a R4 scanner (Hurov et al., 2007). MicroPET images were corrected for decay, but not attenuation or scatter, then stacked regions-of-interest (ROI) of relevant tissues and organs were analyzed with AnalyzePC 6.0 software. ROI counts were converted to counts per gram of tissue (nCi/g), assuming a tissue density of 1 g/ml. Data for accumulation of $^{18}$F-FDG on microPET images were expressed as standard uptake values (SUV), representing counts per gram of tissue divided by injected dose of radioactivity per gram of animal weight (Sharma et al., 2007).
After statistical analysis using a one-way ANOVA and Student’s t-tests, comparison between genotypes and experiments was enabled by normalization of the SUV to the corresponding WT littermates, data for Par-1a and Par-1b disrupted mice against their littermate WT as normalized average SUV ± SEM; errors were propagated (see below).

**18F-FDG biodistribution experiments.** As previously reported, white adipose tissue (WAT) cannot be analyzed by noninvasive microPET imaging (Hurov et al., 2007). As an alternative we examined glucose-uptake of WAT and other organs by traditional 18F-FDG biodistribution (Bigott et al., 2005; Cirrito et al., 2005; Kesarwala et al., 2006; Sharma et al., 2005). Mice were anesthetized with isoflurane (2% vaporized in oxygen), followed by tail vein injection of 18F-FDG (20 μCi in 100 μl saline). Sixty minutes after 18F-FDG injections, mice were sacrificed by cervical dislocation (under anesthesia). Blood, liver, heart, brain, muscle, WAT and BAT were rapidly removed, weighed and their radioactivity was measured using a Beckman 8000 gamma counter. The percent-injected dose per gram of tissue (% ID/g) was calculated \[ \left( \frac{(\text{tissue } \mu\text{Ci})}{(\text{injected Ci})} \right) \times 100 \] and subsequently compared among tissues, genotypes and diets. Biodistribution analysis was performed on 31 HFD-mice (17WT; 8Par-1a/−; 6Par-1b/−) and 19 mice from the CD-arm (age-matched control group; total n=50). Tissue samples from these mice were fresh-frozen and stored at -80°C. Subsequent cryostat sections confirmed tissues, and allowed histomorphological/histochemical comparison with tissues obtained from animals that did not undergo 18F-FDG uptake (n=5; no differences observed) or HFD (see results). For these HFD-trial biodistribution experiments, the 50 mice were prospectively assigned to one of 9 experimental days with an average of 6
animals per experiment (range: 3-10 mice/day). Excluding the 16 weeks on HFD (see above), these experiments were performed over a 10-month-period.

**Starvation experiments.** Starvation experiments consisted of determination of blood glucose as well as ketone levels using a MediSense Precision Xtra Diabetes Glucose and Ketone Monitor System (Abbott, Alameda, CA). After overnight fasting (20 h) measurements were repeated; one group of mice was subjected to extended fasting (additional four hours) and the other group of mice was given an i.p. injection of 2 mg/g glucose for 2 h. Tissues were collected at all time points and samples separated for western blotting, routine histology, electron microscopy and glycogen determination.

**Dissection and Histology.** Organs were excised, weighed, rinsed in PBS; fixed in 10% neutral-buffered formalin, rinsed in PBS, and stored in 70% ethanol. Formalin fixed tissues were paraffin embedded (FFPE) and sectioned at (~5 μm) using standard procedures. For morphologic evaluation and fat content H&E and Oil-red-O staining was performed on FFPE and fresh-frozen sections (stored at -80°C), respectively. Morphological analysis was performed using multiple independent samples per site/organ (13-26 samples per genotype) as well as all 55 animals on the HFD-trial. We quantified BAT and WAT samples taken from the interscapular and gonadal fat pads, respectively. Quantification was performed using digital image processing via ImageJ (v. 1.37v http://rsb.info.nih.gov/ij/) and Photoshop CS3 (Adobe Systems) according to previously established semi-automatic protocols (Brunt et al., 1999; Gustot et al., 2006). Specifically, Oil-red-O positivity was quantified on ≥20 high-power fields, cell number or lipid vacuoles were quantified on 6-20 randomly chosen images per sample and PAS-staining
was quantified using integrated density measurements (IntDen: equivalent to the product of area and mean grey value).

**Glycogen determination.** Fifty milligrams of liver and skeletal muscle samples were extracted with 0.9 N perchloric acid and 99% ethanol to precipitate glycogen. The glycogen from the pellet was dialysed, and digested to free glucose with amyloglucosidase. Glucose concentrations were measured by the glucose oxidase method (Glucose Analyzer II; Beckman Instruments, Fullerton, CA).

**Electron microscopy (EM).** EM was performed according to established protocols (Schmidt et al., 2009). Tissues were fixed overnight at 4°C in modified Karnovsky’s fixative containing 3% glutaraldehyde and 1% paraformaldehyde in sodium cacodylate buffer pH 7.4. After rinsing in sodium cacodylate buffer, samples were postfixed in phosphate cacodylate-buffered 21% OsO4 for 1h, dehydrated in graded ethanols with a final dehydration in propylene oxide and embedded in Embed-812 (Electron Microscopy Sciences, Hatfield, PA). One-micron thick plastic sections were examined by light microscopy after staining with toluidine blue. Ultrathin sections (90 nm thick) of individual samples were cut onto formvar coated slot grids, which permit visualization of larger cross sections. Sections were post stained with uranyl acetate and Venable’s lead citrate and viewed with a JEOL model 1200EX electron microscope (JEOL, Tokyo, Japan). Digital images were acquired using the AMT Advantage HR (Advanced Microscopy Techniques, Danvers, MA). Plastic sections as well as ultrastructural review was performed blinded to the genotype.

**Digitization and image analysis.** Analyses were performed using an Olympus DP70 digital camera (Olympus, Tokyo, Japan) connected to an Olympus BX51 light
microscope or a Scanscope XT whole slide scanner/ImageScope v10.0.36.1805 (Aperio, Vista, CA). Image and pixel quantification employed established semiautomatic threshold algorithms (Lennerz et al., 2008) using the software package ImageJ [http://rsb.info.nih.gov/ij/; (Abramoff et al., 2004; Rasband, 1997-2007)]. Statistical testing between groups was performed using Student’s t-test and Wilcoxon two-sample test.

**Statistics.** Chi-squared, t-tests (two tailed), the non-parametric alternative Mann-Whitney U test, 1-way- and 2-way ANOVA, and Bonferroni post-tests to compare replicate means were used when appropriate. In experiments with small n’s (defined as n≤12) the D’Agostino-Pearson omnibus K2 normality test (D’Agostino, 1986) was used to approximate Gaussian distribution of values. Therein P values of <0.05 indicate non-Gaussian distribution and we assessed these experiments with both parametric and non-parametric tests; both P values are provided when applicable. In all studies, the accepted level of significance was P<0.05 and data are reported as mean ± SEM. In selected experiments and for comparison between strains and experiments, values were normalized to the corresponding WT and errors were propagated according to the following formula:

\[
\sqrt{\left[ \frac{\sigma_{\bar{x}_{WT}}}{\bar{x}_{WT}} \right]^2 + \left( \frac{\sigma_{\bar{x}_{KO}}}{\bar{x}_{KO}} \right)^2} \cdot \bar{x}_{NKO} \quad \text{adapted after (Meyer, 1975)}
\]

\[\sigma_s = \text{standard error of the mean}; \bar{x} = \text{average}; \bar{x}_N = \text{normalized average}\]

Error propagation and statistical tests in general were chosen assuming the most pessimistic situation (Rabinovich, 2000). All data were analyzed using Prism 5.0b (GraphPad Software Inc., La Jolla, CA) or Microsoft Excel 2008 (Version 12.1.9; Microsoft Corporation, Redmond WA).
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**Fig. 4.1 Targeted disruption of Par-1a in mice and tissue distribution of Par-1a and Par-1b.**

A. Par-1a locus (top), targeting vector (middle) and chromosomal organization after recombination (bottom). The Par-1a gene was disrupted by insertion of a neomycin (neo) phosphotransferase cDNA as a selectable marker. Sizes of upstream (2 kb) and downstream (3.8 kb) homologous arms are indicated (grey boxes), as is exon 2 (open box). Black boxes indicate positions of the probes for genomic Southern blotting; triangles depict locations of PCR primers for genotyping. Abbreviations: P=Pst I; B=Bgl II; S=Sac I; Hp=Hpa I; N=Neo I; H=Hind III.  

B. Southern blot analysis demonstrating homologous recombination at the Par-1a locus. Genomic DNA was digested with Pst I and subjected to Southern blotting using the 5’ and 3’ probes indicated in panel A. Genotypes are indicated (WT: wild-type mouse; HET: heterozygous, KO: homozygous).  

C. PCR analysis of mouse DNA (tail), amplified with 3 PCR primers (a, b, and c) shown in panel A. The WT-allele produces a 300 bp PCR product and null alleles produce a 400 bp PCR product.  

D. Western Blot analysis of Par-1a protein in brain lysates prepared from C57BL/6-mice.  

E. Tissues from wild-type, Par-1a/- and Par-1b/- mice were isolated. Protein lysates from the indicated tissues were resolved by SDS-PAGE and subjected to Western blotting for the indicated proteins: Brain (0.2 mg), BAT=brown adipose tissue (1.1 mg), WAT=white adipose tissue (1.1 mg), colon (0.4 mg), Stom=stomach (0.4 mg), Thym=thymus (0.4 mg), Test=testis (0.4 mg), SPLN=spleen (0.4 mg), SKM=skeletal muscle (0.4 mg), Liver (0.13 mg), W=wild-type; K=knock-out.
Fig. 4.2 Par-1a/--mice are growth retarded and hypermetabolic.

A. Body weights of mice from birth to 30 weeks of age (n=4-14 per genotype per time point). B. Body weights at 6 and 20 weeks. Body weights of Par-1a+/+ (black bars), Par-1a+/- (striped bars), and Par-1a -/- (open bars) mice at 6 weeks of age (number of animals per sex indicated). C. Metabolic rate, D. energy expenditure, E. respiratory quotients (RQ=VCO2/VO2), and F. food intake in WT- (black bars) and Par-1a null mice (open bars). G. Insulin tolerance tests were performed by i.p. injection of 0.30 unit/kg insulin into WT and Par-1a-/--mice (n as indicated); tail bleeds were obtained and glucose levels were monitored at indicated time points. Glucose levels plotted as % blood glucose at time 0 before injection.
Fig. 4.3 Resistance of Par-1a-/- mice to high-fat diet induced metabolic changes.

A. Body weights during 15 weeks HFD starting at 3 weeks of age in male mice (n per genotype as indicated; P value designations: **, P<0.005; *, P<0.001). B. Body weight gain (in %) indicated above weight comparisons of control group (black/white/grey) and HFD-arm of the trial (red) in 18-week-old male mice. C. Glucose tolerance testing was performed by i.p. injection of D-glucose at 1mg/g body weight into all genotypes on chow and high-fat diet (n as indicated). Glucose levels were monitored as indicated and expressed in % of initial glucose normalized to their littermates on chow; for comparison a dotted band shows the time course for WT and Par-1a-/- on chow diet. Statistical testing was performed using original values and P-values are provided for testing between genotypes on HFD (red) and between WT-HFD vs. WT-CD (black), when significant. D. Serum insulin levels as determined by ELISA for the first 4 timepoints of the glucose tolerance test after HFD.
**Fig. 4.4 Reduced adiposity and decreased glucose uptake in Par1a--/-- mice.**

A. Tissues weights normalized to total body weight in indicated tissues from 18-week old male WT- (black bar) and Par-1a--/-- mice (open bars) fed with chow diet (black) or HFD (red; selected tissues); n≥7 samples per tissue. Labeling a-e see results. B. Hematoxylin and eosin (H&E) stain of WAT from Par-1a--/-- and Par-1a+/+-mice; insets, gonadal fat pads. C. Weight comparisons of WAT between WT, Par-1a--/-- and Par-1b--/--mice on chow (black, white, hatched, respectively) and after 16 weeks of high-fat diet (red); weights normalized to WT on chow diet. Statistical tests and indicated P values derive from original weights (provided below bars). D. Invasive biodistribution experiments following injection of 18F-FDG and dissection of gonadal WAT 1h post-injection; values expressed as % injected dose per gram of tissue (%ID/g). E. H&E stain of BAT from Par-1a--/-- and Par-1a+/+-mice; insets, interscapular fat pads. F. Weight comparisons of BAT between WT, Par-1a--/-- and Par-1b--/--mice on chow (black, white, hatched, respectively) and after 16 weeks of high fat diet (red); weights normalized to WT on chow diet. Statistical tests and indicated P values derive from original weights (provided below bars). G. Representative coronal section microPET images of 18F-FDG uptake in Par-1a--/-- (top) and Par-1b--/--mice (bottom) 1 h after 18F-FDG injection.
Fig. 4.5  Liver findings in high-fat diet- and starvation experiments.

A. Morphologic comparison of Oil-red-O stained sections shows resistance to hepatic steatosis in Par-1a-/-mice. Inset: Abundant macrovesicular steatosis.  B. Semi-automated pixel quantification, expressed as percent of Oil-red-O-positive structures (fat) per high-power field (HPF); genotypes, number of animal and diet indicated. C. Periodic acid Schiff (PAS) staining of liver of 20-week-old WT, Par-1a-/- and Par-1b-/- littermates following overnight fasting or 2 h after administration of 2 mg/g glucose by i.p. injection. Images are representative of 96 fields from 8 animals. D. WT-mice show a physiologic decreased in blood glucose in response to overnight starvation with a nadir at 24 + 4 h (top) and corresponding increased ketone production (bottom); note restoration of blood glucose and ketones, 2 h after intraperitoneal glucose injection (2 mg/g). Par-1a-/- mice show significantly lower blood glucose levels at both timepoints with associated hypoglycemia at 24 + 4 h. Fasting hypoglycemia is accompanied by a significant decrease in ketone levels (hypoketotic hypoglycemia). Par-1b-/- mice maintained glucose levels and exhibited expected lower ketone levels. E. Glycogen content was measured in livers from 20-week-old Par-1a-/- (open bars), Par-1b-/- (grey bars), and WT (black bars) littermates following overnight fasting or 2 h after i.p. injection of 2 mg/g glucose. Tissues were extracted and acid hydrolyzed, and glycosyl units were assayed using an amyloligosidase/glucose oxidase method (see Methods). F. Western Blot for glycogen synthase (GS) and phosphorylated GS (pGS) in overnight fasted liver samples of 4 different animals per genotype; weight markers GS/pGS 100kDa (top), 75kDa (bottom); actin (37kDa); arrows indicate Par-1a-/- G. Ultrastructureal characterization of liver tissue from WT (a-c) and Par-1a-/- littermates (d-f) after nutrient starvation for 24 h. Ga.
Toluidine blue-stained plastic section of starved WT liver at low magnification showing accumulation of lipid droplets (open arrow) and glycogen (arrow). Gb. Electron microscopic examination of WT hepatocytes at low-power magnification showing large number of variable-sized lipid vacuoles (open arrow) and glycogen islands (arrow). Gc. Detail of cytoplasmic organization with lipid droplets (open arrow) surrounded by numerous mitochondria (asterisks). Gd. Toluidin blue-stained plastic section of starved Par-1a-/- at low magnification shows absence of lipid droplets and glycogen. Ge. Electon microscopic examination of Par-1a-/- hepatocytes at low-power magnification showing absence of glycogen and lipid vacuoles. Gf. Detailed EM view of cytoplasmic organization with numerous autophagosomes (arrows) consisting of isolated membranes surrounding cytosolic material (top: so-called myelin figures; bottom ingested rough endoplasmic reticulum). Note the close proximity of autophagocytic vacuoles and mitochondria (asterisk).
A

Offspring Ratio (%)

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<td>+/-</td>
<td>+/-</td>
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<tr>
<td>Par1a</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
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<td>15</td>
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<td>25</td>
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<td>30</td>
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<td>25</td>
<td>15</td>
<td>10</td>
<td>5</td>
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B

Normalized Weight (%WT)

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<th>26</th>
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<tr>
<td>WW</td>
<td>100</td>
<td>90</td>
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<tr>
<td>KH</td>
<td>100</td>
<td>90</td>
<td>80</td>
<td>70</td>
<td>60</td>
</tr>
</tbody>
</table>

P-values:
- WW vs. WK: P=0.0001
- WW vs. KW: P=0.0003
- WW vs. HK: P=5.5e-08
Fig. 4.6 Par-1b⁻/⁻/Par-1a⁻/⁻--double knock-out mice are not viable and breeding experiments support ranked redundancy between isoforms.

A. Survival analysis of the offspring from matings of double-heterozygous Par-1a/b mutant mice. The black bars plot the observed frequency of the indicated genotypes as percentage of the total, whereas the gray background indicates the expected frequency based on Mendelian inheritance (n=2466). B. Comparison of body weights for five of the obtained genotypes from B. Weight is normalized to WT-littermates [HK, n=6 mice at 15-20 weeks vs. control WT (n=6); KH (E17dpc) n=12 vs. age-matched control WT (n=8; from same litter)].
<table>
<thead>
<tr>
<th>A. Regulators</th>
<th>Function (‘upstream’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LKB1</td>
<td>Wnt-signaling; Peutz-Jeghers syndrome, Insulin signal transduction, pattern formation</td>
<td>(Alessi, 2001; Lizcano et al., 2004; Spicer et al., 2003)</td>
</tr>
<tr>
<td>TAO1</td>
<td>MEK3/p38 stress-responsive mitogen-activated protein (MAP) kinase pathway</td>
<td>(Hutchison et al., 1998)</td>
</tr>
<tr>
<td>MARKK</td>
<td>Nerve growth factor signaling in neurite development and differentiation</td>
<td>(Timm et al., 2003)</td>
</tr>
<tr>
<td>aPKC</td>
<td>Ca(^{2+})/DAG-independent signal transduction, cell polarity, glucose metabolism</td>
<td>(Brajenovic et al., 2004; Goransson et al., 2006; Hurov et al., 2004; Kusakabe and Nishida, 2004; Ossipova et al., 2005; Suzuki et al., 2004)</td>
</tr>
<tr>
<td>nPKC/PKD</td>
<td>DAG-dependent, Ca(^{2+})-independent signal transduction (GPCR)</td>
<td>(Watkins et al., 2008)</td>
</tr>
<tr>
<td>PAR-3/PAR-6/aPKC(-)</td>
<td>regulates Par-1; assembly of microtubules, axon-dendrite specification</td>
<td>(Chen et al., 2006)</td>
</tr>
<tr>
<td>GSK3β</td>
<td>(-) tau-phosphorylation; Alzheimer’s dementia; energy metabolism, body patterning</td>
<td>(Kosuga et al., 2005; Timm et al., 2008)</td>
</tr>
<tr>
<td>Pim-1 oncogene</td>
<td>(-) G2/M checkpoint; effector of cytokine signaling and Jak/STAT(3/5)</td>
<td>(Bachmann et al., 2004)</td>
</tr>
<tr>
<td>CaMKI</td>
<td>(-) Ca(^{2+})-dependent signal transduction; neuronal differentiation</td>
<td>(Uboha et al., 2007)</td>
</tr>
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<table>
<thead>
<tr>
<th>B. Substrates</th>
<th>Function (‘downstream’)</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>Cdc25C</td>
<td>Regulation of mitotic entry by activation of the cdc2-cyclin B complex</td>
<td>(Dalal et al., 1999; Ogg et al., 1994; Peng et al., 1998; Zhang et al., 1997)</td>
</tr>
<tr>
<td>Class II HDAC</td>
<td>Control of gene expression and master regulator of subcellular trafficking</td>
<td>(Dequiedt et al., 2006; Kao et al., 2001)</td>
</tr>
<tr>
<td>CRTC2/TORC2</td>
<td>Gluconeogenesis regulator via LKB1/AMPK/TORC2 signaling, PPAR(^{1}) coactivator</td>
<td>(Jansson et al., 2008)</td>
</tr>
<tr>
<td>Dlg/PSD-95</td>
<td>Synaptogenesis and neuromuscular junction; tumor suppressor</td>
<td>(Woods and Bryant, 1991; Zhang et al., 2007)</td>
</tr>
<tr>
<td>Disheveled</td>
<td>Wnt-signaling; translocation of Dsh from cytoplasmic vesicles to cortex</td>
<td>(Ossipova et al., 2005; Sun et al., 2001)</td>
</tr>
<tr>
<td>KSR1</td>
<td>Regulation of the Ras-MAPK pathway</td>
<td>(Muller et al., 2001; Muller et al., 2003)</td>
</tr>
<tr>
<td>MAP2/4/TAU</td>
<td>‘Dynamic instability’ (Mitchison and Kirschner, 1984; Sammak and Borisy, 1988) of microtubules; Alzheimer’s dementia (Drewes, 2004) (Biernat et al., 2002; Drewes et al., 1998; Drewes et al., 1997; Drewes et al., 1995; Illenberger et al., 1996; Murphy et al., 2007; Tian and Deng, 2009)</td>
<td></td>
</tr>
<tr>
<td>Mib/Notch</td>
<td>Mind bomb (Mib) degradation and repression of Notch signaling = neurogenesis</td>
<td>(Krahn and Wodarz, 2009; Ossipova et al., 2005; Rubio-Aliaga et al., 2007)</td>
</tr>
</tbody>
</table>
Par3/OSKAR/Lgl  Cytoplasmic protein segregation, cell polarity and asymmetric cell division (Benton et al., 2002; Betschinger et al., 2003)

Pkp2  Desmosome assembly and organization; nuclear shuttling (Muller et al., 2001; Muller et al., 2003)

PTPH1  Linkage between Ser-/Thr- and Tyr-phosphorylation dependent signaling (Zhang et al., 1997)

Rab11-FIP  Regulation of endocytosis (Cullis et al., 2002); trafficking of E-cadherin (Ducharme et al., 2006)

LKB1 also known as Par-4; MARKK also known as Ste20-like; (-): inhibitory/negative regulation has been shown; GPCR: G-protein coupled receptors; MARKK is highly homologous to the thousand and one amino acid kinase (TAO-1) (Hutchison et al., 1998)
Table 4.2 Terminology and Localization of the Mammalian Par-1 Family Members

<table>
<thead>
<tr>
<th>Synonyms</th>
<th>Subcellular localization</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Par-1a</strong>, MARK3, C-TAK1, p78/KP78, 1600015G02Rik,</td>
<td></td>
</tr>
<tr>
<td>A430080F22Rik, Emk2, ETK-1, KIAA4230,</td>
<td>basolateral(^1)/apical(^2)</td>
</tr>
<tr>
<td>mKIAA1860, mKIAA4230, M80359</td>
<td></td>
</tr>
<tr>
<td><strong>Par-1b</strong>, EMK, MARK2, AU024026, mKIAA4207</td>
<td>basolateral</td>
</tr>
<tr>
<td><strong>Par1c</strong>, MARK1</td>
<td>basolateral</td>
</tr>
<tr>
<td><strong>Par1d</strong>, MARK4, MARKL1</td>
<td>not asymmetric(^3)</td>
</tr>
</tbody>
</table>

Par: partitioning defective [not to be confused with protease-activated receptor 1 (PAR1; (Di Serio et al., 2007)]; C-TAK1: Cdc twenty-five C-associated kinase; MARK: microtubule affinity regulating kinase; MARKL: MAP/microtubule affinity-regulating kinase like 1; \(^1\)to a lesser degree in comparison to Par-1b (Goransson et al., 2006); \(^2\)human KP78 is asymmetrically localized to the apical surface epithelial cells (Parsa, 1988); \(^3\)variant that does not show asymmetric localization in epithelial cells when overexpressed (Suzuki et al., 2004).
Table 4.3 Metabolic characteristics of Wild-type (WT), Par-1a<sup>−/−</sup> and Par-1b<sup>−/−</sup> animals.

<table>
<thead>
<tr>
<th></th>
<th>Insulin (ng/ml)</th>
<th>Glucagon (pg/ml)</th>
<th>Leptin (ng/ml)</th>
<th>Adiponectin (µg/ml)</th>
<th>Triglycerides (mg/dl)</th>
<th>Cholesterol (mg/dl)</th>
<th>NEFA (µmol/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Male</strong></td>
<td>0.65 ± 0.10</td>
<td>45.5 ± 11.7</td>
<td>3.68 ± 0.26</td>
<td>9.8 ± 0.66&lt;sup&gt;§&lt;/sup&gt;</td>
<td>17.9 ± 1.7</td>
<td>74.9 ± 3.46</td>
<td>83.27 ± 2.4</td>
</tr>
<tr>
<td><strong>Female</strong></td>
<td>17.9 ± 1.7</td>
<td>74.9 ± 3.46</td>
<td>83.27 ± 2.4</td>
<td></td>
<td></td>
<td></td>
<td>1303 ± 65.8</td>
</tr>
<tr>
<td><strong>P</strong></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td><strong>WT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Par-1a−</strong></td>
<td>0.61 ± 0.08</td>
<td>31 ± 4.33</td>
<td>3.64 ± 0.16</td>
<td>16.5 ± 1.6</td>
<td>18.4 ± 2.2</td>
<td>73.1 ± 3.77</td>
<td>74.95 ± 2.87</td>
</tr>
<tr>
<td><strong>P</strong></td>
<td>0.74</td>
<td>0.79</td>
<td>0.008*</td>
<td>0.87</td>
<td>0.74</td>
<td>0.03*</td>
<td>0.84</td>
</tr>
<tr>
<td><strong>Par-1b−</strong></td>
<td>0.37 ± 0.04</td>
<td>34 ± 2.25</td>
<td>3.02 ± 0.16</td>
<td>11.4 ± 1.3</td>
<td>11.7 ± 0.84</td>
<td>62.2 ± 3.39</td>
<td>73.45 ± 2.81</td>
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<tr>
<td><strong>P</strong></td>
<td>0.04</td>
<td>0.28</td>
<td>0.005*</td>
<td>0.013*</td>
<td>0.011*</td>
<td>0.11</td>
<td></td>
</tr>
</tbody>
</table>

Twenty-week-old mice on regular chow diet were fasted overnight (n≥11 per genotype; all male, unless otherwise indicated); serum values are expressed as the mean ± SEM of measurements obtained. <i>P</i> represents <i>P</i> value of Student’s <i>t</i> tests comparing <i>Par-1</i> mice with WT (asterisk indicates significance). <sup>§</sup> indicates significantly lower adiponectin levels in male mice (<i>P</i>=0.0003); note: this gender difference is lost in both <i>Par-1</i> mice; NEFA: non-esterified fatty acids.
References


Fig. 4.S1  UCP2 and LC3 protein levels in livers from mice on high fat diet and overnight starvation.

Western blot for UCP2 (A.) and LC3 (B.) in overnight fasted liver samples of four different animals per genotype. Weight markers: UCP2, 30kDa; LC3, 15 kDa; actin, 37 kDa.
CHAPTER 5

Summary, Discussion, and Future Directions
Summary

Establishing and maintaining cellular polarity are critical for the homeostasis of unicellular and multicellular organisms. The major goal of my thesis work was to investigate functional interactions between the Par-1b protein kinase and the RNF41 E3 ubiquitin ligase. Atypical Protein Kinase C (aPKC) phosphorylates Par-1b on a conserved threonine residue (T595), and I participated in studies demonstrating that novel Protein Kinase C (nPKC) activates Protein Kinase D (PKD) to directly phosphorylate Par-1b on serine 400 (S400), a residue that is conserved in all four mammalian Par-1 kinases as well as the fly ortholog. Phosphorylation of Par-1b on T595 and S400 causes Par-1b to relocate from membranes to the cytosol and to bind 14-3-3 proteins. In 2004, Brajenovic, et al. isolated human Par-1d along with associated proteins using tandem affinity purification (TAP) (Brajenovic et al., 2004). Nrdp1/RNF41, a RING finger E3 ligase was identified in their screen along with 14-3-3 and aPKC. I found that Par-1b also binds to RNF41 and identified RNF41 as a novel cell polarity determinant. I also showed that Par-1b phosphorylates RNF41 on S254 both in vitro and in vivo. My work has demonstrated that phosphorylation of RNF41 on S254 by Par-1b is necessary for establishing epithelial apical-basal cell polarity.

Discussion

Cell polarity is a structural and functional specialization whose significance is ubiquitous throughout biology. It is especially common across phyla, thus reflecting a fundamental requirement to localize different cellular components to distinct regions of a cell, especially when individual cells come together to form complex multicellular tissues.
The specialized domains of plasma membrane that result from polarization determine cell orientation, function, and fate. For example, cell polarization enables long-range communication in the immune system, transport of ions across epithelial cells, and niche-specific orientation of stem-cell division, which specifies the developmental fate of daughter cells (Mellman and Nelson, 2008).

Par-1b/MARK2 is essential in the establishment of polarity. In the early *C. elegans* embryo, mutation of Par-1, among several other partitioning proteins, disrupts asymmetric divisions, blastomere fates, localization of P granules, and mitotic spindle orientation (Kemphues et al., 1988; Kirby et al., 1990). Some Par-1 mutants have mutations in their kinase domain, suggesting that kinase activity of Par-1 is necessary to establish polarity (Guo and Kemphues, 1995). Additionally overexpression of Par-1b/MARK2 in MDCK cells inhibits apical-basolateral polarization by altering apical protein transport (Cohen and Musch, 2003).

My thesis work has helped identify and investigate a new phosphorylation site (S400) on Par-1b (Watkins et al., 2008). This work demonstrated that phosphorylation on S400 is upregulated with the PKC activator phorbol-12-myristate-13-acetate (PMA) stimulation. PMA activates nPKC, which then phosphorylates PKD. PKD subsequently phosphorylates Par-1b on S400. Par-1b is phosphorylated through two arms of the PKC pathway on T595 and S400 and phosphorylation regulates Par-1b localization and binding to cytoplasmic 14-3-3/Par-5 proteins (Hurov et al., 2004; Watkins et al., 2008). Moreover, our studies using polarized breast epithelial cells (MCF-10A) grown in three-dimensional (3D) culture, we observed enhanced phosphorylation of Par-1b on both S400 and T595 in 3D cultures relative to the two dimensional (2D) cultures. In the future it
would be critical to test if phosphorylation of Par-1b on S400 and T595 are essential for establishing apical/basal epithelial cell polarity or if phosphorylation of Par-1b on S400 and T595 is a result of established cell polarity.

Another goal of my thesis project was to study the Par-1b and RNF41 protein-protein interaction. I identified a new Par-1b substrate, the E3 ubiquitin ligase RNF41 and demonstrated that Par-1b phosphorylates RNF41 both in vivo and in vitro on S254. Knockdown of RNF41 in MCF-10A breast epithelial cells using shRNA revealed a novel role for RNF41 as being essential for establishing apical-basal cell polarity. Moreover, expression of wild-type RNF41 in these cells rescued apical-basal polarity; however, expression of the S254A mutant of RNF41 was not able to do so, suggesting that phosphorylation on S254 is necessary for establishing apical-basal cell polarity and continuing the theme of phosphorylation regulating polarity proteins. Interestingly, orthologs of every Par gene, except the RING finger containing Par-2, have been identified in mammals. Par-2 functions to regulate and stabilize cell polarity in *C. elegans* and shares homology with RNF41 in their RING finger domain and part of their C-terminal domain, leaving open the interesting possibility that RNF41 is an ortholog of Par-2.

We were unable to detect phosphorylation of endogenous RNF41 on S254 when cells were grown in 2D cultures. Given the critical role played by Par proteins in establishing and maintaining apical-basal polarity, we investigated the phosphorylation status of RNF41 in MCF-10A mammary epithelial cells grown in 3D culture and observed enhanced phosphorylation on endogenous RNF41 S254. The observation that cells polarized in 3D culture illustrated the link between phosphorylation of RNF41 on
S254 and establishment of epithelial cell polarity. Furthermore, this observation emphasizes the importance and influence of the cell microenvironment, since cellular context clearly can modulate Par protein function. Another example of this is seen with Par-6 and aPKC, as both of these proteins are concentrated at tight junctions of epithelial cells grown in 2D cultures, but are spread over the apical surface in 3D cultures. Within the much more complicated environment of an organism, such influences are likely to be widespread and might alter signaling inputs to and outputs from the polarity regulating proteins such as Par-1 and RNF41.

Cells can adopt different functions in response to specific physiological contexts, and the plasticity of the loss and re-establishment of cell polarity suggests that common mechanisms are used but executed differently depending on the physiological context. In disease states such as metastatic cancers, epithelial cells lose polarity (one way is through epithelial-mesenchymal transition (EMT)), disengage from multicellular interactions, migrate, and then reintegrate into a second tissue, in which they undergo reorganization to reside at the new site (Thiery and Sleeman, 2006; Wodarz and Nathke, 2007). Cells expressing RNF41 shRNA with and without expression of the S254A mutant of RNF41 do not express the classical protein markers for EMT (low E-cadherin and high Vimentin and SNAIL protein levels); however, these cells may be undergoing a partial EMT and warrant further examination since we have observed that these cells are not able to establish apical-basal polarity in 3D culture.

RNF41 has been studied for its role as an E3 ubiquitin ligase in regulating steady state neuregulin receptor ErbB3 levels (Diamonti et al., 2002). ErbB3 is overexpressed in a subset of breast cancers. Moreover, there is a correlation seen between decreased
RNF41 levels and higher levels of ErbB3 in both an *in vivo* transgenic mouse model of ErbB2-induced mammary tumorigenesis and in a panel of primary breast tumors (Yen et al., 2006). While one mechanism for proliferation and establishment of these tumors may be due to increased levels of ErbB3, loss of RNF41 and thus lack of cell polarity, may also contribute to this process.

Over 85% of fatal malignancies in adults in the USA arise from epithelial tissues (Bryant and Mostov, 2008), and loss of polarity is a hallmark of increased malignancy. Furthermore, acute injury of major epithelial organ systems is collectively one of the most important causes of death worldwide (Fausto et al., 2006; Liano and Pascual, 1996; Matthay and Zimmerman, 2005). Understanding polarization of epithelia, therefore, is important in analyzing the response of a tissue to injury and in establishing prospects for regenerative medicine. Many organs, such as the kidney, lung, and liver, can recover from injury. In the case of the kidney, this involves the local proliferation of epithelial cells which replace their dead neighbors in a process that involves partial EMT (Bryant and Mostov, 2008). Learning how to improve the response to injury by controlling the polarity state of cells offers enormous possibilities to enhance human health.

Understanding cell polarization is one of the major goals of cell biology and will inevitably have a broad impact not only at the level of basic science but also in understanding diseases such as cancer. Work from many laboratories has uncovered a complicated web of signaling systems that surround and intersect with the Par proteins, yet we still understand very little about what the Par proteins do, how they are localized, how their various interactions are regulated, and which signaling components operate in which contexts. After all, the organization of a polarized cell is a complicated process.
that involves cytoskeletal remodeling, membrane traffic, and protein complex assembly and disassembly, with feedback to gene expression and protein turnover.

**Future Directions**

The C-terminus of RNF41 binds to the Rhodanese domain of USP8 (the enzyme which deubiquitinates and thus stabilizes RNF41 protein) and the crystal structure of this heterodimer has previously been published (Avvakumov et al., 2006). After examining the crystal structure of the USP8 binding pocket on the C-terminus of RNF41, we noticed that S254 on RNF41 resides in this pocket and hypothesize that phosphorylation on S254 may affect USP8 binding. Therefore, I will immunoprecipitate wild-type and the S254A mutant of RNF41-FlagHis6 from MCF-10A cells grown in 3D culture, and I will Western blot for USP8 in order to compare the levels of binding of USP8 to RNF41. I hypothesize that phosphorylated RNF41 may bind less USP8 than wild-type or the S254A mutant. If this is true, then the levels of phosphorylated RNF41 should be lower than the non-phosphorylated form. However, if the S254A mutant of RNF41 prevents phosphorylation and still binds to USP8, then its protein levels should be higher than wild-type RNF41. An alternative hypothesis is that phosphorylated RNF41 may bind more USP8 than wild-type or the S254A mutant of RNF41. If this is the case, then the phosphorylated form of RNF41 should be stabilized and the S254A mutant of RNF41 should bind less USP8, and therefore its levels should be lower than the phosphorylated form of RNF41.

During my thesis work I have shown that Par-1b binds and phosphorylates RNF41 on S254 *in vitro*. Furthermore, RNF41 is phosphorylated on S254 *in vivo* and overexpression of kinase active Par-1b enhances this phosphorylation *in vivo* while
kinase dead Par-1b does not. I have generated MCF-10A cells which stably express two separate shRNA hairpins for human Par-1b and have shown that Par-1b protein levels are reduced by greater than 90% in these cells. Thus, I will culture these cells in 3D and subsequently Western blot for pS254 RNF41. I hypothesize that pS254 RNF41 levels will decrease in these cells compared to pS254 levels in control MCF-10A cells; however, it is possible that I may not detect a decrease in the level of pS254 RNF41 due to compensation by other mammalian Par-1 orthologs. We know from our study and from work by others that both Par-1b and Par-1d bind to RNF41 (Brajenovic et al., 2004). Thus, it is possible that other mammalian Par-1 proteins may also bind to RNF41 and there likely may be some compensation by these other Par-1 proteins when Par-1b is depleted in cells.

After examining our work with Par-1b and RNF41, still the question remains, how does RNF41 regulate apical-basal cell polarity? Recently, work by others has shown that Par-1b kinase activity and protein localization is essential for regulating basement membrane placement. Furthermore, this study also showed that establishment of the correct localization of basement membrane is required for the coordination of tissue polarity (Daley et al, 2012.). Epithelial cell organization and function require positional information from the surrounding microenvironment (Bryant and Mostov, 2008); such cues include contacts with both adjacent cells and the basement membrane. I have preliminary data which suggests that loss of RNF41 results in the loss of Laminin V deposition (basement membrane) on the cell surface of the MCF-10A acini where it is needed to establish apical-basal polarity. Moreover, Laminin V accumulation is rescued when wild-type RNF41 is overexpressed; however, not when cells are rescued with the
S254A mutant of RNF41 suggesting that phosphorylation of RNF41 on S254 is necessary for Laminin V deposition. In the future, I will test if accumulation of other basement membrane proteins (such as collagen IV, fibronectin, and perlecan) is affected by the loss of RNF41. This will be determined by Western blot, immunofluorescence microscopy, and qPCR for basement membrane proteins and genes. I hypothesize that the loss of apical-basal polarity observed in cells expressing RNF41 shRNA is due to the loss of endogenous basement membrane accumulation. Thus, I will culture the MCF-10A cell lines expressing Luc shRNA, RNF41 shRNA, RNF41 shRNA + wild-type RNF41, or RNF41 shRNA + S254A RNF41 in 3D culture using Matrigel instead of collagen type I and stain for apical and basal polarity markers in order to determine if apical-basal polarity is rescued when these cells are supplemented with exogenous basement membrane proteins.

Another possible explanation for the lack of established apical-basal cell polarity in cells expressing RNF41 shRNA and the S254A mutant of RNF41 is that S254A RNF41 is mislocalized. Preliminary data indicates that wild-type RNF41 overexpressed in MCF-10A cells cultured in collagen type I matrix is localized to basolateral membranes; however, the S254A mutant of RNF41 is mislocalized and accumulates in the cytoplasm. Future experiments will also be directed at determining if S254 phosphorylation regulates the intracellular localization of RNF41. The failure of the S254A mutant of RNF41 to localize properly or to bind to key substrates would account for its failure to rescue polarity in RNF41 deficient MCF-10A cells.

Finally, in the future I propose to determine how RNF41 regulates basement membrane (Laminin V) deposition. Previous work has demonstrated that Par-1b kinase
activity is essential for cell surface assembly of laminins by regulating one of the laminin receptors, the dystroglycan (DG) complex (Masuda-Hirata et al., 2009). I propose to investigate if RNF41 plays a role in laminin receptor assembly and/or function in order to regulate basement membrane accumulation on the cell surface. Determining how RNF41 regulates cell polarity and basement membrane deposition is critical for numerous reasons; however, one implication may be in cancer as loss of apical-basal polarity is a characteristic of tumor cells. Also, RNF41 protein levels have previously been shown to be low in some breast cancers. Preliminary data suggests that low RNF41 protein levels results in loss of basement membrane proteins; thus one might hypothesize that without basement membrane tumor cells not only are incapable of establishing apical-basal polarity, but may also be more likely to escape and metastasize.
References


