

Diacylglycerol Kinase ι Mediates Actin Cytoskeletal Reorganization by Regulating the Activities of RhoC and Rac1

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Authorization

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Abstract

Cell migration is required for a number of physiological processes and is implicated in pathologies such as tumor metastasis. Cell motility is dependent upon dynamic actin reorganization, and is regulated by the Rho family of small GTPases. Rho GTPases are molecular switches that cycle between their active and inactive conformations. The best-studied members of this family are Rac1, RhoA, and Cdc42. Each is responsible for the formation of specific actin structures. Diacylglycerol kinases (DGKs) act at the membrane to convert diacylglycerol (DAG) and phosphatidic acid (PA), maintaining the balance of these two lipid second messengers. Previous studies from our lab have demonstrated that the ζ isoform of DGK facilitates the release of Rac1 and RhoA from their inhibitor, RhoGDI. Here we studied a closely related isoform, DGK ι , using mouse embryonic fibroblasts (MEFS) in which the gene for DGK ι had been deleted. Aberrations in cell morphology, spreading, and migration were identified in DGK ι -null MEFs. We showed that the activity of Rac1 and RhoC, but not RhoA, was impaired in the absence of DGK ι , yet only RhoC protein levels were affected. Reduced activation of these Rho GTPases was accompanied by defects in Rac1- and RhoC-related actin structures. These data demonstrate that DGK ι , in addition to DGK ζ , contributes to the regulation of GTPase activation and remodeling of the actin cytoskeleton.

La migration cellulaire est essentielle pour de nombreux processus physiologiques tels que les métastases tumorales. De plus, la motilité cellulaire dépend

de la réorganisation dynamique du cytosquelette d'actine régulé par la famille des GTPases Rho. Les GTPases Rho sont des aiguillages moléculaires qui cyclent entre leurs conformations actif et inactif. Les membres les plus caractérisés de cette famille sont Rac1, RhoA, et Cdc42; chacun est responsable de la formation des structures d'actine spécifiques. Les kinases diacylglycerol agissent sur la membrane pour convertir le diacylglycérol (DAG) en acide phosphatidique (PA) maintenant un équilibre entre ces deux lipides. Les études antérieures menées par notre laboratoire ont démontré que l'isoforme ζ de la kinase diacylglycérol facilite la libération de Rac1 et RhoA de leur inhibiteur, RhoGDI. Nous avons donc étudié l'isoforme diacylglycérol ι , étroitement liés à l'isoforme ζ , dans des fibroblastes embryonnaires murins dans lesquelles le gène pour la kinase diacylglycérol ι a été supprimé. Des aberrations dans la morphologie cellulaire, la propagation, et la migration ont été observé dans les fibroblastes. Nous avons également démontré que l'activité de Rac1 et RhoC, mais pas l'activité de RhoA, a été affectée par l'absence de la kinase diacylglycérol ι . Par contre, seuls les niveaux de RhoC ont été atteints. Particulièrement, une réduction de l'activation des GTPases Rho a été accompagnée par des défauts dans les structures d'actine, qui est liée à l'activité de Rac1 et RhoC. Ces résultats démontrent que la kinase diacylglycérol ι , comme la kinase diacylglycérol ζ , contribuent à la régulation de l'activation des GTPases et la réorganisation du cytosquelette d'actine.

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List of Abbreviations

bp	Base pair
cDNA	Complementary deoxyribonucleic acid
DAG	Diacylglycerol
DGK	Diacylglycerol kinase
DMEM	Dulbecco's modified Eagle's medium
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
F-actin	Filamentous actin
G-actin	Globular actin
GAP	GTPase activating protein
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
HA	Hemagglutinin
HRP	Horseradish peroxidase
IB	Immunoblot
Ig	Immunoglobulin
IP	Immunoprecipitation
KD	Kinase dead
LPA	Lysophosphatidic acid
MARCKS	Myristoylated alanine-rich C kinase substrate

MEF	Mouse embryonic fibroblast
PA	Phosphatidic acid
PAK1	p21-activated kinase 1
PBD	p21-binding domain
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDZ	Postsynaptic density 95, Discs Large, zonula, occludens (ZO-1)
PDZ-bm	PDZ-binding motif
PFA	Paraformaldehyde
PKC α	Protein kinase C α
PKN-B	Protein kinase B
PVDF	Polyvinylidene fluoride
RhoGDI	Rho guanine nucleotide dissociation inhibitor
RNA	Ribonucleic acid
RBD	Rho-binding domain
ROCK	Rho-associated protein kinase
RSB	Reducing sample buffer
S, Ser	Serine
SEM	Standard error of the mean
TBS-Tween	Tris-buffered saline Tween
WT	Wild type

Acknowledgements

While this project has been filled with challenges, it has taught me many lessons about determination, perseverance, and the value of hard work. I have greatly enjoyed my time spent uncovering the mysteries of DGK α , and I look forward to the future knowing that this experience has prepared me well for what is to come.

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Chapter 1

Introduction

Cell migration and the actin cytoskeleton

Cell migration is essential to a number of physiological processes including embryogenesis, development, wound healing, and immune response (Lauffenburger and Horwitz, 1996; Burridge and Wennerberg, 2004). Furthermore, aberrant cell migration is implicated in pathogenesis, as occurs with tumor metastasis (Yamaguchi et al., 2005; Condeelis et al., 2005). Cell motility is dependent upon rearrangement of the actin cytoskeleton, a network of microfilaments beneath the plasma membrane (Wickstead and Gull, 2011). Actin microfilaments are composed of globular actin (G-actin) monomers that polymerize to form filamentous actin (F-actin; Holmes et al., 1990; Dominguez et al., 2011). During migration, actin filaments are rapidly reorganized to form cellular structures that facilitate motility in response to signals from the extracellular environment.

The leading and trailing edges of motile cells, regions at the front and rear of the cell, respectively, are each associated with the formation of specific actin-rich structures and cause cells to adopt a polarized cell morphology that allows for their translocation (Figure 1; Lauffenburger and Horwitz, 1996). Actin polymerization at the leading edge is required for the formation of protrusive structures that explore the extracellular environment and form new attachments to the underlying matrix, responding to extracellular chemotactic cues to guide the direction of cell migration (Small et al., 2002; Ballestrem et al., 1998; Xue et al., 2010; Krause and Gautreau, 2014). These structures include lamellipodia, filopodia, and peripheral membrane ruffles. Lamellipodia are broad, sheet-like projections that contain a highly branched network of F-actin (Small et al., 2002; Krause and Gautreau, 2014). Peripheral membrane ruffles are often observed along

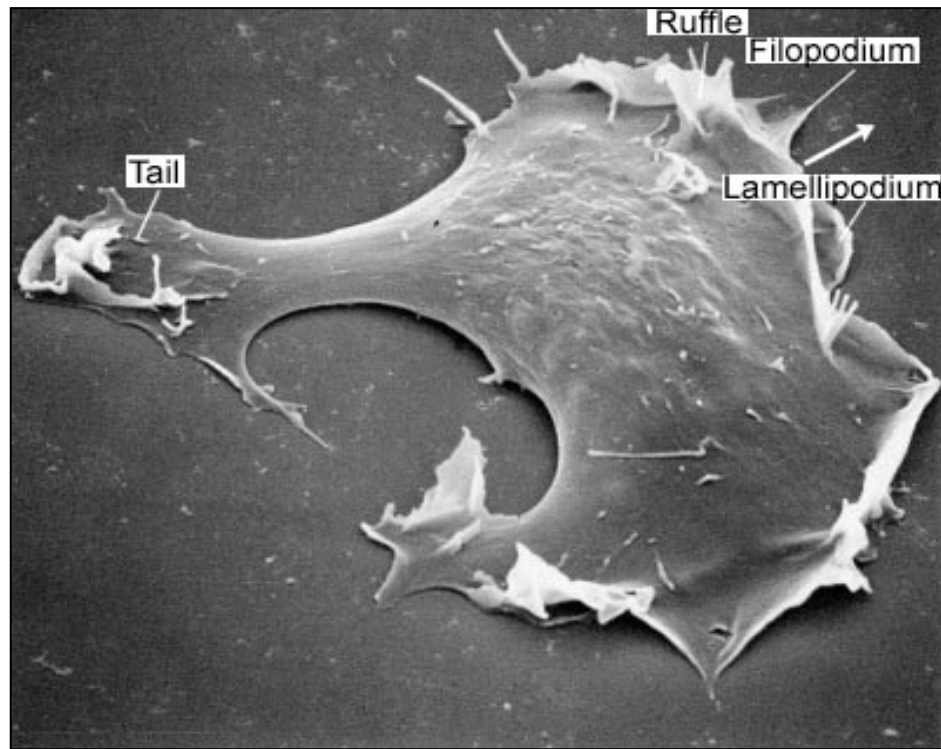


Figure 1. Specific cellular structures promote cell motility. The leading and trailing edges of migrating cells are associated with specific actin structures that facilitate motility. Lamellipodia, membrane ruffles, and filopodia are produced at the leading edge to guide migration and form new attachments to the substrate. Contractile actin stress fibers are formed within the body of the cell and contract during migration bringing the trailing edge forward.

the edges of growing lamellipodia, as rapid actin reorganization causes the plasma membrane to lift from the cytoskeleton (Abercrombie et al., 1970; Ridley et al., 1992). Lastly, filopodia are narrow, finger-like projections that extend from lamellipodia to guide migration (Hall, 1998; Xue et al., 2010). Together, these structures drive protrusion at the leading edge of migrating cells and form new attachments to the substrate to initiate forward cellular movement.

Actin structures within the cell body coordinate trailing edge retraction with the protrusive activity at the leading edge. Contractile bundles of F-actin known as stress fibers span the length of the cell and contract during migration, bringing the trailing edge forward. At the ends of stress fibers, dynamic multi-protein complexes known as focal adhesions assemble to form attachments to the extracellular matrix that provide traction during migration by anchoring the cell to the substrate (Hu et al., 2007). Focal adhesions are most abundant along the cell periphery and act as sites of integrin signaling where information is exchanged between the intracellular and extracellular environments (Geiger et al., 2009; Parsons et al., 2010). During migration, signal transduction pathways that regulate actin structure formation are spatiotemporally integrated to ensure leading edge protrusion is coordinated with stress fiber contraction and focal adhesion detachment at the trailing edge (Lauffenburger and Horwitz, 1996).

Rho GTPases regulate actin reorganization

Actin cytoskeletal reorganization is regulated by the Rho family of small GTPases (Etienne-Manneville and Hall, 2002; Charest and Firtel, 2007). In addition to migration, Rho GTPases are involved in the regulation of cellular processes such as mitosis,

apoptosis, and proliferation (Ridley and Hall, 1992; Ridley et al., 1992; Norman et al., 1994; Ridley et al., 1994; Chrzanowska-Wodnicka and Burridge, 1996; Machesky and Hall, 1997; Subauste et al., 2000). The best-studied members of the Rho GTPase family are Rac1, RhoA, and Cdc42. Early studies in which constitutively active Rho GTPase mutants were expressed in cultured fibroblasts showed that each Rho GTPase is responsible for the formation of specific actin structures (Madaule and Axel, 1985; Hall, 1998). Active Rac1 induces the formation of lamellipodia and membrane ruffles (Ridley et al., 1992), while RhoA stimulates the assembly of stress fibers and focal adhesions (Paterson et al., 1990; Ridley et al., 1992), and Cdc42 promotes filopodia extension (Figure 2; Nobes and Hall, 1995).

Initial models of cell migration localized the activities of Rac1 and Cdc42 to the leading edge during lamellipodia and filopodia extension, and RhoA activity within the cell body during actin contraction (Nobes and Hall, 1999; Wang et al., 2002; Yamana et al., 2006; Chrzanowska-Wodnicka et al., 1996). More recent studies using Rho GTPase biosensors have shown that active forms of all three Rho GTPases are present within micrometers of the leading edge to regulate actin dynamics during cell protrusion (Machacek et al., 2009; Pertz et al., 2006). The activities of Rac1, RhoA, and Cdc42 within this region are precisely regulated, both spatially and temporally, through feedback mechanisms and Rho GTPase crosstalk, indicating that Rho GTPase signaling during migration is much more complex than initially described (Spiering and Hodgson, 2011).

Increased Rho GTPase expression is associated with increased activity and occurs in a number of human cancers (Karlsson et al., 2009). Overexpression of Rac1 and RhoA

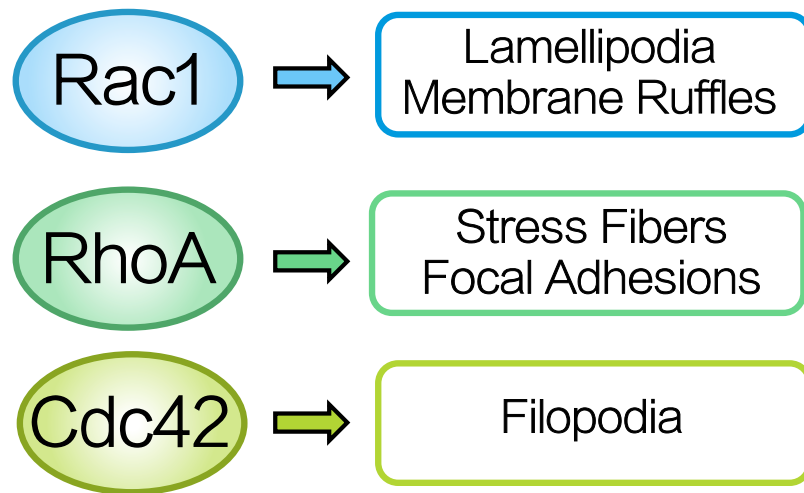


Figure 2. Rho GTPases regulate the formation of specific actin structures. The best-studied Rho GTPases are Rac1, RhoA, and Cdc42. When active, each is thought to be responsible for the formation of specific actin structures. Active Rac1 induces the formation of lamellipodia and membrane ruffles, while RhoA activity induces stress fiber and focal adhesion formation and Cdc42 activation results in the extension of filopodia.

is implicated in cell transformation and tumor metastasis due to the involvement of these Rho GTPases in both cell cycle progression and cell migration (Karlsson et al., 2009; Jaffe and Hall, 2005; Friedl and Wolf, 2003). RhoA shares 85% amino acid identity with RhoB and RhoC, two Rho isoforms that are also involved in cancer progression (Wheeler and Ridley, 2004). RhoC activity promotes metastasis through the formation of invadopodia, extensions that degrade the extracellular matrix to promote invasion (Bravo-Cordero et al., 2011). In contrast to RhoA and RhoC, increased RhoB expression is thought to suppress tumor formation by negatively regulating cell cycle progression, indicative of differential roles for structurally similar Rho GTPases during pathogenesis (Chen et al., 2002; Liu et al., 2001; Ridley, 2013).

Regulation of Rho GTPase activation

Rho GTPases are molecular switches that cycle between their active and inactive conformations (Figure 3; Bourne et al., 1991; Hall, 1992). When bound to GTP, Rho GTPases are in their active conformation and stimulate downstream effectors to produce changes in actin dynamics (Nobes and Hall, 1994). Hydrolysis of GTP to GDP causes Rho GTPases to become inactive, incapable of stimulating actin reorganization (Nobes and Hall, 1994). The cycling of Rho GTPases between their active and inactive conformations is mediated by a large number of regulatory proteins, including guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), and Rho guanine nucleotide dissociation inhibitor (RhoGDIs). GEFs stimulate the activation of Rho GTPases by facilitating the exchange of GDP for GTP (Rossman et al., 2005), while GAPs increase the intrinsic hydrolytic activity of Rho GTPases to induce their

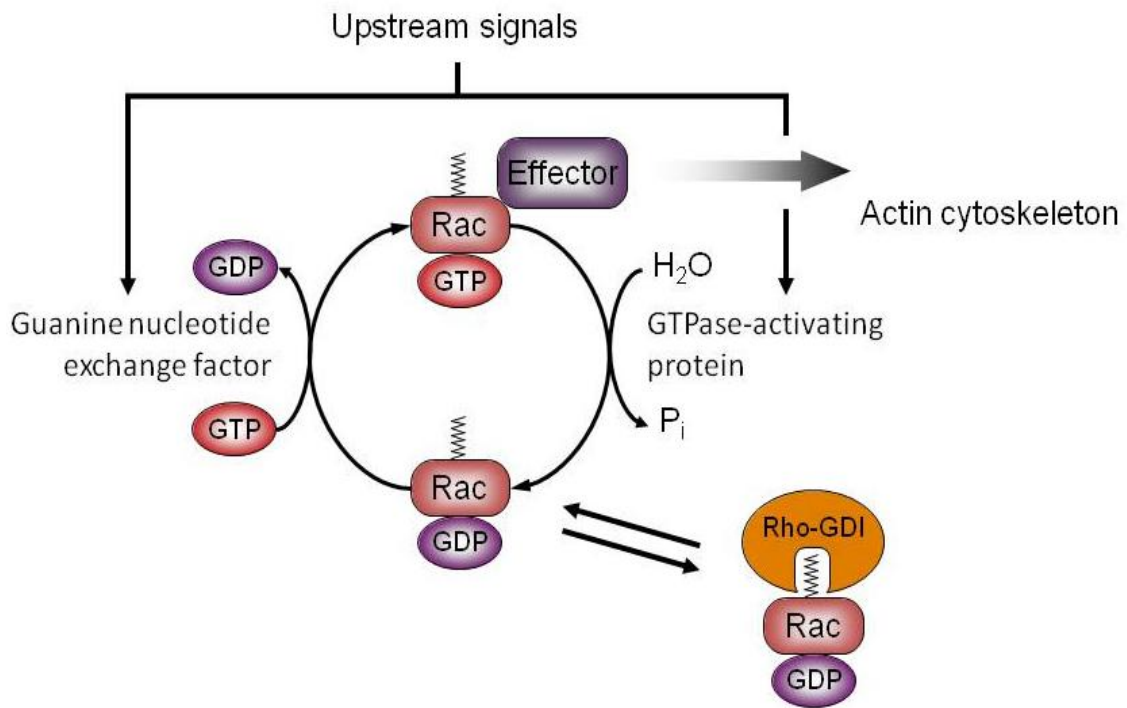


Figure 3. Rho GTPase cycle of activation. Rho GTPase activation is regulated by guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), and Rho guanine nucleotide dissociation inhibitor (RhoGDI). GEFs increase Rho GTPase activation by facilitating the exchange of GDP for GTP, while GAPs increase the intrinsic hydrolytic activity of Rho GTPases to induce their inactivation. RhoGDI binds and sequesters Rho GTPases in inactive, cytosolic complexes. Upon activation, Rho GTPases are capable of interacting with their effectors to stimulate changes in actin dynamics. (Reproduced with permission from Dr. Stephen Gee).

inactivation (Gibbs et al., 1990). RhoGDI provides a third level of regulation over Rho GTPase activation by binding and sequestering these proteins in inactive, cytosolic complexes (Hart et al., 1992; Chuang et al., 1993; DerMardirossian et al., 2005). Over 70 GAPs and 80 GEFs have been identified as contributing to the regulation of Rho GTPase activation, while only three mammalian isoforms of RhoGDI are known (Tcherkezian et al., 2008; Rossman et al., 2005; Garcia-Mata et al., 2011).

Membrane localization is required for Rho GTPase activation and is facilitated by a post-translational modification in which an isoprenyl lipid moiety is attached to the C-terminus of these proteins (Figure 4; Winter-Vann et al., 2005; Clarke, 1992). Rho GTPases contain a C-terminal C-A-A-X sequence in which C represents a cysteine residue, A represents an aliphatic residue, and X represents any amino acid (Hancock et al., 1989). During isoprenylation, a geranylgeranyl lipid moiety is covalently attached to the cysteine residue and the -A-A-X portion of the peptide is cleaved (Mitin et al., 2012). Mutations within the C-A-A-X sequence that prevent isoprenylation inhibit both the activation and membrane localization of Rho GTPases and lead to a cytosolic accumulation of these proteins in their inactive conformation (Winter-Vann et al., 2005).

While necessary for their membrane localization, isoprenylation reduces the stability of Rho GTPases in the aqueous cytosol and causes them to be targeted for degradation by the proteasome (Boulter et al. 2010; Boulter and Garcia-Mata, 2012). The crystal structures of RhoGDI in complex with Rac1, RhoA, and Cdc42 demonstrate RhoGDI inhibits each through a conserved physical interaction (Fig. 5; Wei et al., 1997; Longenecker et al., 1999; Grizot et al., 2001; Hoffman et al., 2000; Scheffzek et al., 2000; Boulter and Garcia-Mata, 2012). RhoGDI consists of two structurally distinct

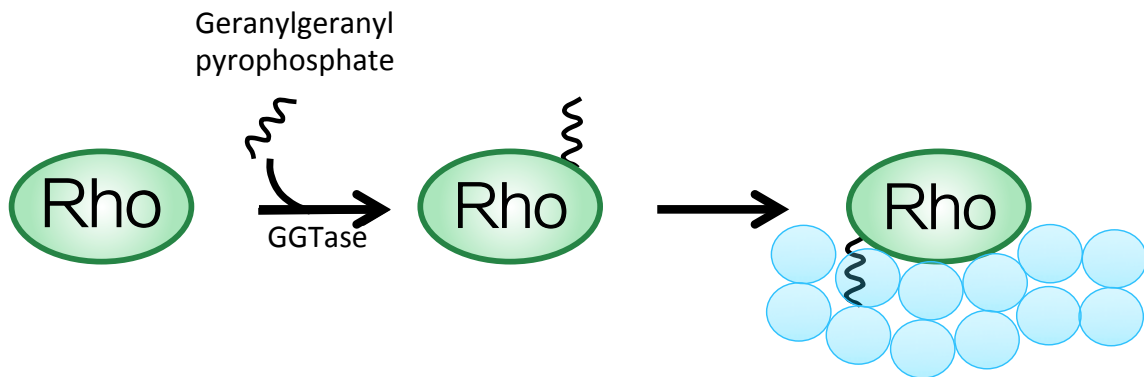


Figure 4. Rho GTPase isoprenylation. Rho GTPases are post-translationally modified through the addition of a geranylgeranyl lipid moiety to their C-terminal –C-A-A-X motif. Isoprenylation occurs by a geranylgeranyl transferase. Upon attachment of the lipid moiety, Rho proteins translocate to the surface of the ER, the –A-A-X portion of the peptide is cleaved, and a C-terminal methyl group is added. RhoGDI then binds to isoprenylated Rho GTPases to localize them to the plasma membrane. (Boulter et al., 2012, reproduced with permission from the FASEB publishing group).

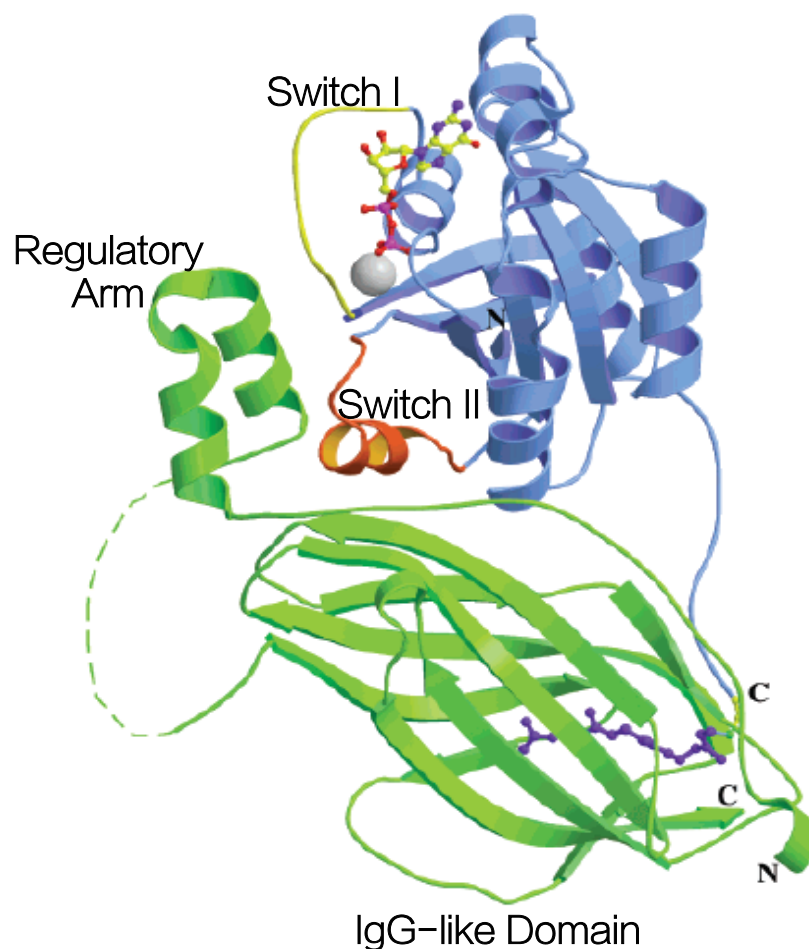


Figure 5. Rac1 binding to RhoGDI. RhoGDI inhibits Rac1, RhoA, and Cdc42 through a conserved physical interaction. Rac1 is represented in blue, with the switch I and switch II domains in yellow and red, respectively. RhoGDI is shown in green. The geranylgeranyl group at the C-terminus of Rac1 is represented in ball and sticks, as is the GDP molecule in contact with the switch domains of Rac1. (Grizot et al., 2001, reproduced with permission from the Biochemistry publishing group).

regions, a flexible N-terminal regulatory arm and a C-terminal immunoglobulin-like (IgG-like) domain (Keep et al., 1997). The regulatory arm of RhoGDI associates directly with the switch I and switch II domains of Rho GTPases responsible for guanine nucleotide binding (Scheffzek et al., 2000). This interaction prevents the exchange of GDP for GTP by inactive Rho GTPases while interfering with GTP hydrolysis by Rho GTPases in their active state (DerMardirossian et al., 2005). Furthermore, the IgG-like domain of RhoGDI creates a hydrophobic pocket into which the C-terminal isoprenyl tail of Rho GTPases is inserted and shielded from the cytosol, effectively increasing Rho GTPase protein stability (Wei et al., 1997; Grizot et al., 2001; Hoffman et al., 2000; Boulter and Garcia-Mata, 2012). Given that the cellular concentration of RhoGDI is similar to that of Rac1, RhoA, and Cdc42 combined, these molecules must compete against one another for RhoGDI-binding to evade degradation (Michaelson et al, 2001; DerMardirossian and Bokoch, 2005). By binding and sequestering these proteins in cytosolic complexes, RhoGDI maintains cellular concentrations of inactive Rho GTPases and allows for rapid initiation of actin reorganization in response to extracellular stimulation.

Multi-Protein signaling complexes specifically release RhoA and Rac1 from RhoGDI

Phosphorylation of RhoGDI by protein kinases is a common theme in the regulation of Rho GTPase activation (Garcia-Mata et al., 2011). It has been suggested that phosphorylation of RhoGDI at particular sites may act as a “dissociation code” to signal the release of a particular Rho GTPase while maintaining others in inhibitory complexes (DerMardirossian and Bokoch, 2005). The current working model put forth by

our laboratory proposes that RhoGDI binds and inhibits Rac1 and RhoA within larger multi-protein signaling complexes that are unique to each (Figure 6). The release of Rac1 from RhoGDI is regulated by p21-activated kinase 1 (PAK1), an auto-inhibited homodimer activated in response to platelet-derived growth factor (PDGF) as well as phosphatidic acid (PA; DerMardirossian et al., 2004; DerMardirossian and Bokoch, 2006; Lei et al., 2000; Parrini et al., 2000; Bokoch et al., 1998). PAK1 forms a complex with Rac1 and RhoGDI and, when active, phosphorylates RhoGDI at Ser-101 and -174 (DerMardirossian et al., 2004; DerMardirossian and Bokoch, 2006). Phosphorylation at these sites induces a conformational change that causes RhoGDI to specifically release Rac1, allowing for its activation (DerMardirossian and Bokoch, 2006).

Prior studies identified diacylglycerol kinase (DGK) activity in the Rac1-RhoGDI complex (Tolias et al., 1998). DGKs phosphorylate diacylglycerol (DAG) to yield PA (Rincón et al., 2012), an activator of PAK1 (Bokoch et al., 1996). After identifying a direct interaction between diacylglycerol kinase zeta (DGK ζ) and Rac1 (Yakubchik et al., 2005), our lab demonstrated that DGK ζ interacts with Rac1, RhoGDI, and PAK1 to regulate Rac1 activation within in a larger multi-protein complex (Abramovici et al., 2009). Within this complex the catalytic activity of DGK ζ acts as the source of PA required for PAK1 activation that leads to RhoGDI phosphorylation and Rac1 release (Fig. 7; Abramovici et al., 2009).

The dissociation of RhoA from RhoGDI is regulated by a separate protein kinase, protein kinase C alpha (PKC α), which binds and phosphorylates RhoGDI at Ser-34 (Dovas et al., 2010). Ser-34 phosphorylation induces a conformational change in RhoGDI that specifically dissociates RhoA, leaving Rac1 and Cdc42 inhibition unaffected (Dovas

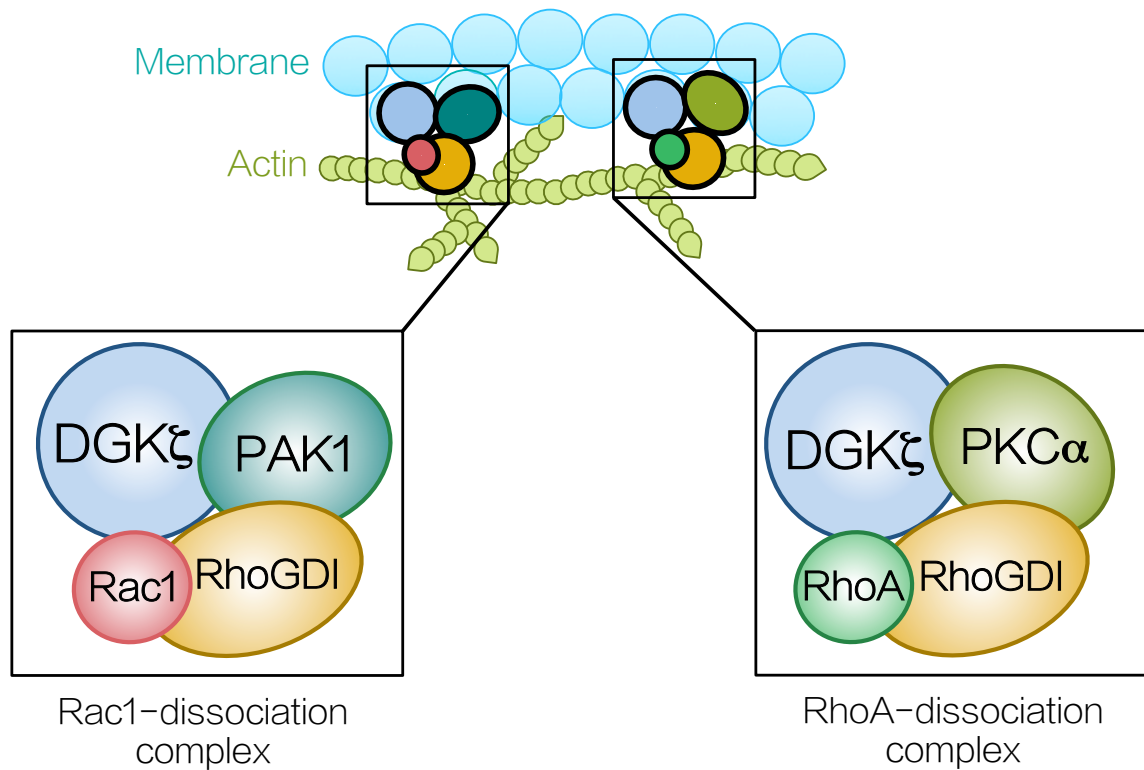


Figure 6. DGK ζ forms multi-protein signaling complexes with Rac1 and RhoA.

DGK ζ acts at the membrane to link lipid signaling to actin cytoskeletal reorganization.

DGK ζ is involved in multi-protein signaling complexes unique to Rac1 and RhoA that stimulate their dissociation from RhoGDI.

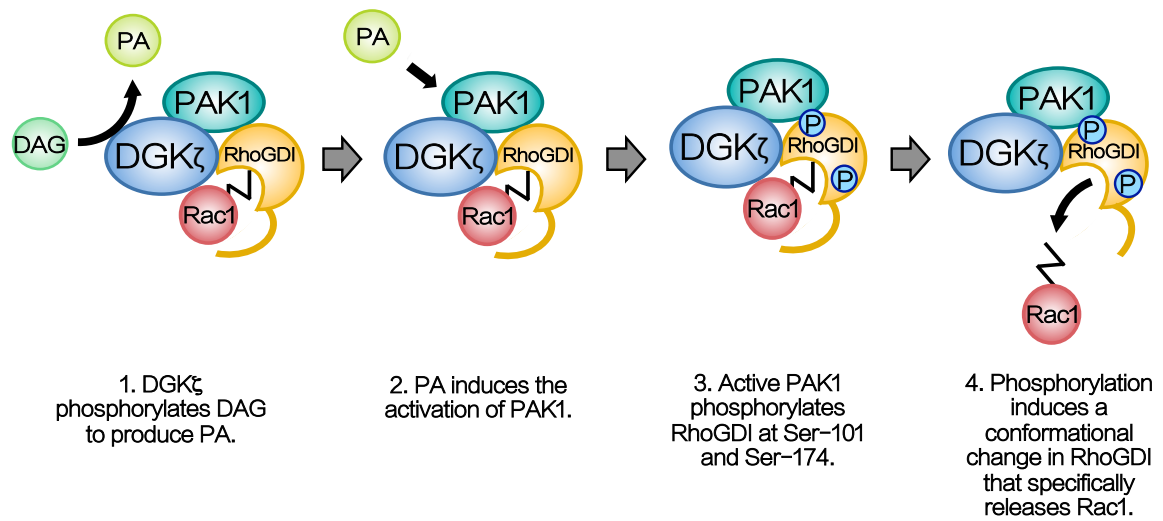


Figure 7. A multi-protein complex specific to Rac1 regulates its release from RhoGDI. DGK ζ associates with PAK1 in a larger protein complex to regulate the specific dissociation of Rac1 from RhoGDI. PA generated by DGK ζ stimulates that activation of PAK1 which then phosphorylates RhoGDI on Ser-101 and -174 to induce a conformational that specifically releases Rac1. Rac1 then localizes to the to be activated by GEFs and stimulate changes in actin dynamics through signaling to downstream effectors.

et al., 2010). Similar to the Rac1-dissociation complex, our lab demonstrated that RhoA, RhoGDI, and PKC α associate within a larger protein complex that also requires DGK ζ (Figure 8; Ard et al., 2012). The catalytic activity of DGK ζ is dispensable for its role in regulating the release of RhoA from RhoGDI, as it acts primarily as a scaffold to facilitate the interaction between other members of the RhoA-specific dissociation complex (Ard et al., 2012). Regulation of Rac1 and RhoA release from RhoGDI by multi-protein signaling complexes unique to each allows for these Rho GTPases to be activated independent of one another, both spatially and temporally, despite their shared inhibitor.

Diacylglycerol Kinases

Currently, ten mammalian genes for DGK have been identified (Figure 9; Rincón et al., 2012). In stimulating the conversion of DAG to PA, DGKs maintain the balance between these lipid second messengers, stimulating signaling by PA-activated pathways while attenuating DAG-related signaling (Figure 10; Mérida et al., 2008). DGKs are divided into five families, or types, based on their shared functional domains (Sakane et al., 2007). All ten DGKs contain a highly conserved catalytic domain as well as two or three C1-domains in their N-terminal region. Type I DGKs, DGKs α , β , and γ , contain an EF hand domain and an N-terminal recoverin homology domain. DGKs δ , η , and κ , the type II isoforms, are characterized by their N-terminal pleckstrin-homology domain. The only type III isoform, DGK ϵ , is identified by its N-terminal hydrophobic motif. The type IV DGKs are DGK ζ and DGK ι , distinguished by their

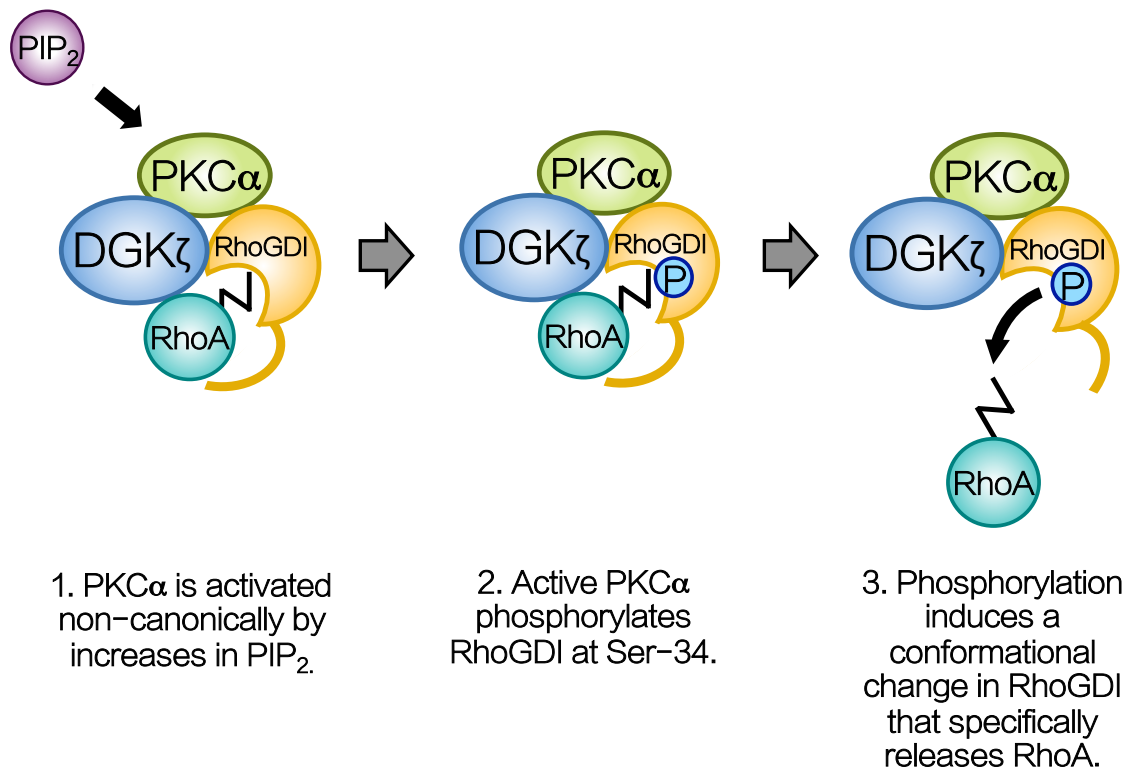


Figure 8. A unique multi-protein complex regulates RhoA release from RhoGDI.

DGKζ associates with PKCα, RhoGDI, and RhoA within a protein complex specific to RhoA that regulates its dissociation from RhoGDI. In this complex, DGKζ acts as a scaffold to facilitate the interaction of other members within the complex. Active PKCα phosphorylates RhoGDI on Ser-34 to induce a conformational change that specifically releases RhoA. RhoA then localizes to the membrane to be activated by GEFs and interact with downstream effectors.

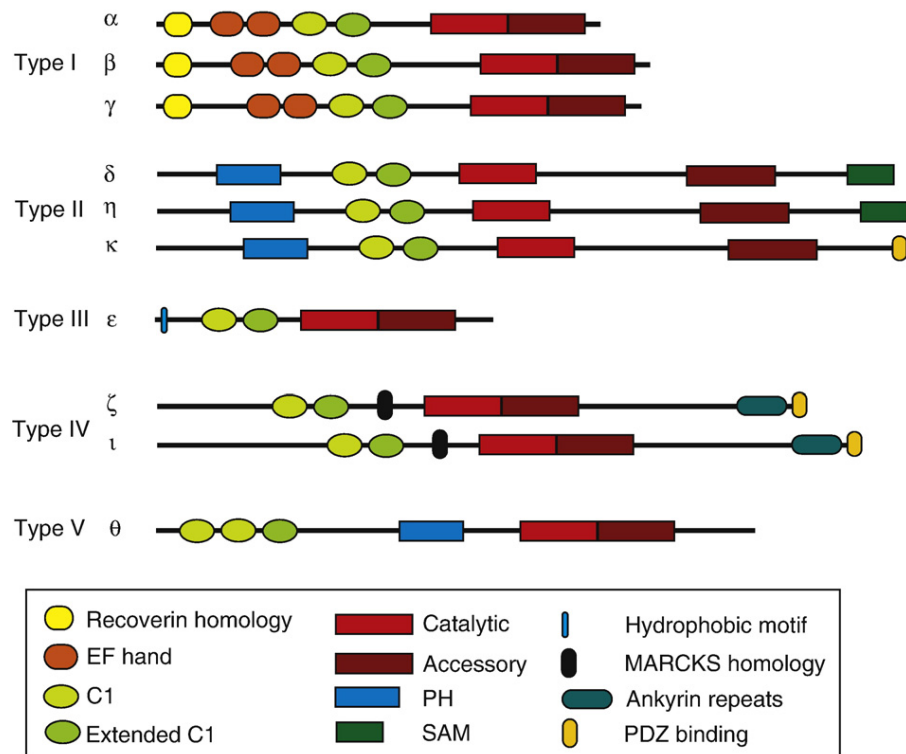


Figure 9. Mammalian DGKs. Ten mammalian DGKs have been identified and categorized into five families based on their shared functional domains. Type IV DGKs, DGK ζ and DGK ι , are those studied by our laboratory. (Cai et al., 2009, reproduced with permission from the Elsevier publishing group).

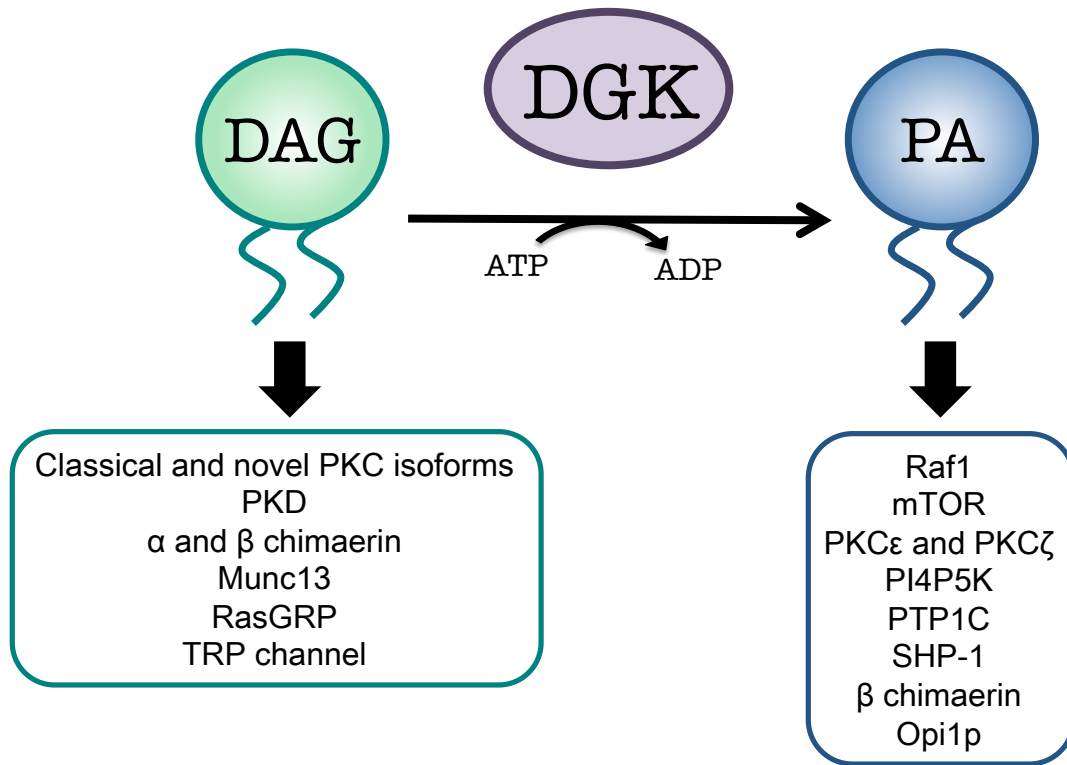


Figure 10. DGKs regulate the balance between DAG and PA lipid second messengers. DGKs phosphorylate DAG to produce PA. Each of these lipid second messengers regulates a distinct set of signaling pathways. In converting DAG to PA, DGKs attenuate signaling by DAG while activating pathways mediated by PA.

myristoylated alanine-rich C kinase substrate (MARCKS) domain, four ankyrin repeats, and a C-terminal PDZ-binding motif. Lastly, DGK θ is the only type V DGK and is identified by its pleckstrin-homology domain in addition to being the only isoform with three C1-domains. While each isoform is the product of a separate gene, many DGKs are alternatively spliced to further increase the diversity of these proteins (Sakane et al., 2007). DGKs are evolutionarily conserved among lower order organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans*, however these organisms have only one DGK isoform of each type suggesting no functional redundancy between DGKs of different types (Shulga et al., 2011).

The catalytic domain is highly conserved among DGK isoforms and is comprised of a catalytic and an accessory subunit (Kano et al., 2002; van Blitterswijk, 2000). These subunits form an uninterrupted catalytic domain in all DGKs except for DGK δ , η , and κ , the type II isoforms, in which the catalytic and accessory subunits are separated by a sequence of amino acids with no known function (Cai et al., 2009). In addition to their catalytic domain, all DGKs possess either two or three C1-domains homologous to those of PKCs (Hurley et al., 1997). In PKCs these domains facilitate binding to DAG (Newton, 2001). While DAG-binding by the C1-domains of DGK was not detected initially (Sakane et al., 1996), these regions in DGK β and DGK γ associate with DAG-analogues such as phorbol esters (Shindo et al., 2003; Santos et al., 2002) and have been speculated to act as weak DAG sensors that allow the catalytic domain to be brought in proximity with its substrate (Los et al., 2004). Instead of binding to DAG, the C1-domains of DGK serve as protein-protein interaction domains (Colón-González et al.,

2006), facilitate membrane localization, and are required for DGK activation (Santos et al., 2002; Los et al., 2004).

Type IV DGKs: DGK ι and DGK ζ

Type IV DGKs, DGK ι and DGK ζ , have been studied in depth by our laboratory. While DGK ζ is ubiquitously expressed (Bunting et al., 1996; Liu et al., 2001; Ding et al., 1997) DGK ι expression is restricted to the brain and retina (Ding et al., 1998; Yang et al., 2011). These isoforms regulate lipid signaling within the nucleus and at the plasma membrane (Topham et al., 1998). The subcellular localization of DGK ζ is determined by phosphorylation of the MARCKS domain, a region homologous to the phosphorylation-site domain of the myristoylated alanine-rich C kinase substrate (MARCKS) protein (Bunting et al., 1996; Ding et al., 1998). This domain contains PKC α phosphorylation sites that, when phosphorylated, exclude DGK ζ from the nucleus and restrict its localization to the cytosol (Luo et al., 2003). Similarly, co-transfection of epitope-tagged PKC α with DGK ι leads to reduced levels of nuclear DGK ι (Ding et al., 1998). The homology between DGK ι and DGK ζ (Figure 11) suggests the altered localization of DGK ι in the presence of PKC α results from phosphorylation of multiple serines within its MARCKS domain (Luo et al., 2003a).

MARCKS domain phosphorylation excludes DGK ζ from the nucleus and restricts its localization to the cytosol (Luo et al., 2003). Similarly, co-transfection of epitope-tagged PKC α with colored according to their conservation between these sequences. In addition to regulating the subcellular localization of type IV DGKs, phosphorylation of the MARCKS domain reduces the catalytic activity of DGK ζ and leads to an

		1		80
DGK ₁ hum	(1)	MDAAGRGCHLLPLPAARGPARAPAAAAAASPPGPCSGAACAPSAAAGAGAMN	SSSSAGEKKGATGGSSSSCSGAGSCC	
DGK _{ζ1} hum	(1)	-----	MBERDGSPEARSSDSEASASS-----	
		81		160
DGK ₁ hum	(81)	LGAEGGADPRGAGSAAAGAAALDEFAAGQKEKDEALEEKL	NLTFRKQVSYRKATSRAGLOHLAPAHPLSLPVANGPA	
DGK _{ζ1} hum	(23)	-----SSGERDAGPEPDKAFRRLNK-----	RRFPGLRLFGHRKATKSGLOHLAPPPPTPGAPCSESE	
		161		240
DGK ₁ hum	(161)	KEPRATLDWSENNAVNGEHLWLETNVSGDLCTLGEENGQVRF	FAKSALRRKCAVCKIVVHTACIEQLEKINFRCKPTFREGC	
DGK _{ζ1} hum	(81)	RQIRSTVDWSESATYGEHWFETNVSGDFCYVGEQYGVARML	KSVSRKCAACKIVVHTPCIEQLEKINFRCKPSPRESG	
		241		320
DGK ₁ hum	(241)	SRSPRE-NFVRHHWVHRRQEGKCKQCGKGFQQKFSFHSKEI	VAISCSWCKQAHNKVTCFMLHHIEEPCSLGAHAIV	
DGK _{ζ1} hum	(161)	SRNVREPTFVRHHWVHRRQDGKCRHCGKGFQQKFTFHSKEI	VAISCSWCKQAMHSKVS CFMLQQIEEPCSLGVHAIV	
		321		400
DGK ₁ hum	(320)	PPTWILIKVKKPQNSLKASNRKKKRTSFKRKASKRGMENKGR	PFFVTKPISSPLMKPLLVFVNPKSGGNQGTQVLMFMW	
DGK _{ζ1} hum	(241)	PPTWILRARRPQNTLKAS-KKKKRASFRRSSKKGPE-EGRW	RPFIIIRPTFSPLMKPLLVFVNPKSGGNQGAIIQSFLW	
		401		480
DGK ₁ hum	(400)	YLNPRQVFDLSQEGPKDALELYRKVENLRLACGGDGTVGWIL	SILDELQLSPQPPVGVLPPLGTGNDLARTLNWGGGYTD	
DGK _{ζ1} hum	(319)	YLNPRQVFDLSQGGPKFALEMYRKVENLRLACGGDGTVGWIL	STDLQLRKPPPPVAILPLGTGNDLARTLNWGGGYTD	
		481		560
DGK ₁ hum	(480)	EPVSKILQVEDGTVVQLDRWNLHVERNPDLFPEE-IEDGV	CKLPLNVFNNYFSLGFDAAHVTLFESREANPEKFN	
DGK _{ζ1} hum	(399)	EPVSKILSHVEEGNVVQLDRWDLHAFNPPEAGPEDRDEG	ATDRPLPLVFNNYFSLGFDAAHVTLFESREANPEKFN	
		561		640
DGK ₁ hum	(559)	RNKMFYAGAAFSDFLQSSRDLSKHVKVCDGIDLTPKIQEL	KFOCVFLNIPRYCAGTMPWGNPGDHHDFEPQRHDDGY	
DGK _{ζ1} hum	(479)	RNKMFYAGTAFSDFLMGSSKDLAKHIRVVCDDGIDLTPKIQ	ELKFOCVFLNIPRYCAGTMPWGNPGDHHDFEPQRHDDGY	
		641		720
DGK ₁ hum	(639)	IEVIGFTMASLAALQVGGHGERLHQCREVVLTKSKAIPVQ	DGEPCKLAPAMIRISLRNQANMVOKSKRRTSMPLLNDFQ	
DGK _{ζ1} hum	(559)	IEVIGFTMTSLAALQVGGHGERLHQCREVVLTKSKAIPVQ	DGEPCKLAASRIALRNQATMVQAKRRSAAPLHSDQQ	
		721		800
DGK ₁ hum	(719)	SVPDRLRIRVNKISLDYEGGFHYDKEKLREASISDWLRTI	AGELVQSFGAIPLGILVVRGDCDLET	CRMYIDRLOEDLQS
DGK _{ζ1} hum	(639)	FVPEQLRIQVSRVSMHDYFALHYDKEQLREAS-----	V--P---LGTVVVVGDSDLLET	CRAHIERLQOE
		801		880
DGK ₁ hum	(799)	VSSCSQRVHYQDHETSFPRALSAQLSPRCFLDDRSQEH	LHFVMEISQDEIFILDPDMVVSQPAGTPPGMPDLVVEQ	
DGK _{ζ1} hum	(701)	AGAKSPTCQKLSPKWCFLDATTASRFYR-----IDRA	QEHLMVTEIAQDEIYILDPPELLGASAR-----	PDLPTPTSP
		881		960
DGK ₁ hum	(879)	GISDWNNEALRRMLSDSGLGMIAPYYEDSDLKDLSHSR	VLQSPVSSDHAILQAVIAGDLMKLTIESYKNGGSLLIQ	
DGK _{ζ1} hum	(770)	LPTSPCSETPRSLQGDAPP-----	QGEELIQAARNDCKLOELHRAGGDLMHREDEQ	
		961		1040
DGK ₁ hum	(959)	HCSLLHYAAKTCNGEIVKYLIDHCFSELDMADSETCET	ALHKAACQRNRAVCQLLVDAASLRKTD	SKCKTPQERAQQA
DGK _{ζ1} hum	(823)	SRLLHHAIVTGSKDVRVYLLDHAFPEILLAVE-ENGET	CLHQAALGQRTICHYIIVAGASLMKTD	QQGDTFRQRAEKA
		1041		1067
DGK ₁ hum	(1039)	GDPELAAYLESRONVKVIGHEDLETAV		
DGK _{ζ1} hum	(902)	QDTELAAYLENROHYQMIQREDQETAV		

Figure 11. Alignment of DGK₁ and DGK_{ζ1} amino acid sequences. Amino acid sequence alignment of DGK₁ and DGK_{ζ1} splice variant of DGK_ζ. Residues in black are identical in DGK₁ and DGK_{ζ1}, with similar amino acids in grey.

accumulation of cellular DAG (Luo et al., 2003b). Furthermore, preliminary evidence from our lab suggests MARCKS domain phosphorylation causes DGK ζ to dissociate from Rac1 (Yakubchuk et al., 2005) and preferentially bind to RhoA (Gee lab, unpublished results). The catalytic activity of DGK ζ is required for its role in the activation of Rac1 but is dispensable for RhoA activation, which suggests MARCKS-domain phosphorylation may be the mechanism by which DGK ζ regulates the balance between Rac1 and RhoA signaling (Ard et al., 2012).

DGK ζ and DGK ι each contain four ankyrin repeats. Studies using DGK ι splice variants have shown that these domains are required for the maximal catalytic activity of DGK ι , as DAG phosphorylation by a variant truncated between the catalytic domain and ankyrin repeats is impaired despite its fully intact catalytic domain (Ito et al., 2004). Similarly, the catalytic activity of DGK θ is reduced as a result of truncating mutations outside of the catalytic domain (Los et al., 2004). Regions outside of the catalytic domain likely influence the catalytic function of DGKs through their effects on protein folding.

PDZ-domains (postsynaptic density 95: PSD-95, Discs Large: DLG, zonula occludens: ZO-1) are protein motifs abundant in the eukaryotic genome that bind to short C-terminal sequences known as PDZ-binding motifs (PDZ-bm), and both DGK ι and DGK ζ contain a PDZ-bm in their C-terminal region. In neurons, DGK ι binds to the PDZ-domain of PSD-95 family proteins (Yang et al., 2011). This interaction localizes DGK ι presynaptically where it metabolizes DAG and regulates neurotransmitter release (Yang et al., 2011). Preliminary data from our lab suggests the PDZ-bm of DGK ι also binds to syntrophins, a family of PDZ-domain containing scaffold proteins associated with the actin cytoskeleton (Gee lab, unpublished). Consistent with these findings is the ability for

syntrophins to bind to the PDZ-bm of DGK ζ (Hogan et al., 2001). Through this interaction, syntrophins stabilize DGK ζ localization at the plasma membrane where it regulates cellular processes like myoblast fusion and neurite outgrowth (Abramovici et al., 2003; Yakubchuk et al., 2005).

During neurite outgrowth, binding to syntrophins localizes catalytically active DGK ζ to growth cones where it regulates Rac1 activation and induces neurite-associated actin reorganization (Yakubchuk et al., 2005; Kozma et al., 1997). Similarly, during myofiber formation in skeletal muscle a syntrophin-DGK ζ -Rac1 complex localizes to regions where intercellular contacts are formed between neighboring myoblasts that facilitate their fusion (Abramovici and Gee, 2005). In both neurons and myoblasts, the interaction between DGK ζ and syntrophins is required to localize Rac1 activity to regions of dynamic actin reorganization.

DGK ζ regulates Rac1 and RhoA activation through its involvement in multi-protein signaling complexes that are unique to each Rho GTPase (Abramovici et al., 2009; Ard et al., 2012). Studies conducted in DGK ζ -knockout MEFs identified aberrations in actin structure formation associated with reduced Rac1 activity, including impaired lamellipodia formation and membrane ruffling (Abramovici et al., 2009). Defects in the formation of RhoA-regulated structures were also observed in these cells and included altered actin stress fiber organization and differences in the distribution of focal adhesions (Ard et al., 2012). Furthermore, DGK ζ -null cells showed defects in cell migration and altered cell morphology during spreading on extracellular matrix substrates (Abramovici et al., 2009; Ard et al., 2012). Despite its homology with Rac1 and RhoA, the activation of Cdc42 was unaffected in cells lacking DGK ζ , indicative of DGK ζ

specificity for particular Rho GTPases. While little is known about the role of DGK ι in the regulation of actin, its homology with DGK ζ and preliminary studies by a previous student in our lab suggest that, like DGK ζ , DGK ι contributes the regulation of actin dynamics.

Objectives and overview

Studies in mouse embryonic fibroblasts (MEFs) elucidated the molecular mechanisms by which DGK ζ regulates the specific release of Rac1 and RhoA from RhoGDI to stimulate changes in actin dynamics (Abramovici et al., 2009; Ard et al., 2012). Given that DGK ζ shares 61% amino acid identity with DGK ι (Fig. 11) we hypothesized that DGK ι might be involved in similar signaling pathways that regulate actin structure formation, and that through these mechanisms DGK ι may contribute to cellular processes dependent upon actin reorganization such as spreading and migration. Our objectives were to study the role of DGK ι in actin organization, and identify possible actin defects primarily using DGK ι -null MEFs. My studies show aberrations in actin structures such as stress fibers, peripheral membrane ruffles, and focal adhesions, as well as differences in cell shape. In functional studies, DGK ι -null cells showed increased migration in response to wounding and an altered phenotype during spreading on extracellular matrix substrates. To elucidate the molecular mechanism by which DGK ι regulates actin rearrangement, the activities of Rac1, RhoA, and RhoC were measured in DGK ι -null cells. The activities of both Rac1 and RhoC were reduced in the absence of DGK ι , yet only RhoC protein levels were affected. Despite its homology with RhoC, RhoA expression and activation were unaffected in DGK ι -null cells. Furthermore, a

strong interaction was detected between DGK ι and RhoC, however DGK ι was unable to associate with RhoA and Rac1 with the same avidity.

Taken together, my findings support a model in which DGK ι specifically stimulates RhoC activation through a direct protein-protein interaction that may also increase RhoC protein stability. Furthermore, my data suggest Rac1 activity is stimulated indirectly by DGK ι , perhaps by acting through proteins upstream of Rac1 activation. Given its similarity with DGK ζ , it is possible that DGK ι regulates RhoC and Rac1 activity within multi-protein signaling complexes.

Chapter 2

Materials and Methods

Reverse transcriptase PCR

Total RNA was collected from cells using TRIzol® (Life Technologies). cDNA was synthesized from total RNA using Qiagen's QuantiTect® Reverse Transcription (Venlo, Limburg, Netherlands). PCRs were then performed with Taq DNA polymerase in 20ul reactions to detect cDNA levels. For DGK₁, cDNAs were amplified using the primer pair 5'-AGAGGAACTGAGCAGGAAACC-3' (forward) and 5'-AGGTTCCAGCGATCCAACTG-3' (reverse). For RhoC, cDNAs were amplified using the primer pair 5'-ATATGAATTCATGGCTGCAATCCGAAAGAAGCT-3' (forward) and 5'-ATATCTCGAGCTAGAGAATGGGACAGCCCCTCCGACGCTT-3' (reverse).

Antibodies

Monoclonal mouse and polyclonal rabbit anti-HA antibodies were purchased from Sigma Aldrich (St. Louis, MO), as were monoclonal mouse anti-tubulin. Monoclonal anti-c-myc antibody was purchased from Roche Applied Science (Indianapolis, IN). Antibodies against Rac1 and RhoA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), while antibodies for Cdc42, PAK1, phospho-PAK1(Ser144), cofilin, and phospho-cofilin(Ser3) were acquired from Cell Signaling Technology (Boston, MA). Mouse anti-paxillin was purchased from BD Biosciences (Franklin Lakes, NJ). HRP-conjugated anti-rabbit and anti-mouse secondary antibodies were acquired from Jackson ImmunoResearch Laboratories (West Grove, PA). Alexa Fluor 488- and 596-conjugated phalloidin secondary antibodies, as well as DAPI, were obtained from Invitrogen (Carlsbad, CA).

Cell culture, transfection, and infection

Immortalized MEF cell lines (described in Regier et al., 2005) were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin. Cells were maintained at 37°C in 5% CO₂. Transfections were performed with Attractene (Qiagen, Venlo, Limburg, Netherlands) following the protocol in the manufacturer's instructions. For adenoviral overexpression experiments, cells were infected at a multiplicity of infection of 100 for 1 hour at 37°C and incubated for 24-48 hours under standard conditions.

Cell-spreading assays

Cells were serum starved overnight, washed twice with phosphate-buffered saline (PBS), pH 7.4, and harvested using 2ml of trypsin-EDTA solution. Cell suspensions were transferred to 2 ml of 1mg/ml soybean trypsin inhibitor, and cells were collected by centrifugation, washed three times and resuspended in 5ml DMEM containing 1% bovine serum albumin (BSA) in PBS. Cells were held in suspension by rotation for 1 hour at 37°C. Cells were then plated at a density of 1.5×10^5 on fibronectin-coated cover slips and incubated at 37°C in 5% CO₂ for times as indicated. Cells were fixed with 4% paraformaldehyde (PFA) and stained for immunofluorescence microscopy.

Immunofluorescence microscopy

Cells were washed twice with phosphate buffered saline (PBS), pH 7.4, before fixing with 4% PFA for 15 minutes. After fixing, cells were permeabilized with 1% Triton X-100 in PBS for 10 minutes, then blocked for 30 minutes using 1% BSA in PBS.

F-actin was stained using Alexa Fluor 488-conjugated phalloidin, and focal adhesions were visualized using a monoclonal mouse anti-paxillin antibody (BD Biosciences, Franklin Lakes, NJ), followed by an Alexa Fluor 594-conjugated goat-anti-mouse secondary antibody. Cells expressing HA- or myc-tagged constructs were stained with either monoclonal mouse anti-HA or polyclonal rabbit anti-HA primary antibody (Sigma Aldrich, St. Louis, MO), or monoclonal mouse anti-c-myc primary antibody (Roche Applied Science, Indianapolis, IN), followed by Alexa Fluor 594-conjugated goat anti-mouse or goat anti-rabbit secondary antibody (Invitrogen, Carlsbad, CA). Cells were mounted on cover slips with Fluoromount GTM (SouthernBiotech, Birmingham, AL) and imaged with an Axioskop 2 microscope (Carl Zeiss, Jena, Germany). All steps were carried out at room temperature.

For membrane ruffling experiments, cells were first serum starved for 16 hours at 37°C either in serum-free media alone or in the presence of the PAK1-inhibitor IPA-3 (Sigma Aldrich, St. Louis, MO). IPA-3 was diluted in DMSO and used at a final concentration of 30 μ M. After serum-starvation, cells were stimulated with 50 ng/ml PDGF in serum-free media along, or containing IPA-3 for PAK1 inhibition experiments, for 5 minutes at 37°C. Cells were fixed and stained as described above immediately after stimulation.

Cell area and perimeter measurements

Cell area and perimeter analysis measurements were acquired with images taken using a Axioskop 2 microscope (Carl Zeiss, Jena, Germany), and images were analyzed in ImageJ. Cells were highlighted by thresholding, and the ‘wand’ tool was used to

outline cells of interest. Next, area and perimeter were selected under the measurements tab and values were collected and analyzed in Excel.

Immunoblotting

Cells were lysed on ice with cold lysis buffer (50mM Tris-Cl, pH 7.4, 50mM NaCl, 5mM MgCl₂, 1% Triton X-100, protease inhibitors, phosphatase inhibitors). Lysates were cleared by centrifugation at 10 000 x g for 10 minutes at 4°C, and protein concentrations were measured by Bradford assay. Equivalent amounts of protein were resuspended in RSB, heated to 98°C, and separated by gel electrophoresis. Proteins were transferred to PVDF membranes and incubated with antibodies diluted in 5% milk prepared in TBS-Tween. Membranes were incubated with primary antibodies for one hour at room temperature followed by three 15-minute washes in TBS-Tween. An HRP-conjugated secondary antibody was applied for an hour at room temperature, followed by three 15-minute washes in TBS-Tween. SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL) was applied to membranes and signals were captured by a LI-COR Odyssey Fc Imager (LI-COR Biotechnology, Lincoln, NE).

Rho GTPase activity assays

Levels of active Rac1 were measured using a Rac effector pull down assay (Pellegrin and Mellor, 2008), in which GST-fused p21-binding domain (PBD) of PAK1 was incubated with cell lysates to capture GTP-Rac1. Cells were serum starved overnight and stimulated for 5 minutes with 50ng/ml PDGF diluted in DMEM at 37°C, 5% CO₂. The medium was removed and cells were lysed on ice with cold lysis buffer (50 mM

Tris-Cl, pH 7.2, 1% Triton X-100, 500 mM NaCl, 10mM MgCl₂, protease inhibitors), collected by scraping, and centrifuged for 5 minutes at 15 000 x g, 4°C. Roughly equal amounts of total protein were incubated with GST-PBD for 30 minutes at 4°C with rotation. Beads were collected by centrifugation and washed three times in cold wash buffer (50 mM Tris-Cl, pH 7.2, 1% Triton X-100, 150 mM NaCl, 30mM MgCl₂, protease inhibitors). Binding of active Rac1 was determined by immunoblotting.

Levels of RhoA were measured using a Rho effector pull down assay, in which GST-fused Rho-binding domain of Rhotekin (RBD) was incubated with cell lysates to capture active RhoA (Ren et al., 1999). Following serum-starvation overnight, cells were stimulated for 3 minutes with 10% FBS-containing DMEM at 37°C, 5% CO₂. The same procedure was followed as that used in Rac1-activity assays with differences in the composition of the lysis buffer (50 mM Tris-Cl, pH 7.5, 500 mM NaCl, 0.1% SDS, 10 mM MgCl₂, 1% Triton X-100, protease inhibitors) and wash buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 50 mM MgCl₂, 1% Triton X-100, protease inhibitors). Rather than using equivalent amounts of total protein, roughly double the amount of DGK₁-null lysates was used relative to wild type to compensate for the reduced abundance of total RhoC in cells lacking DGK₁.

Glutathione S-transferase pull-down assays

Cells expressing HA-tagged wild type DGK₁ were lysed on ice using cold lysis buffer (50mM Tris-Cl, pH 7.4, 50mM NaCl, 5mM MgCl₂, 1% Triton X-100, protease inhibitors). Lysates were collected and cleared by centrifugation at 10 000 x g for 10 minutes at 4°C and the protein concentration was measured by Bradford assay. Equal

amounts of protein (500-1000 µg) were incubated with GST-fusion proteins immobilized on Glutathione Sepharose 4B beads for 1 hour at 4°C with rotation. Beads were collected by centrifugation and washed three times in cold lysis buffer (50mM Tris-Cl, pH 7.4, 50mM NaCl, 5mM MgCl₂, 1% Triton X-100, protease inhibitors) and eluted proteins were resuspended in reducing sample buffer (RSB), separated by gel electrophoresis, and identified by immunoblotting.

Immunoprecipitations

Cells expressing HA-tagged wild type DGK₁ were lysed on ice using cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 50 mM MgCl₂, and protease inhibitors). Lysates were cleared by centrifugation at 10 000 x g for 10 minutes at 4°C and the protein concentration was determined by Bradford assay. 1 mg of total protein was incubated with 5 µg of a polyclonal antibody against HA or IgG at 4°C for 3 hours. 50 µl of a 50% slurry of protein A/G-sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA) were used to capture immune complexes. Beads were then collected by centrifugation at 1000 x g for 3-5 minutes and washed 5 times in cold lysis buffer. Eluted proteins were resuspended in reducing sample buffer (RSB), separated by gel electrophoresis, and identified by immunoblotting.

Cell attachment assays

Cells were plated on 50ug/ml fibronectin-coated cover slips at a density of 200 000 cells/ml, incubated at 37°C, 5% CO₂ for 20 minutes, and washed three times with PBS, pH 7.4. Cells were fixed with 4% PFA and cover slips were mounted on

microscope slides with Fluoromount G (SouthernBiotech, Birmingham, AL). The number of cells per field of view was counted using a 20X objective, and five fields of view were counted per cover slip for eight cover slips of each cell type.

Wound healing assays

Cells were plated in 96-well lock plates (Essen Bioscience, Ann Arbor, MI) and incubated at 37°C, 5% CO₂ until a confluency of approximately 95% was reached. Cells were wounded using a 96-well WoundMaker (Essen Bioscience, Ann Arbor, MI), washed three times with PBS pH 7.4, and imaged in phase contrast with the IncuCyte™ Zoom imaging system (Essen Bioscience, Ann Arbor, MI). Images of the wounded areas were captured every hour for 6 hours. Analysis was performed by Incucyte's 96-Well Migration/Invasion software Application Module. This software identified the wounded region of each image based on three representative phase-contrast images chosen by the user. From this, the area of the wound was measured and expressed as a percentage of the initial wound, the relative wound density, for times as indicated and raw data was exported to Excel for analysis.

Chapter 3

Results

Altered morphology of DGK ι -null fibroblasts

Previous work from our laboratory demonstrated a role for DGK ζ in the regulation of actin dynamics. Studies in mouse embryonic fibroblasts (MEFs) elucidated a molecular mechanism by which DGK ζ contributes to the specific release of Rac1 and RhoA from their inhibitor, RhoGDI, through its involvement in multi-protein signaling complexes unique to each Rho GTPase (Abramovici et al., 2009; Ard et al., 2012). In doing so, DGK ζ increases Rac1 and RhoA activation and induces the remodeling of the actin cytoskeleton. Given that DGK ι shares 61% amino acid identity with DGK ζ (Figure 11), we hypothesized that it might also be involved in signaling pathways that regulate actin dynamics. To test this, we used immortalized DGK ι -null MEFs (Regier et al., 2005). I first verified by that there was no detectable DGK ι mRNA by RT-PCR. Using primers for DGK ι , a single PCR product was generated from wild type MEF total mRNA (449 bp) that was absent from DGK ι -null cells, indicating that the gene for DGK ι had been successfully deleted (Figure 12A). I next analyzed the phenotype of DGK ι -null cells plated on plastic substrates. An overt phenotype was apparent in the DGK ι -null MEFs when compared to wild type cells, as DGK ι -nulls were small and spindly relative to the polygonal, well-spread morphology of wild type cells (Figure 12B). When plated on fibronectin, DGK ι -null cells appeared spindle-like with long, narrow projections, while wild type MEFs were broadly spread in comparison. The F-actin of DGK ι -null and wild type MEFs was visualized by Alexa Fluor 488-conjugated phalloidin staining, and immunofluorescence imaging confirmed that the narrow and elongated morphology of DGK ι -null MEFs visibly contrasted with the appearance of

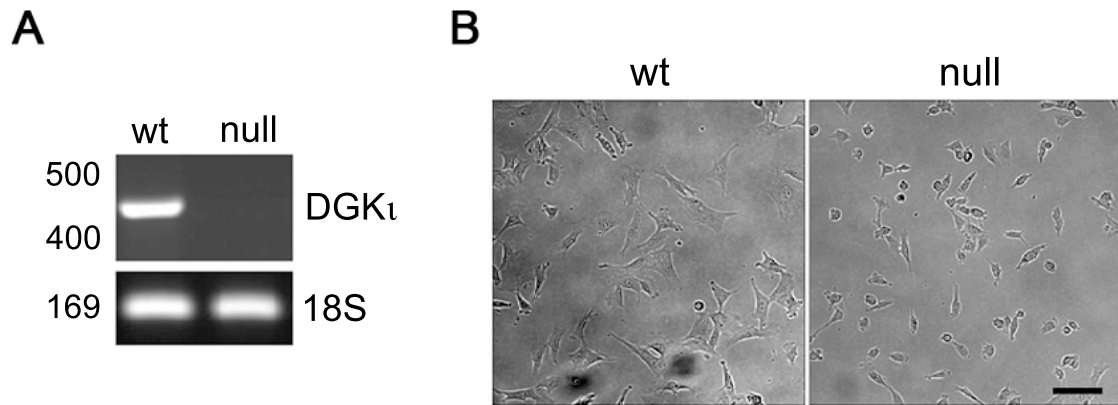


Figure 12. DGK1-knockout MEFs have an altered cell morphology. **A.** RT-PCR was performed using cDNA prepared from wild type (wt) and DGK1-null (null) MEFs using primers for DGK1. The expected 449-bp band was produced only in the wild type sample. Primers for the 18S ribosomal subunit, visible in both samples, were used as a loading control and demonstrate equal amounts of cDNA in each reaction. **B.** Representative DIC images of wt and null MEFs 24 hours after replating on plastic. Shown below are magnified images of the boxed regions. Scale bar, 100 μ m.

wild type MEFs, which were generally polygonal, broadly spread, and often observed with ruffling lamellipodia at their periphery (Figure 13A). The mean cell area of wild type MEFs was $2329 \pm 32 \mu\text{m}^2$, while the mean cell area of DGK1-null cells was only $926 \pm 64 \mu\text{m}^2$ (Figure 13B). While DGK1-null cell perimeter was also reduced relative to wild type, the reduction in perimeter was less drastic than the decrease in cell area as wild type cells had a mean cell perimeter of $370 \pm 27 \mu\text{m}$, compared to a mean perimeter of $269 \pm 7 \mu\text{m}$ in null cells (Figure 13C). These measurements are consistent with the spindle-like appearance of DGK1-null MEFs. The altered cell morphology of DGK1-null MEFs suggests that DGK1 may contribute to the broadly spread cell morphology of fibroblasts, perhaps by stimulating actin reorganization to increase cell area and reduce the prominence of pointed, filopodia-like projections.

The loss of DGK1 affects stress fiber formation but not circular ruffling

To determine the basis for the altered morphology of DGK1-null MEFs, the cells were stained with Alexa Fluor 488-conjugated phalloidin to visualize F-actin and were analyzed by immunofluorescence microscopy. Both wild type and DGK1-null cells formed stress fibers, however those in DGK1-null MEFs were often thin and disorganized. To quantify the appearance of stress fibers, we determined the percentage of cells with stress fibers best characterized by each of the following categories: (1) thick and parallel, (2) thick and disorganized, (3) thin and parallel, or (4) thin and disorganized

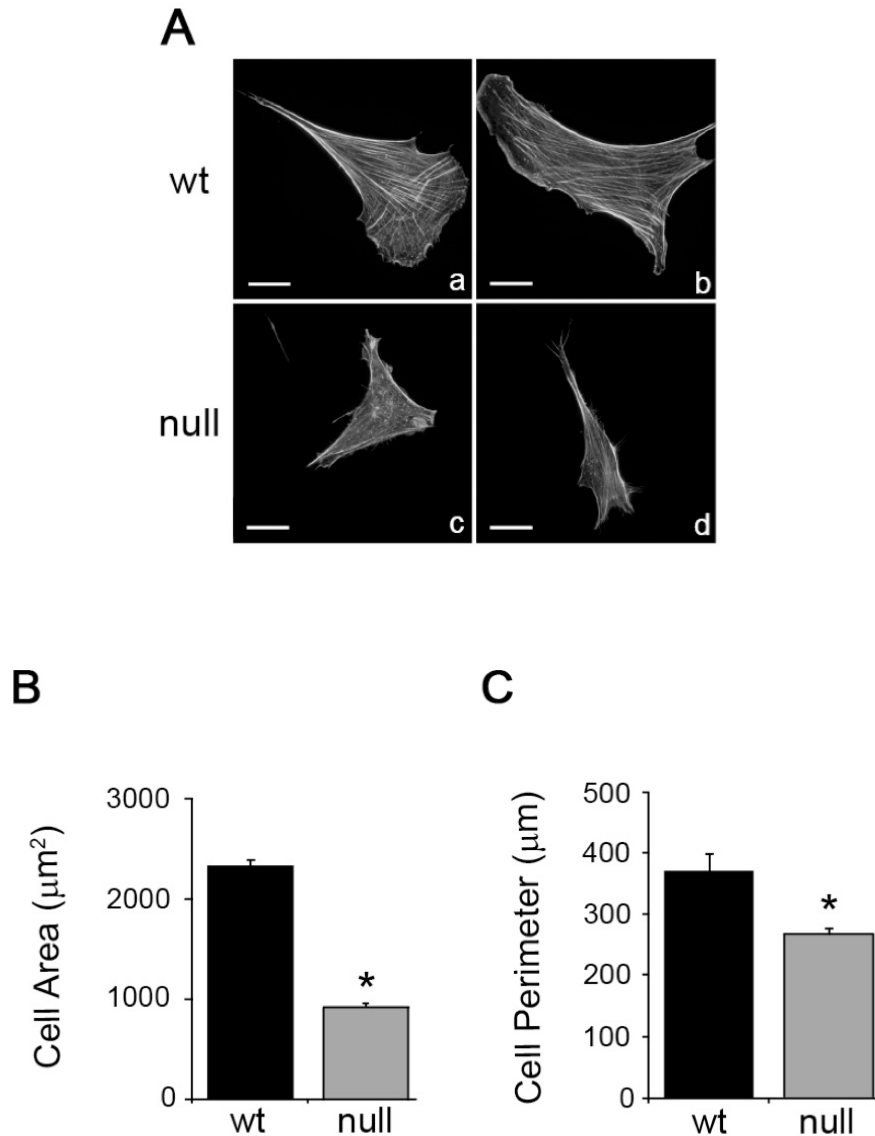


Figure 13. DGK α -knockout MEFs have reduced cell area and perimeter. A. Representative images of wt (a and b) null (c and d) MEFs on fibronectin 24 hours after replating. Cells were stained with AlexaFluor 488-conjugated phalloidin to visualize F-actin. Scale bar, 20 μm . **B and C.** Area and perimeter of wt and null MEFs 24 hours after replating on fibronectin. Values represent the mean $\pm$ of SEM. Asterisk indicates statistical significance, $p < 0.05$.

(Figure 14A). Approximately half ($50.4 \pm 2.3\%$) of the wild type cells had stress fibers that were thick and parallel (Figure 14B). DGK1-null MEFs most frequently had stress fibers that were either thin and disorganized ($39.6 \pm 4.6\%$), or thin and parallel ($36.9 \pm 4.8\%$). Thick and disorganized stress fibers were least common in both wild type and DGK1-null cells, with only $6.1 \pm 0.9\%$ of wild type and $6.9 \pm 1.7\%$ of DGK1-null cells with this stress fiber phenotype. Together, these data suggest DGK1 has a role in the regulation of actin stress fibers.

Circular membrane ruffles form on the dorsal surface of fibroblasts in response to stimulation with PDGF, and are thought to be involved in the initial stages of vesicle formation during macropinocytosis (Mallstrom et al., 1988; Swanson, J.A. and Watts, C., 1995; Dharmawardhane et al., 2000; Hoon et al., 2012). Circular ruffling is regulated by Rac1 signaling, and is reduced in the absence of DGK ζ (Abramovici et al., 2009). To assess whether DGK1 might also be involved in the formation of circular membrane ruffles, wild type and DGK1-null MEFs were stimulated with PDGF for 5 minutes prior to fixing, then stained with Alexa Fluor 488-conjugated phalloidin to visualize F-actin. In the absence of stimulation, these structures were absent from both cell types, however upon treatment with PDGF, prominent membrane ruffles were observed on the dorsal surface of both wild type and DGK1-null MEFs (Figure 14C). The percentage of cells with at least one circular ruffle was quantified for both wild type and DGK1-null cells, and no statistically significant difference was found between cell types with $28.4 \pm 3.6\%$ of wild type and $21.1 \pm 6.1\%$ of DGK1-nulls producing these structures (Figure 14D). This finding suggests DGK1 is not required for signaling pathways that regulate circular membrane ruffling in fibroblasts in response to PDGF stimulation.

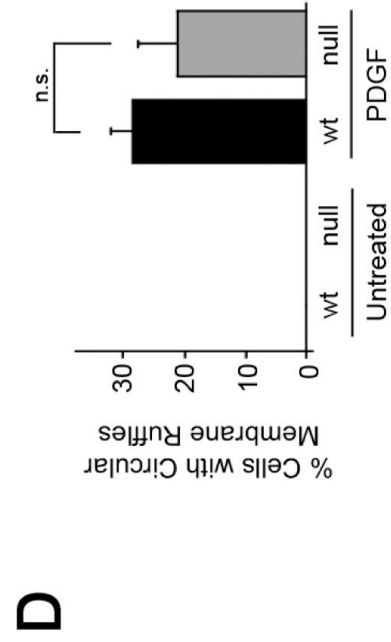
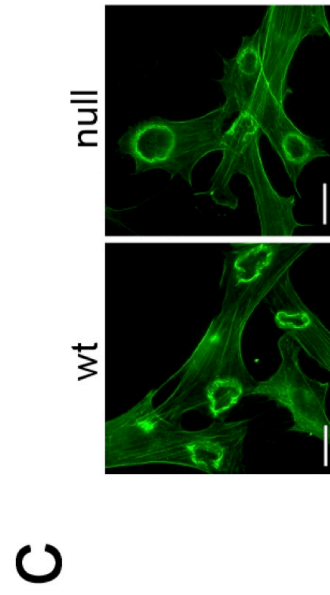
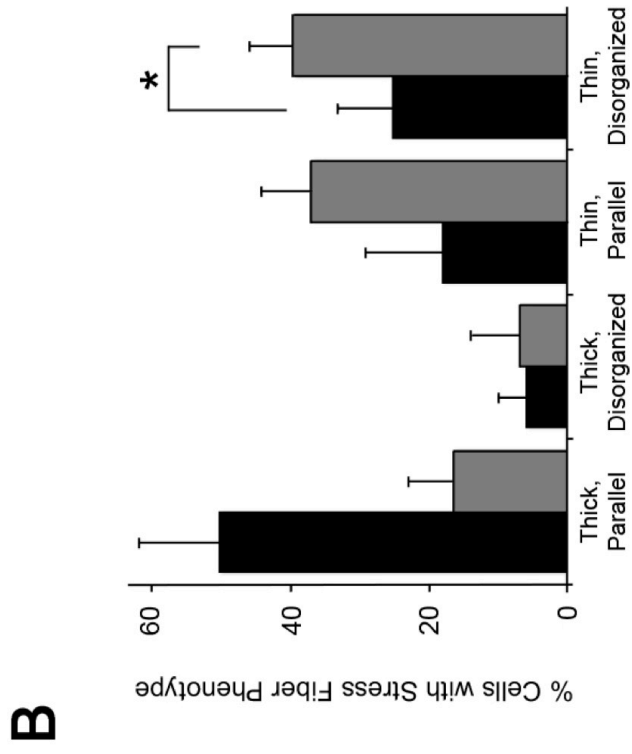
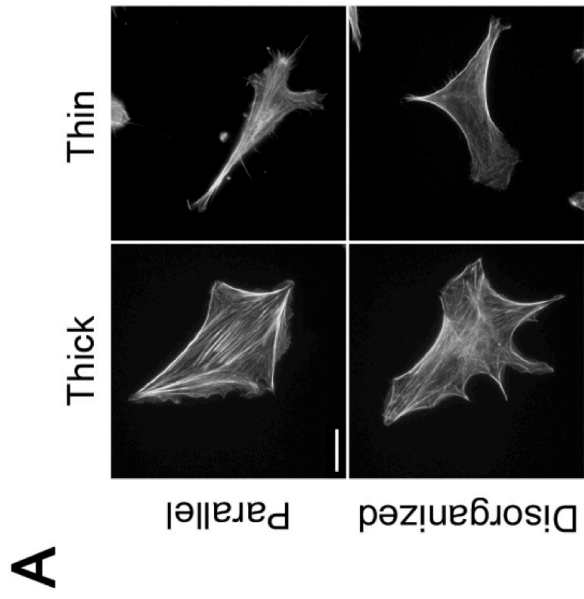


Figure 14. Stress fibers, but not circular membrane ruffles, are affected in DGK α -null MEFs. **A.** Representative images of thick and parallel (a), thin and parallel (b), thick and disorganized (c), and thin and disorganized (d) stress fibers in wt and null MEFs. Scale bar, 20 μ m **B.** Percentage of wt and null MEFs with each stress fiber type represented in A. Values represent the mean $\pm$ SEM of three independent experiments in which a minimum of 80 cells were counted per experiment. Asterisk indicates statistical significance, $p < 0.01$. **C.** Representative images of circular membrane ruffles in wt and null MEFs. Cells plated on fibronectin and 24 hours later were stimulated with PDGF. Cells were then fixed, and stained with AlexaFluor 488-conjugated phalloidin (green) to visualize F-actin. Scale bars, 20 μ m. **D.** Percentage of wt and null MEFs producing at least one circular membrane ruffle after stimulation with PDGF. Values represent the mean $\pm$ SEM of three independent experiments in which a minimum of 200 cells were counted per experiment.

Cell migration is increased in the absence of DGK κ

Cell migration depends on the spatiotemporal regulation of actin structures that facilitate motility (Lauffenburger, D.A. and Horwitz, A.F., 1996; Iijima et al., 2002; Pollard, T.D. and Borisy, G.G., 2003; Carlier, M-F. et al., 2003; Ridley et al., 2003). To determine if cell migration is affected by the changes in actin organization in DGK κ -null cells, scratches were made to cell monolayers and phase contrast images of the wounded region were taken every hour for six hours after wounding. The relative wound density, defined as the cell density within the area of the initial wound relative to the cell density outside of the wound, was determined for each image as outlined in Materials and Methods. An increase in cell migration by DGK κ -null cells was measured at 3 and 6 hours after wounding, and migration by null cells was increased as indicated by a greater increase in the relative wound density of these cells compared to wild type (Figure 15A, B). Wild type cells reached a relative wound density of 43% by the end of the time course compared to 31% for null cells. These data suggest DGK κ may have an inhibitory role during cell motility, leading to increased migration in its absence.

Altered cell spreading by DGK κ -null cells

Since dynamic actin rearrangements also facilitate cell spreading on extracellular matrix (ECM) substrates (Zimmerman et al., 2004), we next sought to determine whether cell spreading might be affected by the absence of DGK κ . To this end, wild type and DGK κ -null MEFs were replated on fibronectin, then fixed at 15 minute-intervals over one hour. After fixing, cells were stained with Alexa Fluor 488-conjugated phalloidin to

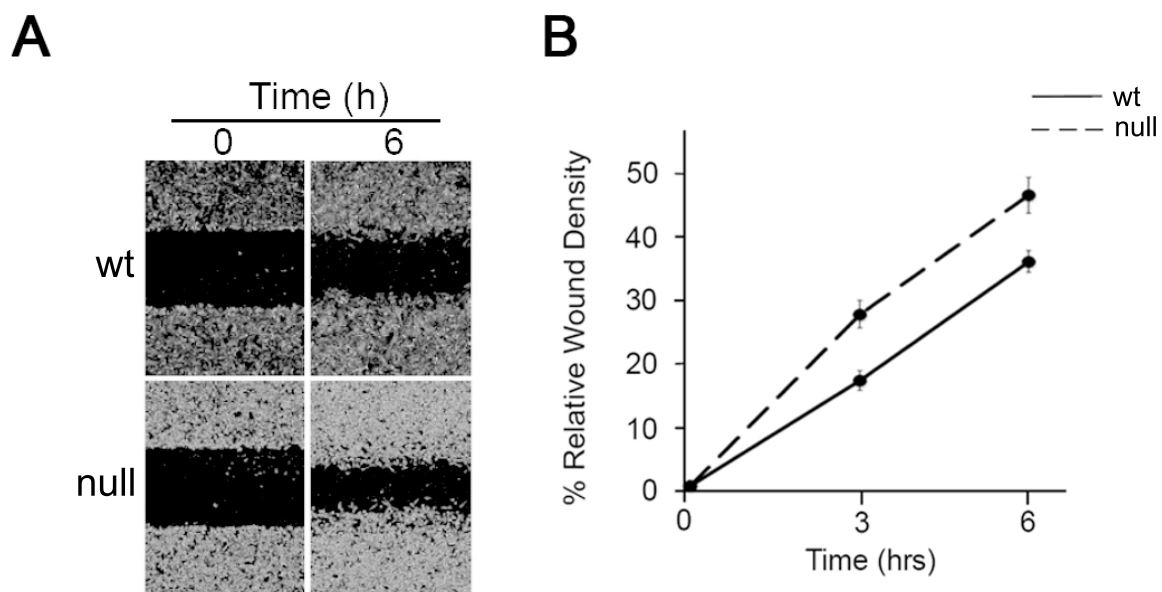


Figure 15. Increased migration of DGK1-null fibroblasts. **A.** Representative images of wt and null fibroblasts immediately after wounding (0 h) and 6 hours into wound closure (6 h). **B.** Quantification of the % relative wound density over time for wt and null MEFs.

visualize F-actin. Wild type MEFs were initially spherical and poorly spread, and gradually flattened to become circular 30 minutes after replating (Figure 16A). Peripheral membrane ruffles produced as a consequence of rapid actin reorganization were observed in many of these cells, and gradually matured into lamellipodia. By 60 minutes after replating, wild type cells finally began to adopt the polygonal morphology typical of MEFs.

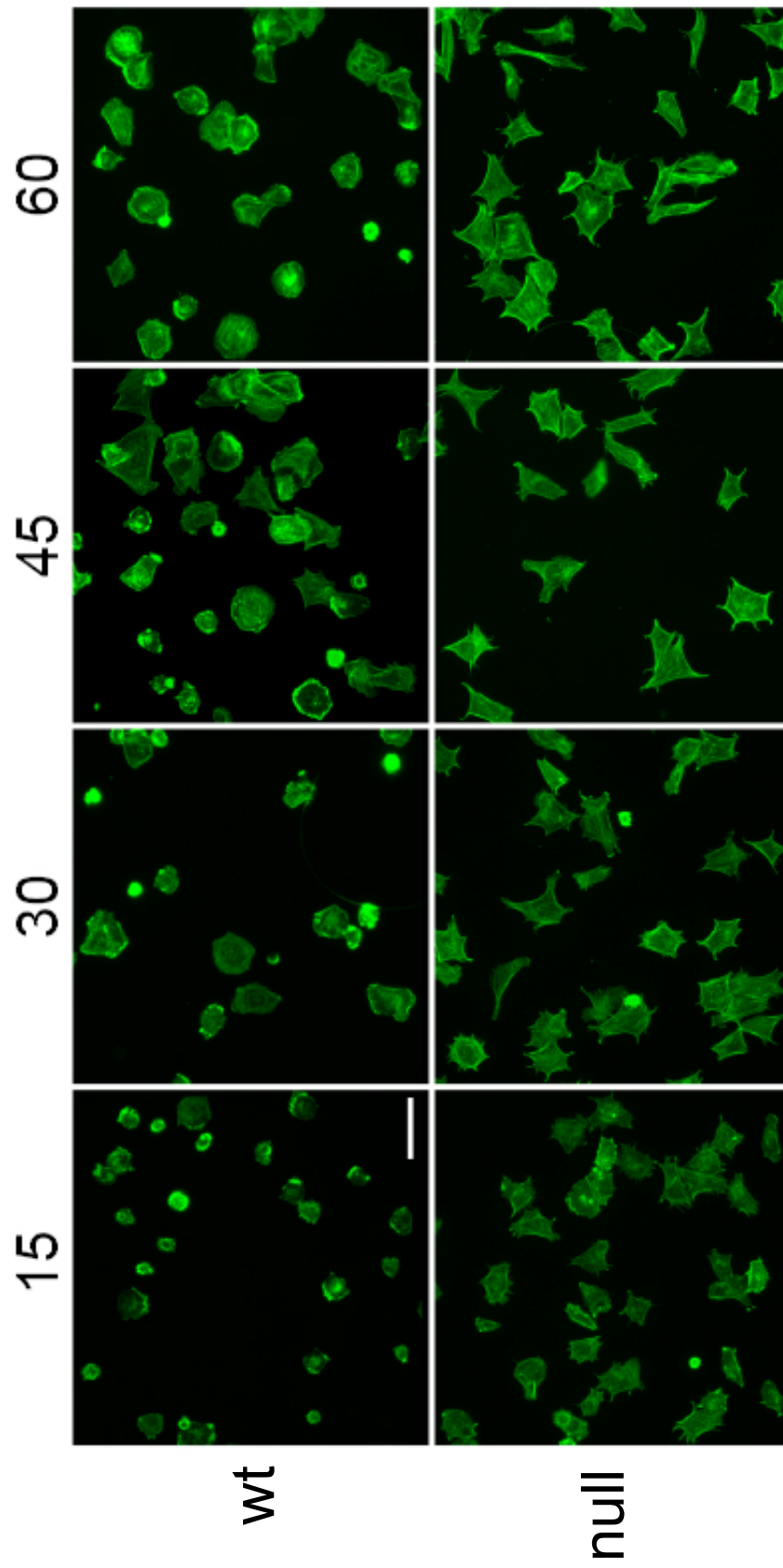
Differences in the morphology of DGK1-null MEFs were apparent as early as 15 minutes after replating. The majority of DGK1-null cells had already flattened after 15 minutes and extended processes from multiple regions along their periphery (Figure 16A). These processes continued to elongate over the 60 min course. Membrane ruffles were drastically reduced in these cells (Figure 16B), and by 60 minutes, the majority of DGK1-nulls maintained their stellate morphology, however some had elongated into a spindle-like morphology.

When replated on fibronectin, DGK1-null MEFs flattened more rapidly than wild type cells and spread by the extension of pointed projections in multiple directions along their periphery. The absence of peripheral membrane ruffles from DGK1-null MEFs suggests DGK1 is likely involved in the formation of these actin structures during cell spreading on ECM substrates.

DGK1 influences focal adhesion localization during cell spreading

Focal adhesions are dynamic protein complexes that couple the ECM to the actin cytoskeleton (Hynes, 1992; Chrzanowska-Wodnicka and Burridge, 1996). Located at the ends of stress fibers, focal adhesions relay mechanical and chemical cues from the

A



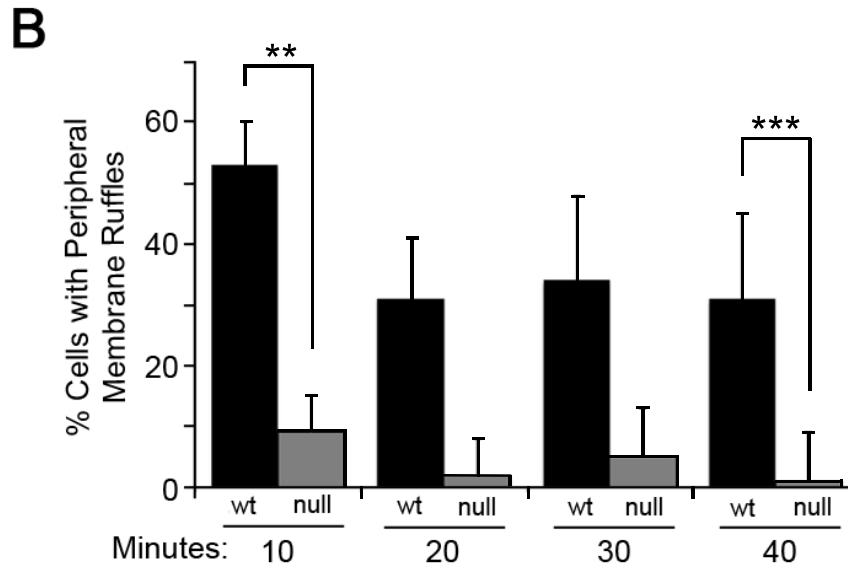


Figure 16. Cell spreading in fibroblasts is affected by the absence of DGK λ . **A.** Representative immunofluorescent images of wt and null fibroblasts 15, 30, 45, and 60 minutes after replating on fibronectin. Cells were fixed at indicated times and stained with AlexaFluor 488-conjugated phalloidin (green) to visualize F-actin. Scale bar, 50 μ m. **B.** Cells were replated on fibronectin and fixed during spreading at the indicated times before staining with AlexaFluor 488-phalloiding to visualize F-actin. The percentage of wt and null cells with peripheral membrane ruffles was quantified at various times after replating. Values represent the mean of three independent experiments $\pm$ SEM. Asterisks indicate statistical significance as determined by Student's T-test, ** = $p < 0.001$, *** = $p < 0.0001$.

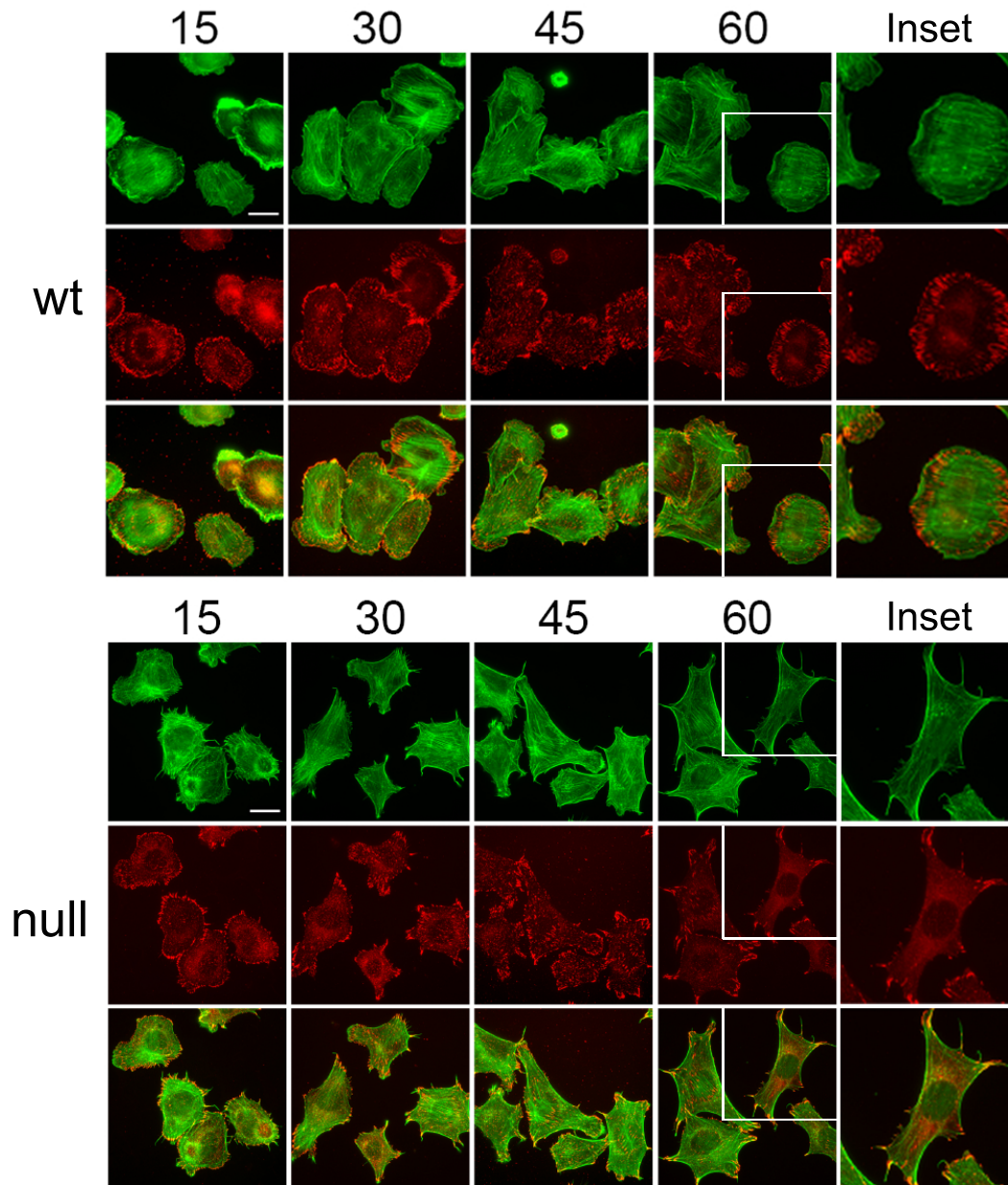


Figure 17. Altered focal adhesion distribution in DGK α -null MEFs. Wt and null fibroblasts were replated on fibronectin and fixed at indicated times. Cells were stained with AlexaFluor 488-conjugated phalloidin (green) to visualize F-actin, as well as with an antibody against paxillin, followed by an AlexaFluor 594-conjugated secondary antibody (red) to visualize focal adhesions. Scale bar, 20 μ m.

extracellular environment during processes like migration and spreading (Geiger, B. et al., 2011). To examine focal adhesion formation in DGK1-null MEFs during spreading, cells were replated on fibronectin and fixed at 15-minute intervals. Cells were costained with AlexaFluor 488-conjugated phalloidin to visualize F-actin and with an antibody against paxillin, a marker of focal adhesion complexes (Turner, 2000), followed by an AlexaFluor 594-conjugated secondary antibody. Cells were then imaged using immunofluorescence microscopy (Figure 17). Paxillin staining in DGK1-null MEFs revealed differences in the subcellular localization of adhesion complexes compared to wild type MEFs. In wild type cells, paxillin staining was most abundant at the cell periphery where it was often evenly distributed. This was especially apparent 15 and 30 min after replating. As in wild type cells, paxillin was mostly confined to the peripheral regions of DGK1-nulls; however, it accumulated at the tips of processes rather than being evenly distributed along the cell perimeter. The localization of paxillin to pseudopodia-like processes in DGK1-null MEFs suggests DGK1 is required for the even distribution of focal adhesions along the periphery of the cell.

Cell attachment is unaffected in DGK1-null MEFs

Studies in fibroblasts have demonstrated that the spatial distribution of focal adhesions can influence the ability for cells to adhere to ECM substrates, and that some focal adhesion arrangements allow for greater cell adhesion than others (Elineni and Gallant, 2011). Here, cell attachment assays were used to determine whether the atypical focal adhesion arrangement in DGK1-null MEFs affected cell attachment to fibronectin. Cells were replated on fibronectin and incubated under standard conditions for 20 min

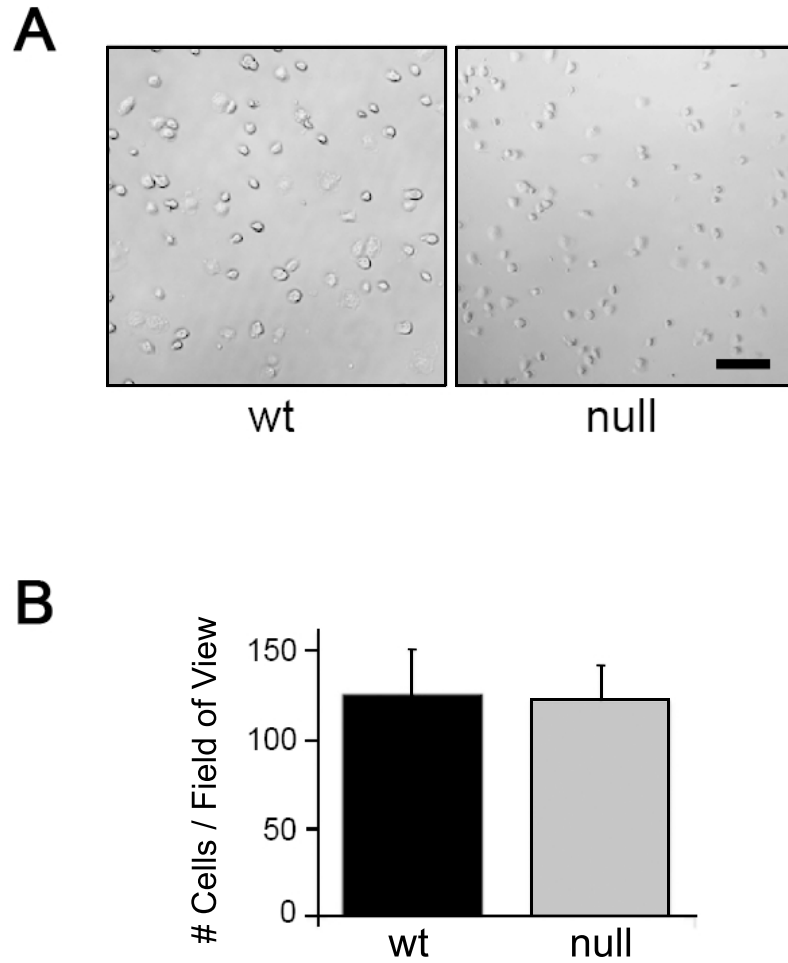


Figure 18. DGK α is not required for cells to form functional attachments to fibronectin. Attachment assays were performed using wt and null fibroblasts on fibronectin. **A.** Representative DIC images of wt and null MEFs 20 minutes after replating on fibronectin. Cells were washed three times with PBS before imaging. Scale bar, 50 μ m. **B.** Quantification of the number of wt and null cells per field of view after three PBS washes performed 20 minutes after replating on fibronectin.

before washing with PBS. The cells that remained were assumed to be those with attachments robust enough to withstand the PBS washes, and were quantified by DIC microscopy (Figure 18A, B). No statistically significant difference in the number of attached cells was found between these two cell types, suggesting focal adhesions in DGK1-null MEFs are functional, despite their altered distribution, and the ability for cells to adhere to fibronectin is unaffected by the alternative placement of focal adhesions in DGK1-null cells.

Rac1 activation is impaired in the absence of DGK1

We showed previously that Rac1 activity is reduced in DGK ζ -null cells, and results in the impaired formation of Rac1-related actin structures (Abramovici et al., 2009). Therefore, we sought to determine whether reduced Rac1 activity might be similarly responsible for the reduced cell area of DGK1-null cells, as well as the absence of peripheral membrane ruffles in these cells during spreading. The level of active Rac1 was measured in lysates of wild type and DGK1-null cells using the p21-binding domain (PBD) of PAK1, which only binds active, GTP-bound Rac1 (Sander et al., 1998; Pellegrin, S. and Mellor, H., 2008). GST-PBD was incubated with lysates from serum-starved cells and the amount of bound Rac1 was determined by immunoblotting. No significant difference in the level of active Rac1 was found between unstimulated wild type and null cells (Figure 19A, B). However, stimulation with PDGF resulted in a 60% increase in GTP-Rac1 levels in wild type cells, but only a 15% increase in null cells (Figure 19A, B). There was no significant difference between the levels of total Rac

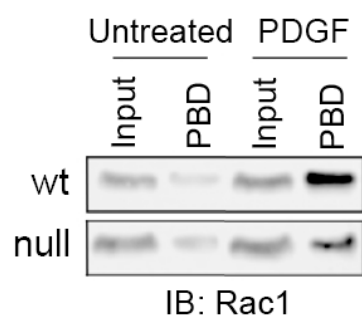
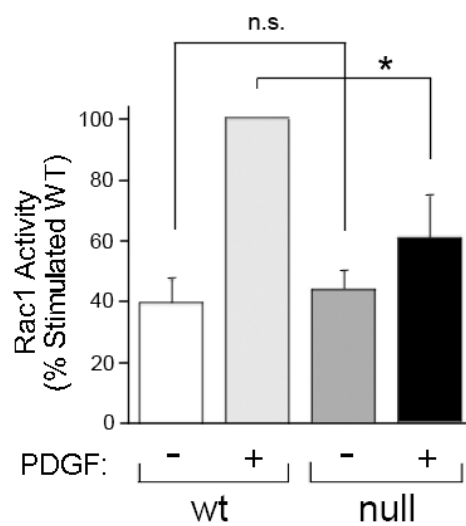
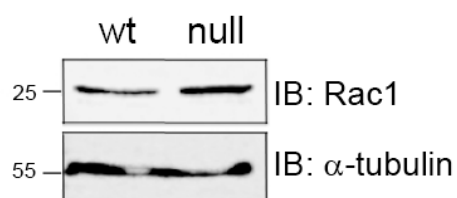
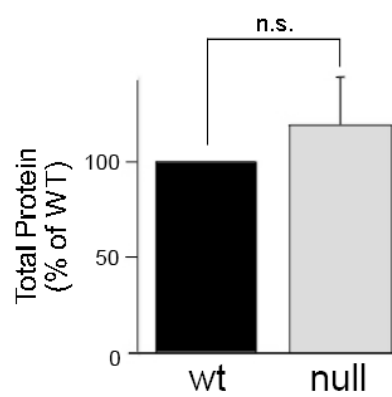
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Figure 19. Decreased PDGF-stimulated Rac1 activation in DGK κ -null cells. **A.** Representative immunoblots (IB) showing global levels of active Rac1 with and without PDGF stimulation. Serum-starved wt and null MEFs were stimulated with either DMEM alone (untreated) or with PDGF for 3 minutes. Lysates were incubated with GST-PBD beads. Binding of active Rac1 to PBD was determined by immunoblotting. Input represents 10% of the lysate used for the assay. **B.** Quantification of active Rac1 before and after PDGF stimulation in both wt and null cell lysates. Values represent the mean of five independent experiments $\pm$ SEM, and are expressed as the percentage of active Rac1 in wild type lysates after stimulation determined by densitometric analysis of immunoblot bands. Asterisk indicates a statistically significance difference, $p < 0.05$. **C.** Representative IB showing levels of total Rac1 in wt and null MEFs. α -tubulin was used as a loading control and demonstrates similar levels of total protein were loaded for each cell type. **D.** Rac1 immunoblot bands from wt and null cell lysates were quantified by densitometry and normalized to α -tubulin. Values represent the mean $\pm$ SEM of four independent experiments, and are expressed as a percentage of wild type. n.s. indicates no statistically significant difference.

protein in wild type versus null cells (Figure 19C, D). These results suggest there is a defect in growth factor-stimulated Rac1 activation in DGK1-null cells.

RhoC, but not RhoA, activation is reduced in the absence of DGK1

The impaired stress fiber formation in DGK1-null cells raised the possibility that RhoA and/or RhoC activation was affected by the absence of DGK1. To measure RhoA/C activity we used an effector pull-down assay (Ren et al., 1999; Ren and Schwartz, 2000). A GST fusion protein comprising the Rho-binding domain (RBD) of Rhotekin, a Rho effector that only binds to GTP-bound RhoA/C (Ren et al., 1999), was incubated with lysates of serum-starved cells and the amount of bound GTP-RhoA/C determined by immunoblotting (Figure 20A). In the absence of stimulation, the levels of active RhoA and RhoC in wild type cells were not significantly higher than in null cells when total amounts of RhoA/C were equal (Figure 20B, C). Stimulation with serum, which contains the RhoA/C activator lysophosphatidic acid (LPA) (Chrzanowska-Wodnicka and Burridge, 1996), lead to a similar increase in active RhoA levels in both wild type and DGK1-null cells (Figure 20A, B). In contrast, the level of active RhoC was significantly reduced in stimulated DGK1-null cells compared to wild type cells (Figure 20 A, B) suggesting DGK1 is specifically required for the stimulated activation of RhoC, but not RhoA.

Decreased levels of RhoC protein in the absence of DGK1

The decreased Rho GTPase activity in DGK1-null MEFs might result from reduced protein expression or stability rather than a defect in GTPase activation, per se.

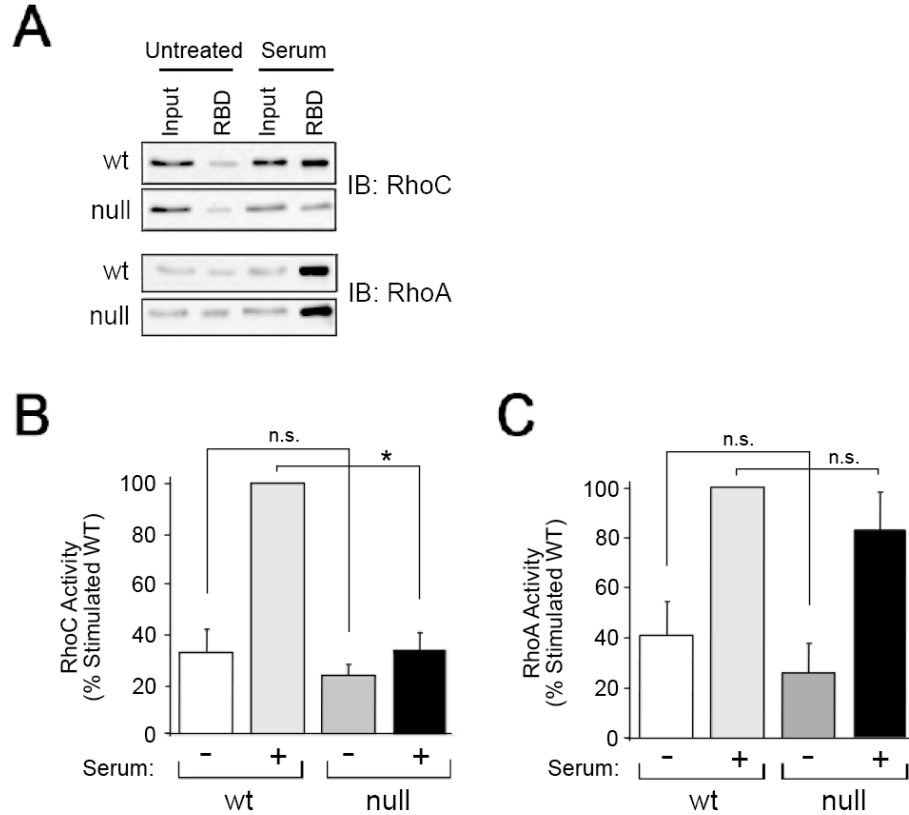


Figure 20. RhoC, but not RhoA, activation is impaired in DGK κ -null cells. A. IB showing levels of active RhoC and RhoA in wt and null MEFs. Serum-starved wt and null cells were incubated for 3 minutes with DMEM alone (untreated) or DMEM containing 10% serum. Cell lysates were incubated with a GST fusion protein containing the Rho-binding domain of Rhotekin (RBD) immobilized to glutathione sepharose beads. Bound RhoA and RhoC were detected by immunoblotting with specific antibodies. Input represents 10% of the lysate used in the assay. **B and C.** Quantification of active RhoA and RhoC, respectively, determined by densitometric analysis of immunoblots. Values are expressed as the percentage of stimulated wild type cells and are the mean $\pm$ SEM of at least five independent experiments. Asterisk indicate a statistically significant difference, $p < 0.05$, while n.s. indicates no statistically significant difference.

To test this idea, the levels of total RhoA and RhoC protein in wild type and DGK ι -null cell extracts were measured by immunoblot analysis (Figure 21A, C). Densitometric analysis of immunoblot bands showed RhoC was significantly decreased in DGK ι -null cell lysates, while RhoA was unchanged (Figure 21B, D). RT-PCR using primers against RhoC produced a 609 bp PCR product from both wild type and DGK ι -null total cell mRNA. This showed RhoC mRNA in DGK ι -null cells was equivalent to wild type cells (Figure 21E), which suggests that DGK ι regulates the abundance of RhoC at the protein level.

DGK ι interacts strongly with PAK1 and RhoC, but not with Rac1 or RhoA

We next asked whether the decreases in Rac1 and RhoC activity in DGK ι -null cells result from the loss of a direct interaction of Rac1 and RhoC with DGK ι . To test the interaction with Rac1, GST-Rac1^{V12} (constitutively active) and GST-Rac1^{N17} (dominant negative) were incubated with wild type MEF lysates infected with HA-tagged DGK ι . A GST fusion with PAK1 was included as a control. HA-DGK ι did not bind appreciably to GST, GST-Rac1^{V12}, or GST-Rac1^{N17} but did consistently bind to GST-PAK1 (Figure 22A). These data suggest DGK ι is unlikely to directly associate with Rac1 but likely binds directly to PAK1. An interaction between PAK1 and DGK ι was further supported by immunoprecipitation assays in which an anti-HA antibody eluted both HA-tagged DGK ι and endogenous PAK1 from infected wild type cell lysates (Appendix, Figure 1). Using the GST protein pull-down assay described above, we tested GST-RhoC^{WT}, as well as constitutively active (GST-RhoC^{V14}) and dominant negative (RhoC^{N19}) mutants for an interaction DGK ι . While all three RhoC fusion proteins bound DGK ι , RhoC^{WT} appeared

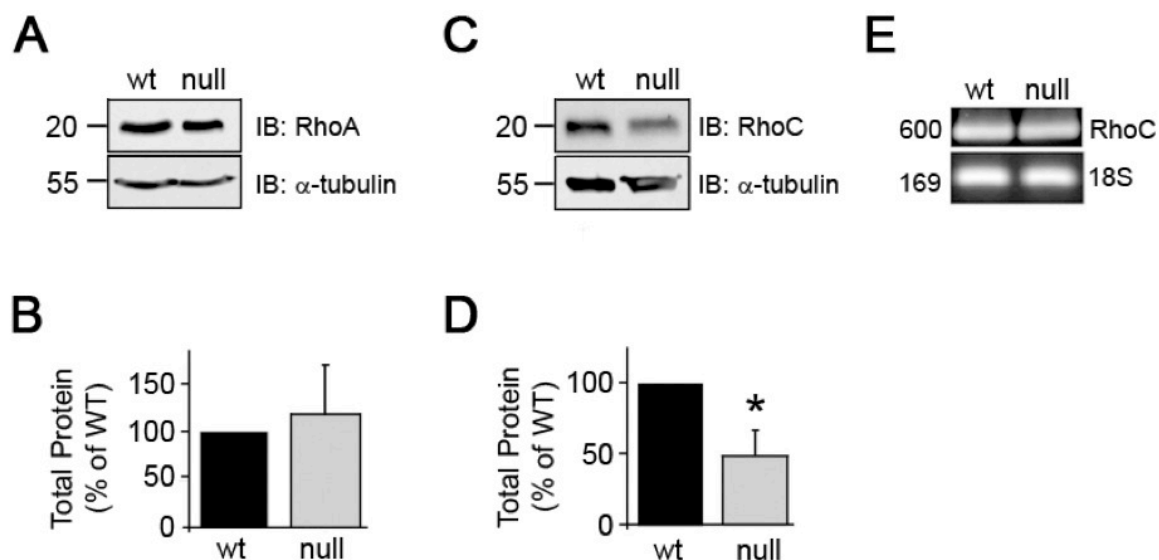


Figure 21. RhoC protein, but not RhoA, is reduced in DGK1-null MEFs. **A and C.** IB showing total protein levels of RhoA and RhoC, respectively, in wild type and DGK1-null MEFs. **B and D.** Quantification of RhoA and RhoC protein levels, respectively. Immunoreactive bands were quantified by densitometry and normalized to α -tubulin. Values represent the mean $\pm$ SEM of four independent experiments and are expressed as a percentage of protein in wild type cells. Asterisk indicates statistical significance as determined by Student's t-test, $p < 0.05$. **E.** RT-PCR was performed using total mRNA from wild type and null cells lysates using primers designed for RhoC. Primers for the 18S ribosomal subunit were used as a loading control and demonstrate equal amounts of cDNA in each reaction.

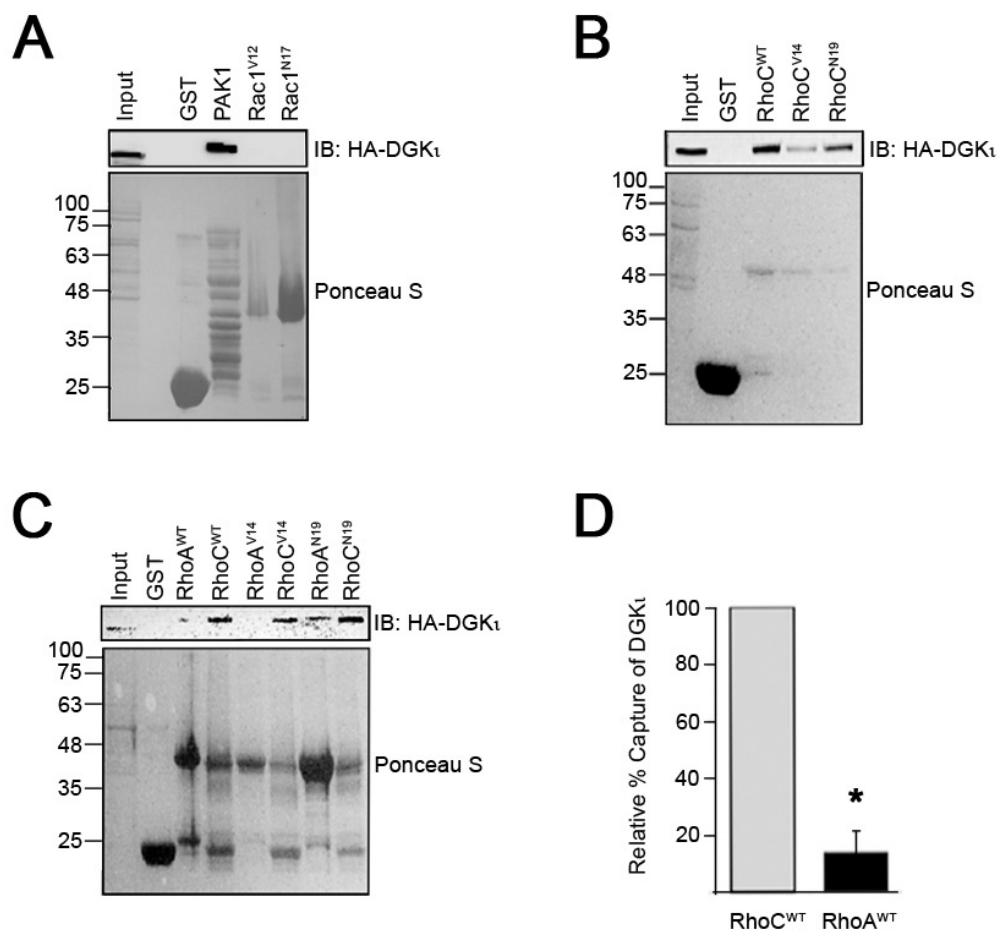


Figure 22. DGK ι associates with PAK1, but not Rac1. Lysates from wt MEFs expressing HA-tagged wild type DGK ι were incubated with the indicated GST-fusion proteins. Association of DGK ι was determined by IB. Relative amounts of fusion protein are shown in the Ponceau S blot below. **A.** GST pull down assay in which PAK1, Rac1^{V12} (constitutively active), and Rac1^{N17} (dominant negative), and wild type PAK1 were tested for binding to DGK ι . **B.** GST pull down assay in which wild type RhoC (RhoC^{WT}), and constitutively active (RhoC^{V14}) and dominant negative (RhoC^{N19}) mutants RhoC activation in DGK ι -null cells.were tested for binding to DGK ι . **C.** GST pull down assay in which wild type RhoA (RhoA^{WT}), constitutively active (RhoA^{V14}) and dominant negative (RhoA^{N19}) RhoA mutants were tested for binding to DGK ι and compared to binding by RhoC constructs. **D.** Quantification of DGK ι captured by RhoA^{WT} and RhoC^{WT}. Asterisk indicates a statistically significant difference determined by Student's T-test, $p < 0.001$.

to interact more strongly than RhoC^{V14} or RhoC^{N19} (Figure 22B). In contrast, HA-DGK ι bound only weakly or not at all to the equivalent RhoA constructs, even when more fusion protein was loaded on the beads (Figure 22C). Quantification of the bound protein revealed that RhoA bound only 15% as much DGK ι as RhoC (Figure 22D). These data suggest DGK ι specifically binds RhoC and are consistent with the specific decrease in RhoC activation in the null cells.

PAK1 activation is reduced in the absence of DGK ι

Binding of active Rac1 to PAK1 relieves its auto-inhibition and causes PAK1 to phosphorylate itself (Parrini et al., 2002; Chong et al., 2001). Such autophosphorylation, which occurs at a number of sites including ser-144, stabilizes PAK1 in its active conformation (Chong et al., 2001; Lei et al., 2000). To determine if DGK ι affects PAK1 phosphorylation, the level of Ser-144-phosphorylated PAK1 (pPAK1(S144)) was measured in lysates of wild type and DGK ι -null cells by immunoblotting and compared to total PAK1 (Figure 23A). While the level of PAK1 protein was unchanged, pPAK1(S144) was substantially reduced in DGK ι -null cells. Quantification of the signal intensities revealed a 60 % decrease in the level of pSer-s144 in DGK ι -nulls (Figure 23B) suggesting PAK1 phosphorylation, and hence its activity, are significantly impaired in DGK ι -null cells.

Cofilin phosphorylation is impaired in the absence of DGK ι

By severing actin filaments, cofilin stimulates actin polymerization by increasing the number of barbed ends and the concentration of free G-actin monomers in the cytosol (Bravo-Cordero et al., 2013). To restrict cofilin activity to areas of dynamic actin

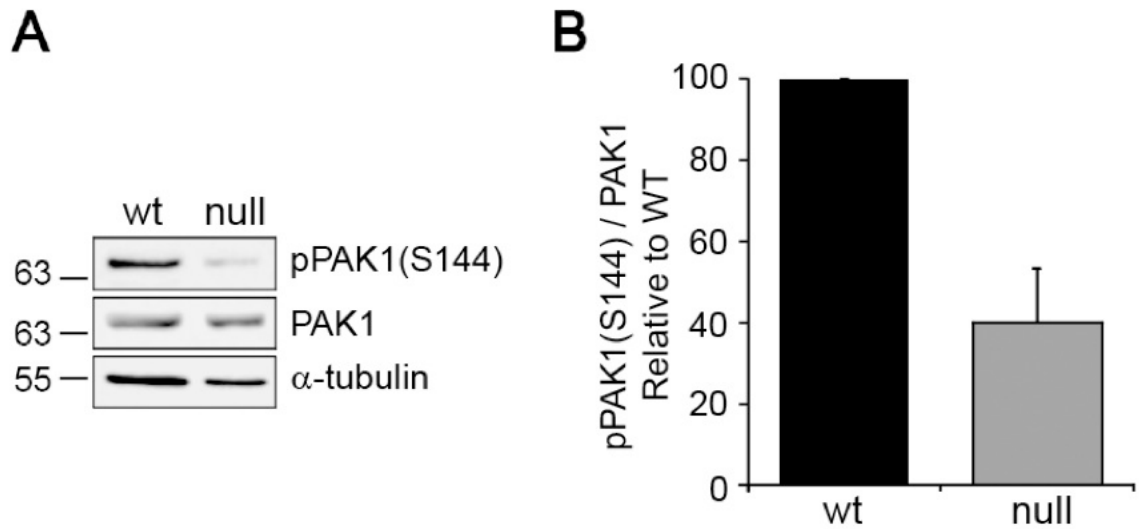


Figure 23. PAK1 Phosphorylation is reduced in DGK1-null cells. **A.** IB of Ser-144 phosphorylated PAK1 (pPAK1(S144)), total PAK1, and α -tubulin in wt and null cells. Approximately equal amounts of total protein were loaded for each cell type. **B.** Quantification of pPAK1(S144) in wt and null cells expressed as a percent of total PAK1 normalized to α -tubulin and expressed as a percentage of wt. Values are the mean $\pm$ SEM of two independent experiments.

rearrangement, RhoC acts behind the leading edge to activate its effector Rho-kinase, which phosphorylates LIM-kinase (LIMK) (Maekawa, 1999). LIMK then phosphorylates cofilin on Ser-3, inhibiting F-actin severing (Arber et al., 1998; Yang et al., 1998). To determine if the reduced RhoC activity of DGK ι -null cells affects cofilin regulation, the level of Ser-3-phosphorylated cofilin (pCofilin(S3)) was measured in DGK ι -null cell lysates and compared to lysates from wild type cells. Figure 24 shows that both p-Ser3 cofilin and total cofilin were substantially reduced in DGK ι -null cells. Quantification of the immunoblot signals and normalization to α -tubulin revealed that 60% of cofilin was phosphorylated in wild type cells, whereas only 25% of cofilin was phosphorylated in DGK ι -null cells. Together, these data suggest DGK ι is required for optimal cofilin phosphorylation and also for the maintenance of normal cofilin protein levels.

DGK ι is not required for Rho GTPase function downstream of activation

Recent results from our lab indicate that constitutively active mutants of Rac1 and RhoA, which produce well-characterized, stereotypical actin cytoskeleton changes in normal cells, fail to induce such reorganization in cells lacking DGK ζ . These results were unexpected because previous results suggested DGK ζ functions “upstream” of Rac1 and RhoA (Abramovici et al., 2009; Ard et al., 2012). Thus, even once Rac1 and RhoA become active, they still require the continued presence of DGK ζ to function correctly. To determine if active Rho GTPases similarly require the continued presence of DGK ι , wild type and DGK ι -null MEFs were transfected with epitope-tagged, constitutively active mutants of Rac1, RhoA, and RhoC (myc-Rac1^{V12}, myc-RhoA^{V14}, and HA-

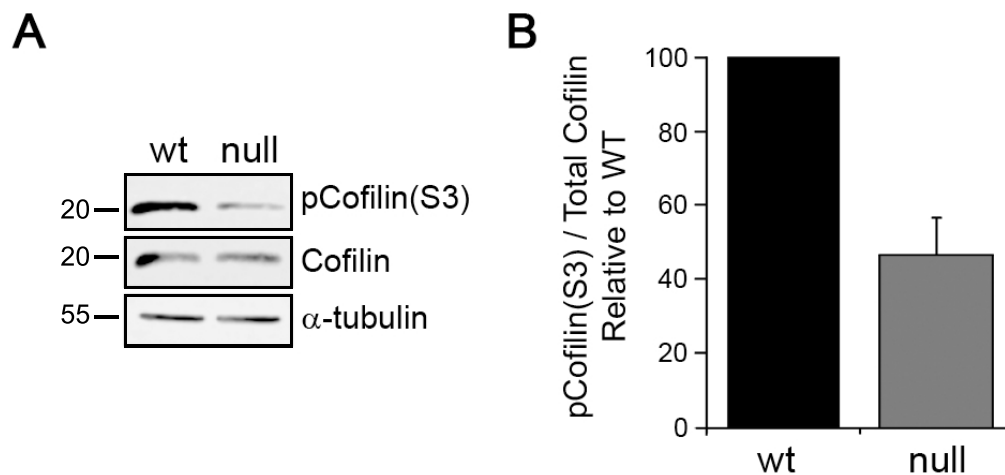


Figure 24. Phosphorylation of cofilin on Ser3 is impaired in DGK1-null cells. **A.** IB showing cellular levels of Ser-3 phosphorylated (pCofilin(S3)) and total cofilin in wt and null cells. **B.** Quantification of pCofilin(S3) in wt and null cells expressed as a percent of total cofilin normalized to α -tubulin and expressed relative to levels in wt cells. Values are the mean $\pm$ SEM of two independent experiments.

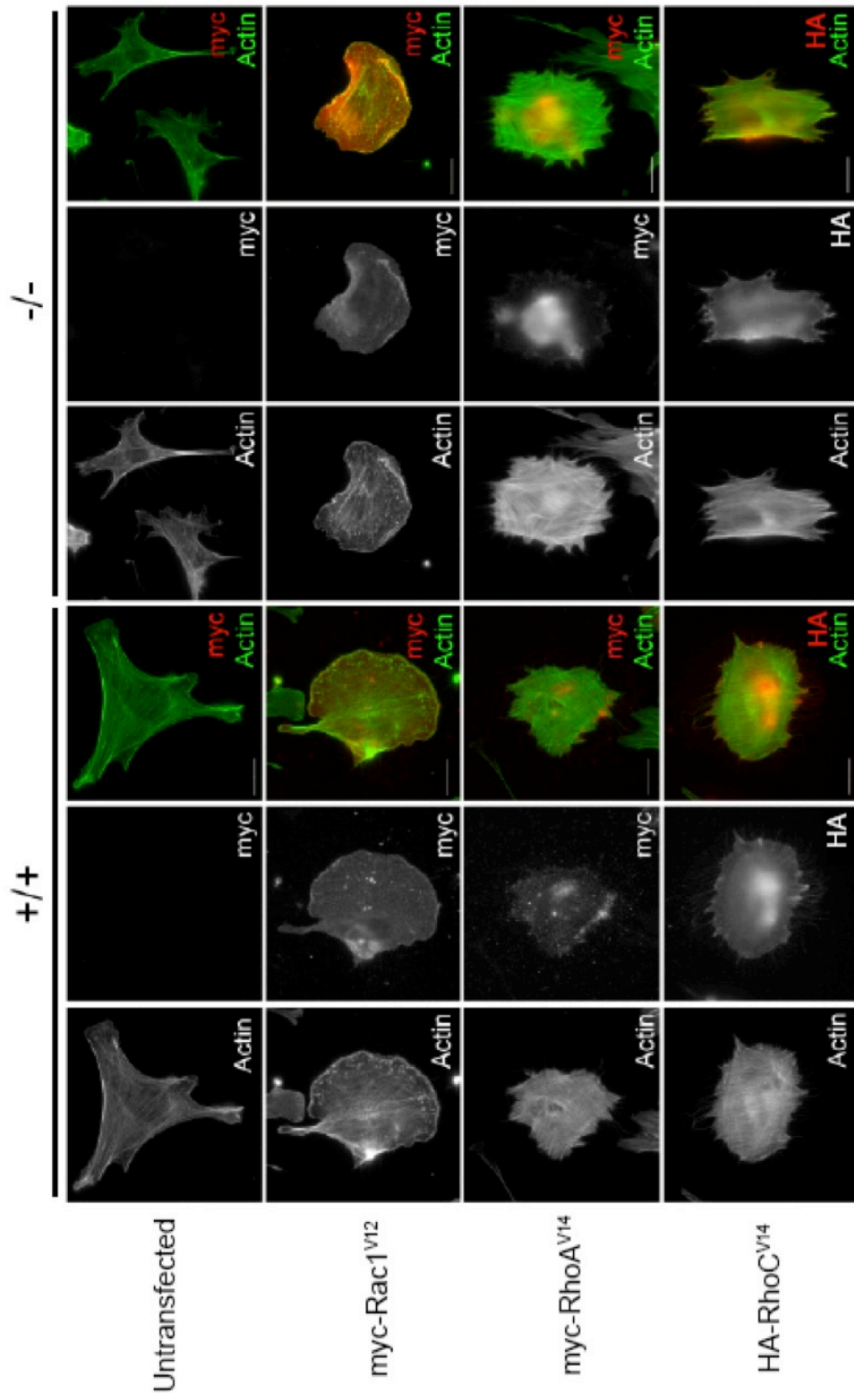


Figure 25. Constitutively active mutants of Rac1, RhoA, and RhoC function normally in DGK α -null cells. Representative images of wt and null fibroblasts either untransfected or transfected with plasmids encoding epitope-tagged, constitutively active mutants of Rac1 (myc-Rac1^{V12}), RhoA (myc-RhoA^{V14}), or RhoC (HA-RhoC^{V14}). Cells were fixed 24 hours after transfection and stained with anti-myc or anti-HA antibodies, followed by AlexaFluor 594-conjugated secondary antibody (red) and with AlexaFluor 488-conjugated phalloidin (green) to visualize F-actin. Scale bars, 20 μ m.

RhoC^{V14}, respectively). After 24 h, the cells were stained with Alexa Fluor 488-conjugated phalloidin to visualize F-actin and with anti-myc or -HA antibodies followed by Alexa Fluor 594-conjugated secondary antibodies to visualize transfected protein. As shown in Figure 25, Rac1^{V12} induced broad lamellipodia and peripheral membrane ruffles in both wild type and DGK ζ -null cells. RhoA^{V14} and RhoC^{V14} produced dense, parallel F-actin stress fibers that spanned the length of the cell in both wild type and DGK ι -null cells. The ability of these constitutively active Rho GTPases to stimulate actin reorganization in both wild type and DGK ι -null MEFs suggests DGK ι is not required downstream of Rho GTPase activation and differentiate it from DGK ζ .

Rac1 binding to PAK1 is unaffected in DGK ι -null fibroblasts

We formulated two hypotheses to explain why DGK ζ is required for Rac1 and RhoA to function even once they are active: (1) It is necessary for active Rac1/RhoA to interact with downstream effector proteins or (2) that it is required for targeting them to the correct subcellular domain(s). We tested the first of these ideas and found that DGK ζ is required for the interaction of Rac1^{V12} with PAK1 (Gee lab, unpublished results). The altered phenotype of DGK ι -null MEFs lead us to ask whether DGK ι might also be required for PAK1 binding to active Rac1. To this end, GST-Rac1^{V12} was incubated with wild type and DGK ι -null MEF cell lysates and the amount of bound PAK1 assessed by immunoblotting. GST-Rac1^{V12} captured similar amounts of PAK1 from lysates of wild type and null cells (Figure 26), suggesting DGK ι is not required for the interaction of active Rac1 with PAK1 in MEFs. As expected, GST-Rac1^{N17} did not bind appreciably to PAK1 in either cell type.

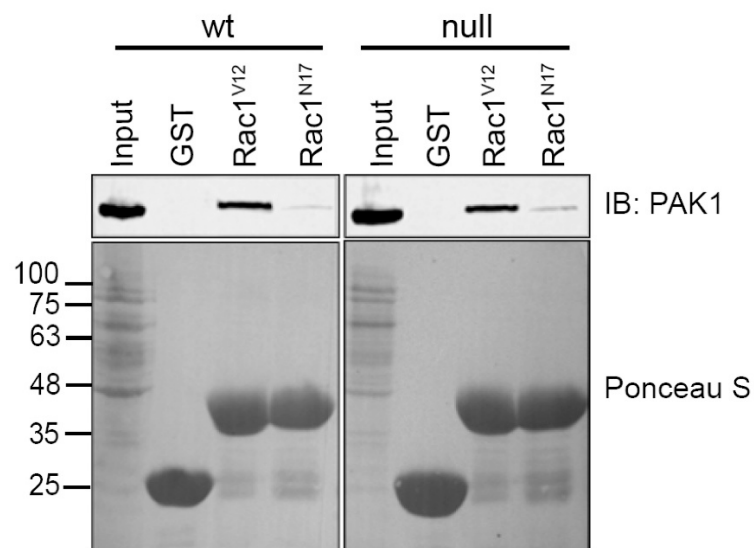


Figure 26. Active Rac1 binding to PAK1 is preserved in DGK α -null cells. IB showing the interaction between Rac1 and PAK1 in wt and null MEFs. GST-fusion proteins of constitutively active (Rac1^{V12}) and dominant negative (Rac1^{N17}) mutants of Rac1 mutants immobilized on glutathione-Sepharose beads were incubated with lysates of wt or null MEFs. Bound PAK1 was detected by IB. Approximately equal amounts of fusion protein were loaded, as shown in the Ponceau S stained blot below.

Chapter 4

Discussion

DGK ι regulates actin dynamics

The Rho family of small GTPases stimulates dynamic actin reorganization in response to a variety of extracellular signals (Etienne-Manneville and Hall, 2002). Through signaling to their downstream effectors, Rho GTPases regulate the formation of F-actin structures involved in cell migration to facilitate a number of physiological processes that are dependent upon cell motility. Rho GTPases are molecular switches that alternate between their active and inactive conformations, and their activation is highly regulated, both spatially and temporally, to prevent precocious cell migration.

RhoGDIs are important regulators of Rho GTPase signaling. These inhibitors bind and sequester Rho GTPases in inactive, cytosolic complexes, preventing their activation and association with downstream effectors (Garcia-Mata et al., 2011). Our lab previously showed DGK ζ regulates actin reorganization by facilitating the release of Rac1 and RhoA from RhoGDI (Abramovici et al., 2009; Ard et al., 2012).

We hypothesized that the closely related isoform, DGK ι , would regulate actin dynamics by similar molecular mechanisms. Indeed, I found that DGK ι -null cells had thinner stress fibers, misplaced focal adhesions, and reduced peripheral membrane ruffling. These changes in actin structure formation were accompanied by differences in cell spreading and increased migration. In addition, I found that DGK ι regulates the activities of Rac1 and RhoC, but not RhoA. Moreover, my results suggest DGK ι directly interacts with and regulates RhoC but indirectly regulates Rac1 activity through its association with the Rac1 effector PAK1. While additional studies are needed to precisely define the molecular mechanisms by which DGK ι regulates RhoC and Rac1, my results strongly suggest the functions of DGK ι and DGK ζ are not redundant. As

discussed below, it is likely that these two DGK isoforms function cooperatively to fine tune actin organization.

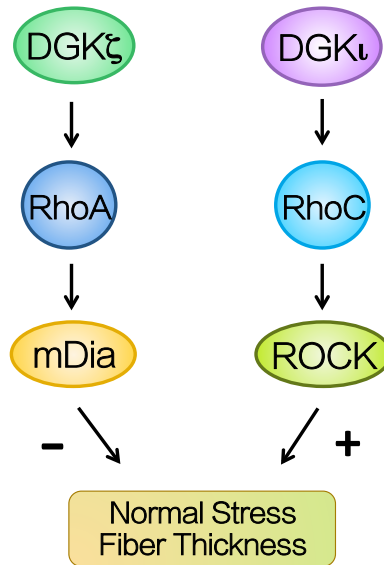
DGK ι regulates F-Actin organization

The balance between the Rho effectors ROCK and mDia regulates stress fiber thickness and organization: ROCK activity increases the thickness of these F-actin filaments (Leung et al., 1996; Amano et al., 1997), while mDia reduces it (Watanabe et al., 1999). Thus, decreased stress fiber thickness and organization DGK ι -null cells likely result from an imbalance in the activities of ROCK and mDia. An interesting possibility is that by stimulating RhoC, which has a greater affinity for ROCK than RhoA (Wheeler and Ridley, 2004), DGK ι increases the activity of ROCK relative to mDia and increases stress fiber thickness (Figure 27A).

Previous studies by our lab showed that decreased RhoA activity in DGK ζ -null cells increases the thickness of F-actin stress fibers due to increased ROCK signaling relative to mDia (Ard et al., 2012). Given their opposing effects on stress fiber morphology, it is likely that DGK ι and DGK ζ act cooperatively to regulate the balance between RhoC and RhoA signaling and the activity of their effectors. It is possible that DGK ι specifically stimulates RhoC activity to maintain cellular levels of active ROCK, increasing stress fibers thickness, while DGK ζ regulates RhoA activation to increase mDia activity, reducing the thickness of F-actin stress fibers (Figure 27A). Together, DGK ι and DGK ζ may maintain the balance in signaling by these Rho effectors to regulate the organization of F-actin. This model predicts that in the absence of DGK ι , impaired RhoC-ROCK signaling decreases the thickness of stress fibers (Figure 27B),

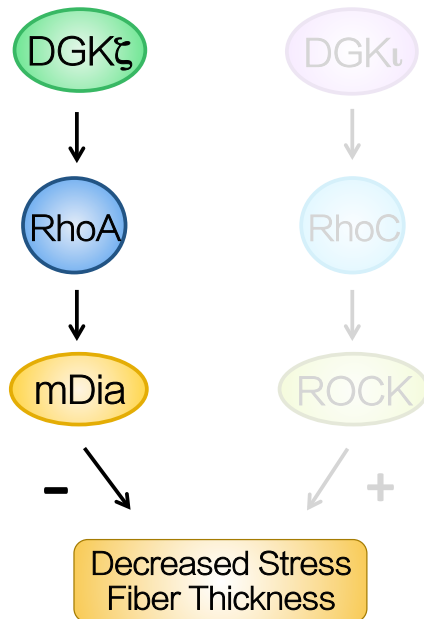
A

Stress Fiber Morphology



B

DGK ι -null



C

DGK ζ -null

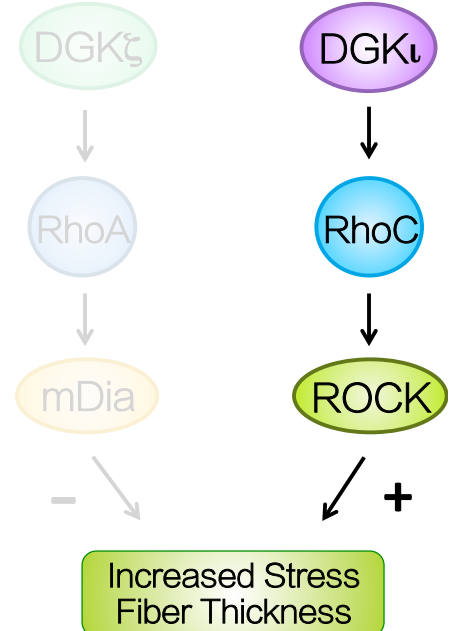


Figure 27. Balanced signaling by DGK ι and DGK ζ maintains stress fiber morphology. The Rho effectors ROCK and mDia determine the thickness of F-actin stress fibers. ROCK increases the thickness of these structures, while mDia reduces their thickness. RhoA activity downstream of DGK ζ may be required to specifically maintain the activity of mDia. This pathway may be RhoC activity downstream of DGK ι to increase ROCK activity. Decreased stress fiber thickness in DGK ι -null cells may result from increased mDia signaling relative to ROCK, while increased ROCK activity relative to mDia increases stress fiber thickness in cells lacking DGK ζ . By regulating the activities of their respective Rho isoforms, DGK ι and DGK ζ may maintain the balance between ROCK and mDia signaling and maintain stress fiber thickness.

while DGK ζ depletion leads to impaired RhoA-mDia activity, increasing stress fiber thickness (Figure 27C).

DGK ι regulates focal adhesion formation

Focal adhesions form stable attachments to the substrate at the ends of actin stress fibers and facilitate signaling between the intracellular and extracellular environments (Ridley and Hall, 1992; Nobes and Hall, 1994). The localization of focal adhesions to the tips of pseudopod-like extensions in DGK ι -nulls is consistent with decreased Rac1 and RhoA/C activation, as signaling by each of these Rho GTPases is required for normal adhesion distribution along the cell perimeter (Vidali, 2005; Ridley and Hall, 1992; Ard et al., 2012). Attachment to fibronectin by DGK ι -null cells is unaffected, which suggests DGK ι regulates the distribution of focal adhesions but not their ability to form stable attachments. A previous study by our lab showed cells lacking DGK ζ have an increased number of centrally located focal adhesions with a corresponding decrease in peripheral adhesions (Ard et al., 2012). Thus, both DGK ι and DGK ζ are required for normal focal adhesion distribution, as neither isoform alone is sufficient to maintain these structures along the cell perimeter, yet each isoform regulates a different aspect of focal adhesion localization.

Changes in F-actin organization and Rho GTPase activity also likely explain why DGK ι -null MEFs are longer and narrower than wild type MEFs. Reduced Rac1 and RhoC activity in DGK ι -null cells may cause filopodia, which are regulated by Cdc42, to become more apparent, leading to a more spindle-like morphology. Moreover, decreased RhoC activity may give rise to tail-retraction defects. Tail retraction is regulated by

ROCK, a RhoA/C effector that couples stress fiber contraction in the cell body with focal adhesion detachment at the trailing edge (Worthylake et al., 2001; Raftopoulou and Hall, 2004; Fukata et al., 2001; Totsukawa et al., 2000). Impaired ROCK signaling prevents the trailing edge from following the leading edge during migration, causing cells to elongate as stress fibers contract while focal adhesions at the rear of the cell remain attached to the substrate (Worthylake et al., 2001). Although I did not directly measure ROCK activity in DGK1-null MEFS, impaired RhoC activation is known to decrease ROCK signaling and thus would be predicted to cause tail-retraction defects, resulting in the elongated morphology characteristic of DGK1-deficient cells. Furthermore, PAK1 signaling has also been shown to be involved in focal adhesion turnover (Manser et al., 1997), and its reduced activity in DGK1-null cells may also interfere with the detachment of adhesions at the trailing edge, contributing to the elongated appearance of these cells.

DGK1 stimulates the activity of RhoC, but not RhoA

The activity of both RhoA and RhoC contribute to the regulation of stress fiber assembly (Ridley and Hall, 1992; Ridley, 2007). A surprising finding of my studies was that the level of active RhoC, but not RhoA, was dramatically decreased in DGK1-null MEFs. Thus, it is likely to be solely responsible for changes in Rho-regulated F-actin structures. Previously, we showed that cells lacking DGK ζ have impaired RhoA activation, which leads to aberrations in Rho-regulated actin structures (Ard et al., 2012). While effector-protein pull-down assays provide an indication of global changes in Rho GTPase activity, this approach does not reflect the spatiotemporal modularity by which Rho GTPase signaling occurs (Pertz, 2010). Further experiments that identify the precise

subcellular regions where DGK ι stimulates RhoC activation will provide further insight into the molecular mechanisms by which DGK ι mediates actin reorganization.

Decreased RhoC activation in DGK ι -null cells is accompanied by a ~50% decrease in RhoC protein but not RhoC mRNA, suggesting DGK ι regulates post-translational mechanisms that affect RhoC abundance. In addition, because RhoC activity measurements (Figure 20A, B) were normalized to total RhoC protein, the ~50% decrease observed in pull-down assays likely underestimates the actual amount of active RhoC in DGK ι -null cells. Thus, in contrast to DGK ζ , which regulates the activity but not the level of RhoA, DGK ι appears to regulate both the activity and abundance of RhoC. As discussed below, this latter activity may reflect the direct interaction of DGK ι with RhoC via a putative binding site located in the latter half of the protein, which is not found in DGK ζ .

In the absence of stimulation, most Rho GTPases are bound to RhoGDI in the cytoplasm, and through this interaction are maintained in an inactive state (Adamson et al., 1992). In addition, recent evidence suggests RhoGDI increases Rho GTPase stability and prevents degradation by the proteasome (Boulter et al., 2010). Indeed, targeted depletion of RhoGDI markedly decreases the protein levels of RhoA, RhoC, Rac1 and Cdc42, but surprisingly, the amount of active protein either remains the same (Rac1 and Cdc42) or is increased (RhoA) (Boulter et al., 2010; Ren, Kiosses, and Schwartz, 1999). In a preliminary experiment, the level of RhoGDI was also markedly decreased in DGK ι -null cells (Appendix, Figure 2), raising the possibility that the decrease in RhoC protein is secondary to decreased RhoGDI. However, the activity of RhoC was also substantially

decreased, which would not be expected if RhoGDI were responsible for the decrease in total RhoC.

The decrease in Rho GTPase abundance in DGK κ -null cells appears to be specific to RhoC because the levels of RhoA and Rac1 were unchanged. This further argues against a RhoGDI-dependent mechanism because a decrease in the level of one Rho GTPase generally increases the level of others by allowing them greater access to RhoGDI, which increases their stability (Figure 28; Ho et al., 2011).

DGK κ interacts with RhoC, possibly through a direct protein-protein interaction

Our lab previously showed DGK ζ binds directly to Rac1 and RhoA through its N-terminal C1 domains (Yakubchyk et al., 2005; Ard et al., 2012). The findings presented here suggest DGK κ is unlikely to associate with Rac1, yet interacts with RhoA and RhoC in pull-down assays. The interaction between DGK κ and RhoC is considerably more robust than that between DGK κ and RhoA. Functional differences between closely related Rho GTPases are attributed to differences in their hypervariable C-terminal region (Figure 29; Michaelson et al., 2001; Wennerberg and Der, 2004), and it is possible that differences in this region of RhoC and RhoA allow DGK κ to distinguish between the two, conferring stronger binding to RhoC than RhoA.

DGK κ contains a putative RhoC-binding site in its C-terminal region consisting of amino acids 744-815. This region was identified through an amino acid sequence alignment with the RhoC-binding domains of PKN-B, Rhotekin, and Rhophilin (Figure 30). DGK ζ , included in this alignment, contains no such RhoC-binding site. Taken together, our findings suggest DGK κ binds to RhoC directly and through this interaction

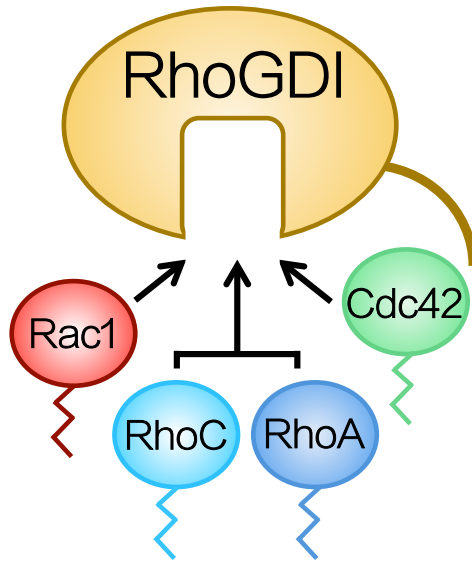


Figure 28. Rho GTPases compete for binding to their shared inhibitor, RhoGDI.

RhoGDI binding maintains cellular levels of inactive Rho GTPases by increasing their stability and protecting them from degradation. While all Rho GTPases compete against one another for binding to their shared inhibitor, RhoA and RhoC must compete against one another directly before competing against the others. Thus, a decrease in the abundance of RhoC increases the availability of RhoGDI for binding to RhoA, thereby increasing its stability and abundance within the cell.

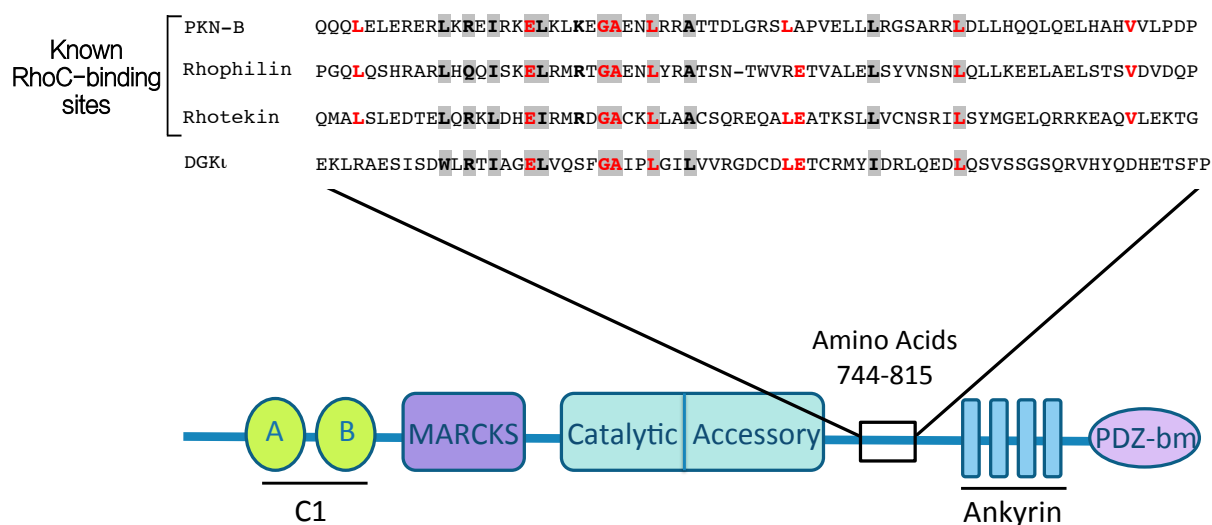


Figure 30. DGK ι amino acid sequence alignment with known Rho-binding domains. The amino acid sequences of Rho-binding domains for PKN-B, Rhophilin, and Rhotekin were aligned with the entire sequence of DGK ι . These domains aligned with a sequence composed of amino acids 744-815 in the C-terminal region of DGK ι . Aligned residues are colored according to their conservation in these sequences. Bolded red residues are identical residues, while bolded black residues are similar. Residues highlighted in grey are those that are either identical or similar in all four sequences. Figure adapted from Reid et al., 1996.

increases the protein stability of RhoC while facilitating its activation. Differential binding to Rho GTPases by DGK ι and DGK ζ provides further evidence that these closely related isoforms diverge functionally during actin reorganization.

Our results suggest DGK ι -RhoC binding regulates RhoC activity, yet it is possible that the reciprocal is also true. A binding site for GTP-RhoA has been identified in the catalytic domain of DGK θ . When bound to this region, RhoA inhibits the kinase activity of this DGK (Houssa et al., 1999). RhoC binding to the putative RhoC-binding site identified in DGK ι may have a similar, inhibitory affect on the catalytic activity of this isoform. Although this putative RhoC-binding site lies outside of DGK ι 's catalytic region, a conformational change introduced by RhoC binding may still affect the structure and function of the catalytic domain. Phosphorylation of DGK ζ 's MARCKS domain has been shown to reduce its kinase activity (Luo et al., 2003) and demonstrates that modifications to regions outside of DGKs catalytic domain have the potential to influence DAG phosphorylation by these enzymes.

DGK ι indirectly stimulates Rac1 activity to regulate actin reorganization

Rac1 signaling induces the formation of protrusive actin structures that regulate cell morphology and facilitate spreading (Ridley et al., 1992). The spindle-like morphology and decreased cell area of DGK ι -nulls is consistent with insufficient Rac1 activity, as are the extension of narrow, pointed protrusions and the aberrant localization of focal adhesions during spreading (Vidali et al., 2011). Targeted RhoC depletion results in unregulated Rac1 activity along the cell perimeter and leads to the formation of broad lamellipodial and extensive peripheral membrane ruffling (Vega et al., 2011). The

decreased Rac1 activity in DGK ι -null cells likely prevents these structures from forming in response to decreased active RhoC, and thus contributes to the elongated morphology of these cells. Defects in the formation of Rac1-related actin structures, such as lamellipodia and peripheral membrane ruffles, were previously identified in cells lacking DGK ζ (Abramovici et al., 2009). Thus, both DGK ι and DGK ζ are required for optimal Rac1 activation, as either isoform alone is insufficient to maintain normal levels of active Rac1. It is possible that each DGK isoform is responsible for activating a distinct pool of cellular Rac1 that may be determined by differences in subcellular localization that allow DGK ι and DGK ζ to associate with unique signaling mechanisms upstream of Rac1 activation.

The molecular mechanism by which DGK ζ stimulates Rac1 activity involves direct binding between DGK ζ and Rac1 within a larger signaling complex (Abramovici et al., 2009). The findings presented here show DGK ι is also required for optimal Rac1 activation, but an interaction between DGK ι and Rac1 was not detected. It is possible that DGK ι associates with Rac1 through a transient interaction or one too weak to be detected by this approach. However, if Rac1 and DGK ι do not interact, these results suggest DGK ι regulates Rac1 activity indirectly, perhaps by acting through GEFs or other regulators of Rac1 activation. Moreover, they suggest DGK ι and DGK ζ likely regulate Rac1 activation through separate molecular mechanisms, despite their high degree of amino acid identity.

DGK ι may regulate Rac1 signaling by stimulating the activity of PAK1

Rac1 activation involves a complex positive feedback loop in which phosphatidic acid produced by DGK ζ first activates PAK1, which then phosphorylates itself and

RhoGDI to induce Rac1 dissociation from RhoGDI and its subsequent activation by GEFs (DerMardirossian et al., 2004; Abramovici et al., 2009). The binding of active Rac1 induces more PAK1 activation leading to further Rac1 activation (Manser et al., 1994; Daniels and Bokoch, 1999; Lei et al., 2000).

Our findings suggest DGK α is required for the activation of both Rac1 and PAK1. Growth factor stimulation rescues PAK1 autophosphorylation on Ser-144 in DGK α -null cells (Appendix, Figure 3) and induces circular ruffling (Figure 14), yet Rac1 activity remains low (Figure 9A, B). These observations are inconsistent with a model in which PAK1 activity directly stimulates the activation of Rac1 (Abramovici et al., 2009). It is known that PAK1 phosphorylates ArhGAP15, a Rac1-GAP, inhibiting its activity and preventing it from inactivating Rac1 (Radu et al., 2013). It is possible that DGK α is required to localize active PAK1 to the membrane where it can interact with and inhibit GAP activity (Figure 31A). In the absence of DGK α , the inability for PAK1 to associate with Rac1-GAPs would increase GAP activity and reduce levels of active Rac1 (Figure 31B).

Similarly, DGK α may localize PAK1 to Rac1-GEFs to stimulate their activation (Figure 32A). In the absence of DGK α , PAK1 may be unable to associate with and stimulate the activity of GEFs upstream of Rac1, leading to decreased GTP-binding by Rac1 (Figure 32B). Moreover, PAK1 has been shown to compete with Rac1 for binding the Rac1-GEF β -PIX, thereby reducing Rac1 activation (Figure 33A; ten Klooster et al., 2006). An alternative possibility is that PAK1-DGK α binding may sequester PAK1 from β -PIX, increasing the interaction between Rac1 and β -PIX and indirectly stimulate

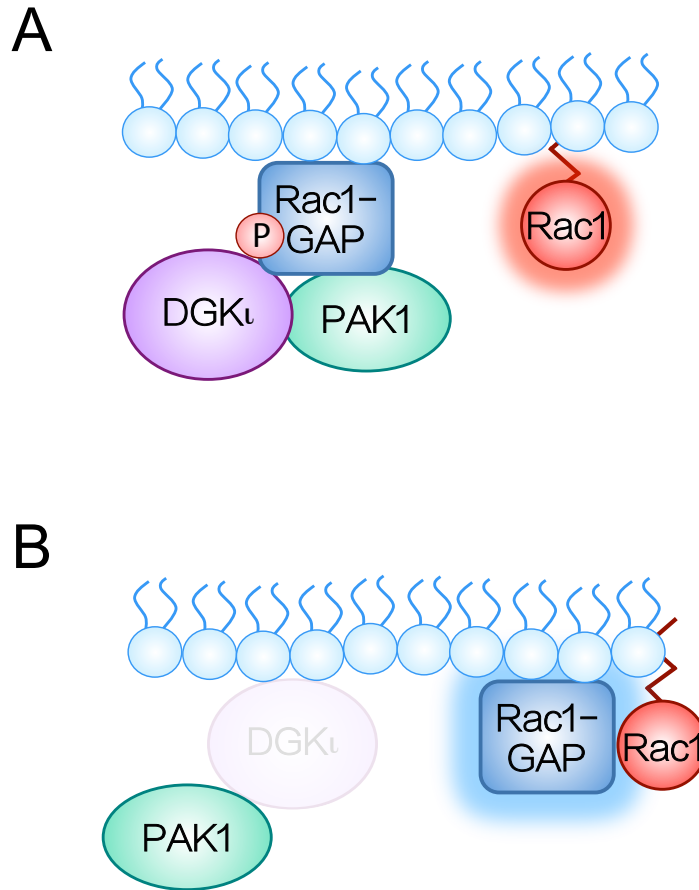
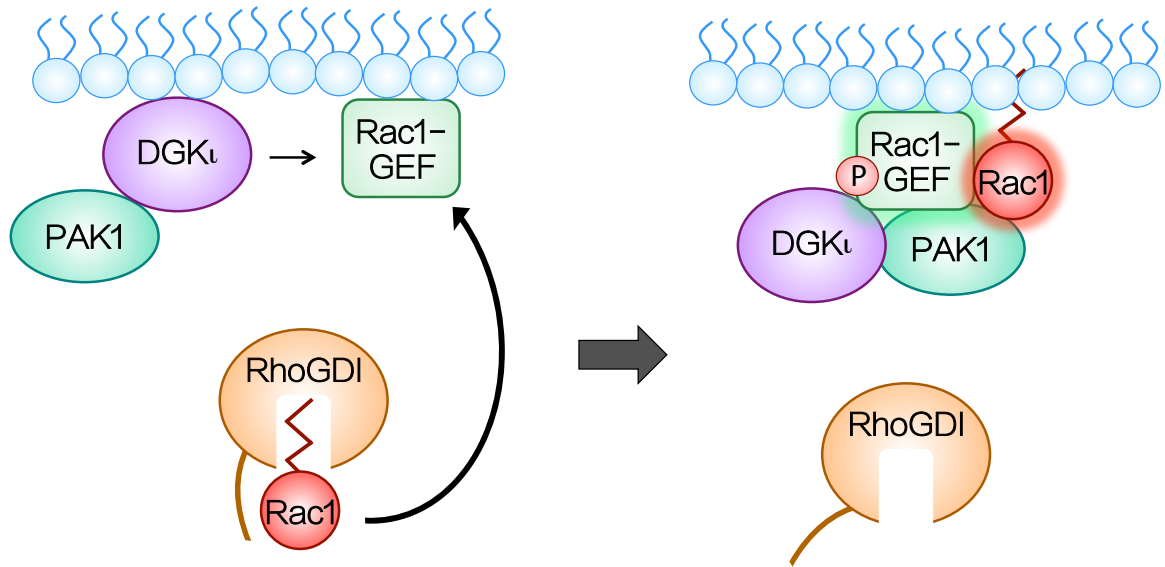


Figure 31. Schematic showing a possible mechanism by which DGKi and PAK1 indirectly stimulate Rac1 activity through the inhibition of GAPs. A. Under normal conditions, DGKi may localize PAK1 to GAPs at the membrane. Phosphorylation of GAPs by PAK1 have been shown to act as an inhibitory signal, preventing these negatively regulators or Rho GTPase activity from associating with and inactivating Rac1. **B.** In the absence of DGKi, PAK1 may be unable to associate with and phosphorylate GAPs to negatively regulate their activity, resulting in increased Rac1 inactivation.

A



B

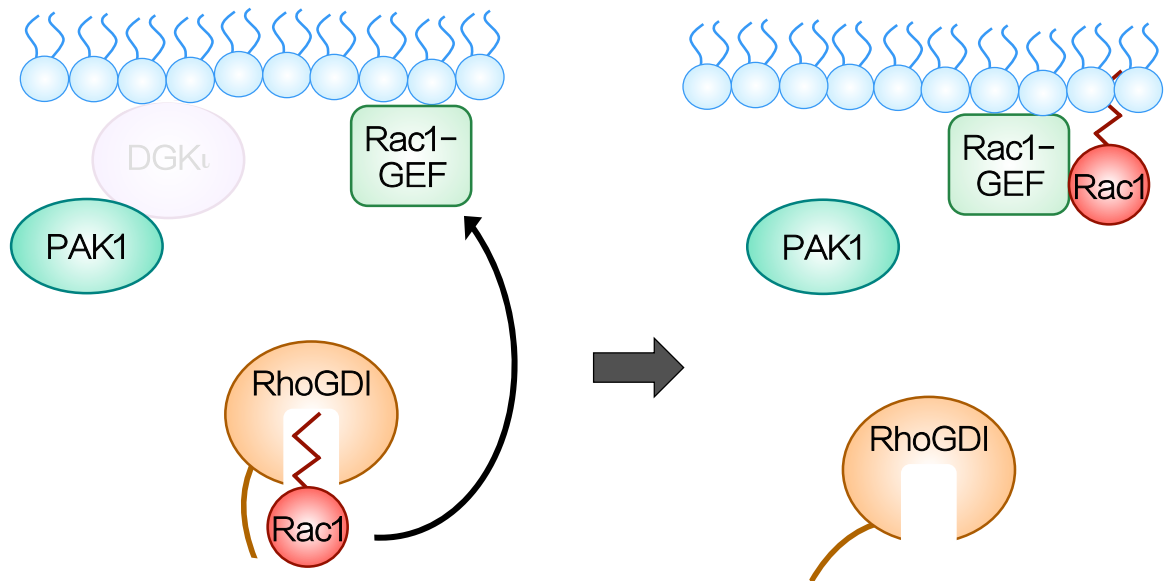


Figure 32. Schematic showing a possible mechanism by which DGK ι facilitates the activation of GEFs by PAK1 to increase Rac1 signaling. **A.** Under normal conditions, DGK ι may interact with PAK1 to facilitate its membrane localization, stimulating the activities of Rac1-GEFs. Active GEFs can then induce the activation of Rac1 upon its release from RhoGDI. **B.** In the absence of DGK ι , PAK1 may not be sufficiently localized to the membrane. As a result, GEF activity may remain low, preventing Rac1 from becoming active once dissociated from RhoGDI.

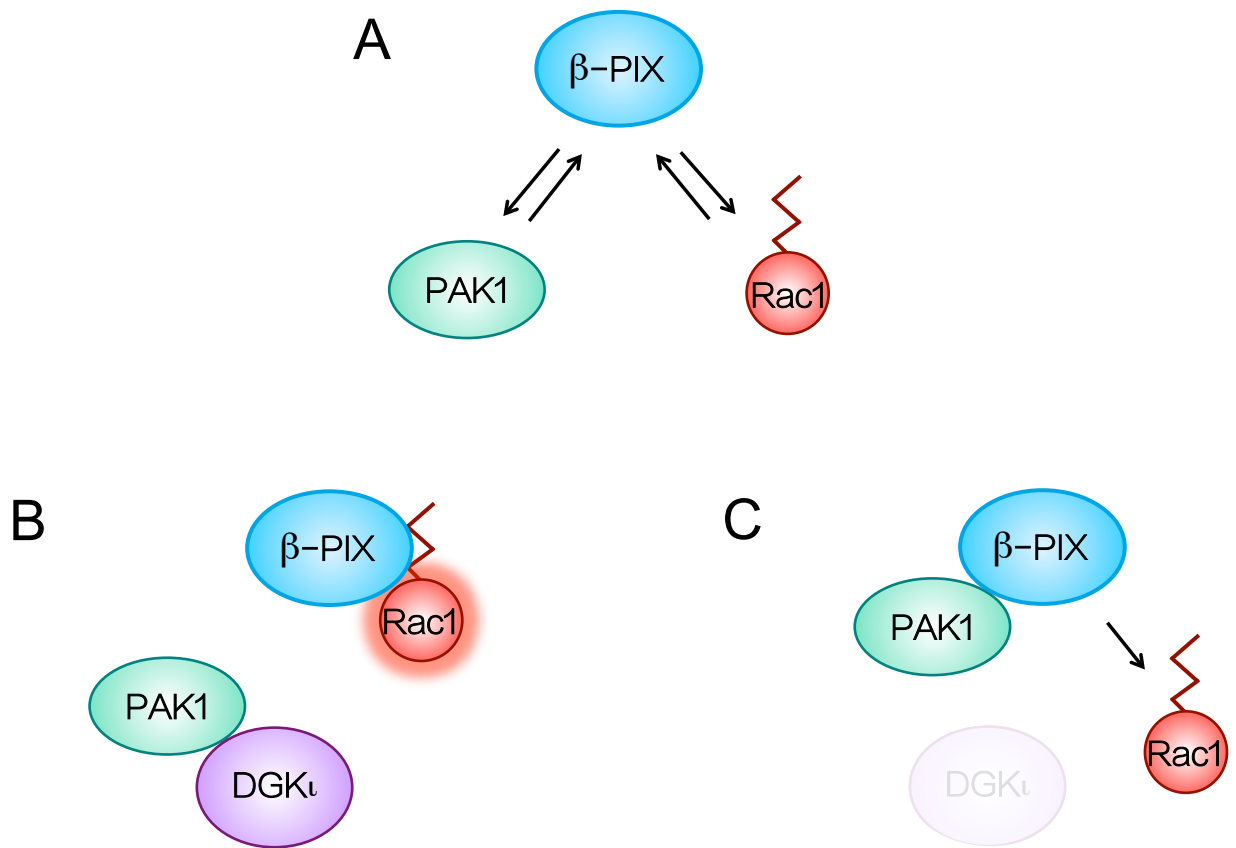


Figure 33. Schematic depicting an alternative mechanism in which DGK ι indirectly stimulates Rac1 activation by sequestering PAK1 from the GEF β -PIX. **A. PAK1 and Rac1 compete for binding to β -PIX, a Rac1-GEF. **B.** In this model, DGK ι binding to PAK1 sequesters PAK1 from β -PIX, allowing β -PIX to associate with and activate Rac1. **C.** In the absence of DGK ι , PAK1 binding to β -PIX is increased and displaces Rac1, preventing its activation.**

Rac1 activity (Figure 33B). Thus, in the absence of DGK ζ , PAK1 binding to β -PIX may increase, reducing the pool of this GEF available for binding to and activating Rac1 (Figure 33C).

PDGF stimulates circular ruffling in DGK ζ -null cells, despite reduced levels of GTP-Rac1

PDGF signals via a Rac1-PAK1 pathway to induce the formation of circular membrane ruffles on the dorsal surface of fibroblasts (Dharmawardhane et al., 1997; Sells et al., 1997; Sells et al., 2000). PDGF-induced circular ruffling, peripheral ruffling and lamellipodia formation are impaired in DGK ζ -null MEFs, consistent with reduced Rac1 and PAK1 activation (Abramovici et al., 2009). In contrast, PDGF-induced circular ruffling persisted in DGK ζ -deficient cells, despite the dramatic reduction in Rac1 and PAK1 activity. That circular ruffling in DGK ζ -null cells was blocked by Rac1 and PAK1 inhibitors (Appendix, Figure 4) suggests it occurs downstream of Rac1-PAK1 signaling and not by an alternative mechanism. Perhaps the residual Rac1 activity in DGK ζ -null cells exceeds a threshold for the formation of circular ruffles, but not for peripheral ruffles. Alternatively, PDGF may bypass Rac1 and directly stimulate PAK1 activity. This model is supported by our finding that PAK1 phosphorylation on Ser-144 is reduced in cells lacking DGK ζ , yet is largely rescued by PDGF treatment (Appendix, Figure 3).

PAK1 is an auto-regulated homodimer in which one subunit binds and inhibits the kinase activity of the other (Lei et al., 2000). Binding of active Rac1 or Cdc42, or stimulation by PA, introduces a conformational change that allows one monomer to phosphorylate the other at multiple sites, stabilizing the enzyme in its active conformation (Bokoch et al., 1998; Lei et al., 2000). Many autophosphorylation sites

have been identified (Chong et al., 2001), and Thr-423 and Ser-144 are among the first to be phosphorylated (Parrini et al., 2002; Parrucello et al., 2006). PAK1 phosphorylation on Ser-144 is substantially decreased in DGK α -nulls suggesting PAK1 activation is impaired in the absence of DGK α . My results showing DGK α and PAK1 interact in cells supports the idea that DGK α -derived PA directly contributes to the stimulation of PAK1 activity. However, my finding that a catalytically inactive DGK α is able to generate lamellipodia and peripheral ruffling, which occur downstream of PAK1 activation, raises the possibility of alternative mechanisms. Thus, additional experiments are required to more fully explore how DGK α regulates PAK1 activity.

DGK α acts upstream of Rho GTPase activation to regulate actin dynamics

Both Rac and Rho signaling pathways regulate the activity of cofilin, an actin-remodeling protein that cleaves actin filaments (Figure 34). RhoA and RhoC signal to ROCK, which stimulates the activity of LIMK-kinase (Riento and Ridley, 2003). LIM-kinase then phosphorylates cofilin on Ser-3, inhibiting its ability to bind and sever F-actin (Arber et al., 1998). Cofilin inhibition downstream of ROCK and LIM-kinase is thought to be regulated by RhoA in normal cells (Worthylake and Burridge, 2003) and RhoC in cancer cells (Bravo-Cordero et al., 2011; Bravo-Cordero et al., 2013). Through a separate signaling pathway, Rac1 signals to its effector, PAK1, which induces the activation of LIM-kinase and leads to the inhibition of cofilin (Edwards et al., 2000). Through their respective signaling pathways, Rac1, RhoA, and RhoC confine cofilin activity to areas of

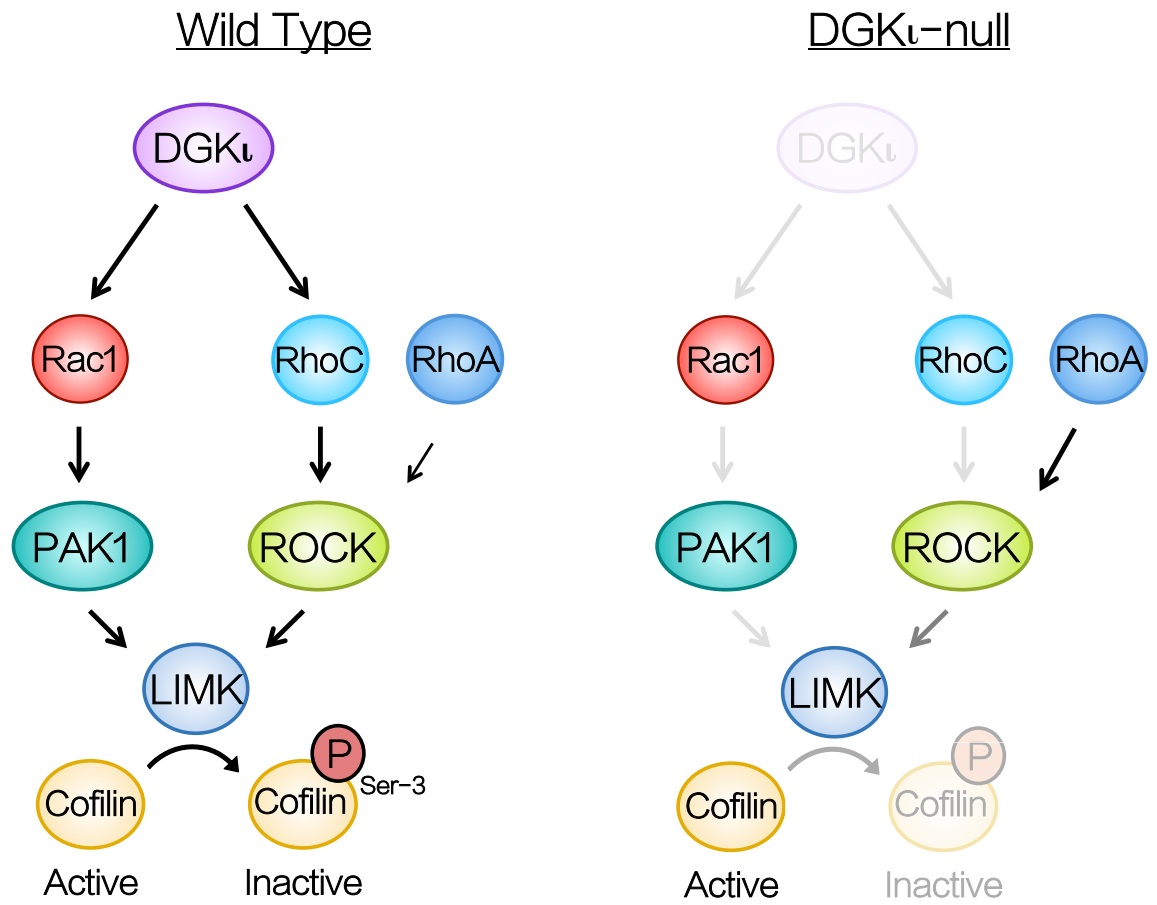


Figure 34. DGK ι regulates cofilin activity through Rac1 and RhoC signaling. The activity of cofilin, an F-actin severing protein, is regulated by Rac1 and RhoC. On the left, Rac1 activates its effector, PAK1, which then stimulates LIM-kinase leading to cofilin phosphorylation on Ser-3. Phosphorylation at this site impairs cofilin binding to F-actin and inhibits its activity. Similarly, RhoC activates its effector, ROCK, to induce cofilin phosphorylation by LIM-kinase. RhoA also stimulates ROCK, however its affinity for this effector is less than that of RhoC, as indicated by a small arrow between RhoA and ROCK. On the right, the absence of DGK ι reduces the activities of both Rac1 and RhoC, preventing the activation of their downstream effectors, reducing LIM-kinase activity and cofilin phosphorylation. Signaling by RhoA is unaffected by the loss of DGK ι , maintaining some regulation over ROCK and LIM-kinase activity but much less than in the wild type condition. As a result, cofilin inhibition is impaired in cells lacking DGK ι , which leads to increased F-actin severing and disrupts the dynamic balance of actin reorganization.

rapid actin reorganization (Yang et al., 1998; Bravo-Cordero et al., 2014). Phosphorylation of cofilin on Ser-3 was substantially decreased in DGK κ -null cells. This indirect measurement of Rho GTPase activity further establishes that DGK κ functions upstream of Rho GTPases to regulate actin dynamics in MEFs. Not only was phospho-cofilin(S3) decreased in DGK κ -null cells but also the level of total cofilin protein was reduced. Interestingly, the reduction in Ser-3 phosphorylated cofilin was greater than the decrease in total cofilin protein suggesting the loss of DGK κ affects the activity and abundance of proteins that function downstream of it. It will be interesting to determine if other actin regulatory proteins are similarly affected.

DGK ζ -null cells are unable to form Rac1- and RhoA-related actin structures upon expression of their respective constitutively active mutants, Rac1^{V12} and Rac1^{V14}, respectively (Gee lab, unpublished results). In contrast, the expression of constitutively active Rac1, RhoA, and RhoC in DGK κ -null cells was sufficient to induce the formation of their respective F-actin structures (Figure 25), indicating that DGK κ is not required downstream of Rho GTPase activation. Consistent with this, Rac1 binding to PAK1, which is substantially decreased by the absence of DGK ζ , was unaffected by the absence of DGK κ . Together, these findings suggest DGK κ functions exclusively upstream of Rho GTPase activation to regulate actin reorganization, and provide further support for a model in which DGK κ and DGK ζ regulate actin dynamics through distinct molecular mechanisms.

Cell migration is increased in cells lacking DGK ϵ

Cell migration is essential to a number of physiological processes (Etienne-Manneville and Hall, 2002). Signaling by Rho GTPases spatiotemporally coordinates the formation of F-actin structures required for motility (Ridley et al., 2003). Rac1 and Cdc42 have traditionally been thought to act at the leading edge of the cell, stimulating cell protrusion through the formation of lamellipodia and filopodia, while RhoA was thought to function at the rear to stimulate contraction and the detachment of adhesions (Ridley et al., 2003). More recent studies of Rho GTPase activation during migration show that spatially discrete zones of active Rac1, RhoA, and Cdc42 are present at the leading edge. Through complex cross-talk, these Rho GTPases spatially and temporally coordinate the activities of each other to regulate protrusion and retraction at the leading edge (Machacek et al., 2009). The increased migration by DGK ϵ -null cells likely results from decreased Rac1 and RhoC activity, leading to an imbalance of their activities relative to other Rho GTPases in the cell, such as RhoA (Figure 35). My thesis work provides evidence that this imbalance affects the formation of different actin-dependent structures and overall cell shape. Ultimately, these changes will impact how the cells move on two- and three-dimensional substrates.

In this study, cell migration on 2D substrates was measured by changes in ‘relative wound density’, which represents the density of cells within the wounded region expressed as a percent of the cell density outside of the wounded region. This method of determining changes in migratory behavior accounts for differences in cell density and allows for a direct comparison to be made between different cell types. The values for relative wound density shown in Figure 15 represent the mean of 24 technical replicates.

The data suggest DGK ι -null MEFs migrate substantially faster than wild type MEFs. Since this experiment comprises one biological replicate, it should be repeated and statistical significance determined. Nevertheless, when taken together with our previous results showing decreased migration of DGK ζ -null cells (Abramovici et al., 2009) the results presented here suggest DGK ι and DGK ζ having opposing effects on migration of MEFs: DGK ζ stimulates, while DGK ι inhibits, migration. Thus, it would appear that the balance of activities of these two DGK isoforms regulates the extent of cell motility, although additional studies are required to validate this conclusion and to extend it to other cell types. In addition to regulating actin reorganization during 2D migration, Rho GTPases also mediate cell invasion through 3D matrices. The molecular mechanisms involved in regulating these processes are distinct due to differences in the F-actin structures involved; yet both require upstream input from Rho GTPases (Khatau et al., 2012; Doyle et al., 2009). It is possible that DGK ι , by regulating Rac1 and RhoC activation, also impinges upon invasion through 3D matrices. Indeed, RhoC signaling is associated with increased tumor cell invasiveness and is absolutely required for metastasis of breast cancer cells in vivo (Hakem et al., 2005; Kleer et al., 2005). Future studies aimed at studying DGK ι 's role in tumor cell invasion will seek to expand the relevance of DGK ι as a possible target for strategies aimed at inhibiting metastasis of metastatic cancer cells.

Conclusions

The findings presented herein demonstrate that DGK ι mediates dynamic F-actin reorganization by stimulating the activities of RhoC and Rac1 and may balance signaling by these Rho GTPases with others involved in actin cytoskeletal remodeling (Figure 35).

DGK ζ , which is structurally similar to DGK ι , was previously shown to facilitate the activation of RhoA and Rac1 through its involvement in multi-protein signaling complexes unique to each (Abramovici et al., 2009; Ard et al., 2012). Aberrant actin structure formation occurs in both DGK ι - and DGK ζ -null cells, yet differences in the morphology of these two cell types suggests DGK ι and DGK ζ are not functionally redundant isoforms despite their identity. Moreover, the findings presented here suggest DGK ι specifically regulates the activities of Rac1 and RhoC. In contrast, DGK ζ regulates Rac1 and RhoA (Abramovici et al., 2009; Ard et al., 2012), further suggesting differential actin regulation by these DGK isoforms. Given that Rho GTPase signaling occurs in the context of larger protein signaling complexes coupled to specific downstream effectors in response to a particular stimulus upstream (Abramovici et al., 2009; Pertz, 2010; Ard et al., 2012), DGK ι and DGK ζ likely associate with separate protein complexes that allow each to regulate the activities of their respective Rho GTPases through distinct molecular mechanisms.

The majority of the findings presented here were acquired using DGK ι -null MEFs. It is possible that DGK ι -null cells up-regulate or down-regulate the expression or activity of additional proteins to compensate for the loss of DGK ι , and that these biochemical changes contribute to the altered morphology of these cells. Additional experiments, such as F-actin characterization upon DGK ι mRNA silencing in wild type cells, would provide further support for the role of DGK ι in Rho GTPase signaling and actin cytoskeletal reorganization. Furthermore, rescue experiments in which exogenous DGK ι is expressed in DGK ι -null cells would also strengthen the arguments that DGK ι is required for the activation of Rac1 and RhoC. Preliminary data shows that the expression

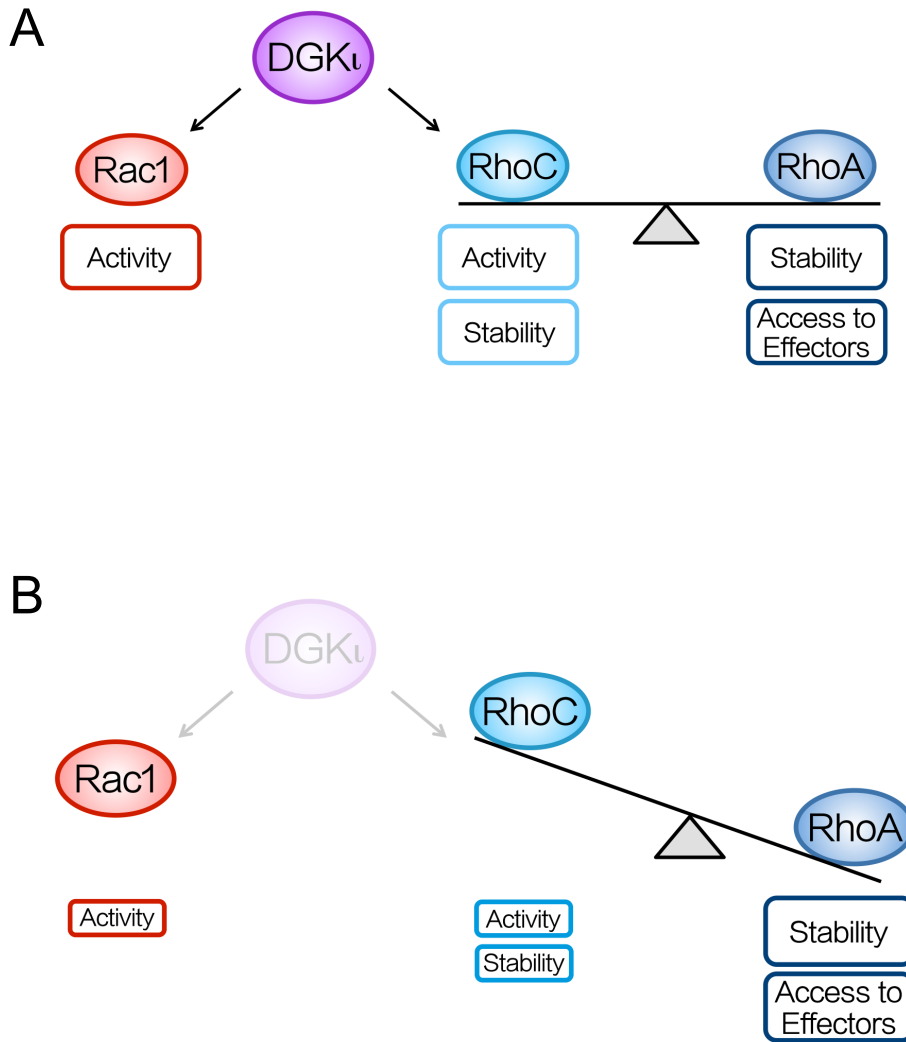


Figure 35. DGK_I regulates Rac1 and RhoC activity, and in doing so maintains the balance between signaling by RhoC and RhoA. **A.** DGK_I regulates the activities of Rac1 and RhoC, although the precise molecular mechanisms by which DGK_I does so remain unknown. In addition stimulating RhoC activation, DGK_I increases cellular levels of RhoC protein, perhaps by binding to it to increase its stability in the cytosol. **B.** In the absence of DGK_I the activities of Rac1 and RhoC are decreased, as is RhoC protein stability. As a result, relative levels of RhoA increase, likely granting RhoA greater access to effectors normally sequestered by RhoC.

of wild type DGK ι (DGK ι^{WT}), as well as a kinase dead mutant (DGK ι^{KD}), is sufficient to induce the formation of broad, ruffling lamellipodia and rescue the morphology of spindle-like ι -null cells (Appendix, Figure 5), suggesting the absence of DGK ι is responsible for defective Rac1 signaling in these cells rather than unrelated biochemical changes. Furthermore, these results suggest the catalytic activity of DGK ι is not required for the formation of Rac1-associated actin structures. While these initial findings are promising, further experiments with additional controls will increase support for our finding that DGK ι regulates the activities of Rac1 and RhoC to mediate changes in actin dynamics.

The activities of Rac1, RhoA, and RhoC are increased in several human cancers (Karlsson et al., 2009). Increased RhoA signaling is implicated in cell transformation, while unregulated signaling by Rac1 and RhoC is associated with tumor metastasis (Ruth et al., 2006; Chen et al., 2014; Bellocin et al., 2006; Simpson et al., 2004; Bid et al., 2013). Our novel finding that DGK ι specifically regulates the activities of Rac1 and RhoC, but not RhoA, may allow for the development of therapeutic agents that specifically target Rho GTPases involved in metastasis and prevent precocious cell migration associated with disease.

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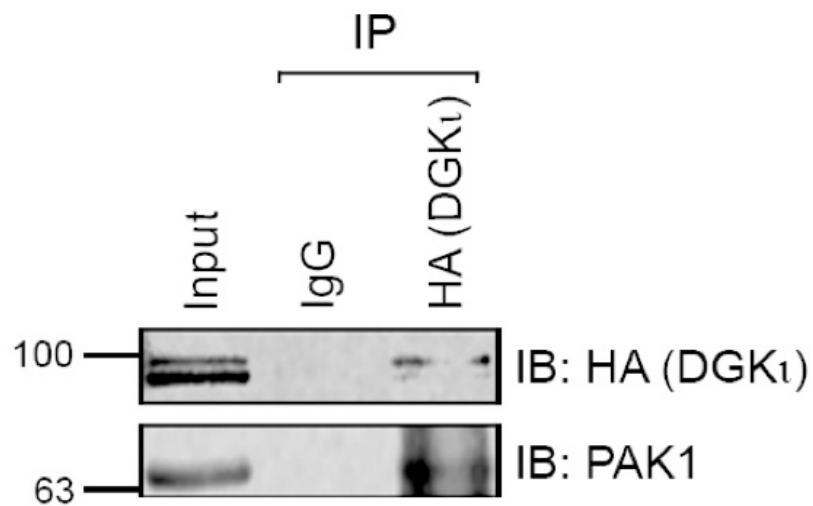
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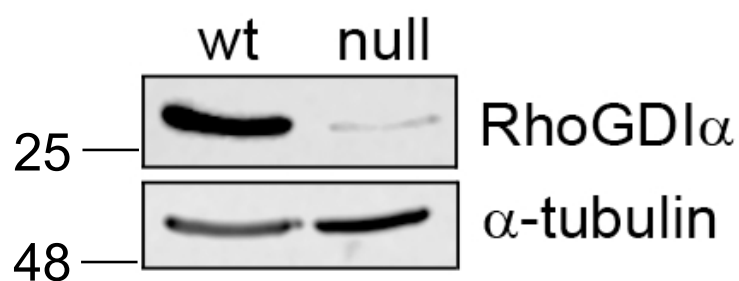
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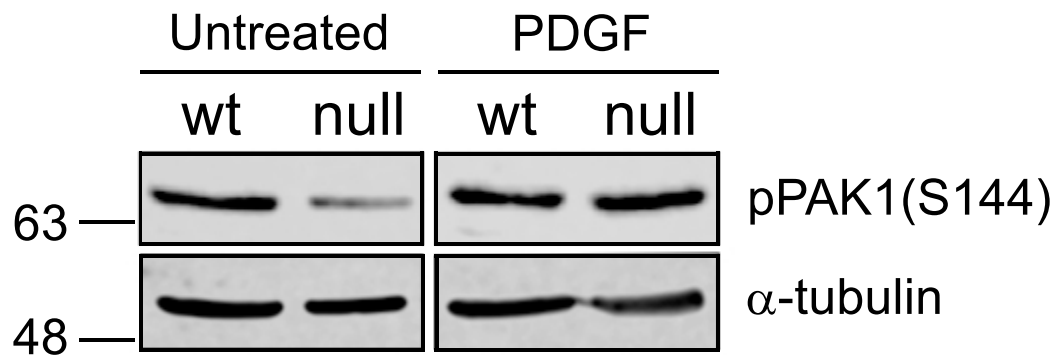
Appendix



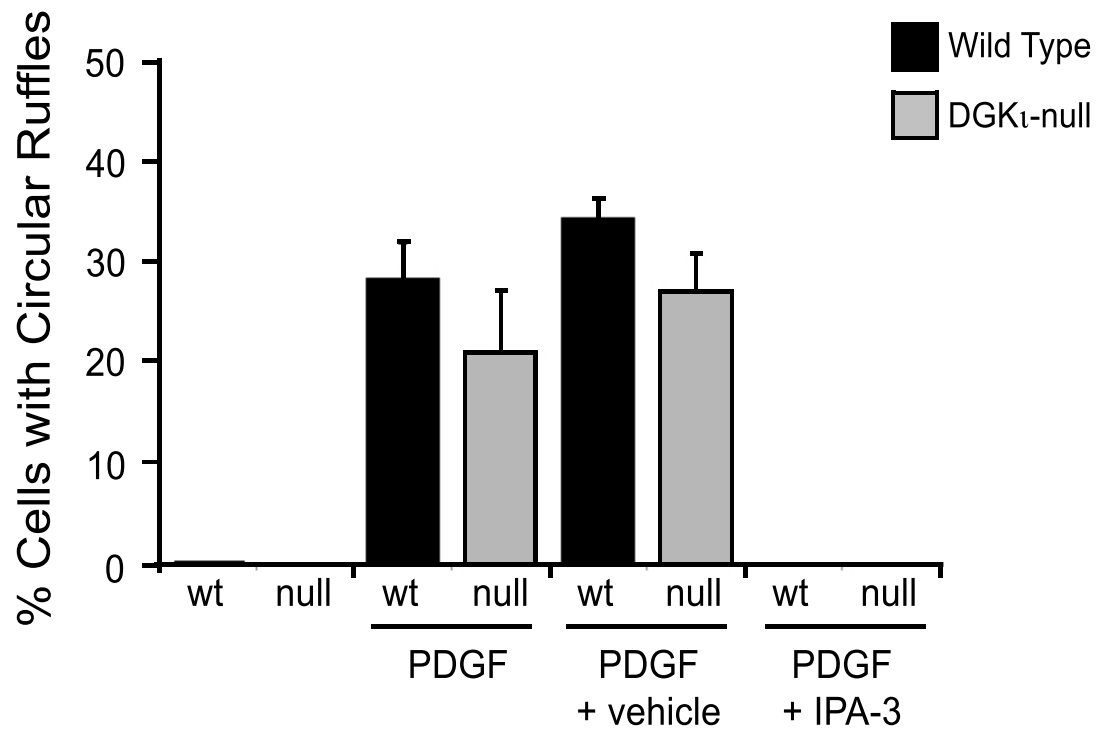
Appendix Figure 1. PAK1 interacts with DGK1 in MEF cell lysates. Endogenous PAK1 co-immunoprecipitates with HA-tagged wild type DGK1. Wild type MEFs were infected with an adenovirus for HA-DGK1. 24 hours after infection these cells were harvest and lysate was immunoprecipitated (IP) with anti-HA antibody or control IgG. Immune complexes were analyzed by immunoblotting using an antibody against endogenous PAK1.



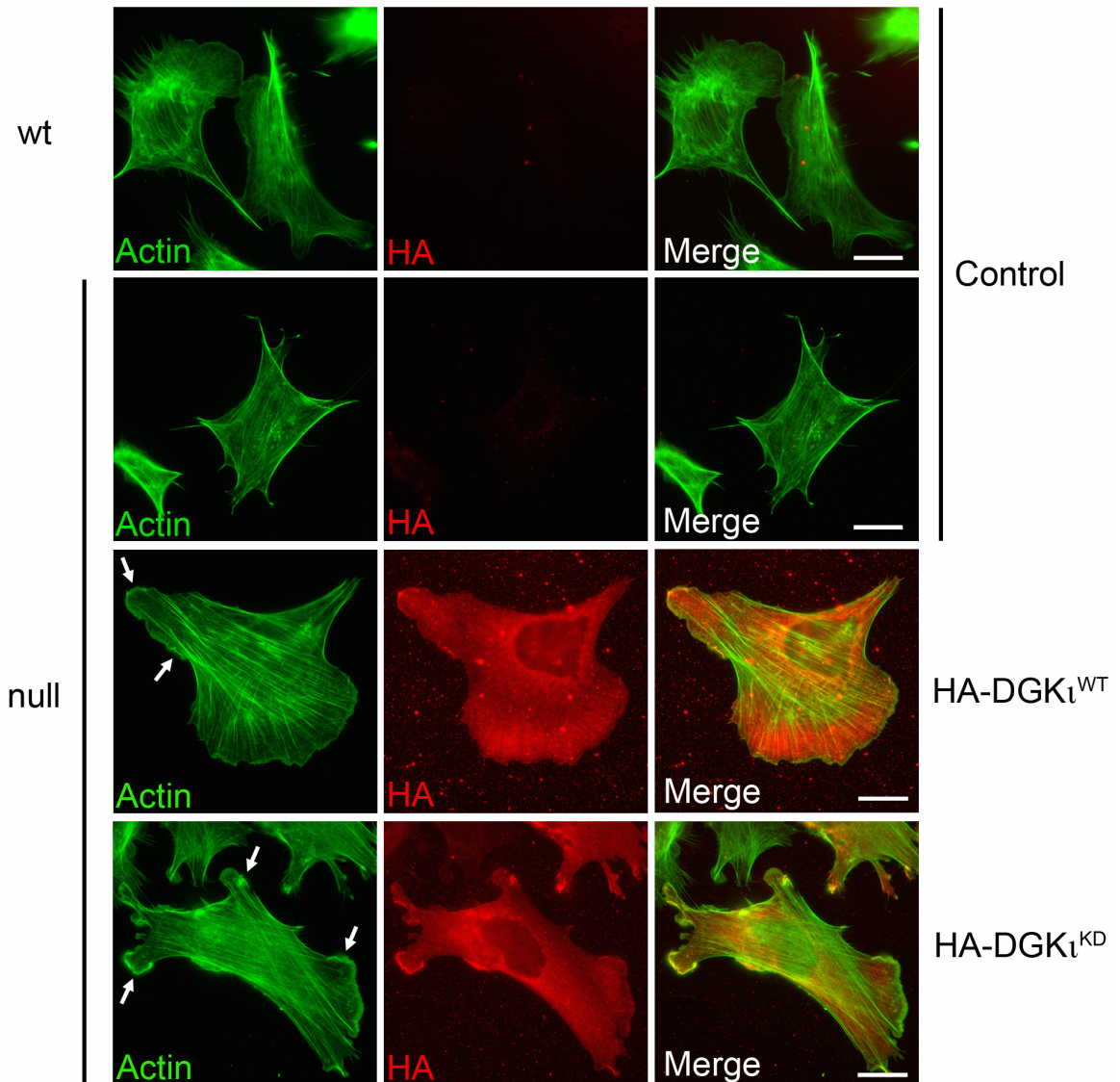
Appendix, Figure 2. RhoGDI protein is reduced in α -null cell lysates. IB showing global levels of RhoGDI in wt and null cell lysates. Cells were grown in standard conditions and the total amount of RhoGDI protein present in cell lysates was determined by immunoblotting using an anti-RhoGDI antibody. α -tubulin was used as a loading control.



Appendix, Figure 3. PDGF stimulation rescues PAK1 autophosphorylation on Ser-144 in DGK κ -null cells. Representative IB showing levels of Ser-144 phosphorylated PAK1 in wt and κ -null cell lysates. Cells were serum-starved for 16 hours before stimulation with 50ng/ml PDGF in serum-free media for 5 minutes. Levels of Ser-144 phosphorylated PAK1 were assessed by immunoblotting using a phosphospecific antibody against Ser-144 phosphorylated PAK1, and α -tubulin was used as a loading control.



Appendix, Figure 4. Inhibition of PAK1 reduces PDGF-induced circular membrane ruffling. Graph showing the percentage of wt and ι -null cells with at least one circular membrane ruffle upon stimulation with 50ng/ml PDGF alone, as well as in the presence of a vehicle (DMSO), and 10 μ M of the PAK1-inhiitor IPA-3. Values represent the average of three independent experiments $\pm$ SEM.



Appendix Figure 5. Wild type and kinase dead DGK ι induce lamellipodia formation and membrane ruffling in DGK ι -null cells. DGK ι -null cells were plated on fibronectin and infected with an adenovirus for HA-tagged wild type DGK ι (HA-DGK ι^{WT}), as well as an HA-tagged kinase-dead mutant (HA-DGK ι^{KD}). Uninfected wt and null cells were used as controls. Cells were stained with Alexa Fluor 488-conjugated phalloidin to visualize F-actin and an anti-HA antibody followed by an Alexa Fluor 594-conjugated secondary antibody to identify infected cells. Scale bar, 20 μm .