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Plakophilin 2: Critical Roles in Intercellular Junction Assembly and Maturation via Regulation of Protein Kinase C and Actin

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ABSTRACT

Plakophilin 2: Critical Roles in Intercellular Junction Assembly and Maturation via Regulation of Protein Kinase C and Actin

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Desmosomes are adhesive complexes found at sites of intercellular contact that are essential for mediating cell-cell adhesion. These junctions undergo regulated assembly and reorganization during processes such as embryogenesis and wound healing. Plakophilins (PKPs) are armadillo family members related to the classic cadherin-associated protein $p120^{ctn}$. PKPs localize to the cytoplasmic plaque of intercellular junctions and participate in linking the intermediate filament (IF)-binding protein desmoplakin (DP) to desmosomal cadherins. During desmosome assembly PKP2 associates with DP in plaque precursors that form in the cytoplasm in response to cell-cell contact, which translocate to nascent desmosomes.

Here we provide evidence that PKP2 governs DP assembly dynamics by scaffolding a DP-PKP2-PKCα complex, which is disrupted by PKP2 knockdown. The behavior of a phosphorylation-deficient DP mutant that associates more tightly with IF is mimicked by PKP2- and PKCα knockdown and PKC pharmacological inhibition, all of which impair junction assembly. siRNA-mediated PKP2 knockdown is accompanied by increased phosphorylation of PKC substrates, raising the possibility that global alterations in PKC signaling may contribute to pathogenesis of congenital defects caused by PKP2 deficiency.

An intact actin cytoskeleton is required for the efficient assembly of these plaque precursors, which closely associate with both actin and IF during assembly. However, the

molecular mechanism underlying actin involvement in the assembly of desmosomes is not well understood. Here we demonstrate that PKP2 directs myosin-dependent actin contractility and actin reorganization. Cells deficient in PKP2 exhibit constitutive myosin activation accompanied by blunted Rho GTPase membrane localization and activation, and the inability to properly reorganize the actin cytoskeleton. These studies demonstrate that Rho activity is required early during junction assembly but must be downregulated later in order for junction maturation to occur. Taken together, our results suggest that PKP2 regulates actin contractility signaling in a temporal and spatially restricted manner that tailors these activities to desmosome assembly-specific events.

The two major desmosome compartments (membrane and plaque) undergo distinct assembly pathways. How PKPs are involved in the coordination of these two pathways is not clear. Here, I show a dramatic defect in desmosomal cadherin assembly (but not adherens junction assembly) in cells deficient for PKP2. Further, siRNA-mediated PKP2 knockdown led to an enhancement in the colocalization of desmosomal plaque component, DP, with desmosomal cadherins and other arm proteins.

Taken together these data suggest that PKP2 is required for efficient desmosome assembly. From this work, I conclude that PKP2 plays an important role in the coordination of desmosomal cadherin and plaque components and in the regulation of signaling pathways important for temporal and spatial control of actin reorganization and DP-IF interactions during desmosome assembly.

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List of Abbreviations

Ab	antibody
Arm	armadillo
ARVC/D	arrhythmogenic right ventricular cardiomyopathy/dysplasia
ARVCF	armadillo gene deleted in velo-cardio-facial syndrome
C-terminus	carboxyl terminus
DAPI	4’6-diamidino-2-phenylindole dihydrochloride
DCM	dilated cardiomyopathy
DMSO	dimethyl sulfoxide
DMEM	Dulbecco’s minimal essential medium
DNA	deoxyribonucleic acid
DP	desmoplakin
Dox	doxycycline
DSC	desmocollin
DSG	desmoglein
E-cadherin	epithelial cadherin
EEA1	early endosome antigen 1
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EM	electron microscopy
ER	endoplasmic reticulum
ERM	ezrin-radixin-moesin

FBS	fetal bovine serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GST	glutathione-S-transferase
HR2	homology region 2
IF	intermediate filament
IP	immunoprecipitation
LEF	lymphoid enhancer-binding factor
LPA	lysophosphatidic acid
MAPK	mitogen-activated protein kinase
MF	microfilament
MLC	myosin light chain
MT	microtubule
MYPT	myosin phosphatase
N-cadherin	neuronal cadherin
NHEK	normal human epidermal keratinocyte
NMMHC	non-muscle myosin heavy chain
N-terminus	amino terminus
PAGE	poly-acrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PG	plakoglobin
PKA	protein kinase A

PKC	protein kinase C
PKP	plakophilin
PMA	phorbol 12-myristate 13-acetate
REF	rat embryo fibroblast
ROCK	Rho-associated coiled-coil-forming kinase
RNA	ribonucleic acid
SCC	squamous cell carcinoma
SDS-PAGE	sodium dodecyl sulfate- polyacrylamide gel electrophoresis
shRNA	short hairpin RNA
siRNA	short interfering RNA
SPPK	striate palmoplantar keratoderma
TBS	tris-buffered saline
TCF	T-cell factor
VASP	vasodilator-stimulated phosphoprotein
WT	wild type

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CHAPTER I

Introduction

Overview and significance

An epithelium is defined as a network of cells either in single layer or multiple layers that serve as an inner or outer lining of an organ. While this is a very simplified definition, the most fundamental quality of an epithelial lining is that it serves as a protective layer and can withstand mechanical stress. Epithelial cells form a resilient protective lining by selectively forming adhesive connections with one another (Niessen and Gumbiner, 2002; Overton, 1977; Runswick et al., 2001). Protein complexes found at sites of cell-cell contact known as desmosomes (also known as *maculae adhaerentes*) and adherens junctions (*zonulae adherens*) serve to mediate adhesion between cells and to anchor different cytoskeletal components to sites of cell-cell contact. These junction complexes and their cytoskeletal connections are responsible for the formation and maintenance of intercellular adhesion.

Desmosome-mediated adhesion is critical for the maintenance of tissue integrity and is not limited to epithelia. In addition to the epidermis, tissues such as myocardium undergo constant mechanical stressors that are generated both internally (heart) and externally (skin). The skin is a protective barrier against external insults including mechanical, toxic, microbial insults, and irradiation and prevents dehydration. The heart requires strong connections between cells to generate the force required to pump oxygen- and nutrient-rich blood to all other tissues. Strong adhesive connections between the cells that make up the tissue are critical for maintaining proper tissue architecture under normal and conditions of stress.

Outside of mechanical stress resistance, cell-cell adhesion plays a major part in maintaining tissue homeostasis. Cell adhesion is important for barrier-formation in skin, kidney, and gut epithelia as well as vascular endothelia (reviewed in (Lai-Cheong et al., 2007)). Intercellular adhesion is modulated during cell migration in processes such as wound healing or embryonic morphogenesis (Green and Simpson, 2007; Tepass et al., 2000) and later during organogenesis and tissue differentiation (Dusek et al., 2007b). Cell-cell junction molecules regulate cell proliferation and apoptosis (Dusek et al., 2007a; Nelson and Chen, 2003; Tinkle et al., 2004; Vasioukhin et al., 2001a; Wan et al., 2007) and cell migration (Ichii and Takeichi, 2007; Sivamani et al., 2007; Sobolik-Delmaire et al., 2007; Wheelock et al., 2008; Yanagisawa and Anastasiadis, 2006).

Loss of adhesion, inappropriate modulation of adhesion or expression of adhesion molecules is one hallmark of tumor invasion and metastasis. This phenomenon is most often described in reference to adherens junction molecule modulation (reviewed in (Hanahan and Weinberg, 2000; Hazan et al., 2004; Wheelock et al., 2008)). Modification of desmosomal molecule expression is seen in numerous tumor types and desmosomal adhesion can have anti-invasive properties (Tselepis et al., 1998). Loss of desmosome-mediated adhesion via autoimmune targeting-, bacterial toxin-mediated cleavage of desmosomal transmembrane components, or genetic mutation desmosomal components leads to compromised heart and skin integrity (reviewed in (Cheong et al., 2005; Green and Simpson, 2007; Lai-Cheong et al., 2007; van Tintelen et al., 2007).

It is known that cell-cell junctions are dynamically regulated during processes such as morphogenesis, wound healing, tumor invasion and metastasis. Descriptions of the temporal

sequence of desmosome protein assembly and turnover have outlined the general calcium dependence of desmosome stability and the order of protein incorporation into what are considered to be desmosome structures (Hennings and Holbrook, 1983; Mattey et al., 1990; Mattey and Garrod, 1986b; O'Keefe et al., 1987; Pasdar et al., 1991; Pasdar and Nelson, 1988a; Pasdar and Nelson, 1988b; Pasdar and Nelson, 1989; Penn et al., 1987a; Penn et al., 1987b; Penn et al., 1989), and numerous studies have described reorganization of the cytoskeleton during assembly. However, little is known about the molecular regulation of these desmosome/cytoskeleton dynamics. Understanding the mechanism of desmosome dynamics and how desmosomes are regulated during junction assembly, disassembly, reorganization and maturation as well as the contribution of these processes in disease pathogenesis is critical for the future development of potential therapeutic and diagnostic tools.

My research goal was to identify and characterize the mechanism by which desmosome assembly is initiated and maintained, with a particular focus on the role of the desmosomal protein plakophilin 2 in regulation of these processes. First, I investigated the requirement of PKP2 in desmosome plaque assembly and its role in recruiting the ubiquitous signaling protein PKC to desmosome precursors (Chapter III). Next, role of PKP2 in governing desmosome plaque assembly via modulation of actin microfilament network organization by contractility signaling was investigated (Chapter IV) and finally, I began to explore the role of PKP2 in coordinating cadherin and plaque assembly (Chapter V). Overall, I will show that PKP2 has multiple unique roles during desmosome assembly. The research presented here demonstrates that PKP2 serves as a scaffold that locally regulates

multiple signaling events and coordination of the separate junction components that are essential for the promotion of desmosome assembly and maturation.

Background

1. Adhering junctions

Desmosomes

Desmosomes are specialized intercellular structures that are essential for mediating cell-cell adhesion. They are prominent intermediate filament cytoskeleton-anchoring adhesive complexes present in all simple and stratified epithelia as well as in the intercalated discs of myocardium (discussed in (Franke et al., 2006)), lymphatic centers (Schmelz and Franke, 1993), and the arachnoid meningeal layer in the central nervous system (Achtstatter et al., 1989). Anchoring of the cytoskeleton to molecular plaques under the plasma membrane at sites of cell adhesion reinforces the adhesive strength of an epithelial cell sheet by providing an interconnected network throughout which mechanical tension is distributed across all cells, a feature necessary for maintaining tissue architecture.

Desmosomes are composed of three major protein families whose primary functions are to provide cell-cell adhesion and to anchor the intermediate filament cytoskeleton to these junctional sites of adhesion. The cadherin family proteins (desmogleins [DSGs] and desmocollins [DSCs]) are single-pass transmembrane glycoproteins that bind through homo and heterodimeric interactions to cadherins on neighboring cells via their extracellular domains in a calcium dependent manner (Chitaev and Troyanovsky, 1997).

The desmosomal cytoplasmic plaque consists of the armadillo-family proteins (plakoglobin [PG] and plakophilins [PKPs]), and the intermediate filament (IF)-binding plakins (desmoplakin [DP]) (reviewed in (Green and Simpson, 2007)). Armadillo proteins

plakoglobin (PG) and plakophilins (PKPs) bind to the cytoplasmic tail of either DSG or DSC where they serve as linker proteins to the intermediate filament (IF)-binding protein desmoplakin (DP) (Godsel et al., 2004; North et al., 1999) (Figure 1.1A).

DP is a crucial component of the desmosomal plaque that is required for anchoring of the IF cytoskeleton to the junction (Bornslaeger et al., 1996; Bornslaeger et al., 1997; Stappenbeck et al., 1993). The strength of the desmosome and cellular integrity relies on intracellular anchoring of the IF cytoskeleton to the desmosomal cytoplasmic plaque, and has been exemplified by decreased adhesive strength and IF-network retraction in cells lacking these plaque components or expressing components deficient in IF-binding (Bornslaeger et al., 1996; Gallicano et al., 1998; Grossmann et al., 2004; Huen et al., 2002). This strong cytoskeletal association is partially responsible for rendering desmosomes highly insoluble and difficult to characterize biochemically (Bass-Zubek and Green, 2007), but also correlates with their high degree of electron density by electron microscopy, and as such, desmosomes are easily identifiable and have a characteristic morphological appearance (Figure 1.1B).

In addition to interacting with DP and desmosomal cadherins, PKP and PG can also bind to one another (Bonne et al., 2003; Bornslaeger et al., 2001; Chen et al., 2002), suggesting that they may also regulate lateral desmosome interactions and clustering. Although PKPs and PG are thought to serve primarily as critical linkers between the cadherin tail and DP, they are also emerging as potential regulators of multiple cell signaling pathways and whose functions will be discussed in more detail below (Bass-Zubek et al., 2008; Chen et al., 2002; Dusek et al., 2007a; Garcia-Gras et al., 2006; Hatzfeld, 2007; Keil et al., 2007; Miravet et al., 2002; Yin et al., 2005a; Yin et al., 2005b)

Several other proteins have been described as being associated with desmosomes including plectin (Eger et al., 1997), envoplakin and periplakin (Ruhrberg et al., 1997), pinin (Ouyang and Sugrue, 1996), corneodesmosin (Simon et al., 1997), desmoyokin (Hieda et al., 1989), desmocalmin (Tsukita, 1985), kazrin (Groot et al., 2004), perp (Ihrie et al., 2005), p0071 (Hatzfeld et al., 2002), and trichoplein (Nishizawa et al., 2005). However, the association of some of these proteins with desmosomes is tissue and differentiation-specific and there is some controversy pertaining to whether some of these proteins are indeed true constitutive desmosome components (Brandner et al., 1997).

Adherens junctions

Adherens junctions are adhesive structures composed of proteins related to desmosomal components and serve to attach the actin microfilament cytoskeleton to the membrane at sites of cell-cell adhesion. Classic cadherins such as E-cadherin or N-cadherin form intercellular attachments by forming homodimers with cadherins on neighboring cells and interact with the armadillo protein β-catenin through their cytoplasmic domains. β-catenin, which is essential for adherens junction adhesion, binds α-catenin, which is classically thought to anchor actin to the junctional plaque either directly or through vinculin or α-actinin ((Drenckhahn and Franz, 1986; Weiss et al., 1998) and reviewed in (Niessen and Gottardi, 2008)) (Figure 1.2). Whether α-catenin actually anchors actin to the β-catenin-E-cadherin complex is unclear due to recent controversial studies suggesting that α-catenin cannot simultaneously bind β-catenin and actin (Pokutta et al., 2008; Yamada et al., 2005). It

is suggested that α-catenin dimerization acts as a switch to regulate its ability to bind E-cadherin/β-catenin and to modulate local actin polymerization (Drees et al., 2005).

$p120^{ctn}$ is an armadillo family protein that binds to the juxtamembrane domain of the classical cadherin and is essential for stabilizing the cadherin at the membrane by preventing its internalization (reviewed in (Xiao et al., 2007)). Plakoglobin is also found to be a naturally occurring E-cadherin partner in adherens junctions and can substitute for β-catenin in E-cadherin stabilization, but is primarily found in desmosomes (Cowin et al., 1986; Piepenhagen and Nelson, 1993). The armadillo family proteins in adherens junctions and desmosomes will be discussed in more detail in the next section.

In addition to the classical junction family members, immunoglobulin (Ig)-like cell adhesion molecules called nectins and their associated actin-binding proteins, afadins, are also considered to be closely associated with adherens junctions and are important for stimulating junction assembly via actin reorganizing signaling pathways, and for regulating higher order epithelial junction organization (i.e. epithelial cell polarization and formation of the apically-located *zonula adherens*) (Hoshino et al., 2005; Okamoto et al., 2005; Sato et al., 2006; Yamada et al., 2004).

Mixed junctions

Mixed adhesive junctions have been observed in a number of tissues where desmosome- and adherens junction-components are found together in a complex. These are found in lymphatic endothelia (Hammerling et al., 2005), vascular endothelia (Dejana, 2004; Kowalczyk et al., 1998; Schmelz and Franke, 1993), cardiac intercalated discs (Borrmann et

al., 2006; Pieperhoff and Franke, 2007), primary lens fiber cells (Leonard et al., 2008), and in some cultured cell lines (Schmelz et al., 1998).

2. Armadillo-family proteins: structure, function, and mechanisms of regulation

The armadillo (arm) protein family is a group of multifunctional proteins homologous to the *Drosophila melanogaster* segment polarity gene *armadillo*. These proteins were first considered to be structural components of adhesive junctions but were quickly shown to exhibit signaling properties. Arm proteins are composed of a variable N-terminal "head" domain, relatively well-conserved central arm repeat domains, and carboxyl-termini of variable length and sequence. The central arm repeat domain can vary from 9-12 repeating units of 42-45 amino acid imperfect repeats. Each repeat consists of three α-helices (Huber et al., 1997). Figure 1.3 compares armadillo family protein structures. In *armadillo*, this central repeat domain is sufficient to induce *Drosophila* embryonic axis development (Funayama et al., 1995). *Armadillo* and its close cousin, β-catenin are classically known to be involved in mediating the cytoplasmic and nuclear function of the canonical wnt/wingless signaling pathway (Peifer et al., 1991).

Arm proteins localize to cell-cell junctions where they play a structural role in promoting junction assembly and stability as well as to the cytoplasm and nucleus where they interact with and modulate numerous signaling factors. The armadillo protein family consists of β-catenin, PG, p120ctn (and related proteins p0071, ARVCF, δ-catenin), and the PKPs (PKP1-3). Although this family of proteins exhibits a relatively high level of sequence

homology, it is clear that they have both overlapping and divergent functions. Armadillo family proteins will be grouped based on their homology, with particular focus on what is known about PKP2 function as it is the focus of this thesis.

PG and β-catenin:

PG and β-catenin: Junctional Function

PG (also known as γ-catenin) and β-catenin are closely homologous arm proteins that contain 12 central arm repeats. PG is primarily a desmosomal protein, but like β-catenin, also associates with adherens junctions (Cowin et al., 1986). In desmosomes, PG is required for recruiting the IF-binding protein DP to the desmosomal cadherin tail and the recruitment of the IF to the sites of desmosomal cadherin cell contact (Troyanovsky et al., 1993; Troyanovsky et al., 1994a; Troyanovsky et al., 1994b). $PG^{-/-}$ mice exhibit keratin retraction, a reduction in desmosome abundance, and altered desmosome morphology (Bierkamp et al., 1996). Desmosomal proteins are less stable in PG-null keratinocytes, and PG re-expression in these cells rescues desmosome membrane association and detergent insolubility (Yin et al., 2005a). These data suggest that PG is required for proper desmosome formation by stabilizing intact desmosome complexes at the membrane.

PG and β-catenin competitively bind to the cadherin C-terminus via their central arm domains (Troyanovsky et al., 1996; Wahl et al., 1996). PG interacts with desmosomal cadherins through arm repeats 1-5, and with E-cadherin through downstream arm repeats (Troyanovsky et al., 1996). In the adherens junction, both PG and β-catenin serve to link α-catenin to the classical cadherin. Although PG can localize normally to adherens junctions,

β-catenin does not associate with desmosomal cadherins unless PG is deficient (Bierkamp et al., 1999; Fukunaga et al., 2005), consistent with the idea that PG may play a unique role in mediating crosstalk between the junction types. However, PG-desmosomal cadherin and PG-classical cadherin complexes are mutually exclusive (Kowalczyk et al., 1997), suggesting that this crosstalk may be spatio-temporally regulated.

PG and β-catenin: involvement in cell signaling

β-catenin is a major player in the canonical WNT signaling pathway. When wnt, a secreted protein, is not bound to its transmembrane receptor Frizzled, cytoplasmic β-catenin is bound by a cytoplasmic complex containing APC, Axin and serine/threonine kinases casein kinase 1 (CK1) and Glycogen Synthase Kinase 3 (GSK3). Phosphorylation of β -catenin by these kinases leads to its targeting to the proteasome for degradation. When WNT is bound to its receptor, Frizzled activates Disheveled, which inhibits phosphorylation of β-catenin by the APC/GSK3 complex, allowing it to accumulate in the cytoplasm. Translocation of β-catenin to the nucleus occurs where it interacts with TCF and transactivates numerous target genes (reviewed in (Clevers, 2006; Gavert and Ben-Ze'ev, 2007)). PG also can localize to the nucleus and activate transcription by binding to sites on TCF distinct from β-catenin sites (Simcha et al., 1998). Recent work suggests that PG can enhance the sensitivity of keratinocytes to apoptosis (Dusek et al., 2007a) and inhibit cell motility (Yin et al., 2005b), but there is still much to be learned about the specific signaling factors that are involved in these processes.

PG and β-catenin: Regulation of function by signaling

Outside of CK/GSK3 serine/threonine phosphorylation of β-catenin during WNT signaling, β-catenin and PG tyrosine phosphorylation regulates adhesive functions by modulating their interaction with cadherins (Hu et al., 2001; Miravet et al., 2003; Piedra et al., 2001; Piedra et al., 2003). V-src, Epidermal Growth Factor receptor (EGFR), Fer and c-met have been implicated in this phospho-regulation of β-catenin-cadherin or PG-cadherin association (Rosato et al., 1998; Roura et al., 1999; Shibamoto et al., 1994). Other β-catenin phosphorylation events affect its protein stability and its interactions with other binding partners such as transcriptional co-activators (reviewed in (Daugherty and Gottardi, 2007)). PG phosphorylation by EGFR disrupts PG-DP interaction (Gaudry et al., 2001), leading to reduced desmosomal adhesive strength (Yin et al., 2005a). These various phosphorylation events appear to be critical for the regulation of desmosome assembly and stability.

p120^{ctn} family

A second set of closely related proteins classified as p120^{ctn} related arm proteins also have adhesive and signaling functions that diverge from those of beta-catenin and PG. This subfamily includes p120^{ctn}, p0071 (also known as PKP4), δ-catenin (also known as neural plakophilin related protein), and ARVCF. These proteins have 10 conserved central arm repeats (exhibiting ~60-80% homology (Hatzfeld, 2007)) that mediate their binding to classical cadherins (Reynolds et al., 1996), and variable N- and C-termini. p120^{ctn} family members exhibit both nuclear and junctional localization patterns. They associate with adherens junctions (p120ctn, δ-cat, ARVCF, p0071) where they bind the juxtamembrane

domain of the classical cadherin (Yap et al., 1998) and to desmosomes (p0071, perhaps $p120^{ctn}$)(Hatzfeld et al., 2002; Kanno et al., 2008).

$p120^{ctn}$: Function in junctions

The most well known function of $p120^{ctn}$ is to stabilize classic cadherins at the plasma membrane and to prevent their internalization (reviewed in (Xiao et al., 2007)). Although ARVCF and p0071 can stabilize cadherins in certain cellular contexts (Hatzfeld et al., 2002), they cannot compensate to prevent E-cad downregulation and loss of adhesion when $p120^{ctn}$ expression is reduced. It has been suggested that in addition to cadherin membrane stabilization, $p120^{ctn}$ is responsible for mediating cadherin membrane trafficking via microtubule based motility. $p120^{ctn}$ colocalizes with microtubules and can bind to kinesin through its N-terminal head domain. $p120^{ctn}$ has been observed to colocalize with N-cad in particles moving towards cell-cell junctions, which requires intact microtubules (Chen et al., 2003; Yanagisawa et al., 2004).

p120ctn: Nuclear Function

p120ctn contains nuclear localization sequences that target it to the nucleus where it binds to kaiso, a nuclear transcription repressor, and relieves kaiso-mediated transcriptional repression by interfering with its ability to bind DNA (Daniel and Reynolds, 1999; Daniel et al., 2002; Kelly et al., 2004). Interestingly, kaiso is related to a number of oncogenes (Daniel and Reynolds, 1999) and has transcriptional activity that plays an important role in embryonic morphogenesis (Kim et al., 2004) and tumorigenesis (Spring et al., 2005). Often,

E-cadherin expression is reduced in tumors, which could lead to aberrant p120-nuclear signaling since E-cadherin expression appears to sequester $p120^{ctn}$ out of the nucleus (van Hengel et al., 1999). Overall, these findings support a critical role for $p120^{ctn}$ in development and in cancer perhaps independent of its junctional function.

$p120^{ctn}$ regulates actin organization via Rho GTPases

p120 has emerged as a major regulator of actin organization by its ability to modulate of RhoA-family small GTPase activity. Several groups demonstrated that fibroblasts or epithelial cells overexpressing $p120^{ctn}$ exhibited a dramatic cell shape change in which numerous membrane protrusions were formed. This morphology change was accompanied by a loss of actin stress fibers, reduction of focal adhesions and increased cell migration. In these studies, the cell morphology change induced by p120 could be reversed by activation of RhoA, inhibition of Rac and Cdc42 or expression of cadherin. These groups showed that $p120^{ctn}$ could directly inhibit intrinsic Rho activity and activate Rac and Cdc42 (Anastasiadis et al., 2000; Grosheva et al., 2001; Noren et al., 2000; Reynolds et al., 1996).

Small molecular weight Rho GTPases are Ras family members whose GTP-GDP hydrolysis cycling acts as a molecular switch within the cell. When bound to GTP, Rho family proteins bind to and activate various effector proteins, which are most well known for their actin modulatory functions. RhoA activity is linked to actin stress fiber formation, Rac is responsible for lamellipodial formation and dynamics, and Cdc42 activity is important in filopodial formation and dynamics (reviewed in (Etienne-Manneville and Hall, 2002)). The ability of $p120^{ctn}$ (or related family member p0071) to modulate Rho (Rac and Cdc42)

activity has significant implications in the control of cell-cell adhesion, migration (Wildenberg et al., 2006) and other cellular functions such as cytokinesis (Wolf et al., 2006). These investigations indicate that other armadillo family proteins (particularly those that are closely related to p120) may also have yet unidentified key roles in the regulation of cell shape, cell migration and cell adhesion.

Plakophilins

Plakophilins (PKPs) are constitutive desmosomal armadillo proteins that are closely related to $p120^{ctn}$ family proteins. There are three PKP isoforms that exhibit differential expression patterns and have partially overlapping functions (PKP1, 2, 3). The PKPs were originally thought to contain 10 arm repeats based on sequence homology, but upon the determination of PKP1 arm domain structure, it was observed that one of the central repeats actually served as a flexible linker lending a sickle-shape to the arm domain (Figure 1.4) (Choi and Weis, 2005). Interestingly, the presence of a basic (positively charged) region in the arm domain of PKP1 that correlates with the protein-protein interaction domain in other arm proteins suggests that it mediates interactions with other proteins (Choi and Weis, 2005); however, no binding partners have yet been identified to interact with the arm domain of any PKPs. All known binding partners associate with the N-terminal "head" domain of the PKP.

PKPs exhibit differential tissue distribution

PKPs localize to desmosomes where they link DP to the cadherin cytoplasmic tail and to the nucleus, where their function is unknown. All three PKPs bind to a broad repertoire of

desmosomal proteins (summarized in Table 1.1) and can recruit DP to sites of cell-cell contact in overexpression experiments (Bonne et al., 2003; Chen et al., 2002; Kowalczyk et al., 1999). The three PKPs exhibit differential tissue distribution patterns. PKP1 is found in more differentiated upper layers of stratified epithelia (Heid et al., 1994; Moll et al., 1997; Schmidt et al., 1997). PKP2 is expressed in all simple, complex and stratified epithelia as well as cardiac myocytes (where it is the only PKP expressed), germinal centers of lymph node follicles, a variety of tumors and numerous cell lines (Mertens et al., 1996; Mertens et al., 1999). PKP3 is more equally distributed among all the epidermal layers and is found in most simple and stratified epithelia except hepatocytes (Bonne et al., 1999; Schmidt et al., 1999). This differential expression pattern of the three PKPs suggests that they may have partially non-overlapping roles in desmosomes.

PKP2: junctional and non junctional functions

PKP2 was first identified in 1996 by Franke and colleagues as a desmosomal protein related to plakophilin 1 (Band 6 protein) and $p120^{ctn}$. PKP2 occurs in two splice variant forms, 2a and 2b. Both isoforms appear to be expressed in numerous cell types. PKP2b contains a insertion of 132 base-pairs in the arm domain that does not encode another arm repeat (Mertens et al., 1996). While there may be functional differences between the two PKP2 isoforms, no studies have yet determined the specificity of PKP2a versus 2b function. All PKP2 cDNA constructs used in this thesis encode PKP2a sequence.

PKP2 interacts with desmosome components through its head domain, which is sufficient to recruit it to cell-cell junctions. The PKP2 Head and Arm are sufficient to

interact with PG, but not as efficiently as full length PKP2. In co-immunoprecipitation and/or Yeast Two-Hybrid experiments using ectopically expressed proteins, PKP2 was shown to interact with DP, several different desmosomal cadherins (summarized in Table 1.1), PG (Chen et al., 2002), and keratin (Hofmann et al., 2000). PKP2 is essential for cardiac morphogenesis

PKP2 also interacts with non-desmosomal proteins including connexin43, (Oxford et al., 2007), β-catenin (Chen et al., 2002) and αT-catenin (Goossens et al., 2007), suggesting a possible function in crosstalk between desmosomes and other types of cell-cell junctions. However, PKP2 does not associate with E-cadherin-bound β-catenin (Chen et al., 2002). This suggests that PKP2 may have a possible role in non-junctional armadillo-related signaling. Indeed, PKP2 potentiates β-catenin/TCF-mediated transcriptional activity (Chen et al., 2002).

In cells that do not form desmosomes, particularly fibroblasts, PKP2 localization is limited to the nucleus (Mertens et al., 1996). PKP2 interacts with RNA polymerase III subunit (RPC155) in cell lysates as well as *in vitro* assays and the two proteins colocalize in nuclear particles (Mertens et al., 2001). RNA pol III is an enzyme that is part of TFIIIB, which is important for transcription of ribosomal and tRNA (Geiduschek and Kassavetis, 2001). PKP2 is a phospho-substrate of Cdc25C-kinase 1 (C-TAK1). This PKP2 phosphorylation regulates its interaction with a 14-3-3 protein important for determining the subcellular localization of PKP2 (Muller et al., 2003). It is possible that via interactions with these and other yet unidentified non-desmosome proteins PKP2 may be involved in a broad range of cellular functions.

PKP2 exhibits promiscuous protein interactions, but very little is known about how these interactions are tailored or organized in different cellular contexts. It is unlikely that a single PKP2 molecule can bind to all of its binding partners at once, and thus these interactions are probably differentially tailored depending on its subcellular localization, adhesion state, or junction assembly status.

3. Desmoplakin (DP)

DP is a member of the plakin family of cytolinkers, whose primary function is to link the IF cytoskeleton to the desmosomal junction. DP is a constitutive member of the desmosome and is required for the attachment of IF to the membrane component of the junction and for desmosome stability and adhesive strength (Green et al., 1990; Huen et al., 2002; Stappenbeck and Green, 1992; Vasioukhin et al., 2001b). DP interacts with IF via regions within its c-terminal domain (Bornslaeger et al., 1997). DP dimerization occurs via the central rod domain, and DP associates with PKPs and PG via its N-terminal domain(Chen et al., 2002; Kowalczyk et al., 1997). In cell-cell adhesion literature, DP is often used as a marker for demonstrating that desmosomes are intact. While it is clear that DP is essential for desmosome formation, the specific mechanism by which its incorporation into desmosomes is regulated and the role of other desmosomal proteins in this process is not fully understood.

4. Congenital mutations in desmosomal molecules are linked to skin and cardiac diseases

Intercellular adhesive junctions such as the desmosome and adherens junction are critical for the protection of load-bearing tissues, such as the epidermis and myocardium, against mechanical stress. Congenital mutations in desmosomal plaque proteins have been shown to result in a constellation of cardiac and skin pathologies such as arrhythmogenic right ventricular cardiomyopathy (ARVC; discussed below), dilated cardiomyopathy (DCM) (Ahmad et al., 2005), striate palmoplantar keratoderma (SPPK)(Armstrong et al., 1999; Wan et al., 2004), Naxos disease (McKoy et al., 2000), Carvajal syndrome (Norgett et al., 2000), ectodermal dysplasia skin fragility (EDSF) ((Hamada et al., 2002), epidermolysis bullosa (EB)(Jonkman et al., 2005) and hypotrichosis (also reviewed in (Godsel et al., 2004; Lai-Cheong et al., 2007; McGrath, 1999; McMillan and Shimizu, 2001; van Tintelen et al., 2007)). Mutations of PG (Naxos disease), DP (SPPK and EB), DSG1 (SPPK), DSG4 (hypotrichosis) and PKP1 (EDSF) lead to skin blistering or fragility, skin dysplasia, epidermal hyperproliferation (skin thickening) and/or hair and nail abnormalities. Mutations of PG (Naxos, ARVC), DP (ARVC, DCM, Carvajal syndrome), DSC2 (ARVC), DG2 (ARVC) and PKP2 (ARVC) lead to cardiac arrhythmias, or dilation and weakening of the cardiac ventricles.

PKP mutations and disease

Mutations throughout the PKP1 gene lead to a syndrome known as EDSF. This is characterized by epidermal fragility, dysplasia (abnormal skin development) and inflammation. Abnormal localization of DP and keratin filament collapse is found in PKP1

deficient skin (McGrath et al., 1997). Although desmosomes are present, they exhibit abnormal ultrastructural organization and a reduction in desmosome abundance and size. Presumably, loss of functional adhesive properties leads to widenening of intercellular spaces (McMillan et al., 2003). Interestingly, PKP2 expression is upregulated in the upper, more differentiated layers of epidermis (McMillan et al., 2003). The presence of multiple PKP isoforms is probably an evolutionary compensatory measure to prevent a severe skin defects upon the loss of one isoform. However, PKP3 and 1 cannot fully compensate for PKP1 in these patients, suggesting that there are distinct functions among isoforms. In tissues only expressing one isoform (i.e. heart), compensation cannot occur when the single functional PKP is lost.

PKP2 and ARVC

Mutations in PKP2 have recently been identified in approximately 25% of patients with autosomal dominant ARVC (Gerull et al., 2004), and have been determined to be a major cause of familial ARVC, ranging up to 70% in affected families (van Tintelen et al., 2006). ARVC is characterized by predominantly right sided cardiac ventricular arrhythmias and fibrofatty replacement of normal heart tissue. Intercalated disc (ID) remodeling, loss of well-preserved desmosome structures, alterations in desmosome length, and widenening of intrajunctional space has been observed in ultrastructural studies of cardiac tissue from PKP2-related ARVC (Basso et al., 2006; Lahtinen et al., 2008) and are likely due to reduced desmosome functionality.

ARVC comprises a major component of sudden cardiac deaths in both sedentary and active young people (under 35 years) (Kayser et al., 2002) with a prevalence of 1/2500 - 1/5000 (Nava et al., 1988; Towbin, 2004). These congenital diseases are often fatal when undiagnosed and can sometimes be asymptomatic until aggravated by physical exertion (reviewed in (Thiene et al., 2007). Recommendations for genetic screening for PKP2 mutations in ARVC patients or people with family history significant for ARVC have been made (Prakasa and H., 2005) and . Since this initial study (Gerull et al., 2004), over 50 mutations have been identified in PKP2 with variable clinical outcome (listed in Table 1.2).

Several mechanisms of ARVC pathogenesis have been proposed. In cardiomyocytes, the adhesive junctions are thought to provide structural support for myofibrillar organization (Luo and Radice, 2003). In PKP2 null mice, homozygous embryos die at embryonic day 13.75 due to heart defects. Immunohistochemistry of $PKP2^{-/-}$ heart sections revealed decreased DP and Dsg2 levels, and displacement of DP from desmosomes (Grossmann et al., 2004). In addition to physical disruption of adhesion, loss of desmosomal adhesion leads to impairment in gap junction formation and altered electrical coupling of cardiomyocytes (Kaplan et al., 2004; Oxford et al., 2007), and altered subcellular localization of desmosomal components leading to modifications of the Wnt signaling pathway (Garcia-Gras et al., 2006). ARVC is characterized by a fibrofatty infiltration and replacement of cardiac tissue (reviewed in (Thiene et al., 2007)) which could perhaps be explained by altered signaling pathways affecting cellular differentiation within the cardiac tissue. The presence of apoptotic cells in degenerating myocardium of ARVC patients (Yamaji et al., 2005) suggests

a potential role for these desmosomal proteins in regulating signaling pathways important for processes such as differentiation or cell survival.

5. Desmosomes in cancer

Although desmosomal protein levels and membrane localization are altered in numerous tumors, there is no overall trend in up or downregulation of desmosomal proteins in tumors. Some desmosomal cadherins are lost in certain types of cancer, some are upregulated (reviewed in (Chidgey and Dawson, 2007)). Reduction in desmosomal adhesion is a key early step in tumor invasion and metastasis, and PG (and perhaps PKP2) is able to influence TCF transcriptional activity (Chen et al., 2002), suggesting that modulation of its nuclear availability could play a role in tumorigenesis.

In many types of head and neck carcinomas, EGFR is highly overexpressed, which has been shown to impair desmosomal adhesion by stimulating the internalization and degradation of desmosomal cadherins and altering PG-DP interactions ((Lorch et al., 2004; Yin et al., 2005a); J. Klessner, B. Desai, and K. Green, manuscript in revision). PG also is involved in regulating cell motility and sensitization of epithelial cells to apoptosis, suggesting multiple potential avenues for desmosome-dependent cellular dysregulation in tumorigenesis, invasion, or metastasis (Dusek et al., 2007a; Yin et al., 2005b).

Reduction of PKP1 and 3 expression is correlated with desmosome instability, increased cell migration, and worse prognosis of metastatic tumors. Increased PKP cytoplasmic localization or loss of membrane staining was found in a number of

oropharyngeal tumors (Kurzen et al., 2003; Moll et al., 1997; Papagerakis et al., 2003; Sobolik-Delmaire et al., 2007; South et al., 2003).

In contrast, increased PKP3 expression was observed in a panel of non-small cell lung cancer tumors and was correlated with cell invasion and proliferation (Furukawa et al., 2005). Likewise, PKP2 expression correlated with poorer prognosis and was suggested to perhaps play an oncogenic role in oropharyngeal tumors (Papagerakis et al., 2003). PKP2 is considered a marker of non-differentiated epithelia as its expression is more prevalent in basal layers of the epidermis and in less differentiated tumors (Mertens et al., 1999). Whether the PKPs actually play causal roles in tumorigenesis remains to be determined, but their varied expression patterns in some tumors suggest a potential functional contribution towards the loss of adhesion.

6. Mechanisms of Desmosome Assembly

Calcium switch

Desmosomes are highly dynamic structures that undergo phases of assembly, disassembly and modification during processes such as wound healing and morphogenesis in which sheets of cells are reorganized. Desmosomes are calcium dependent structures. This functional dependence on extracellular calcium levels approximating 1-2mM (depending on cell type) has been taken advantage of to develop the calcium switch technique, a tool to "synchronize" the induction of junction assembly within a confluent sheet of epithelial cells.

Culturing cells in low calcium containing medium (LCM) leads to disruption of cadherin-cadherin contact (calcium dependent) and induces the splitting and internalization of desmosomes (Mattey and Garrod, 1986b) or internalization of whole desmosome remnants (Mattey and Garrod, 1986a) and a collapse of keratin IF(Hennings and Holbrook, 1983). After a period of time in LCM, switching these cells back to normal calcium containing medium (NCM) induces the re-accumulation of desmosome components and keratin IF at cell-cell borders over time (Hennings and Holbrook, 1983; Watt et al., 1984). Cells require the presence of closely neighboring cells in order for DP cell surface accumulation and tonofilament reorganization and connection to cell surface is established (Jones and Goldman, 1985).

Kinetics of Cadherin vs. Plaque

The desmosomal plaque and cadherin components are assembled in separate subcellular compartments and exhibit distinct assembly kinetics. Desmoplakin is relatively unstable in cultures grown in LCM with a half life about 8h. During its biosynthesis, DP moves from a detergent-soluble pool to an insoluble pool, which is stabilized after the induction of cell contact ($T_{1/2}$ increasing to 72h) (Pasdar and Nelson, 1988a).

The desmosomal cadherin DSG is also titrated from a soluble to insoluble pool during its biosynthetic pathway from ER to Golgi to plasma membrane but its turnover kinetics are very different from those of DP. The insoluble pool of DSG is stabilized when cell contact is induced. This pool of DSG is able to reach the membrane even when cells are cultured in LCM but is unstable there ($T_{1/2}$ is about 4h in LCM). After cell-cell contact occurs, the

insoluble pool is stabilized ($T_{1/2}$ of 24h) (Pasdar and Nelson, 1989). The fact that these two components, DSG and DP exhibit vastly different kinetics and that soluble pools of DSG and DP do not associate with one another (Pasdar et al., 1991) supports the idea that they are assembled in separate compartments that are likely regulated via distinct mechanisms.

Plaque Precursor Assembly

DP-containing electron dense bodies associate with keratin and actin that move towards cell-cell borders after a calcium switch (Green et al., 1987; Jones and Goldman, 1985). It was postulated that these DP-containing bodies were likely macromolecular plaque precursors. Recently, live cell imaging studies involving GFP-DP expressing cells undergoing cell-contact induced junction assembly led to the proposal of a three phase assembly model (Figure 1.5). Phase I: DPGFP accumulates at cell-cell borders within minutes of contact induction. Phase II: DP-GFP-containing plaque precursors form in the cytoplasm near the newly contacting border and are associated with intermediate filaments. Phase III: These precursors then are translocated to the cell-cell border where they are incorporated into the nascent junction (Godsel et al., 2005).

Potential Role of Signaling Pathways in Desmosome Assembly Regulation

Evidence is growing supporting a major contribution that signaling molecules such as Protein Kinase C, Epidermal Growth Factor Receptor (EGFR), and src-family kinases play in the regulation of desmosome assembly (Cowell and Garrod, 1999; Lorch et al., 2004; Miravet et al., 2003; Wallis et al., 2000; Yin et al., 2005a).

In particular, Protein Kinase Cα (PKCα) localizes to cell membranes near edges of re-epithelialization zones in wounded skin (Wallis et al., 2000). PKC is involved in maintaining the calcium dependence of desmosomes, which become calcium independent and hyper-adhesive after attaining confluence in cell culture (Kimura et al., 2007).

PKC activation has been linked to the translocation of DP from the cytoplasm to cell-cell contacts (Sheu et al., 1989) and can even do so in the absence of α-catenin, which was previously shown to be required for the assembly of desmosomes (Taniguchi et al., 2005; van Hengel et al., 1997). Although calcium dependence appears to be important in allowing for desmosome modulation during wound healing, the specific mechanism by which PKC acts on desmosomes is unclear.

Summary and Research Aims

In summary, desmosomes are highly organized essential adhesive junctions that are dynamically regulated during processes involving cell-contact formation or reorganization. The importance of desmosomal adhesion is highlighted by diseases in which desmosomal proteins are targeted. These diseases lead to severe skin and heart defects presumably due to defective cell-cell adhesion. The importance of PKP2 is intercellular adhesion is demonstrated in ARVC, in which loss of PKP2 leads to devastating arrhythmias and cardiac rupture. Studies investigating clinical features of ARVC and mutations in PKP2 have recently become extremely prevalent. However, the role of PKP2 in the desmosome assembly process is poorly understood. PKPs can interact with all desmosomal proteins and recruit DP to junctions, but the current state of knowledge regarding the exact nature of their involvement in overall dynamic desmosome assembly lacks a degree of molecular mechanism.

Based on the ability of PKP2 to colocalize with and recruit DP to junctions, it was hypothesized that **PKP2 is required for DP assembly**. The similarities between PKP2 and p120 and previous studies demonstrating their ability to induce dramatic cell shape changes when overexpressed led to the hypothesis that **PKP2 regulates actin organization.** Finally, the ability of PKP2 to interact with a wide range of desmosomal proteins led to the hypothesis that **it coordinates desmosome component interactions during desmosome assembly.**

To test these hypotheses, siRNA-mediated PKP2 knockdown was utilized in a number of studies to address the following aims:

Aim1: to determine if PKP2 is required for desmosome plaque assembly

Results summary:

PKP2 begins to accumulate at cell-cell contact sites earlier than DP and colocalizes extensively with cytoplasmic DP containing precursors. PKP2 governs DP assembly dynamics by scaffolding a DP-PKP2-PKCα complex, which is disrupted by PKP2 knockdown. The behavior of a phosphorylation-deficient DP mutant that associates more tightly with IF is mimicked by PKP2- and PKCα knockdown and PKC pharmacological inhibition, all of which impair junction assembly. siRNA-mediated PKP2 knockdown is accompanied by increased phosphorylation of PKC substrates and alteration in junctional keratin phosphorylation.

Aim 2: to characterize how PKP2 influences the actin cytoskeleton organization and whether this is important in desmosome assembly

Result summary:

Actomyosin contractility is required for efficient DP assembly independent of adherens junction assembly. PKP2 governs myosin-dependent actin contractility and actin reorganization. Cells deficient in PKP2 exhibit constitutive myosin activation, loss of Rho GTPase membrane localization and activation, and stunted actin reorganization. Rho activity is required for early junction assembly but is inhibitory later during junction maturation.

Aim 3: to determine whether PKP2 is required for desmosomal cadherin assembly and how it coordinates interactions between cadherins and plaque components

Result summary:

siRNA-mediated PKP2 knock down led to a dramatic defect in desmosomal cadherin assembly (but not adherens junction assembly). A mild loss of MTs was observed in PKP2-deficient cells, and the colocalization of the desmosomal plaque component, DP, with desmosomal cadherins and other arm proteins (but not with adherens junction components) was enhanced.

FIGURES

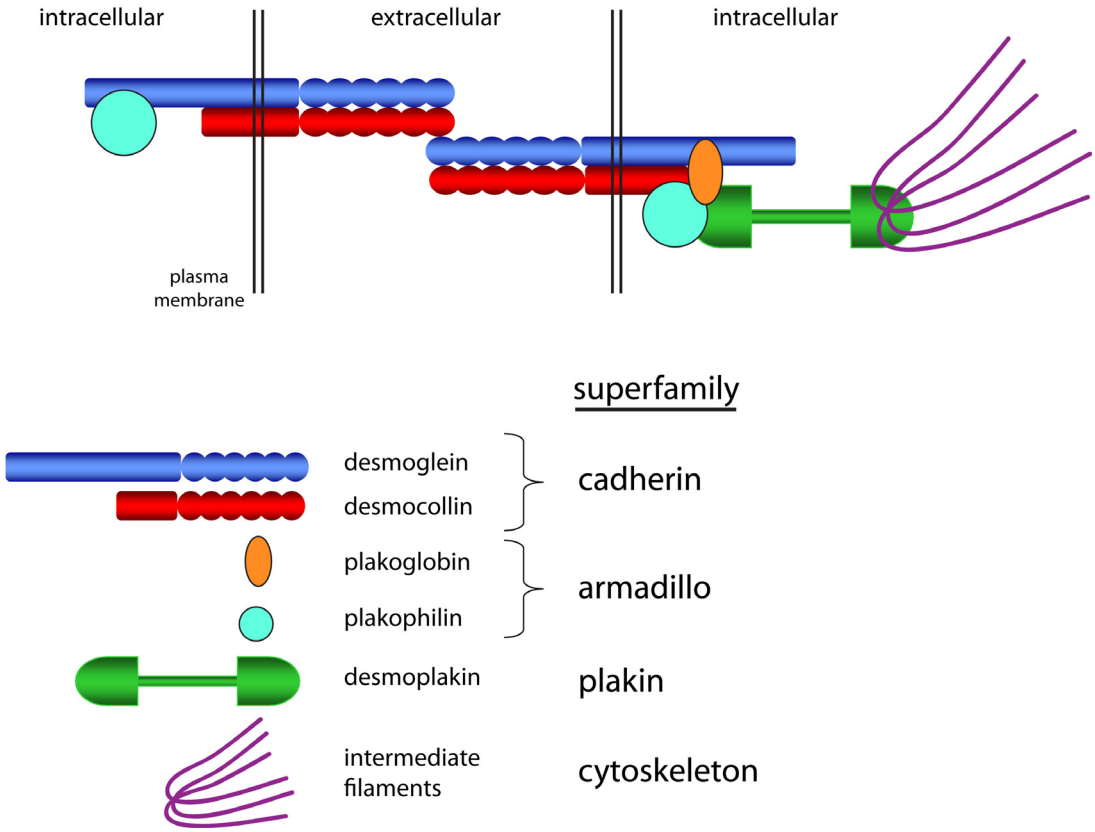
Figure 1.1 Structure of the desmosome

A. Schematic depicting desmosome structure and components.

B. Conventional electron micrograph, from Bovine muzzle epidermis depicting several desmosomes (Bass and Green, 2006).

Figure 1.1

A


intracellular
extracellular
intracellular
plasma membrane
superfamily
desmoglein
desmocollin
cadherin
plakoglobin
plakophilin
armadillo
desmoplakin
plakin
intermediate filaments
cytoskeleton

B

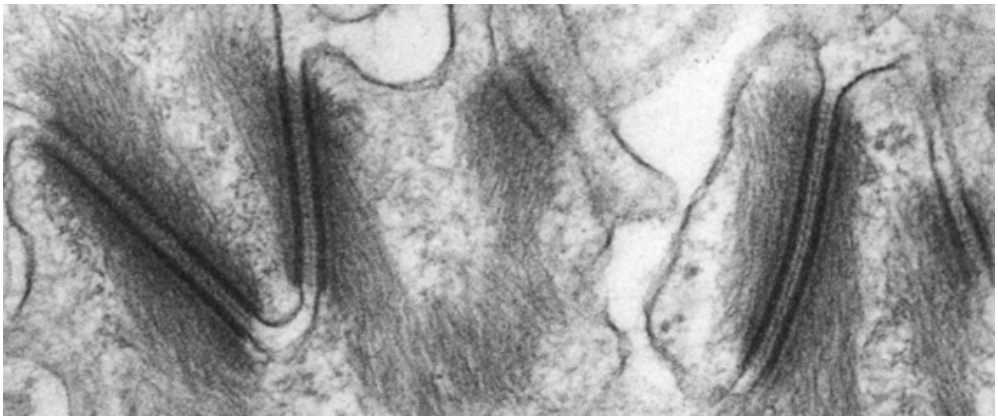


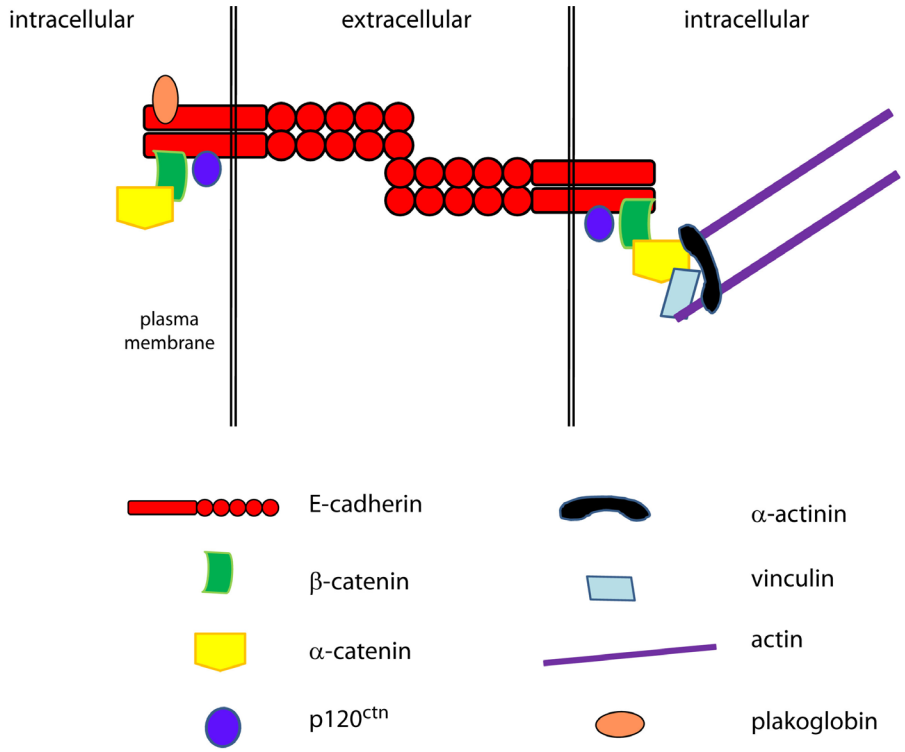
Figure 1.2 Structure of the adherens junction

A. Schematic depicting adherens junction structure and major components.

B. Conventional electron micrograph, from primary mouse keratinocyte adherens junction (Green et al., 1987).

Figure 1.2

A


intracellular
extracellular
intracellular
plasma membrane
E-cadherin
β-catenin
α-catenin
p120ctn
α-actinin
vinculin
actin
plakoglobin

B

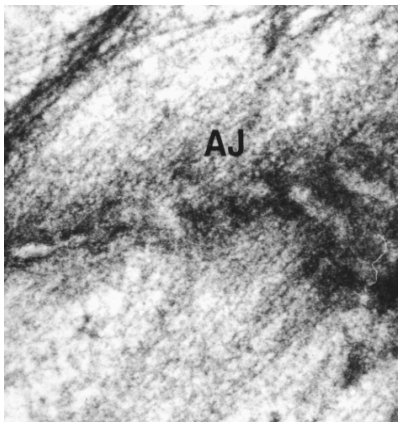

AJ

Figure 1.3 Comparative diagram of armadillo family protein structures

The overall structure of the three armadillo subfamilies are similar. β-catenin/PG contain 12 arm repeats, p120-family proteins contain 10, and PKPs contain 9 arm repeats. Gray boxes indicate highly homologous central arm repeats, white rectangles indicate variable N- and C-termini.

Figure 1.3

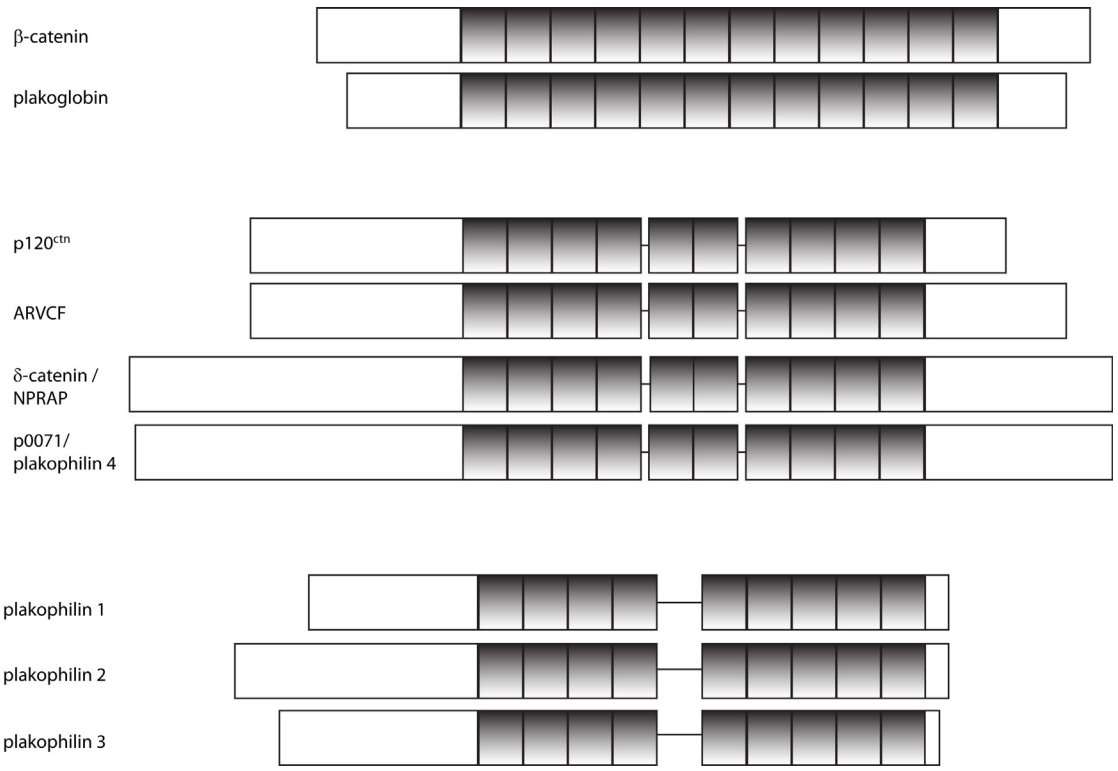

β-catenin
plakoglobin
p120ctn
ARVCF
δ-catenin /
NPRAP
p0071/
plakophilin 4
plakophilin 1
plakophilin 2
plakophilin 3

Figure 1.4 Representation of PKP1 arm domain 3D crystal structure

The central arm domain of PKP1 contains nine arm repeats rather than the predicted ten. This image contains color coded arm domains. In gray is the arm-related insert that induces a bend in the α-helical structure. Reproduced from (Choi and Weis, 2005) with permission of Bill Weis, Stanford University, Stanford, CA.

Figure 1.4

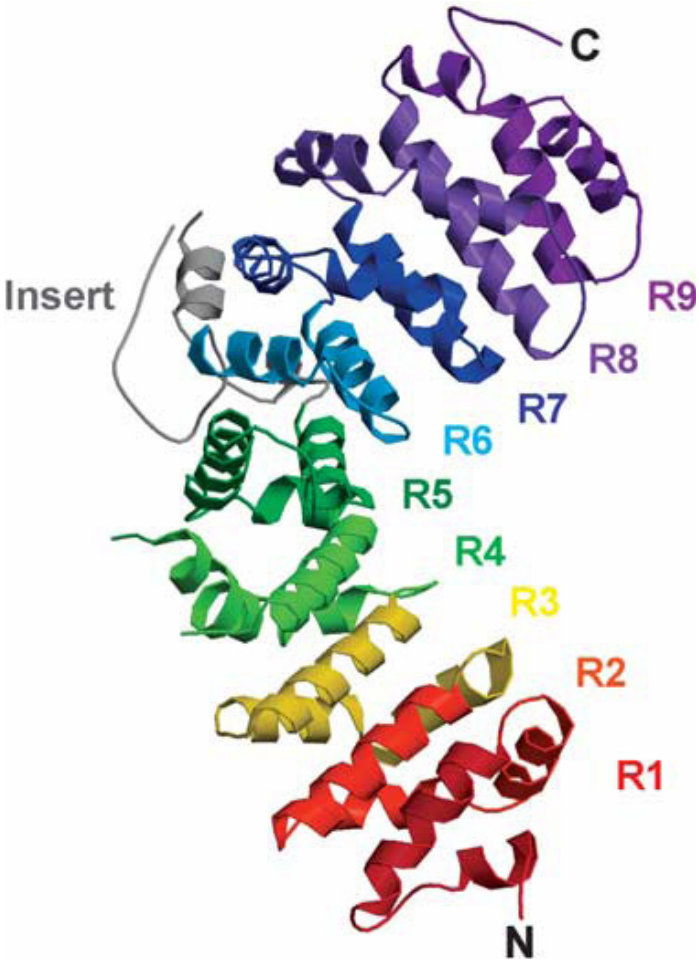


Table 1.1 Table of PKPs and their binding partners

PKPs can interact with desmosome proteins, some adherens junction proteins and a variety of other proteins (Bonne et al., 2003; Bornslaeger et al., 2001; Chen et al., 2002; Furukawa et al., 2005; Goossens et al., 2007; Hatzfeld et al., 2000; Hofmann et al., 2006; Hofmann et al., 2000; Kowalczyk et al., 1999; Mertens et al., 2001; Muller et al., 2003; Oxford et al., 2007).

Table 1.1 Table of PKPs and their binding partners

PKP	Desmosomal/cytoskeleton	Non-desmosomal
Plakophilin 1	DSG1 DSC1 PG DP Keratin Actin Tubulin	αT-catenin
Plakophilin 2	DSG1 DSG2 DSG3 DSC1a DSC2a DP PG Keratin	Cdc25C-associated kinase 1 (C-TAK1) 14-3-3 αT-catenin E-cadherin RNA polymerase III subunits β-catenin Connexin43
Plakophilin 3	DSG1 DSG2 DSG3 DSC1a DSC2a DSC3a DSC3b DP PG	Dynamin1-like protein ras-GAP-SH3-binding protein PolyA binding protein C1 Fragile X Related protein 1 αT-catenin

Table 1.2 A compilation of all PKP2 mutations identified in patients with ARVC.

These mutations are correlated with variable clinical outcomes. Some mutations were found to be homologous, most were heterozygous, a few mutations may be polymorphisms. Δ, deletion; ins, insertion; fs, frameshift; X, unidentified amino acid.

References cited:

1 (Antoniades et al., 2006)

2 (Basso et al., 2006)

3 (Dalal et al., 2006)

4 (Gerull et al., 2004)

5 (Kannankeril et al., 2006)

6 (Lahtinen et al., 2008)

7 (Nagaoka et al., 2006)

8 (Syrris et al., 2006)

9 (van Tintelen et al., 2006)

Table 1.2

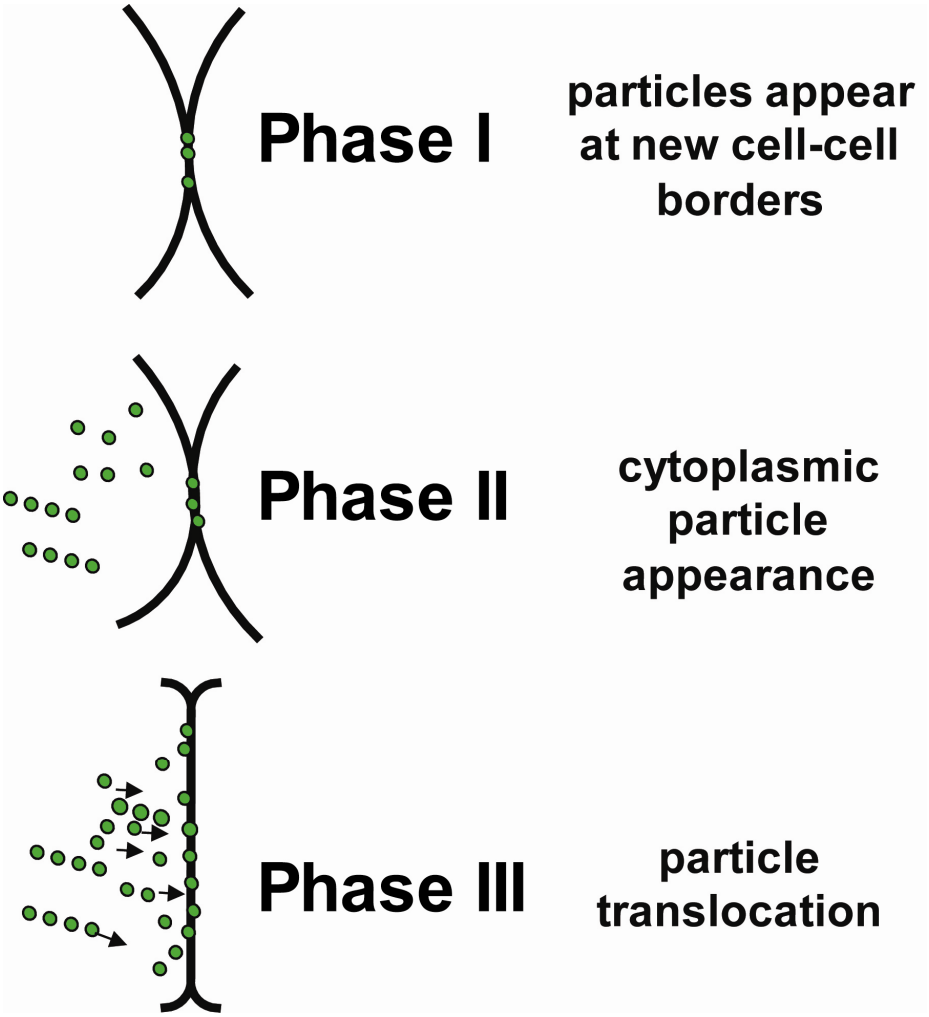
PKP2 mutations in ARVC

Mutation	Protein Result	Reference
2569Δ50	premature stop	1
971-980Δ10	premature stop	1
not described	premature stop V587I	2
not described	premature stop N670fs683X	2
not described	premature stop Q707X	2
1171–2A→G	mutant splice product	3
1271T→C	missense F424S	3
1368ΔA	deletion N456fsX458	3
1613G→A	nonsense W538X	3
2011ΔC	deletion P671fsX683	3
2197-2202insGΔCACACC	deletion A733fsX740	3
1369-1372ΔCAAA	Q457X	4
144-148ΔCAGA	S50fsX110	4
1631-1632insTT	L544fsX563	4
1642ΔG	V548fsX562	4
1912C→T	Q638X	4
1948ΔA	V650fsX655	4
1951C→T	R651X	4
1960A→C	K654Q	4
2076-2077ΔAA	C693fsX741	4
2088insA	K696fsX742	4
2095C→T	Q699X	4
2146–1G→C	mutant splice product	4
216insG	Q74fsX85	4
2176C→T	Q726X	4
2203C→T	R735X	4
235C→T	nonsense R79X	4
2386T→C	missense C796R	4
2393-2401ΔCATTGAACA	N798fsX879	4
2424insA	E809fsX826	4
2489+1G→A	mutant splice product	4
2490-1G→C	mutant splice product	4
2509ΔA	V837fsX930	4
534-535insCT	C179fsX190	4
1844C→T	S615F	4
419C→T	S140F	4
not described	premature stop 740	5
184C→A	Q62K	6
1839C→G	N613K	6
176A→T	Q59L	6
1728_1729insGATG	premature stop R577DfsX5	7
1597-1600ΔATCC	P533fsX561	8
1709ΔC	V570fsX576	8
1755-1756insTTGACTCA	L586fsX658	8
2197-2202ΔCACACCinsG	A733fsX740	8
1237C→T	R413X	8
1211–1212insT	insertion frameshift V406SfsX3	9
148-151ΔACAG	deletion frameshift T50-V51SfsX60	9
1848C→A	nonsense Y616X	9
184C→A	missense: UV E62K	9
2028G→A	nonsense W676X	9
2062T→C	missense S688P	9
2421C→A	nonsense Y807X	9
2544G→A	nonsense W848X	9
258T→G	nonsense Y86X	9
397C→T	nonsense Q133X	9
76G→A	missense D26N	9

Figure 1.5 Three phase model for DP assembly into nascent junctions

DP assembles into cell-cell junctions in three temporally overlapping phases. Phase I: Within minutes of cell-cell contact, DP appears directly at cell-cell borders. Phase II: 15-30 minutes following contact, DP-containing particles coalesce in the cytoplasm near the newly forming border. Phase III: These particles translocate to the nascent junction where they are incorporated into assembling desmosomes (Godsel et al., 2005).

Figure 1.5


Phase I
particles appear at new cell-cell borders
Phase II
cytoplasmic particle appearance
Phase III
particle translocation

Chapter II

Materials and Methods

DNA constructs

DP-GFP, DP^{S2849G}-GFP (Godsel et al., 2005) and PKP2-FLAG, PKP2-H-FLAG, PKP2-A-FLAG (Chen et al., 2002) were described previously. GFP-PKP2 was made by Xinyu Chen. PKP2-H-myc and –A-myc were made by PCR amplifying the PKP2-H and -A into pBK-CMV EcoRI and XbaI sites (generated by Xinyu Chen). mp120^{ctn}, myc.RhoN17, and myc.RhoV14. were provided by A. Reynolds (Vanderbilt University, Nashville, TN). mRFP-actin was kindly provided by W.J. Nelson (Stanford University, Stanford CA). PKCα-FLAG and myr-PKCα-FLAG cDNAs (Rabinovitz et al., 1999) were provided by A. Toker (Beth Israel Deaconess Medical Center, Boston, MA. PKCα-GFP cDNA was provided by Yusuf Hannun (Medical University of South Carolina, Charleston, SC). siRNA-resistant PKP2-FLAG (see Appendix) was made by site directed mutagenesis (Stratagene).

Retroviral constructs: The pSUPER.Retro vector plasmid was a gift from V. Cryns (Northwestern University Feinberg School of Medicine, Chicago, IL). pSUPER.Retro.shPKCα described in (Leirdal and Sioud, 2002) and LZRS-Linker in (Kinsella and Nolan, 1996), LZRS-PKCα and LZRS-PKCαΔ22-28 cDNA constructs were obtained from M. Denning (Loyola University Chicago Stritch School of Medicine, Maywood, IL). LZRS-PKCα was constructed by cloning EcoRI-digested hPKCα cDNA (#65978, ATCC) into the EcoRI sites of LZRS-Linker. Constitutively active PKCα (LZRS-PKCαΔ22-28) was constructed by deleting the pseudosubstrate domain (amino acids 22-29) by site directed mutagenesis, and cloned into the EcoRI sites of LZRS-linker. pSUPER.shPKP2 was a gift from J.K. Wahl, IIIrd (University of Nebraska Medical Center College of Dentistry, Lincoln, NE).

Non-specific siRNA (negative control #1; Cat.# D-001210-01), siGLO (positive transfection control; Cat. # D-001600-01), Smartpool and individual siRNAs against hPKP2 (Cat. # M-012692-01) (all siRNA oligoribonucleotides were purchased from Dharmacon) were used for siRNA experiments.

Cell lines and transfections

SCC9 (gift from J. Rheinwald, Harvard Medical School, Boston, MA), SCC9 DP-GFP, A431 DP-GFP lines, and culture conditions were described in (Godsel et al., 2005). Cos20 cells were grown in DME and 10% FBS. Phoenix amphotropic cells were a gift from G. Nolan (Stanford University, Stanford, CA) and were maintained in DME and 10% hI-FBS. REF52 cells were cultured in 50% DME/ 50% F-12 medium and 10%FBS.

cDNAs were transfected using ExGen 500 (Fermentas) or Lipofectamine2000 (Invitrogen). siRNAs (Dharmacon) were transfected using DharmaFECT1. Cells were analyzed 72-96h after transfection. Retroviruses were packaged and transduced as described (Getsios et al., 2004).

Antibodies and chemical reagents

Rabbit polyclonals NW6 anti-DP; anti-MARCKS (Santa Cruz), anti-phospho-MARCKS (Cell Signaling Technology); anti-PKCα/βII, rIgG (Sigma); anti-FLAG (Affinity BioReagents, Sigma); anti-GFP (Clontech); anti-pSer19 MLC (Chemicon); anti-pERM, anti-ERM (Cell Signal); anti-non muscle Myosin Heavy Chain (BT461) (Biomedical Technologies, Inc.) was kindly provided by R. Chisholm (Northwestern University, Chicago,

IL); anti-pMYPT anti MYPT (Millipore Upstate); anti-α-adducin, anti-phospho-adducin provided by V. Bennett (Duke University, Durham, NC) (Matsuoka et al., 1998); 667 anti-PKP1 provided by M. Hatzfeld; 2026 anti-cMyc, 1905 anti-Dsg3 provided by J. Stanley (University of Pennsylvania, Philadelphia, PA), 8250 anti-pSer33 Keratin18 kindly provided by M.B. Omary (Stanford University, Stanford, CA). Mouse monoclonals 12G10 anti-α tubulin (by J. Frankel and E.M. Nelsen from the DSHB under the NICHD and University of Iowa); anti-GAPDH (Novus); KSB17.2 anti-keratin, 9E10 anti-myc, mIgG (Sigma); MAB6013S anti-PKP2 (Biodesign); RhoA (Santa Cruz); 1G4 anti-DP, 7G6 anti-Dsc2; 6D8 anti-Dsg2; 23E3 anti-PKP3 provided by F. van Roy (University of Ghent, Ghent, Belgium); Chicken polyclonal 1407 anti-PG. Alexa Fluor 568 or 488–conjugated goat IgGs (Invitrogen), HRP- conjugated goat IgGs (KPL; Rockland). Alexa-fluor 568 Phalloidin was used to stain F-actin (Invitrogen Molecular Probes). For kinase inhibition and activation, exhausted media was supplemented with Bisindolylmaleimide I (BIM), PMA, or H-89 (EMD Biosciences) or DMSO vehicle (Sigma). For actin contractility studies, Blebbistatin, LY27632 (Calbiochem), or LPA (Sigma) were supplemented to the media.

Calcium switch

SCC9 cells were incubated in low calcium medium (DME with 0.05 mM $CaCl_2$) for 16-20h, switched to normal growth media containing ~1.2 mM Ca^{2+} to induce cell junction assembly for time periods ranging up to 3 h, and processed for immunofluorescence analysis.

Rho activity assay

Rho activity assay was performed according to manufacturer's (Upstate) protocol. Cells were washed in TBS and lysed in magnesium buffer (125 mM HEPES, pH 7.5; 750mM NaCl; 5% Igepal CA-630; 50mM $MgCl_2$; 5mM EDTA; 10% glycerol). A sample of lysate was removed for input loading control (7% of total lysate). Lysates were cleared and incubated with GSH-agarose beads conjugated to GST-RBD (Rho Binding Domain) Rhotekin for 45-60min at 4°C. Precipitates were washed 3 times, boiled in laemmli buffer + DTT and separated by 12.5% SDS-PAGE. Western analysis of RhoA was performed using anti-RhoA mAb (Santa Cruz). Quantitation of active rho (RBD-pulldown) and total RhoA (input) was performed using ImageJ software.

Cell surface biotinylation and internalization assay

SCC68 cells were seeded onto 60mm dishes and cultured until 20% confluence in Keratinocyte-SFM media (Invitrogen, Carlsbad, CA) at 37°C + 5% CO2. GAPDH and PKP2 were knocked down using siRNA according to the manufacturers' protocol (Dharmacon, Lafayette, CO). Cells were incubated at 37°C for an additional 96 hrs post siRNA transfection. For internalization assay, cells were rinsed in ice-cold PBS+ (2X), (PBS+, 0.25mM Ca), and incubated with 2 mg/ml EZ-Link Sulfo-NHS-SS-biotin (Pierce Chemical Co, Rockford, IL) for 30 min at 4°C. Excess biotin was removed with PBS+ (3X), and internalization of biotin-labeled surface proteins was allowed to proceed at 37°C for 60 mins in pre-warmed culture media. Cells were returned to 4°C and washed with PBS+ (3X).

Residual biotin was stripped from the cell surface by washing with 100 mM sodium 2-mercaptoethanesulfonic acid
(Sigma-Aldrich) in 50 mM Tris-HCl, pH 8.6, 100 mM NaCl, 1 mM EDTA, 0.2% BSA (Sigma-Aldrich) (3X, 20 min). Cells were washed with 120 mM iodoacetamide (1X, 10 min) (Sigma-Aldrich) and PBS+ (3X) to quench residual MESNA and lysed in RIPA buffer containing protease inhibitors. Lysates were vortexed for a minute, centrifuged at 14,000 rpm for 30 min and recovered supernatant was normalized for total protein using the Amido Black assay. 20 µl of lysate was removed to serve as an input. Biotinylated proteins were retrieved with 40 µl UltraLink immobilized streptavidin (Pierce) by end over end rotation at 4°C overnight and washed with RIPA buffer (4X). Complexes were released using reducing Laemmli buffer at 95°C and analyzed using immunblot analysis.

Detergent solubility analysis

Cells were washed in PBS+Ca^{2+} and lysed in TBS (10mM Tris-HCl, pH 7.5; 1.0% TritonX-100; 145mM NaCl; 5mM EDTA; 2mM EGTA; 1.0 mM PMSF). Lysates were triturated and centrifuged. The supernatant (triton soluble pool) was removed to a new tube to which 3X Laemmli buffer (30% glycerol; 3% SDS; 0.1875M Tris-Cl, pH 6.8; 0.009 pyronin-Y.; 5% β-mercaptoethanol) was added. The insoluble pellet was solubilized in an equal volume of urea sample buffer (USB: 8M deionized urea; 1% SDS; 10% glycerol; 63mM Tris, pH 6.8; 0.01% pyronin Y; 0.5% β-mercaptoethanol). Equal volumes of each fraction were resolved using 7.5% SDS-PAGE followed by immunoblotting.

Immuno-precipitation (IP) and -blotting

Lysates were processed for IP as described (Chen et al., 2002) in lysis buffer (10mM TrisCl, pH 7.5; 1% triton x-100; 145mM NaCl; 5mM EDTA; 2mM EGTA; protease inhibitor cocktail (Roche); ± 2% phosphatase inhibitor cocktail IV (Calbiochem). FLAG-tagged proteins were IP'd with anti-FLAG® M2-agarose (Sigma). Endogenous proteins were IP'd with appropriate antibody and Gammabind + Sepharose (Amersham). Lysates were collected in Laemmli or USB +/- Protease Inhibitor. Amido Black assay to normalize protein content. Samples were resolved by 6.5% or 7.5% SDS-PAGE and immunoblotted as described (Chen et al., 2002).

Immunofluorescence analysis, fixed and time-lapse image acquisition

All immunofluorescence, fixed and time-lapse imaging procedures were performed as described (Godsel et al., 2005). SCC9 cells were seeded onto 0.1 mg/ml collagen I-coated (BD Biosciences) glass coverslips. For fixed immunofluorescence analysis, coverslips were washed in PBS, fixed in anhydrous methanol for 2 min at –20°C, and processed for indirect immunofluorescence. When using MAB6013, fixed cells were extracted with 0.5% Triton X-100 for 30 min at 4°C prior to antibody incubation. When staining with phalloidin, cells were fixed using formal saline acetone protocol. Briefly, coverslips were washed in PBS and incubated in 3.7% formaldehyde in PBS followed by washing 30 times in dH_2O. Coverslips were then incubated in ice cold acetone for 2 min and processed for indirect immunofluorescence. When using anti-RhoA antibody, coverslips were washed in 1X PBS

and then fixed following a protocol detailed in (Hayashi et al., 1999). Cells were incubated for 15 minutes in ice cold 10% trichloroacetic acid (100% TCA was dissolved with distilled water to make 100% w/v stock solution that was diluted to 10% distilled water at the time of use). TCA fixed cells were washed 3 X with PBS, incubated for 15 minutes in ice cold 0.2% Triton X-100 in PBS and washed 3X more with PBS. After incubation in appropriate secondary antibody (see "Antibodies and chemical reagents"), coverslips were mounted in polyvinyl alcohol (Sigma-Aldrich).

Fixed cells were visualized with a Leica DMR microscope using 40x (PL Fluotar, NA 1.0) or 63x (PL APO, NA 1.32) objectives, an Orca 100 CCD camera (model C4742-95; Hamamatsu) and MetaMorph 6.1 software (Universal Imaging Corp.) or a Zeiss LSM 510 laser scanning confocal microscope using a 100x objective (PLAN APO CHROMAT, NA 1.4) and LSM 510 software. Images were further processed using Photoshop CS3 and compiled using Illustrator CS3 (Adobe).

For live imaging, 3d post transfection PKP2 siRNA-, control siRNA with siGlo- (to locate siRNA-transfected cells) or un-transfected cells were seeded onto Lab-Tek chambered coverglass slides (Nunc). Cell monolayers were wounded with a 26-gauge needle and incubated in imaging medium (Hanks balanced salt solution, 20 mM Hepes, 1% FBS, 2 mM L-glutamine, 4.5 g/L glucose, 1x amino acids; recipe courtesy of G. Kreitzer, Weill Medical College of Cornell University, New York, NY) at 37°C for 60 min and then processed for time lapse imaging.

DP-GFP fluorescence time-lapse recordings were obtained at consistent time intervals of 1-1.5 min with rapid z-stack acquisition (12–15; 0.4–0.5-μm stacks) using an Application

Solution Multidimensional Workstation (ASMDW; Leica) DMIRE2 inverted microscope fitted with a Coolsnap HQ (Roper Scientific) camera and a 63x objective (HCX PL APO, glycerine, NA 1.3) with a piezo element. Cells were subjected to imaging in a 37°C climate chamber. All images were processed using a blind deconvolution synthetic algorithm and z-stacks were assembled into multi-image projections using ASMDW software. All movies were compiled using MetaMorph 6.1 imaging software (Universal Imaging Corp.).

Quantitation of fluorescence intensity and immunoblot densitometry

Fluorescence pixel intensity at cell borders was determined by multiplying the average pixel intensity by the area. Background intensity was subtracted from border intensity. Calculations were performed using MetaMorph 6.1 software. Densitometric analyses were performed using HP Officejet 5610 scan software and analyzed using Image J (NIH).

Statistical Analysis

Error bars represent SEM; statistical analysis was performed using two-tailed *t* test.

Supplemental material

All movies show DP-GFP dynamics during cell-cell contact initiated junction assembly. Movies 1-3: PKP2 siRNA- (Movies 1, 3) and control siRNA-transfected cells (Movie 2). Movie 4: DP dynamics during PKC inhibition. Movie 5: DP^{S2849G} dynamics.

CHAPTER III

Plakophilin 2: a critical scaffold for PKCα that regulates intercellular junction assembly

Introduction

(Note: With the exception of a few figures, this chapter represents a published report (Bass-Zubek et al., 2008). All movies can be found online at http://www.jcb.org/cgi/content/full/jcb.200712133/DC1)

Armadillo family members are multifunctional proteins that play diverse roles in cell-cell adhesion and signaling. Plakophilins (PKPs) comprise a subgroup of the armadillo protein family, related to the cadherin-associated protein $p120^{ctn}$ (Hatzfeld, 2007). Classically thought to be a constitutive component of the submembrane plaque in intercellular junctions, including desmosomes and the cardiac *area composita* (Franke et al., 2006; Godsel et al., 2004), the PKPs can also localize to the cytoplasm and nucleus (Mertens et al., 1996; Schmidt et al., 1999; Schmidt et al., 1997).

Mutations in desmosomal proteins lead to epidermal fragility and/or cardiac defects (Lai-Cheong et al., 2007). In particular, mutations in PKP2 have been described as a major causative factor for congenital cardiac arrhythmias (Gerull et al., 2004; Lai-Cheong et al., 2007). While compromised junctional integrity is assumed to contribute to disease pathophysiology, the specific etiological mechanism is poorly understood.

PKP2 partners with a number of junction components, including desmoplakin (DP), with which it interacts via its N-terminus (Chen et al., 2002). We recently showed that PKP2 colocalizes with cytoplasmic DP-containing precursors that form in response to cell-cell contact and subsequently translocate to nascent junctions (Godsel et al., 2005). However, the consequences of PKP2 deficiency on precursor formation and dynamics are unknown.

Here I provide evidence that PKP2 facilitates the association of PKCα with DP, which in turn is required for the assembly competence of this junctional plaque protein.

Importantly, PKP2 also regulates the availability of PKC for phosphorylation of other cellular substrates, and thus may have a more global role in cellular homeostasis by serving as a scaffold for a ubiquitous signaling molecule.

Results

Plakophilin 2 is a unique partner for DP in plaque precursors

Using live cell imaging of DP-GFP expressing cells undergoing junction assembly, we previously demonstrated that DP is present in macromolecular particles that form in the cytoplasm in response to cell contact and associate with keratin intermediate filaments. These particles then translocate to junctions where they participate in cell-cell border fluorescence intensification, suggesting that they are incorporated into nascent desmosomes (Godsel et al., 2005). In order to begin to understand the regulation of plaque precursor assembly, we sought to characterize the biochemical composition of these precursors. To determine which desmosome components are present along with DP in plaque precursors, SCC9 cells constitutively expressing DP-GFP were subjected to calcium switch to induce junction assembly. Confocal microscopic analysis of these cells led to the observation that PKP2 colocalized extensively with DP cytoplasmic particles whereas PKP3, PG and DSC2 exhibited less colocalization with DP-GFP particles (Figure 3.1A). The degree of colocalization in cytoplasmic particles was determined by measuring fluorescence intensity per normalized unit area and compared to that found at the cell-cell border where junctions were present (i.e. maximal colocalization). This ratio was then normalized to that of DP-GFP: PKP2 exhibited 98% of maximal colocalization in DP-GFP dots whereas PKP3 exhibited 22%, PG exhibited 44%, and DSC2 exhibited 38% of maximal colocalization (Figure 3.1B).

Whether desmosomes assemble from newly synthesized components that arrive at the plasma membrane separately or from entire or partial desmosomes that have been internalized and then assembled in a vesicular pool in a continuous cycle (Demlehner et al., 1995) is controversial and depends whether on extracellular calcium levels are high enough to stabilize cadherin-mediated adhesion. However, if the latter hypothesis is true, then these DP containing particles would be heavily associated with vesicular membranes. To determine whether these particles are indeed cytoplasmic macromolecular complexes or simply associated with internal vesicles, confocal analysis of cells immunostained for DP and counterstained with an intracellular membrane-specific dye was performed. Very little colocalization of DP particles with intracellular membrane-associated structures near the cell-cell border was observed (Figure 3.1C). This result was verified by testing colocalization of DP particles with a number of other membrane-compartment-specific markers such as EEA1 (early endososmes), LAMP1, and cathepsin D (lysosomes) (data not shown). Ultrastructurally, these DP and PKP2 containing precursors did not appear to be associated with internal vesicles (Godsel et al., 2005). These results are consistent with the notion that these precursors are indeed cytoplasmic, macromolecular, and non-membrane associated complexes.

However, retrospective analysis of DP-GFP expressing cells undergoing wound healing demonstrated that a subpopulation of DP particles move in a retrograde fashion, and a large component of these particles do contain desmosomal cadherins, PKP3 and PG in addition to PKP2. Nonetheless, PKP2 was the only marker to colocalize with DP-GFP in anterograde-directed, assembly-competent precursors (Godsel et al., 2005). Based on these

observations, we hypothesized that PKP2 plays a specific role in facilitating plaque precursor assembly.

Since PKP2 is highly associated with DP during assembly, we next asked whether DP and PKP2 co-assemble. To address this question, a calcium switch was performed on SCC9 parental cells which were fixed after several time points to achieve high temporal resolution. Dual label indirect immunofluorescence against endogenous DP and PKP2 demonstrated that PKP2 appears at cell-cell borders earlier than DP, but strong colocalization occurs in the cytoplasm during assembly and at the membrane once DP begins to accumulate (Figure 3.2). Although PKP2 arrives at the membrane earlier than DP, the two proteins colocalize extensively in the cytoplasm in particles that sometimes appear to align in a filamentous distribution during assembly (Figure 3.3). Indeed, these PKP2/DP precursors were often found at the interstices of keratin intermediate filaments (Godsel et al., 2005), which may serve as the nidus for the cytoplasmic origin of these precursors.

PKP2 is required for efficient assembly of desmoplakin into desmosomes.

To test whether PKP2 is required for DP assembly into junctions, we introduced siRNA pools into parental or DP-GFP expressing SCC9 and A431 epithelial cell lines (present at ≤ 20% and 14% of total DP, respectively), resulting in a ~90% decrease in PKP2. While total levels of desmosome proteins were unaffected, DP border fluorescence was severely decreased compared to control siRNA transfected cells (Figure 3.4A,B) and restored by a silencing resistant PKP2 (Figure 3.5). In addition, DP particles aligned in a striking filamentous pattern in A431 cells (Figure 3.4B), co-localizing extensively with keratin IF

(unpublished data). DP particles also decorated the IF network in PKP2-deficient SCC9 cells, which appeared partially retracted with fewer filaments extending to the membrane (Figure 3.6A, B). Similar results were obtained with three individual siRNAs (unpublished data), supporting the specificity of the response. DP distribution was frequently observed to be more linear in A431 than SCC9 cells, likely reflecting a difference in keratin IF network organization in these lines. In A431 cells IF appeared to be organized in straight radial cables compared with a more sinuous, intersecting network of bundles in SCC9s (Figure 3.6 and data not shown). Interestingly, in wildtype cells (not siRNA transfected) constitutively expressing DP-GFP that were undergoing calcium-induced assembly, PKP2 and DP colocalized in particles that appeared to align along filaments (Fig 3.3). By immunoelectron microscopic analysis, these precursor-like particles that contained PKP2 and DP were confirmed to associate with intermediate filaments near newly forming junctions (Godsel et al., 2005).

To test whether PKP2 knockdown affects the kinetics of DP incorporation into forming junctions, a calcium switch was performed. DP accumulation at SCC9 cell-cell borders was reduced by ~60% at 1hr and ~40% at 3hr (Figure 3.7) whereas E-cadherin and plakoglobin (PG) were minimally affected (unpublished data). To enhance the temporal resolution of analysis, A431 DP-GFP cells were transfected with PKP2 siRNA and live cell imaging of cells coming into contact following scratch wounding was performed. DP border fluorescence appeared within minutes of cell-cell contact in both control and PKP2 SiRNA transfected cells (Figure 3.8; Movies 1-3). However, at 20-30 min, a striking accumulation of DP in an IF-like filamentous pattern was observed (Movie 1; Figure 3.8A; Movie 3). This

time frame corresponds to DP appearance in the cytoplasm as small particles/dots in control cells (Godsel et al., 2005) (Movie 2; Figure 3.8B). DP filamentous accumulation also correlated temporally with the point at which the border fluorescence intensity in PKP2 knockdown cells started to fall off relative to controls (Figure 3.7). While in certain cells a beads-on-a-string type of pattern was observed prior to the beginning of the experiment (Movies 1 and 3), DP fluorescence intensity along these tracks increased in response to cell-cell contact. These results suggest that PKP2 is required for continued accumulation of DP at borders and maturation of junctions during desmosome assembly, possibly by regulating DP's cytoplasmic localization.

To characterize the degree of cytoskeletal association of DP in cells transfected with PKP2 siRNA, a Triton X-100 fractionation experiment was performed. SCC9 cells infected with empty pSUPER retrovirus or pSUPER.retrovirus expressing PKP2 shRNA were lysed and solubilized in triton buffer. In general, Triton x-100-soluble proteins are thought to be cytosolic or membrane associated, and Triton-insoluble proteins are associated with cytoskeletal components. DP^{S2849G} is approximately 5-fold more insoluble than wild type DP (Godsel et al., 2005), and DP in PKP2-deficient cells was slightly more insoluble than in control cells (Figure 3.9). While this is a modest change in solubility, it is possible that the association with IF is a dynamic process and may not be fully reflected by solubility. Additionally, S2849G can be thought of as a constitutive mutation, whereas changes in signaling levels or localization of signaling are often more fluid and transient processes and could lead to a less severe phenotype.

DPser2849 and PKC activity are required for proper DP border localization

The filamentous distribution of DP observed in PKP2 deficient cells was reminiscent of that exhibited by a phosphorylation-deficient point mutant, DP^{S2849G}, which tends to be sequestered along IF and exhibits delayed assembly kinetics (Godsel et al., 2005). Ser2849 falls within a PKA/PKC consensus phosphorylation site in the C-terminus of the DP IF-binding domain. Mutation at this site has been shown to enhance DP interactions with IF 10-fold in yeast two hybrid assays (Fontao et al., 2003; Godsel et al., 2005; Meng et al., 1997; Stappenbeck et al., 1994). Previous work demonstrated that PKC activation can stimulate desmosome formation in the absence of adherens junctions (van Hengel et al., 1997) as well as DP recruitment to cell-cell borders in SCC cells cultured in low calcium (Sheu et al., 1989), both conditions that are normally inhibitory to desmosome formation. Coupled with the similarity between the PKP2-deficient and DP^{S2849G} expressing cells, this suggested that PKP2 may be important for PKC-dependent signaling during assembly.

Supporting this possibility, treatment of cells with the PKC inhibitor BIM severely impaired DP border localization in SCC9 cells (Figure 3.10A), although PKP2 border accumulation was unaffected. However, PKA inhibition by H-89 had no effect on DP border accumulation (Figure 3.10C), *s*uggesting that PKC is more important than PKA in regulating DP incorporation into junctions and that PKC is probably the primary kinase that phosphorylates this site. BIM treatment induced a filamentous DP pattern in A431 cells (Figure 3.10B), similar to that observed in PKP2 deficient cells (Figure 3.4). Further, when A431 cells expressing wild type DP-GFP were treated with BIM and then analyzed by live cell imaging, DP particles that formed in the cytoplasm underwent a dramatic filamentous

alignment, recapitulating the phenotype of DP^{S2849G} (Figure 3.11A) and PKP2 knockdown (Figure 3.11B and C; Movies 4 and 5).

To test whether loss of potential phosphorylation site Ser2849 interferes with PKP2 association with DP, DP^{S2849G}-GFP or DP-GFP expressing SCC9 cells were immunostained for PKP2 and subjected to confocal analysis. PKP2 was able to colocalize with both WT and DP^{S2849G}, although PKP2 did not fully decorate but only studded the IF-like DP^{S2849G} filaments (Figure 3.11C). These results suggest that while mutation of DP Ser2849 to Gly alters its association with IF (Godsel et al., 2005), phosphorylation of DP at Ser2849 is not required for PKP2 association. Interestingly, PKP2 still formed discrete structures, suggesting that it does not partner with the entire DP population but a particular subset. It may be the case that these discrete PKP2 particles are associating with endogenous DP, which can interact with DP^{S2849G}. Further analysis of the particular conditions that regulate PKP2 and DP interactions will be necessary to better understand their co-dynamics.

PKCα is the only calcium-dependent classical PKC expressed in keratinocytes (Denning, 2004), localizes to desmosomes (Garrod et al., 2005; Jansen et al., 2001), and modulates their sensitivity to calcium depletion and adhesive state (Kimura et al., 2006; Wallis et al., 2000). However, the potential mechanistic role of this specific kinase in desmosome assembly is unknown. To test whether PKCα is required for DP assembly into borders, we infected cells with retrovirus-encoded PKCα shRNA and performed a calcium switch. DP border localization was dramatically impaired (67% decrease) in PKCα deficient cells compared to control cells (Figure 3.12A, B), while total desmosome protein levels were unaffected (Figure 3.12C). These studies demonstrate that PKCα regulates DP assembly into

desmosomes, possibly through modulation of DP phosphorylation at Ser2849 and regulating its association with IF.

PKP2 regulates PKC signaling by recruiting it to DP

Based on the similarity in DP behavior under PKP2- and PKC-deficient conditions, we set out to test whether loss of PKP2 affects PKC signaling. Lysates from cells transfected with PKP2 siRNA were probed with phospho-specific antibodies against known PKC substrates. Relative levels of phosphorylated MARCKS and adducin were increased by over 70-fold and 3-fold, respectively, in PKP2 siRNA transfected cells (Figure 3.13). MARCKS phosphorylation was increased in PKP2 deficient cells even when cultured in low calcium (Figure 3.14), suggesting that cell-cell contact is not required for PKP2-regulation of PKC signaling. However, PKCα phosphorylation at Thr638 was not consistently increased (data not shown) suggesting that total cellular levels of PKC activity are not affected by PKP2 knockdown. Based on these findings, we hypothesized that PKP2 serves as a scaffold that recruits PKC locally to control the proper assembly and behavior of DP precursors, and that in its absence, PKC is free to phosphorylate other substrates.

PKC specificity is regulated by scaffolding proteins that determine its subcellular localization thereby bringing certain subpopulations of PKC molecules in close proximity to specific substrates (Schechtman and Mochly-Rosen, 2001). The PKP2 head domain, but not the central armadillo repeats, co-precipitated with PKCα-FLAG in HEK293 cells (Figure 3.15). Endogenous DP also co-precipitated with PKCα raising the possibility that the three proteins form a cytoplasmic complex (Figure 3.16A). Importantly, the level of DP that co-

precipitated with PKCα in PKP2-deficient cells was reduced by 95%, consistent with the idea that PKP2, directly or indirectly, serves as a scaffold for PKC (Figure 3.16B).

PKC activation restores DP desmosome incorporation

If PKP2-dependent recruitment of a limiting pool of active PKC to DP is required for proper regulation of DP-IF interactions, then global activation of PKC might bypass the need for PKP2. Indeed, PMA treatment restored DP borders in PKP2 siRNA treated cells that had been reduced to 40% of the control (Figure 3.17). This observation suggests that hyper activation of PKC leads to an increased probability that DP will be properly phosphorylated by PKC, facilitating its incorporation into desmosomes. Further, ectopic expression of wild-type PKCα and, even more so, constitutively active PKCα in PKP2-deficient cells shifted the proportion of borders from those that exhibit weakly localized DP to those that appear more robust, continuous, and mature (Figure 3.19). The fact that restoration was not complete may reflect a requirement for PKC-independent structural and/or signaling functions contributed by PKP2 in desmosome assembly or maintenance. Interestingly, PKC activation induced a partial enhancement of DP^{S2849G}–GFP accumulation at cell-cell borders but could not rescue its aggregation to IFs (Figure 3.18). This finding suggests that PKC-dependent phosphorylation of DP at Ser2849 is necessary for modulation of its interaction with IF, but PKC may also play another role independent of its ability to phosphorylate Ser2849 to stabilize a subpopulation of DP already localized to junctions. PKP2 could also be important for regulating signaling events at junctions as well. Supporting this idea, phosphorylated Keratin18 (phospho-Ser33) accumulation at cell-cell contacts was abolished in PKP2-

deficient cells (Figure 3.20). This particular phosphorylation site is important for interaction of K18 with 14-3-3 proteins when phosphorylated and regulates both keratin network organization and 14-3-3 nuclear accumulation during mitosis (Ku et al., 2002). Interestingly, keratin phosphorylation was enhanced in the nucleus in PKP2-deficient cells, suggesting another potential role of nuclear PKP2 in regulating signaling that may be important for regulation of nuclear events such as modulation of cell cycle progression. Consistent with this, PKP2 phosphorylation by C-TAK1, a cell cycle-associated kinase (Cdc25-associated), is critical for its interaction with 14-3-3 proteins. This PKP2-14-3-3 interaction regulates PKP2 subcellular localization by preventing its nuclear accumulation PKP2 (Muller et al., 2003).

Discussion

Collectively, these data support a role for PKP2 in coordinating signals required for assembling the DP-rich desmosome plaque (Figure 3.21A, B). In particular, DP behavior during the later phases of assembly is aberrant in PKP2-deficient cells. Instead of coalescing into assembly-competent particles (described in (Godsel et al., 2005)), DP and/or DP-containing particles appear to coat filaments all along their length and fail to efficiently translocate to cell-cell interfaces. The data are consistent with a model whereby cell contact triggers a signal (possibly PKC activation (van Hengel et al., 1997), and that PKP2, either directly or in conjunction with other scaffolding proteins, is required for communicating this signal by recruiting activated PKC to DP, which in turn phosphorylates DP at Ser2849. While the molecular mechanism is not yet known, it seems possible that the observed reduction in affinity for IF exhibited by phosphorylated DP may be due to alterations in the conformation of adjacent regions making up the IF binding domain. A more dynamic association with IF may be necessary for ultimately promoting plaque precursor assembly while maintaining precursors locally concentrated near the cortex for proper delivery to junctions. Specific localized changes in IF phosphorylation state (due to PKC activity or other signaling pathways) may also play a role in the regulation of DP-IF interactions at the membrane as well as modulating IF organization.

Somewhat surprisingly, while PKP2 appears at borders earlier than DP during the assembly process (Figure 3.1), the loss of PKP2 did not completely abrogate DP clustering at cell borders during assembly (Figure 3.6), suggesting that its initial accumulation does not require PKP2. PG and/or PKP3 may partially compensate for the loss of PKP2 and stabilize

DP near the membrane, particularly in tissues where PKP2 is not the primary PKP expressed. However, as the non-membrane bound assembly-competent dots that appear in the cytoplasm after cell contact contain PKP2 but not PKP3 or PG, the role of PKP2 at this intermediate stage may be more critical (Godsel et al., 2005). The fact that DP border accumulation is impaired but PKP3 expression does not change suggests that its presence cannot completely compensate for loss of PKP2. Indeed, DP border intensity is reduced at steady state in all cultured cell types tested, suggesting that compensatory mechanisms are insufficient for proper maturation and/or stability of junctions.

PKP2 determines DP-IF affinity

Our data are consistent with observations performed in PKP2-null mice (Grossmann et al., 2004). The authors reported a loss of DP from cell-cell junctions and the presence of cytoplasmic DP-containing aggregates that were intimately associated with desmin IF of the cardiac muscle. Knockdown of PKP2 in primary cardiomyocytes grown in culture mimics desmosome phenotypes seen in the murine PKP2 knockout model (Pieperhoff et al., 2008), although studies of desmosome dynamics or downstream signaling events were not characterized in the cardiac myocyte system. Along with the fact that Ser2849 phosphorylation also regulates DP-desmin IF interactions (Lapouge et al., 2006), these observations support a similar role for PKP2 in other cell types. It was proposed that mechanical defects due to loss of junctional integrity underlie the observed cardiac rupture and embryonic lethality in PKP2 knockout mice, and may also contribute to human cardiac arrhythmias associated with PKP2 mutations. The relative contributions of PKPs in different

cell contexts may be different, as patients with PKP2 mutations and prominent heart defects have not been reported to exhibit loss of keratinocyte adhesion. However, there may be yet unidentified dominant negative mutations PKP2 that result in a much more severe phenotype leading to embryonic lethality that could have effects in skin adhesion and/or development.

PKP2 regulates PKC activity

In addition to promoting junction assembly and maintenance by locally harnessing PKC activity, the observation that PKP2 prevents aberrant phosphorylation of PKC substrates suggests that it may have broader cellular functions. Certainly, that PKP2 is present in the nucleus and can bind RNA pol III (Mertens et al., 2001) and that we observe dramatic changes in keratin phosphorylation in PKP2-deficienct cells (Figure 3.20) potentially implicates PKP2 in the regulation of nuclear signaling, transcription and/or cell proliferation. Such global changes in the distribution of active PKC could contribute to disease pathogenesis in patients with PKP2 deficiency.

The PKC substrates investigated in this report and others are actin binding proteins (Larsson, 2006). Thus, this PKP2-PKC signaling pathway could also contribute to actin remodeling important for desmosome assembly, an idea that is consistent with our previous observation that DP translocation is actin-dependent (Godsel et al., 2005). Coupled with recently ascribed roles for $p120^{ctn}$ and p0071 in actomyosin contraction signaling and regulation of Rho GTPases (Hatzfeld, 2005; Wolf et al., 2006), the possibility that PKP2 scaffolds PKC suggests an emerging common function for these armadillo proteins in locally coordinating signals that direct cytoskeletal remodeling and regulate junction dynamics.

FIGURES

Figure 3.1 PKP2 colocalizes extensively with DP in cytoplasmic particles during junction assembly.

A. SCC9 cells expressing DP-GFP were incubated in medium containing 0.05mM Ca^{2+} (LCM) for 12-16 h and then switched to medium containing normal calcium (NCM). Cells were counterstained using indirect immunofluorescence against endogenous PKP2, PKP3, PG or DSC2 and confocal microscopic analysis was performed.

B. Quantification of colocalization: PKP2, PKP3, PG, or DSC2 fluorescence intensity within a region of normalized area was measured in DP-GFP cytoplasmic particles and then compared to the fluorescence intensity at the membrane. Values were plotted as a ratio of intensity in cytoplasmic dots to intensity at the border that was then normalized to the ratio of values for DP-GFP.

C. Confocal image of SCC9 cells immunostained for DP and counterstained with an intracellular membrane-specific dye.

Bar = 10μm

Figure 3.1

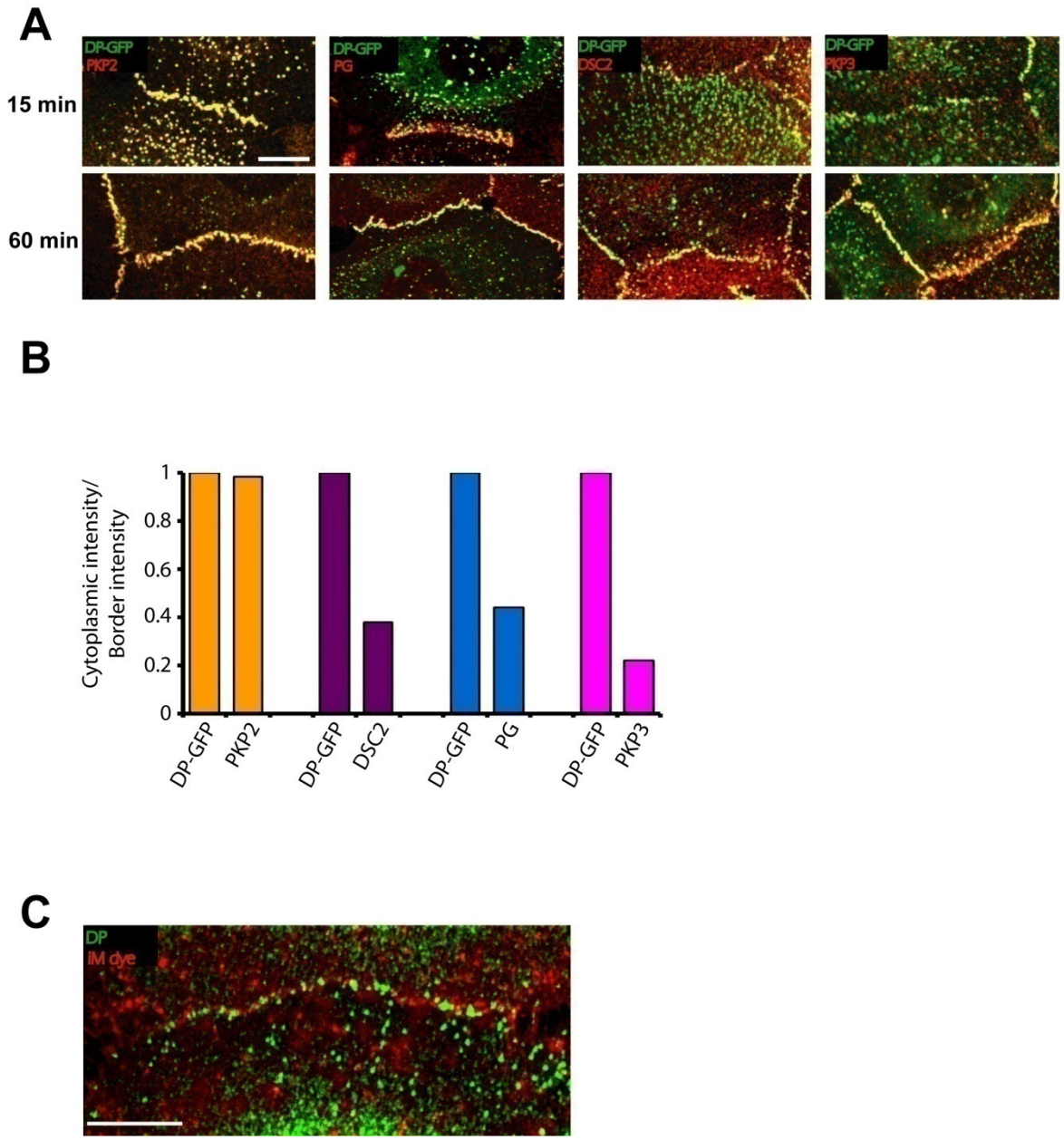

A
DP-GFP
PKP2
DP-GFP
PG
DP-GFP
DSC2
DP-GFP
PKP3
15 min
60 min
B
Cytoplasmic intensity/
Border intensity
1
0.8
0.6
0.4
0.2
0
DP-GFP
PKP2
DP-GFP
DSC2
DP-GFP
PG
DP-GFP
PKP3
C
DP
IM dye

Figure 3.2 PKP2 assembles at cell-cell junctions before DP

SCC9 cells undergoing calcium switch, followed by dual label indirect immunofluorescence of endogenous PKP2 and DP. In fixed cells, PKP2 is visible at cell-cell borders within 10 minutes of cell contact whereas DP begins to arrive at cell borders by 20 min. DP and PKP2 border accumulation appear to equalize by 3h in NCM.

Figure 3.2

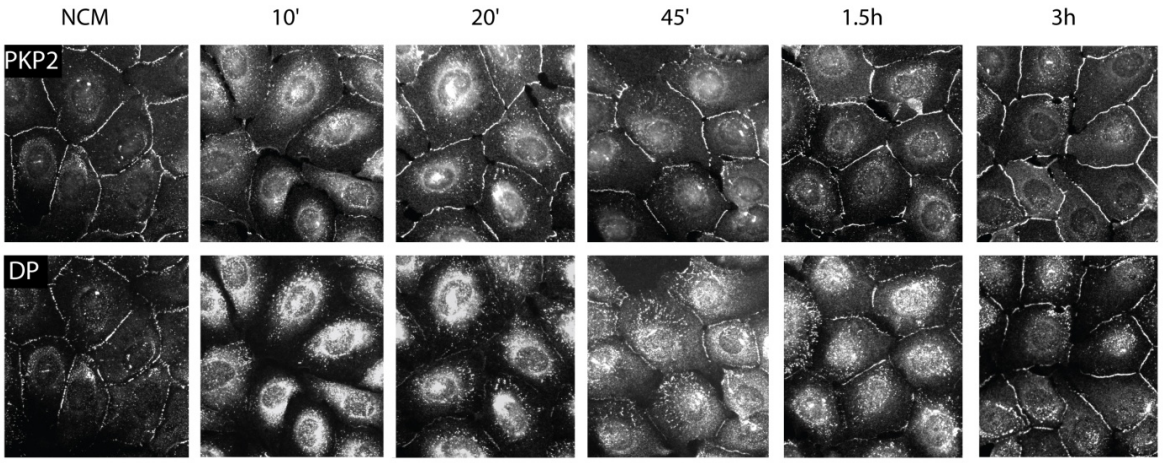


Figure 3.3 PKP2 and DP colocalize in cytoplasmic particles that align in filamentous pattern

SCC9 cells undergoing calcium switch were fixed and stained for PKP2 and DP. At 10', there are a number of cytoplasmic particles that contain PKP2 and DP that align in a filamentous pattern (inset depicts magnification of boxed area). By 45' in NCM, many cytoplasmic dots have been cleared and borders appear more robust, linear and therefore mature.

Figure 3.3

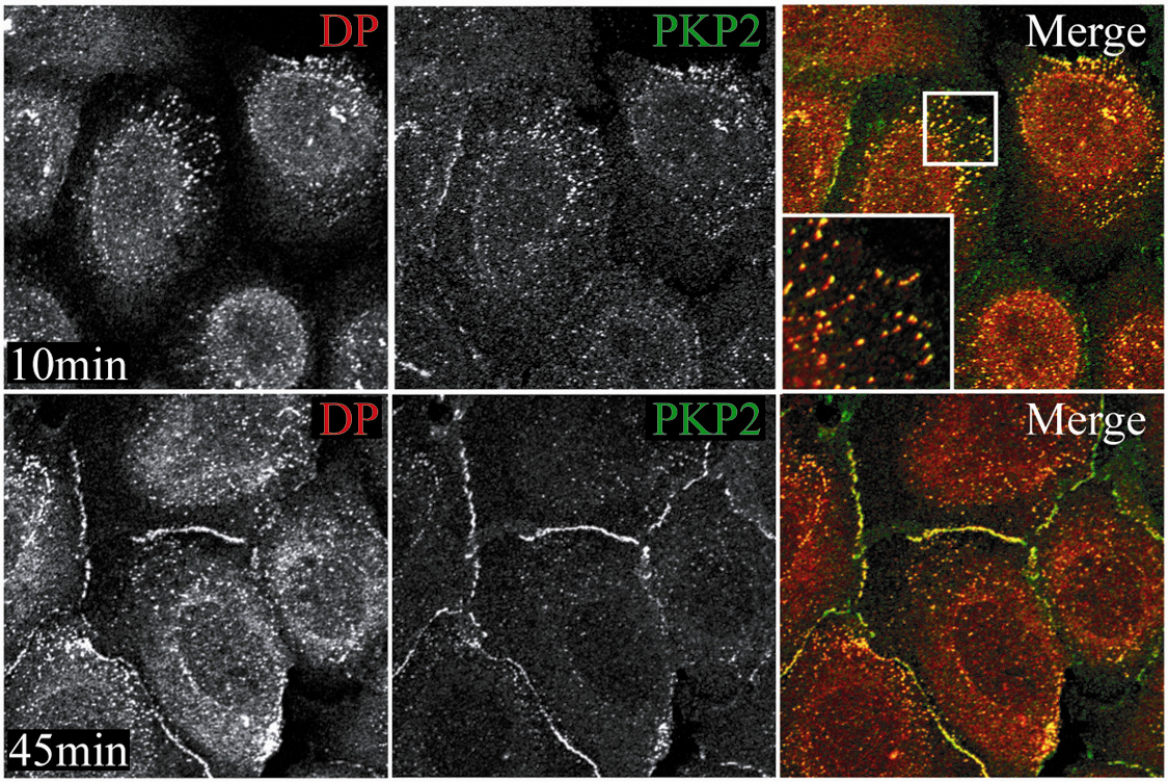


Figure 3.4 Impaired DP border localization in PKP2-deficient cultures.

A, B. SCC9 cells (A, widefield microscopy) or A431 cells (B, confocal microscopy) expressing pooled siRNAs against human PKP2 or non-targeting (NT) control, immunostained for endogenous DP and PKP2. Immunoblot analysis of SCC9 (A, right panels) or A431 (B, right panels) from NT or PKP2 siRNA transfected cells to detect total levels of PKP2, PKP1, PKP3, DP, DSC2, DSG2, DSG3, α-tubulin. PG protein levels were unaffected (unpublished data). Arrows depict cell-cell border. Bar = 10μm

Figure 3.4

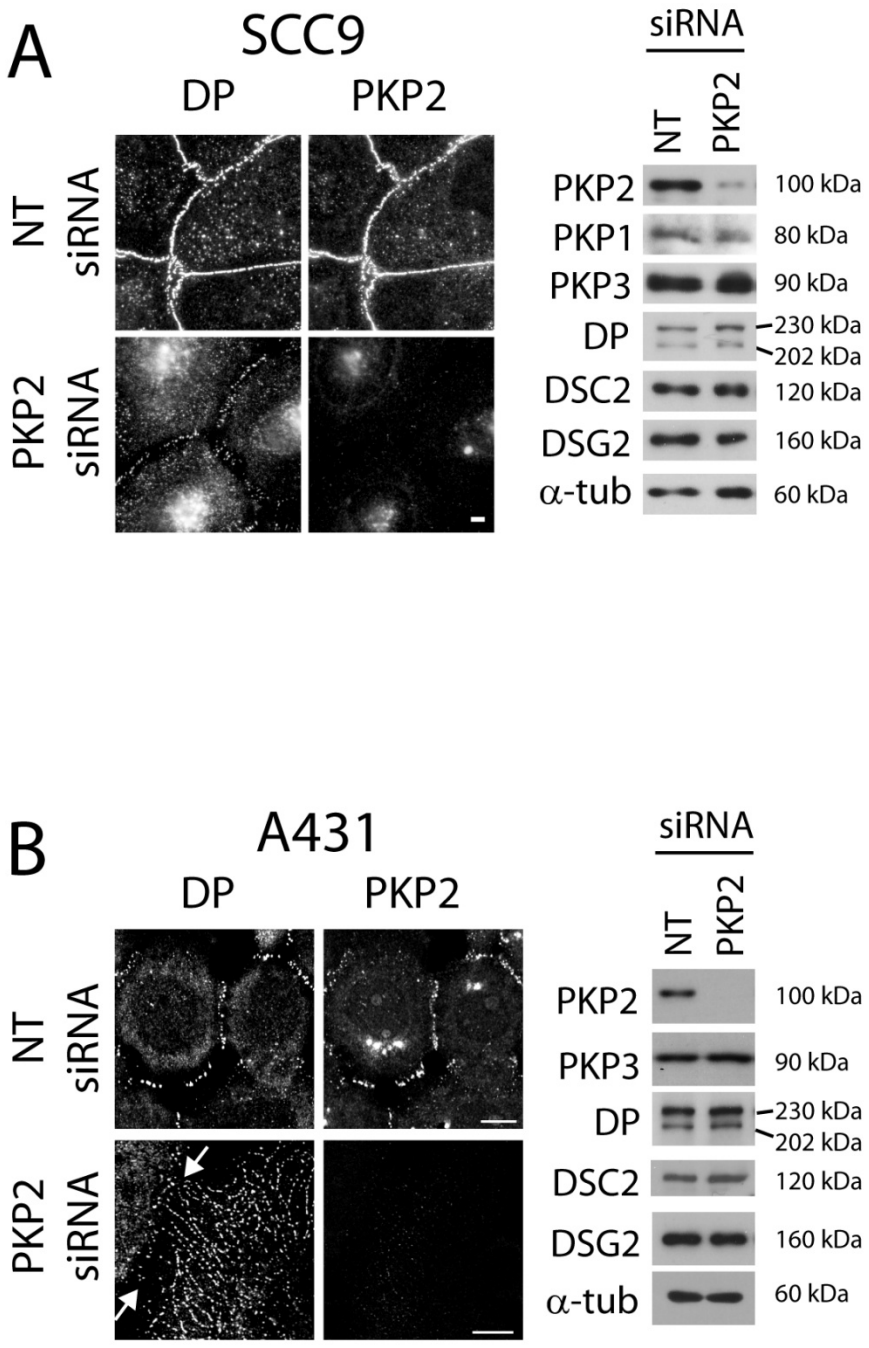

A
SCC9
DP
PKP2
NT siRNA
PKP2 siRNA
siRNA
NT
PKP2
PKP2
PKP1
PKP3
DP
DSC2
DSG2
α-tub
100 kDa
80 kDa
90 kDa
230 kDa
202 kDa
120 kDa
160 kDa
60 kDa
B
A431
DP
PKP2
NT siRNA
PKP2 siRNA
siRNA
NT
PKP2
PKP2
PKP3
DP
DSC2
DSG2
α-tub
100 kDa
90 kDa
230 kDa
202 kDa
120 kDa
160 kDa
60 kDa

Figure 3.5 PKP2 re-expression enhances DP border localization during PKP2 knockdown.

A. Silencing-resistant FLAG-tagged PKP2 was coexpressed with PKP2 or NT siRNA in SCC9 cells. Cells were stained for endogenous DP, and FLAG and PKP2 (using same anti-mouse secondary antibody). Border between two transfected cells: green arrowhead; border between two untransfected cells: red arrow. Bar = 10μm.

B. Intensity of DP border fluorescence at borders between pairs of transfected cells or pairs of untransfected cells. * $p<0.001$.

Figure 3.5

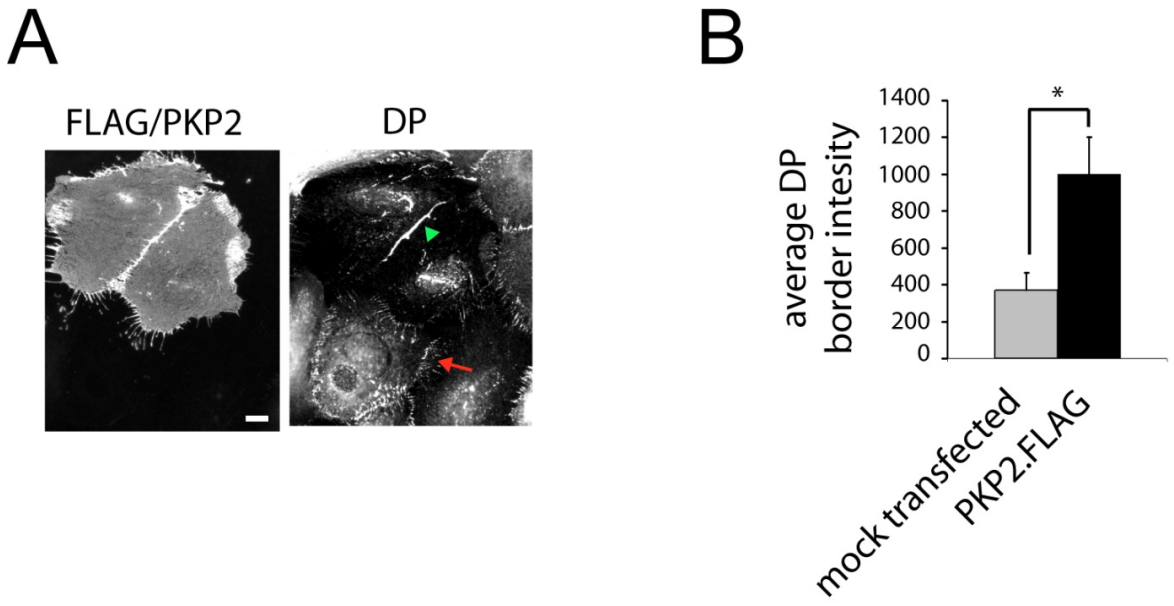

A
FLAG/PKP2
DP
B
average DP
border intesity
1400
1200
1000
800
600
400
200
0
*
mock transfected
PKP2.FLAG

Figure 3.6 DP particles colocalize with keratin IF during PKP2 knockdown

Confocal images of SCC9 cells expressing control nontargeting (NT) siRNA (A) or PKP2 siRNA (B) immunostained for DP (green) and keratin (red). Aa' and Ba' uncropped images taken with 100x objective using confocal laser scanning microscopy. Ab' and Bb' cropped and magnified images from areas in white boxes from Aa' and Ba'.

Bar = 10μm.

Figure 3.6

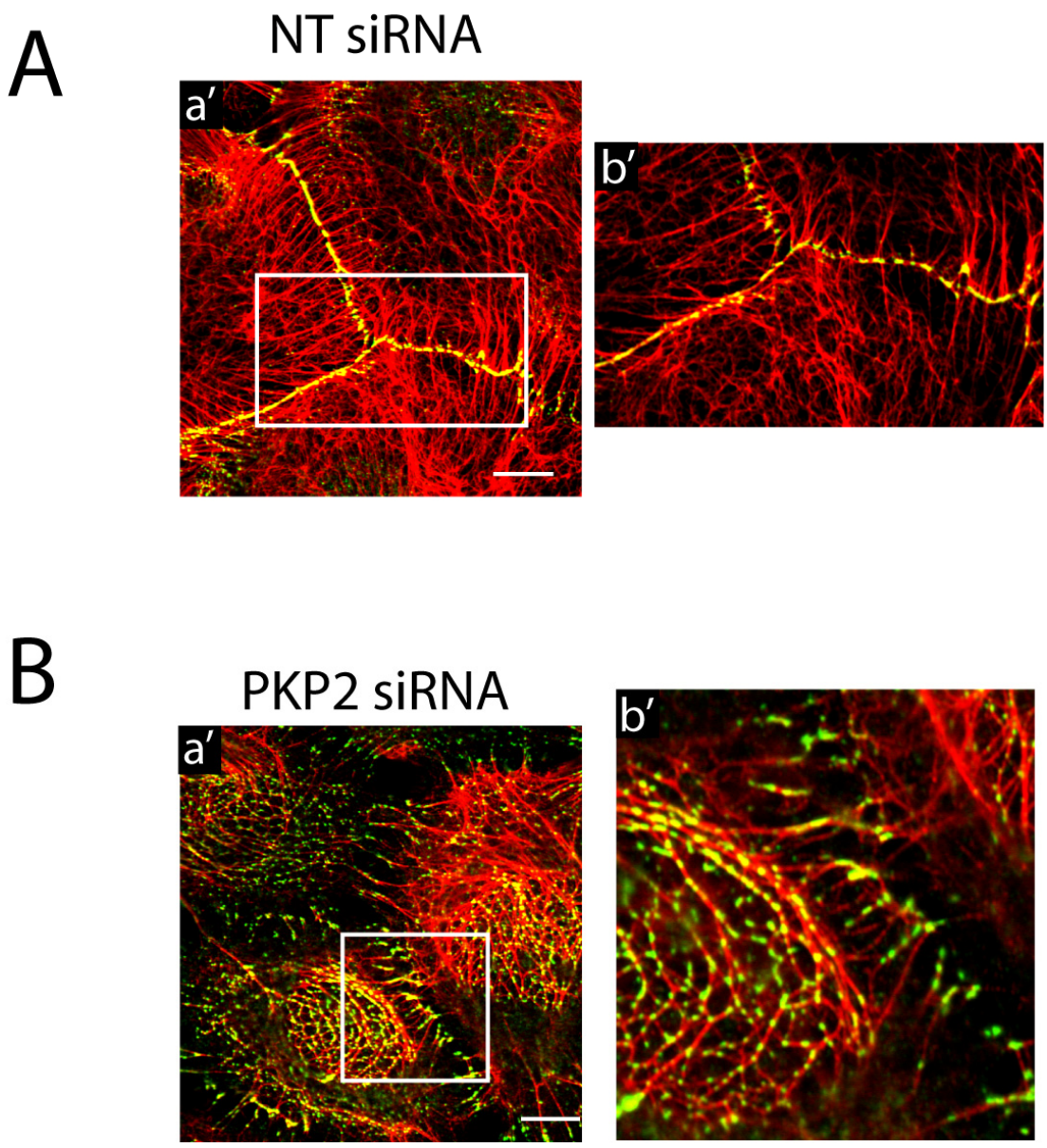


Figure 3.7 DP assembly is impaired during PKP2 knockdown

A. Calcium switch of SCC9 cells transfected with siRNA against PKP2 or NT + siGlo, endogenous PKP2 or DP staining. Arrows depict siGLO particles. Bar = 10μm

B. DP border fluorescence intensity was measured, normalized to background and plotted in D, right panel. Quantification performed with help from Nicholas J. Garcia.

Figure 3.7

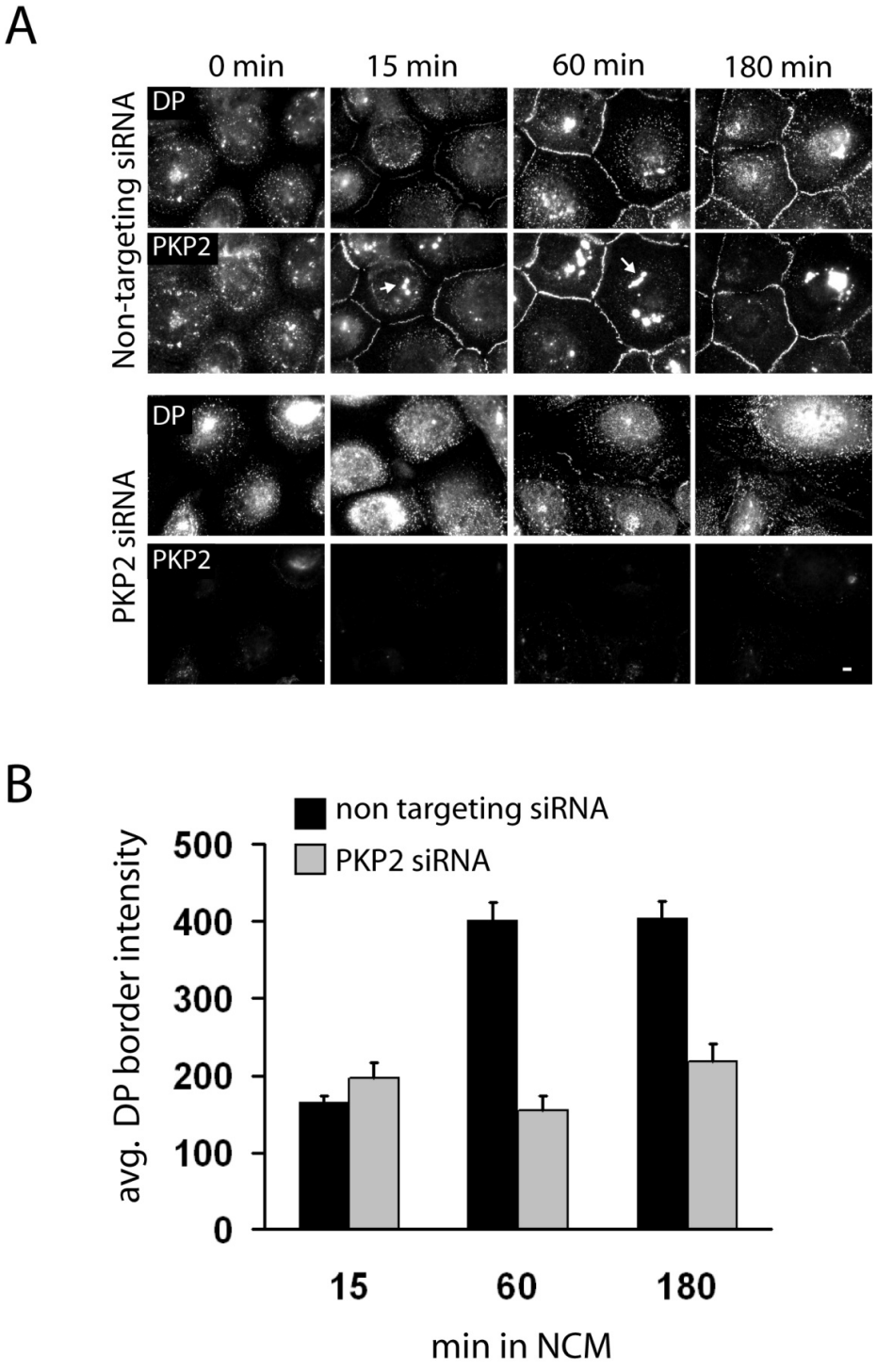

A
0 min
15 min
60 min
180 min
Non-targeting siRNA
DP
PKP2
PKP2 siRNA
DP
PKP2
B
non targeting siRNA
PKP2 siRNA
avg. DP border intensity
500
400
300
200
100
0
15
60
180
min in NCM

Figure 3.8 DP accumulates in a filamentous cytoplasmic pattern during PKP2 knockdown.

Stills from Movies 1 and 2 of PKP2-(A) or NT siRNA-transfected (B) A431 DP-GFP cells. Arrows depict where new borders are being formed. Time points indicate time lapsed from beginning of movie. Red arrowheads show DP accumulation on IF. Experiment performed with Evangeline Amargo.

Bar = 10μm

Figure 3.8

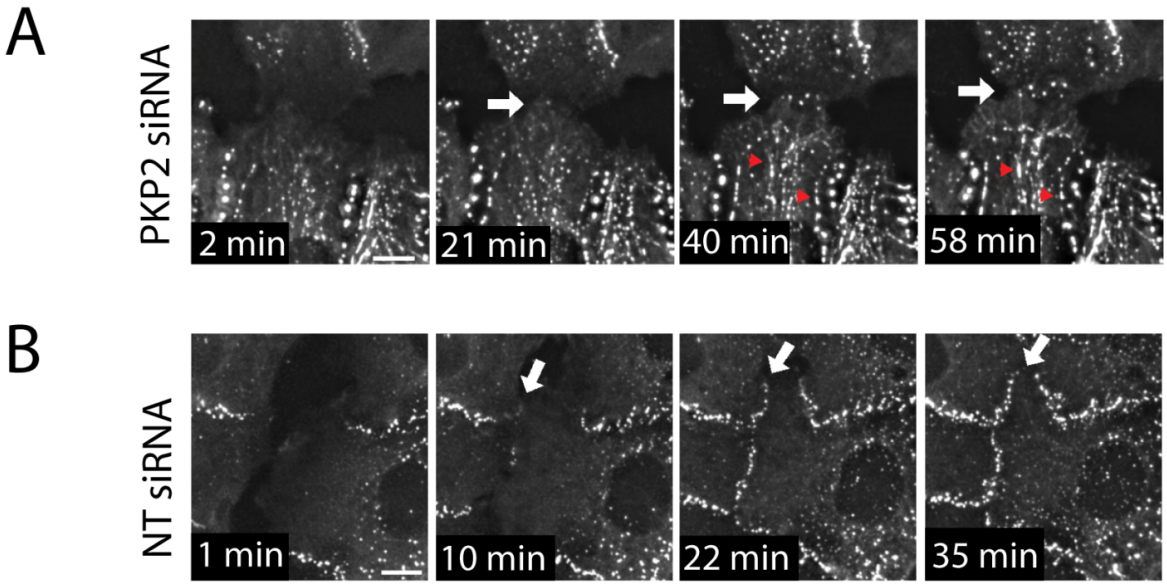

A
PKP2 siRNA
2 min
21 min
40 min
58 min
B
NT siRNA
1 min
10 min
22 min
35 min

Figure 3.9 DP becomes more insoluble during PKP2 knockdown

SCC9 cells infected with retrovirus expressing PKP2shRNA or empty virus were lysed and fractionated into two pools: Triton x-100 soluble (S) and insoluble (I). Western blot probing fractions for endogenous DP, PKP2, keratin 18 (marker for insoluble pool) and α-tubulin (marker for soluble pool).

Figure 3.9

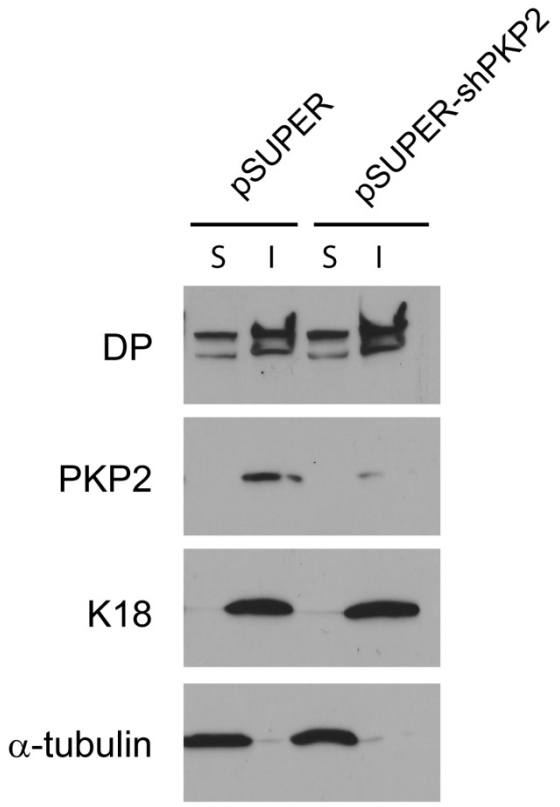


Figure 3.10 PKC but not PKA activity is required for proper DP border localization

PKC inhibition impairs DP border localization in SCC9 cells (A) and induces a filamentous pattern in A431 cells (B). Cells were treated with 12.5 μM BIM or DMSO for 30 min and immunostained for endogenous DP. Nicholas J. Garcia assisted with panel B image acquisition.

C. PKA inhibition does not affect DP border localization. SCC9 cells in low calcium treated with 10 μM H-89 or DMSO and switched to high calcium for 3h, immunostained for endogenous DP. Quantitative analysis of border fluorescence intensity confirmed that DP localization was comparable in H-89 treated cells. This experiment was performed by Sherry N. Hsieh.

Bar = 10μm

Figure 3.10

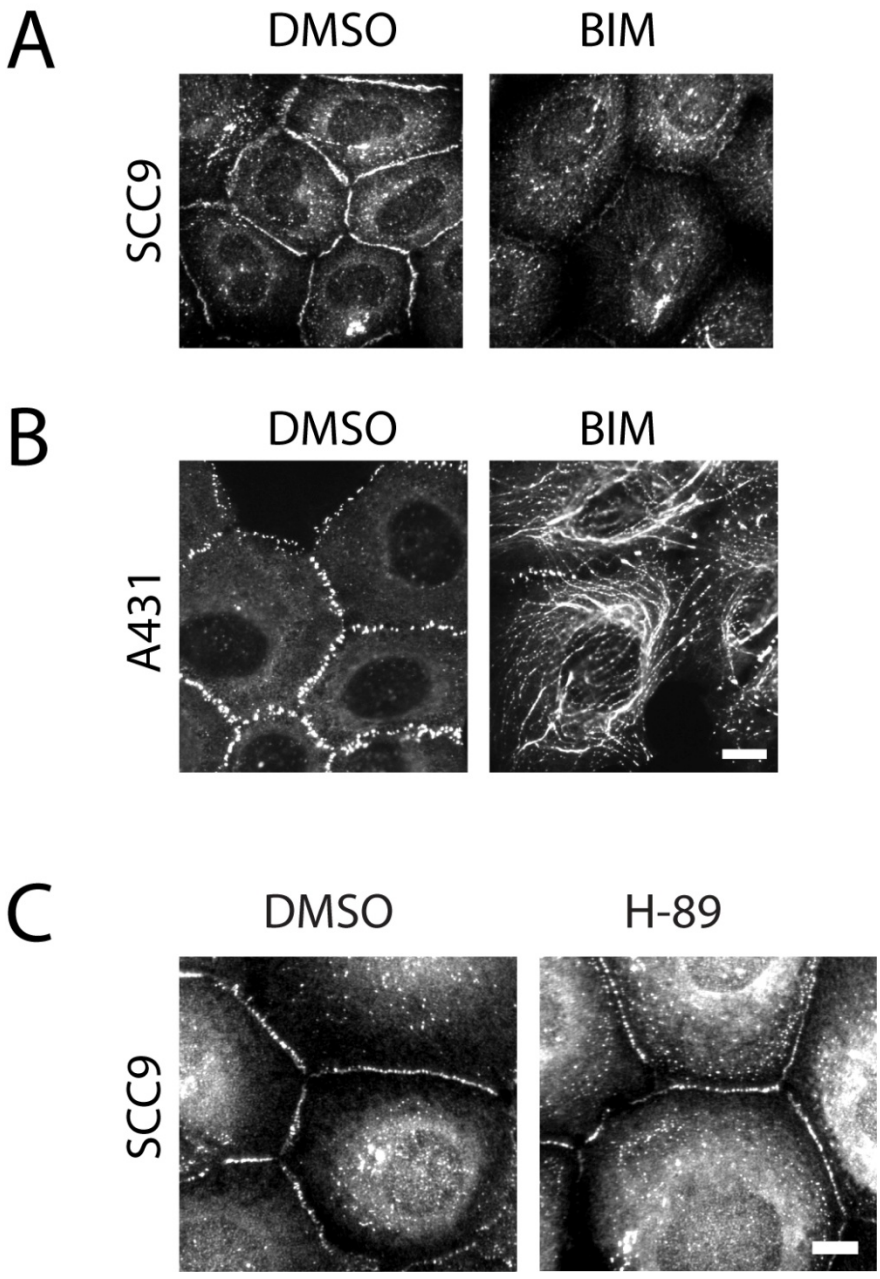


Figure 3.11 Mutation of Ser2849 results in IF alignment.

A. Fixed A431 cells expressing DP-GFP (left panel) or DP^{S2849G}-GFP (right panel) for comparison with Figure 2.11B.

Ba'. Selected stills from Movie 4 of A431 DP-GFP cells treated with 12.5 μM BIM. Drug was added at beginning of movie and maintained throughout the 90 min time course. Bb'. Stills from Movie 5 of untreated A431 DP^{S2849G}.GFP. Arrows depict areas where new cell-cell contacts occur. Note variability in DP^{S2849G}-GFP pattern ranging from particulate to continuous filaments (compare with Figure 2.11A).

C. High magnification of areas of cell-cell borders between SCC9 cells stably expressing DP-GFP (left panel) or DP^{S2849G}-GFP (right panel) and immunostained for endogenous PKP2.

Bar = 10μm

Figure 3.11

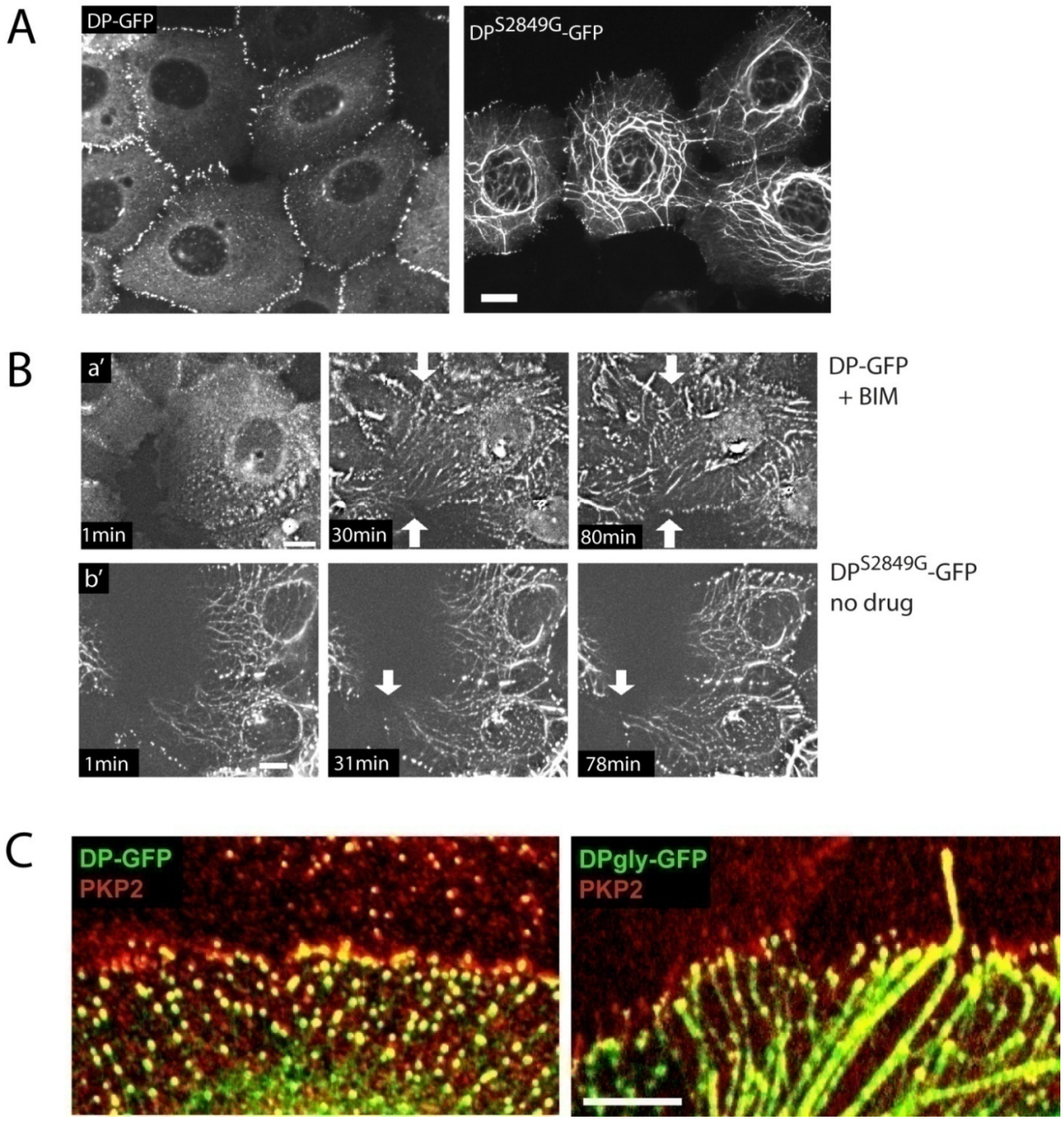

A
DP-GFP
DP^{S2849G}-GFP
B
a′
1min
30min
80min
DP-GFP
+ BIM
b′
1min
31min
78min
DP^{S2849G}-GFP
no drug
C
DP-GFP
PKP2
DPgly-GFP
PKP2

Figure 3.12 Impaired DP border localization during PKCα knockdown

A. Calcium switch of SCC9 cells infected with PKCα shRNA retrovirus or empty virus immunostained for DP. Bar = 10μm.

B. DP border fluorescence was measured, background was subtracted and values plotted against calcium switch time point.

C. Immunoblot analysis of PKC deficient cells for DP, PKP2, DSG2, PKCα or GAPDH loading control.

This experiment was performed by Ryan P. Hobbs.

Figure 3.12

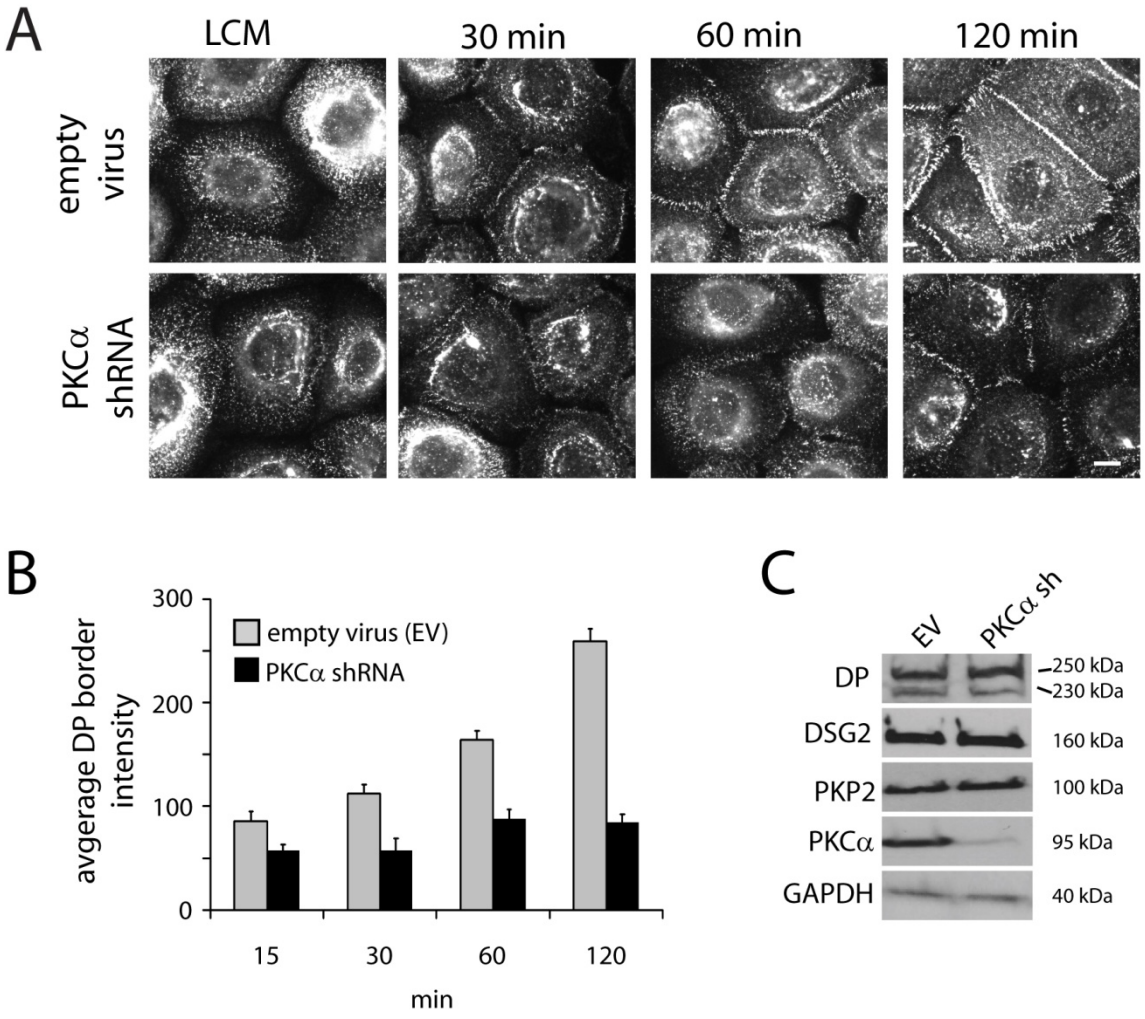

A
LCM
30 min
60 min
120 min
empty virus
PKCα shRNA
B
avgerage DP border intensity
300
200
100
0
empty virus (EV)
PKCα shRNA
15
30
60
120
min
C
EV
PKCα sh
DP
250 kDa
230 kDa
DSG2
160 kDa
PKP2
100 kDa
PKCα
95 kDa
GAPDH
40 kDa

Figure 3.13 Enhanced PKC substrate phosphorylation during PKP2 knockdown.

SCC9 cell lysates from NT or PKP2 siRNA transfected cells or cells treated with DMSO, 15 nM PMA or 12.5 μM BIM for 30 min were probed for PKC substrates phospho-MARCKS, -adducin, total MARCKS, total adducin, PKP2 or α-tubulin (loading control). Densitometry numbers below blots represent ratios of phospho-protein normalized to total protein from treatment samples relative to NT siRNA sample.

Figure 3.13

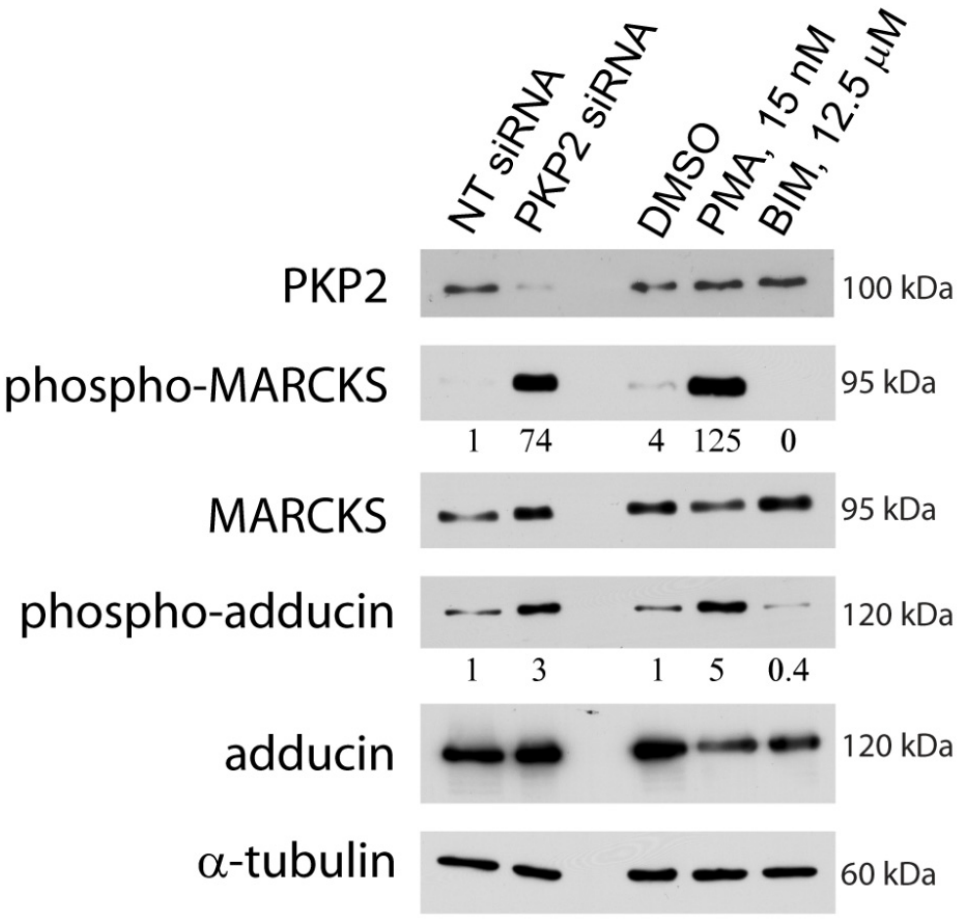

NT siRNA
PKP2 siRNA
DMSO
PMA, 15 nM
BIM, 12.5 μM
PKP2
100 kDa
phospho-MARCKS
95 kDa
1 74 4 125 0
MARCKS
95 kDa
phospho-adducin
120 kDa
1 3 1 5 0.4
adducin
120 kDa
α-tubulin
60 kDa

Fig 3.14 PKP2 knockdown leads to enhanced MARCKS phosphorylation throughout calcium switch

Lysates from SCC9 cells expressing PKP2 siRNA or control siRNA during calcium switch, PMA (TPA)- or DMSO-treated cells were probed with phospho-MARCKS or α-tubulin (loading control) antibodies.

Figure 3.14

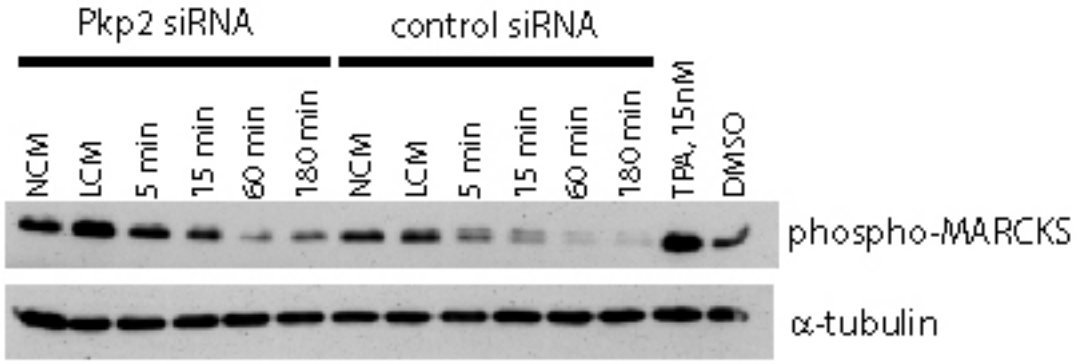

Pkp2 siRNA
control siRNA
NCM
LCM
5 min
15 min
60 min
180 min
NCM
LCM
5 min
15 min
60 min
180 min
TPA, 15nM
DMSO
phospho-MARCKS
α-tubulin

Figure 3.15 PKP2-Head but not -Arm co-immunoprecipitates with PKCα

A. Schematic of Myc-tagged PKP2 constructs used. PKP2-H is the N-terminus fragment of PKP2; PKP2-A is the terminus containing central arm-repeats and the carboxy-tail.

B. FLAG-tagged PKCα and myc-tagged PKP2-Head or –Arm were co-transfected into HEK293 cells and subjected to anti-FLAG immunoprecipitation (IP). Blots were probed with anti-myc or anti-FLAG antibodies. 5% input blots represent 5% of triton lysate from which IP was performed, removed prior to IP for immunoblot analysis.

Figure 3.15

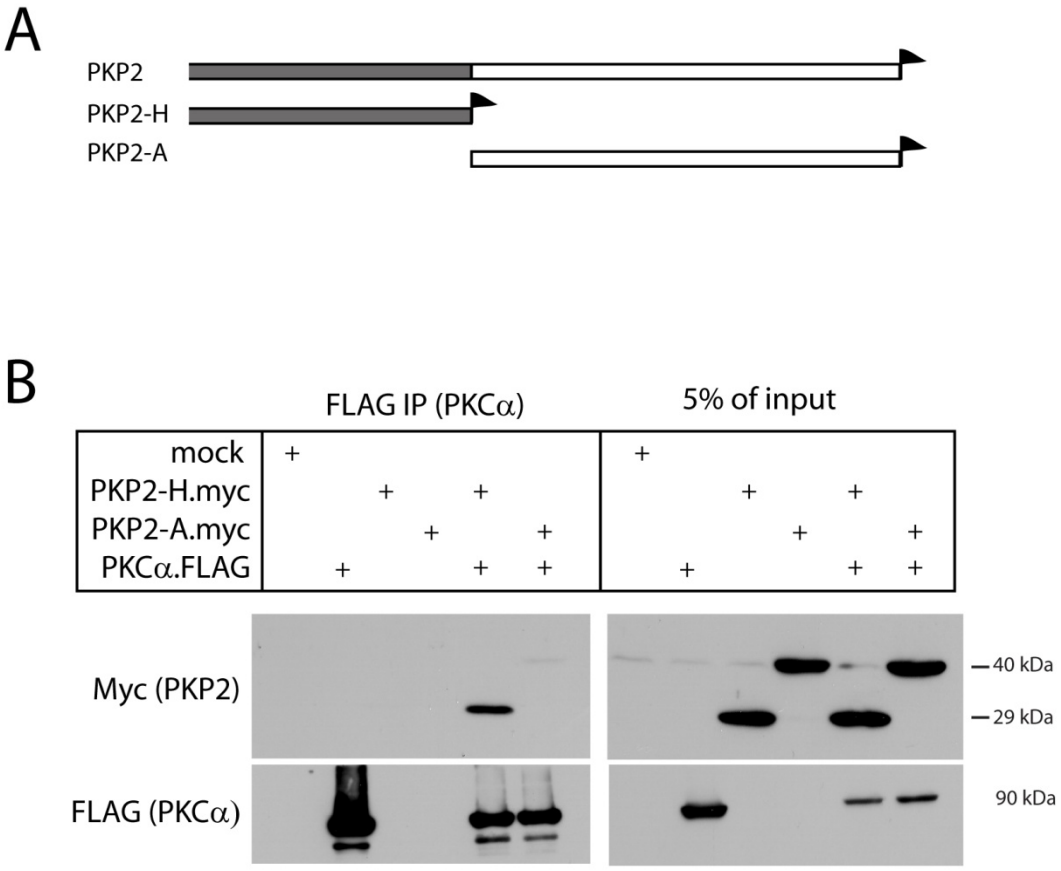

A
PKP2
PKP2-H
PKP2-A
B
FLAG IP (PKCα)
5% of input
mock
PKP2-H.myc
PKP2-A.myc
PKCα.FLAG
Myc (PKP2)
FLAG (PKCα)
40 kDa
29 kDa
90 kDa

Figure 3.16 PKP2 is required for DP-PKC association

A. PKCα coIPs endogenous DP in HEK293 cells. FLAG tagged wild type PKCα, or myristylated (constitutively active) PKCα were transfected into HEK293 cells and subjected to FLAG IP. Precipitates and lysates were probed for endogenous DP or FLAG. Data is representative of 3 independent experiments.

B. PKP2 is required for PKC-DP interaction. PKCα-FLAG was IP'd from HEK293 cells transfected with PKP2 or NT siRNA. Endogenous DP, PKP2 and FLAG were probed. Data is representative of 3 independent experiments.

C. Densitometry of panel B reveals 95% reduction in DP coIP'd with PKCα in PKP2-deficient cells.

Figure 3.16

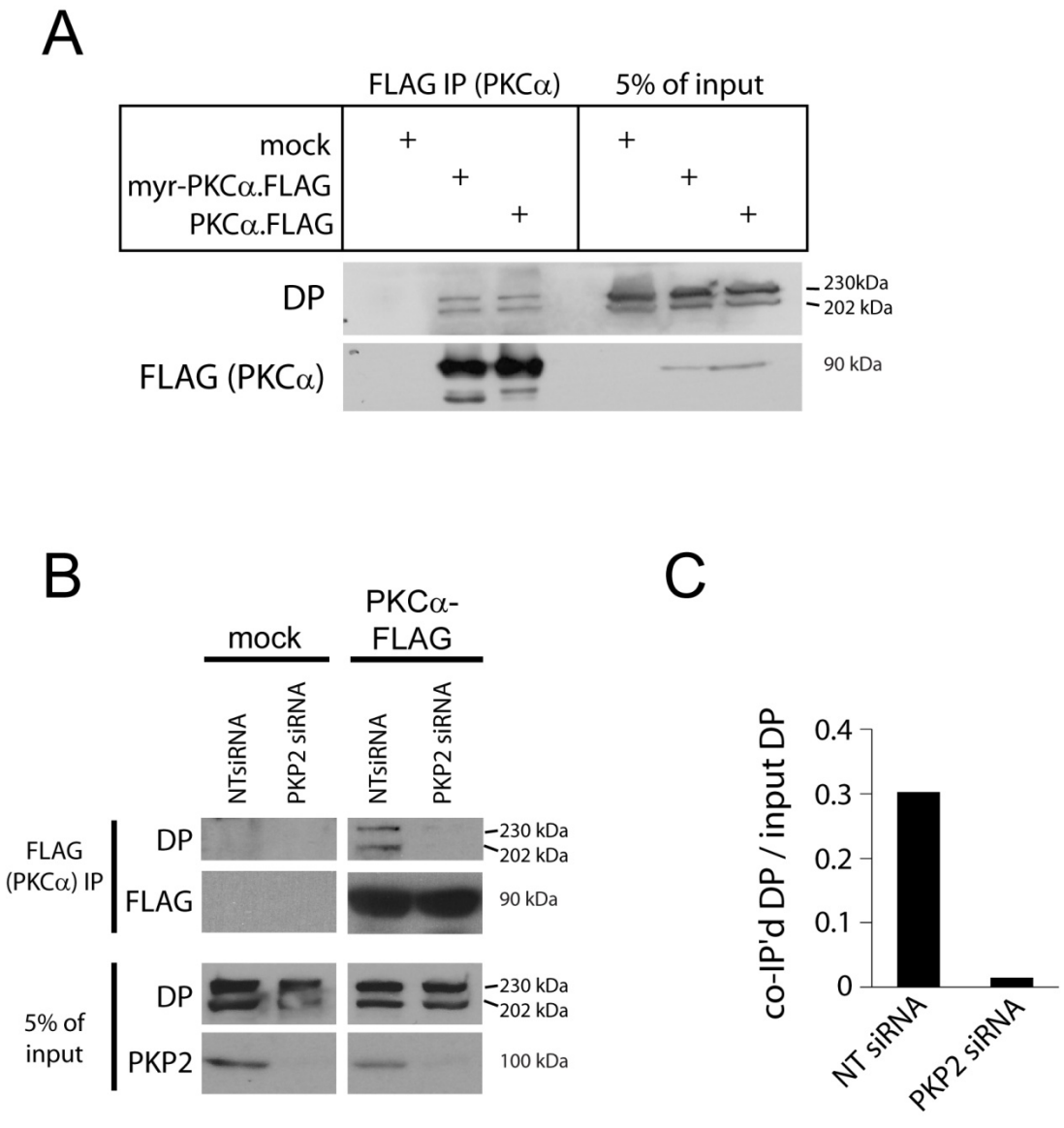
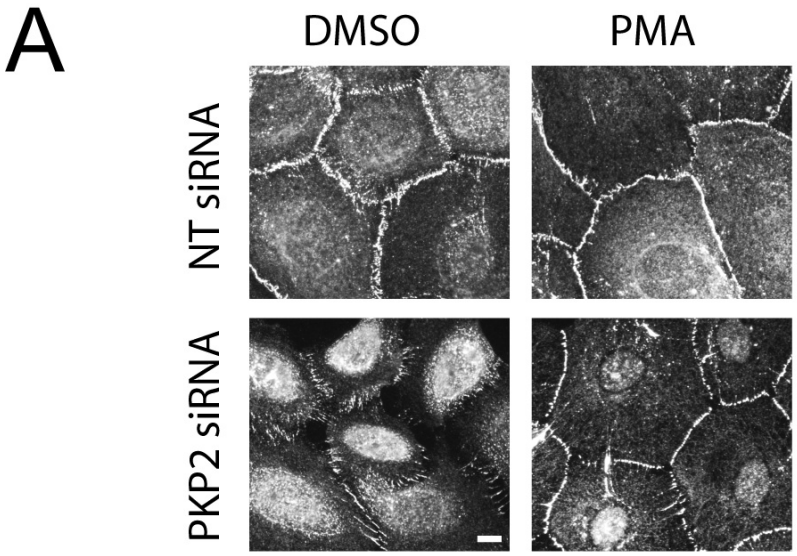

A
FLAG IP (PKCα)
5% of input
mock
myr-PKCα.FLAG
PKCα.FLAG
DP
FLAG (PKCα)
230kDa
202 kDa
90 kDa
B
mock
PKCα-FLAG
NTsiRNA
PKP2 siRNA
NTsiRNA
PKP2 siRNA
FLAG (PKCα) IP
DP
FLAG
230 kDa
202 kDa
90 kDa
5% of input
DP
PKP2
230 kDa
202 kDa
100 kDa
C
co-IP'd DP / input DP
0.4
0.3
0.2
0.1
0
NT siRNA
PKP2 siRNA

Figure 3.17 PKC activation rescues DP border localization during PKP2 knockdown.

A. PKP2 siRNA- or NT siRNA-cells were treated with DMSO or 15nM PMA for 30 min and stained for DP. Bar = 10 μm.

B. DP border fluorescence quantitation of data in panel A. * and ** $p<0.001$.s

Figure 3.17


A
DMSO
PMA
NT siRNA
PKP2 siRNA

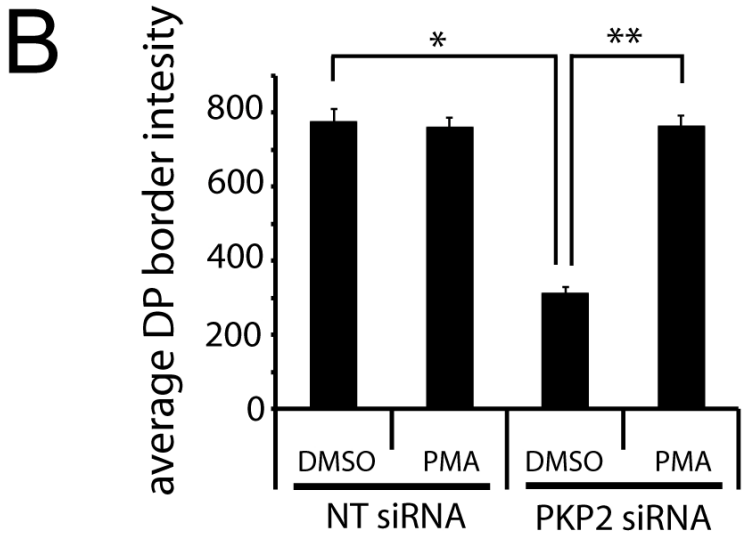

B
average DP border intesity
800
600
400
200
0
*
**
DMSO
PMA
DMSO
PMA
NT siRNA
PKP2 siRNA

Figure 3.18 PKC activation is unable to drive DP^{S2849G}–GFP off of filaments but increases DP^{S2849G}-GFP border accumulation

A. A431 cells inducibly expressing DP^{S2849G}-GFP were treated with DMSO, 12.5μM BIM (not shown) or 15nM PMA for 30'.

B. DP^{S2849G}-GFP border fluorescence was quantitated and plotted for each condition. PMA treatment leads to a 25% enhancement of DP^{S2849G} at borders but cannot drive DP^{S2849G} off of IF. BIM impairs DP^{S2849G} border accumulation by 22%.

Image acquisition and measurement were done with help from Nicholas J. Garcia.

Figure 3.18

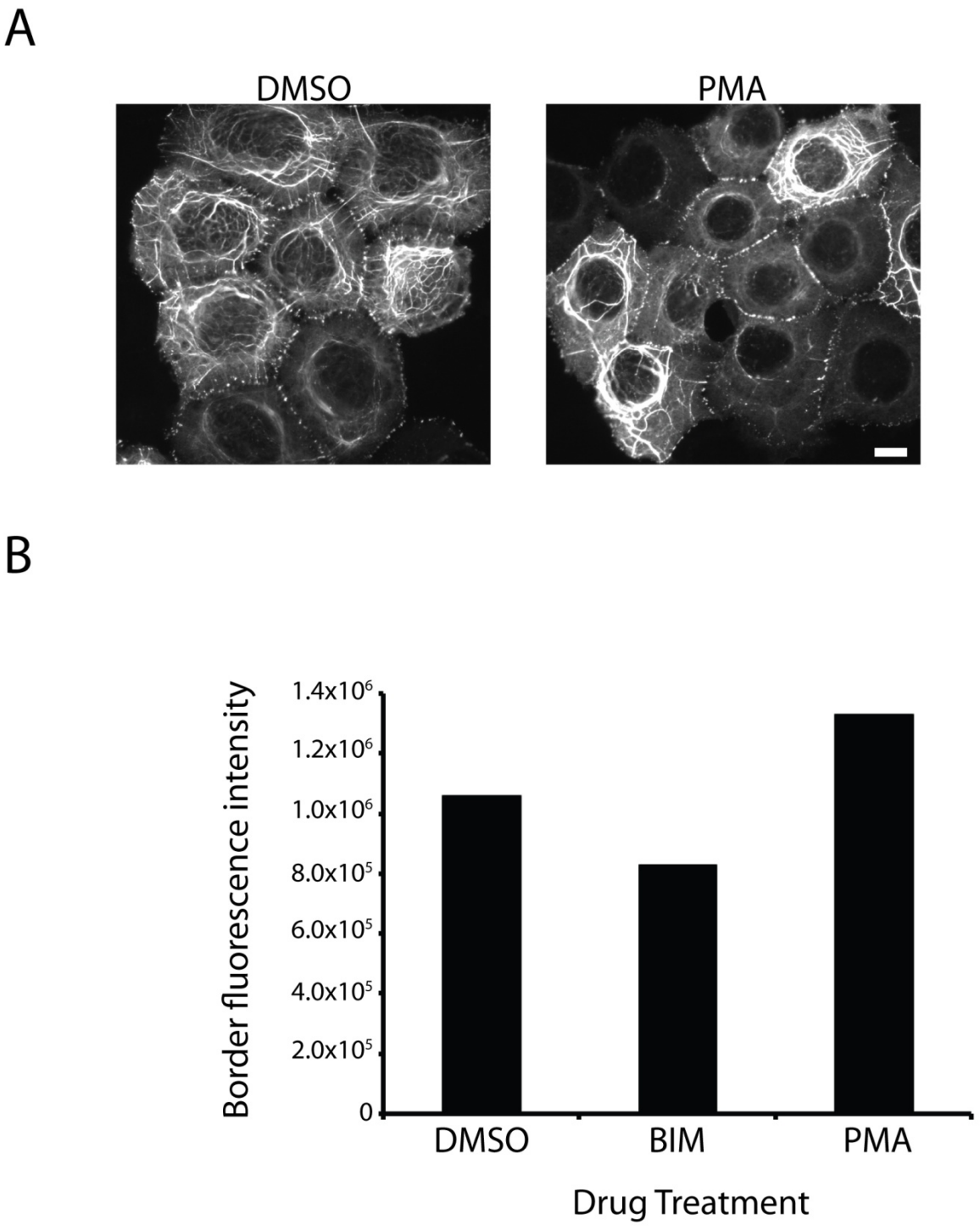

A
DMSO
PMA
B
Border fluorescence intensity
1.4x10^6
1.2x10^6
1.0x10^6
8.0x10^5
6.0x10^5
4.0x10^5
2.0x10^5
0
DMSO
BIM
PMA
Drug Treatment

Figure 3.19 PKC expression rescues DP border localization during PKP2 knockdown

A. SCC9 cells transfected with PKP2 or NT siRNA were infected with wild type PKCα or constitutive active PKCα retrovirus and stained for PKP2 and DP. Overexpression of PKCα rescues DP border localization.

B. DP localization at cell-cell borders was assessed for robustness by taking into account percent occupied border, border continuity and fluorescence intensity. Borders were scored on a graded scale of 1-5 and plotted as percentages of total borders counted. Representative borders scored from 1 to 5: 5 represents most mature border with most continuous and intense DP fluorescence; 1 represents least mature border with minimal to no DP fluorescence.

C. Graphs represent average scored values from three independent experiments as described in B. NT and PKP2 siRNA experiments were performed at same time but separated in graph for clarity.

Figure 3.19

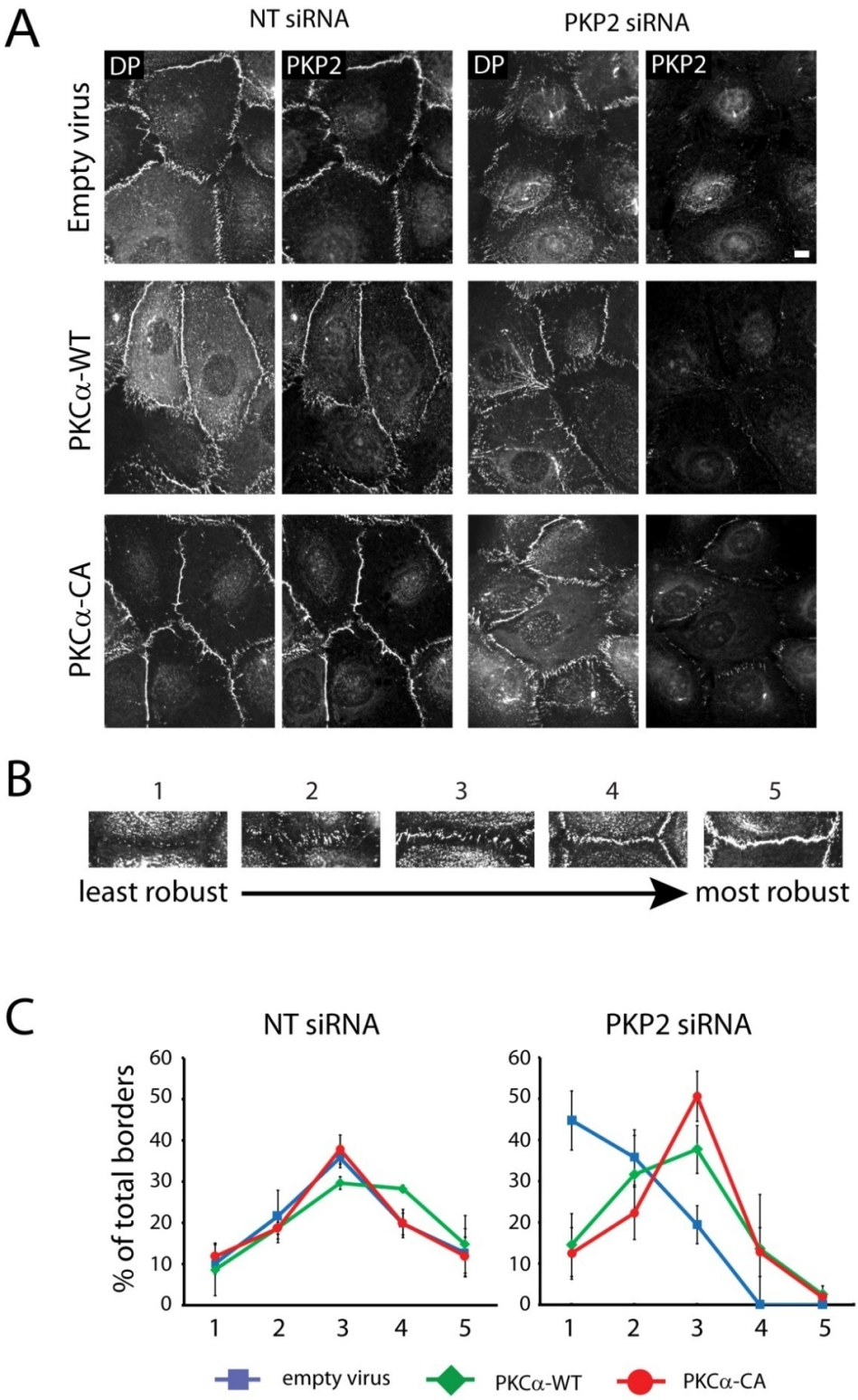

A
NT siRNA
PKP2 siRNA
DP
PKP2
DP
PKP2
Empty virus
PKCα-WT
PKCα-CA
B
1
2
3
4
5
least robust
most robust
C
NT siRNA
PKP2 siRNA
% of total borders
60
50
40
30
20
10
0
1
2
3
4
5
empty virus
PKCα-WT
PKCα-CA

Figure 3.20 PKP2-deficient cells exhibit reduced keratin phosphorylation at cell-cell borders SCC9 cells transfected with siRNA against PKP2 or NT control, endogenous PKP2 or pSer33 Ker18 staining. Knockdown of PKP2 leads to complete loss of phospho-keratin staining at borders and increased nuclear phospho-keratin staining.

Figure 3.20

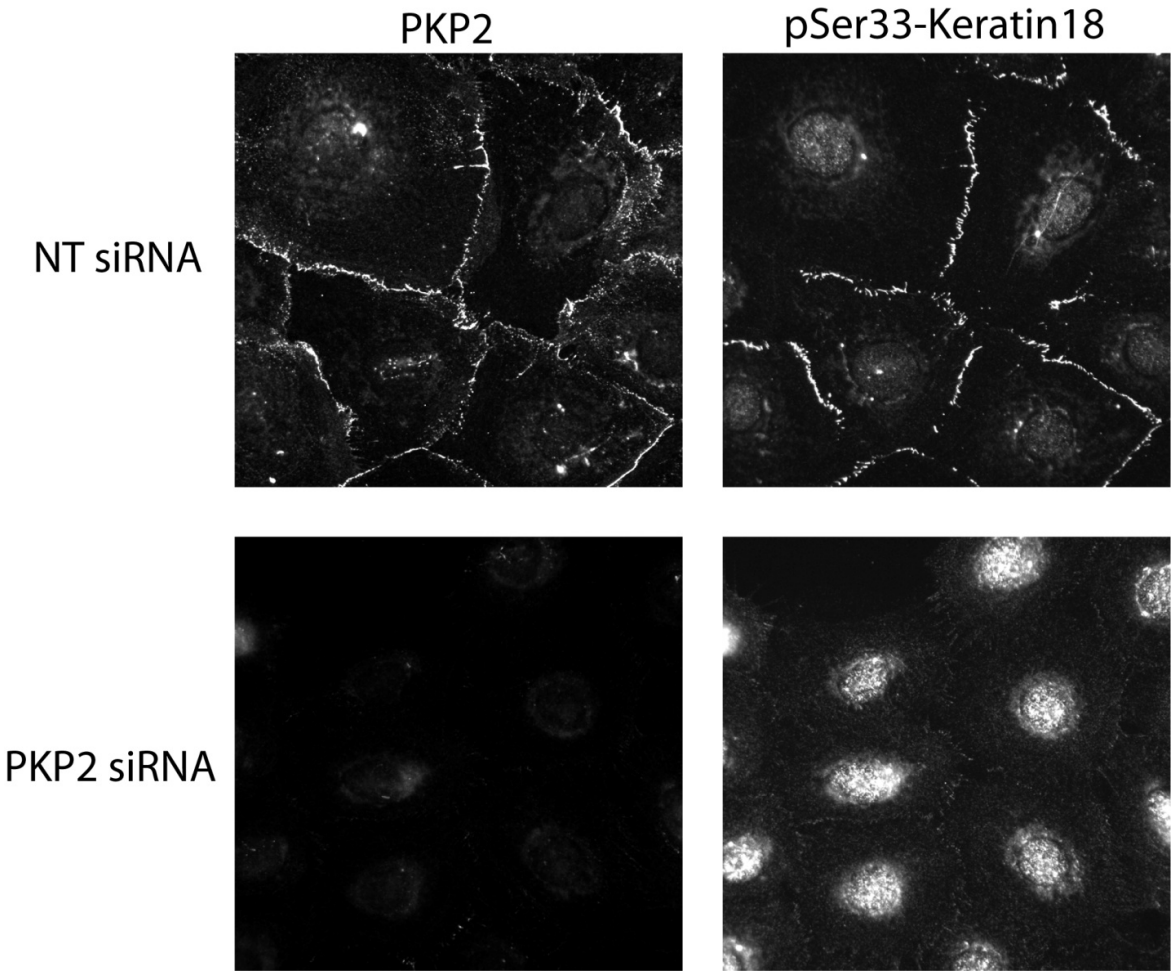


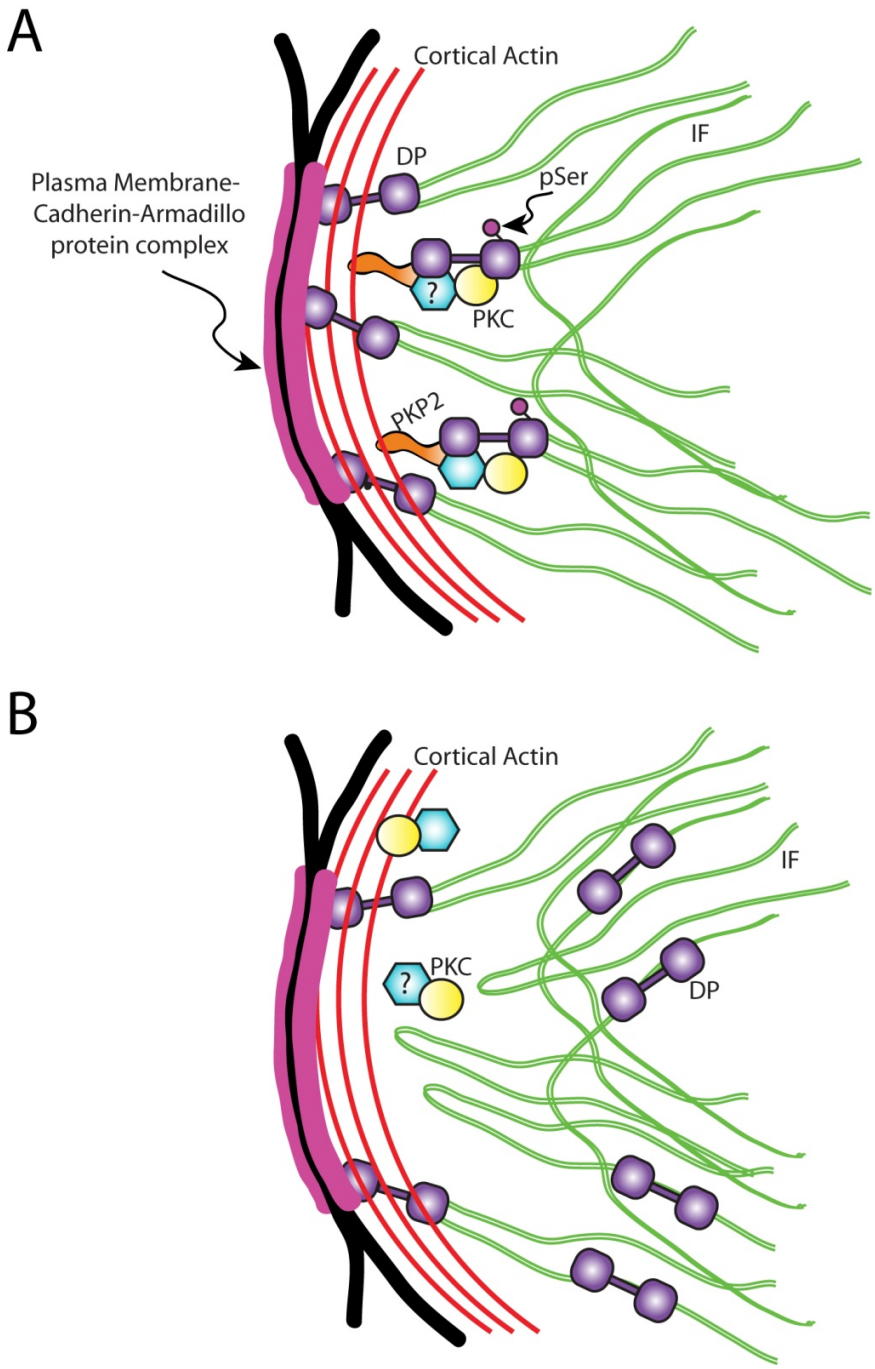
Figure 3.21 Model of role of PKP2 in PKC-regulated DP assembly.

A. Wildtype; PKP2 recruits PKC to DP cytoplasmic complexes where phosphorylation of DP at Ser2849 modulates its interaction with IF and allows DP assembly into cell-cell junctions.

B. PKP2 deficiency; PKC is no longer recruited to DP complexes and is free to phosphorylate other substrates, leading to aberrant accumulation of DP along IF and impaired DP assembly.

Nicholas J. Garcia assisted with this figure.

Figure 3.21


A
Cortical Actin
IF
DP
pSer
Plasma Membrane-
Cadherin-Armadillo
protein complex
?
PKC
PKP2
B
Cortical Actin
IF
PKC
?
DP

CHAPTER IV

Desmosome Assembly and Maturation Requires PKP2-Mediated Regulation of Myosin II-Dependent Actin Reorganization

Introduction

The desmosome and related adhesive complex, the adherens junction, undergo regulated dynamic disassembly and assembly during wound healing and morphogenesis, processes in which sheets of cells are reorganized. Interactions with their cytoskeletal network components (keratin intermediate filaments and actin microfilaments, respectively) are critical for proper assembly of these complexes into functional mature junctions (Adams et al., 1996; Green and Simpson, 2007; Huen et al., 2002; Vasioukhin et al., 2001a). The organization of the actin cytoskeleton and keratin network are closely interconnected (Green et al., 1987; Weber and Bement, 2002) and keratin dynamic properties depend on crosstalk with the actin cytoskeleton (Woll et al., 2005). IF attachment is essential for desmosome formation (Bornslaeger et al., 1996)), however, this close association of IF and actin strongly suggests that both cytoskeletal networks are involved in the regulation of desmosome assembly. Whether the actin cytoskeleton is in fact directing desmosome assembly and what the contribution of signaling pathways involved in these specific events are questions we address in this report.

Desmosomes are highly specialized intercellular structures essential for mediating cell-cell adhesion. They are prominent complexes that anchor the cytoskeleton to cell membranes at sites of cell contact, a function that reinforces their adhesive strength by providing an interconnected network of cells throughout which mechanical tension is distributed. Desmosomes consist of three major protein families whose primary functions are to provide cell-cell adhesion and to anchor the intermediate filament cytoskeleton to these

junctional sites of adhesion. The cadherin family proteins (desmogleins [DSGs] and desmocollins [DSCs]) span the membranes and bind through homo and heterodimeric interactions to cadherins on neighboring cells in a calcium dependent manner. The desmosomal cytoplasmic plaque consists of the armadillo-family proteins (plakoglobin [PG] and plakophilins [PKPs]), and the intermediate filament (IF)-binding plakins (desmoplakin [DP]). The armadillo proteins are thought primarily to serve as critical linkers between the cadherin cytoplasmic tail and desmoplakin and are important for the proper formation of desmosomes (reviewed in (Godsel et al., 2004)).

The desmosomal plaque and cadherin components are assembled in separate subcellular compartments and exhibit distinct assembly kinetics (Pasdar et al., 1991; Pasdar and Nelson, 1988a; Pasdar and Nelson, 1988b; Pasdar and Nelson, 1989). We previously showed that desmoplakin-containing plaque precursors coalesce in the cytoplasm in response to cell-cell contact, are associated with intermediate filaments and plakophilin 2, but exclude membrane components such as desmosomal cadherins and other desmosomal armadillo proteins. These new precursors then translocate to sites of cell-cell contact in an actin dependent manner (approximately 10 fold slower than conventional microtubule (MT) based motility) where they are incorporated into the nascent junction (Godsel et al., 2005). Earlier studies on desmosome assembly suggest that IF-associated DP-containing plaque precursors closely align with radially-oriented microfilaments during calcium switch-induced junction assembly (Green et al., 1987). These observations suggest a potential role for actin in desmosome plaque assembly, but the specific mechanism by which this actin-dependent assembly occurs is unclear.

Plakophilin 2 is a member of the p120^{ctn}-related subset of armadillo proteins. In addition to their adhesive functions within junctions, armadillo family proteins have the ability to modulate a number of signaling pathways that can lead to phosphorylation of junctional proteins and regulate desmosome assembly and disassembly (Bass-Zubek et al., 2008; Chen et al., 2002; Cowell and Garrod, 1999; Dusek et al., 2007a; Garcia-Gras et al., 2006; Hatzfeld, 2007; Keil et al., 2007; Lorch et al., 2004; Miravet et al., 2003; Miravet et al., 2002; Wallis et al., 2000; Yin et al., 2005a; Yin et al., 2005b). PKP-related proteins p120^{ctn} and p0071 have been shown to affect actin organization via the Rho small GTPases (reviewed in (Hatzfeld, 2005))(Wolf et al., 2006). Since PKP2 is essential for proper DP assembly, we sought to determine whether PKP2 directs desmosome plaque assembly via regulation of actin reorganization signaling pathways.

In this chapter, using siRNA mediated knockdown of PKP2 in conjunction with fixed imaging studies of calcium-induced desmosome assembly, I demonstrate that DP plaque precursor assembly requires myosin II activity and that PKP2 directs DP precursor junction assembly by regulating actomyosin contractility and modulation of both RhoA activity and subcellular localization. These data further support a model in which PKP2 plays an important role as an adaptor protein for specific actomyosin contractile signaling molecules that regulate their activity and subcellular localization, ultimately directing cytoskeletal remodeling events essential for desmosome plaque assembly. These studies implicate dysregulation of actin reorganization and contractility as potential contributing factors in the pathogenesis of PKP2-deficient disease states.

Results

DP precursor assembly coordinates with dynamic actin reorganization

The circumferential actin network undergoes reorganization when two cells come into contact (Krendel and Bonder, 1999; Vaezi et al., 2002; Vasioukhin et al., 2000; Zhang et al., 2005). Reorganization of actin from a diffuse circumferential network to an outwardly expanding ring is essential for proper membrane closure and for adherens junction formation and E-cadherin clustering (Maddugoda et al., 2007; Scott et al., 2006; Tamada et al., 2007; Vaezi et al., 2002; Vasioukhin et al., 2000). We previously observed that polymerized actin is required for proper desmosome precursor translocation during contact-dependent junction assembly (Godsel et al., 2005). However, the mechanistic basis underlying actin reorganization and desmosome behavior assembly is not well characterized.

To begin to demonstrate how desmosome plaque precursor assembly and actin reorganization are functionally linked, a calcium switch was performed to analyze actin and DP remodeling during junction assembly (Fig 4.1A). As previously described (Green et al., 1987; Vaezi et al., 2002), radially-oriented, thick actin bundles form in the absence of cell-cell contact (i.e. when cultured in low calcium containing medium (LCM)). These radial fibers have been characterized as “dorsal stress fibers”, which insert into vinculin-containing focal contacts at the cell periphery and then connect to a concentric network of bundled actin rings (Hotulainen and Lappalainen, 2006). The radial actin fibers (red lines in Figure 4.1C) persisted after calcium-induction of cell-cell contact (Figure 4.1A, 15min) and were broken down between 30-60min (Figure 1A). Concomitantly, the series of concentric

circumferential actin rings (blue rings in Figure 4.1C) became more closely positioned to one another and to the membrane as the junctional contact matured over time in high calcium (Figure 4.1A, 3h), a process thought to be due to actomyosin contractile activity (Tamada et al., 2007; Zhang et al., 2005). Throughout early (0-15min) and later stages (30min-3h) of assembly, actin fluorescent puncta were also found directly at sites of cell-cell contact (termed junctional actin (Zhang et al., 2005)), which indicate sites of cadherin-mediated cell-cell contact (Adams et al., 1998; McNeill et al., 1993).

In the early stage of desmosome assembly (15min in NCM), DP began to accumulate at cell-cell contacts and numerous cytoplasmic DP-containing particles appeared within the cortical region (Figure 4.1B, 15'). Later (3h), DP accumulation at borders was much more robust and there was a clearing of cortical cytoplasmic particles, presumably due to translocation of the cytoplasmic particles towards the membrane where they have been shown participate in nascent junction maturation (Figure 4.1B, 3h and (Godsel et al., 2005)). Live imaging of DP-GFP and mCherry-actin expressing cells undergoing wound healing is currently in progress to better characterize the co-dynamics of these proteins with high 4-dimensional resolution. Confocal analysis of DP, keratin and actin revealed DP cytoplasmic particles (that aligned with keratin IF) interspersed within the network of actin rings (Figure 4.2). Based on the timing of the cortical DP clearing and previous DP translocation studies (Godsel et al., 2005), we hypothesized that reorganization of the actin cytoskeleton could provide the means necessary for DP particle incorporation into nascent junctions.

Actomyosin contraction is required for DP assembly

Actomyosin contractility is essential for the formation and reorganization of the actin ring during cell-cell contact initiation (Hotulainen and Lappalainen, 2006; Shewan et al., 2005; Zhang et al., 2005) and for wound closure (Tamada et al., 2007). The observations that DP particles closely associated with radial actin bundles (Green et al., 1987) and the concentric actin network (Figure 4.2) as well as the relatively slow velocity at which these DP precursors have been shown to translocate (Godsel et al., 2005) all suggest that actin contraction could be responsible for the assembly of these particles. Indeed, particle speeds were observed to be approximately 10-fold slower than most conventional microtubule-based motors (Godsel et al., 2005). During cell contact formation, the concentric array of actin bundles undergoes myosin-dependent packing towards the cell periphery (Zhang et al., 2005). One possibility for the mechanism of DP plaque precursor translocation could be through passive flow during this actin reorganization. Although radial actin fibers at cell-cell contacts (Kametani and Takeichi, 2006) and concentric actin rings generally exhibit rearward flow in single motile cells, this rearward concentric actin flow halts upon cell-cell contact induction (Krendel and Bonder, 1999).

Myosin II contractility is known to be important for the formation and stabilization of the adherens junction complex in epithelia (Miyake et al., 2006; Shewan et al., 2005) and myoblast cultures (Lambert et al., 2007). Initial accumulation of E-cadherin at sites of cell-cell contact is followed by and required for stable actin accumulation (McNeill et al., 1993; Vaezi et al., 2002). Adherens junction assembly is thought to precede desmosome assembly (O'Keefe et al., 1987). There is an interdependence between the two structures for their

stabilization, which is particularly important for the integrity of the epidermis (Vasioukhin et al., 2001a; Vasioukhin et al., 2001b). These two junctions are often found next to one another and closely associated (Green et al., 1987), which suggests that perhaps their assemblies are at least temporally and spatially linked.

To begin to characterize whether actomyosin contraction-mediated actin reorganization plays a role in desmosome assembly, SCC9 cells undergoing calcium switch-induced junction assembly were treated with blebbistatin, a specific inhibitor of myosin II ATPase activity (Limouze et al., 2004). The effect of myosin inhibition on desmosome and adherens junction components was examined. Whereas no significant impairment of E-cadherin border fluorescence accumulation was observed, a 75% reduction in DP border fluorescence was observed when cells were treated with 50μM blebbistatin for 15min (Figure 4.3A,B, L. Godsel) and DP impairment was observed even in low concentrations of blebbistatin (10μM) (data not shown). Although its total fluorescence intensity was not significantly reduced, E-cadherin border fluorescence morphology appeared less linear than in the DMSO treated cells, suggesting that adherens junction maturation may be impaired by myosin inhibition in these cells. While other groups have observed that myosin inhibition blocks E-cadherin membrane assembly (Shewan et al., 2005), the concentration that was required to achieve a negative effect on E-cadherin border localization in that report was 2-10 times higher than was used to disrupt DP in the experiments reported here.

Myosin inhibition impaired DP assembly at all time points during a calcium switch (Figure 4.3C, L. Godsel) but did not impair E-cad border accumulation (data not shown), suggesting that actomyosin contraction is important for both DP particle junctional

translocation and maintenance and that its role in the assembly of adherens junctions and desmosomes may be occurring via spatially or temporally separate actomyosin contraction events. These results are consistent with the idea that the assembly of the desmosomal plaque component is more sensitive to changes in myosin activity than adherens junctions, and myosin-II mediated actin contractility is required for the efficient assembly of desmosomes independent of E-cadherin-membrane accumulation.

PKP2 modulates actin organization during cell-cell junction formation

Since there is a disparate effect of myosin inhibition on DP assembly versus E-cadherin assembly in this system, we hypothesized that a desmosome-specific protein may be involved in regulating actin organization during assembly. We previously demonstrated that PKP2 is a major partner for DP in plaque precursors and is essential for proper desmoplakin assembly (Bass-Zubek et al., 2008; Godsel et al., 2005). PKPs contain a central armadillo repeat domain that shares approximately 50% homology to that of $p120^{ctn}$ (Hatzfeld, 2007), an essential armadillo component of the adherens junction. $p120^{ctn}$ can modulate actin organization and induce a dendritic or branching cell morphology when ectopically expressed in fibroblasts (Grosheva et al., 2001; Noren et al., 2000; Reynolds et al., 1996). We hypothesized that if PKP2, actin, and myosin activity are required for proper DP assembly, PKP2 could be modulating actin organization by regulating signaling pathways similar to $p120^{ctn}$. These potential signaling pathways eventually lead to myosin activation, resulting in actin reorganization.

To determine if PKP2 regulates actin organization, SCC9 cells were transfected with siRNA against PKP2 or non-targeting control siRNA and a calcium switch was performed. Cells deficient in PKP2 contained more radially-oriented actin bundles (similar to control cells at 15 min, see Figure 4.1), which persisted in later stages of assembly and were even present in cells cultured at steady state calcium levels (which approximates a long term assembly time point). The circumferential actin bundles exhibited more fluorescence intensity throughout the assembly time course in cells deficient for PKP2 (Figure 4.4A). Quantification of the maximal fluorescence in the circumferential actin bundles in PKP2 siRNA versus control siRNA transfected cells resulted in a 30% increase in fluorescence intensity of the circumferential actin ring (Figure 4.4B). These actin bundles sometimes appeared to be pulled away from the cell-cell border in the PKP2 knockdown cells, suggesting an overall increase in actin contraction. Although, DP particles could still form in the cytoplasm after cell cell contact (live cell imaging not shown), the cytoplasmic particles appeared to be concentrated interior to the actin ring, as though they could not move through the thick bundle of actin fibers (Figure 4.4B).

Previous studies have shown that adherens junctions require association with the actin cytoskeleton for proper assembly and E-cadherin mediated cell-contact stabilization (Vasioukhin et al., 2000) but actin reorganization after cell-cell contact also can be stimulated by cadherin ligation (reviewed in (Mège et al., 2006)). To investigate whether PKP2-deficiency induced disruption of normal actin organization during cell contact formation impairs adherens junction assembly, cells transfected with PKP2 siRNA or control siRNA undergoing calcium switch were immunostained against E-cadherin and α-catenin.

Surprisingly, although actin organization was visibly altered when PKP2 is knocked down, assembly of E-cadherin (Figure 4.5) and α-catenin (data not shown) was minimally affected although the morphology of E-cadherin contacts did appear less mature and less linear than those in control cells. These results suggest that PKP2-deficiency leads to changes in actin organization consistent with an immature assembly state but these changes do not generally impair border accumulation of adherens junction proteins.

PKP2 regulates myosin II localization and activity

Cytoplasmic desmoplakin particles were observed to be enmeshed in the circumferential actin network (Figure 4.2), which is known to dramatically reorganize during cell-cell contact initiation and junction assembly (Adams et al., 1996; Krendel and Bonder, 1999; Vaezi et al., 2002; Vasioukhin et al., 2000; Zhang et al., 2005). DP assembly requires both intact actin (Godsel et al., 2005) and myosin activation (Figure 4.3). Since we observed that PKP2 is essential for proper desmoplakin assembly and that PKP2 can modulate actin reorganization, we hypothesized that PKP2 could be regulating actin dynamics via modulation of myosin activity.

Myosin phosphorylation at Ser19 (and Thr18) leads to activation of its ATPase and therefore actomyosin contraction (Bresnick et al., 1995; Kamisoyama et al., 1994). To test whether myosin activation was altered in PKP2-deficient cells during junction assembly, PKP2-deficient cells lysates or control lysates were probed with phospho-specific antibodies against myosin light chain (MLC). During calcium switch, myosin phosphorylation at its primary site (Ser19) increased over time in both control and PKP2-deficient cells. pMLC

levels (relative to GAPDH) were augmented in PKP2 knockdown cells compared with control cells (Figure 4.6A,B). To test whether myosin localization is altered during PKP2 knockdown, SCC9 cells were transfected with PKP2 siRNA and subjected to calcium switch and processed for indirect immunofluorescence using anti-phospho-MLC or anti-total myosin II antibodies. In control siRNA transfected cells, myosin expression exhibited a relatively diffuse pattern across the cytoplasm with a slight concentration in the cytoplasmic region that reflected circumferential actin bundle localization (Krendel and Bonder, 1999; Zhang et al., 2005). By 15 min in NCM, activated myosin (pSer19 MLC) formed straight bands in the submembrane region that were more abundant in PKP2-deficient cells (Figure 4.6C) and total myosin was enriched in the cortical region of PKP2 deficient cells (Figure 4.6D). These data suggest that PKP2 knockdown leads to enhanced actin contractility via localized myosin activation.

PKP2 overexpression induces branching cell morphology

To begin to characterize the mechanism by which PKP2 modulates actomyosin contraction, colocalization- and overexpression-studies were performed. PKP1 has been previously demonstrated to associate with actin indirectly (Hatzfeld et al., 2000) and the PKP2-head domain can also align along actin stress fibers when ectopically expressed in PtK2 cells (X. Chen, unpublished observations). Consistent with this, endogenous PKP2 colocalizes with junctional F-actin (and endogenous E-cadherin) at cell-cell junctions in SCC9 cells both undergoing assembly and at steady state (Figure 4.8). Full length PKP2 aligned with actin filaments when expressed in REF52 cells (fibroblast cell line) that do not

form desmosomes (Figure 4.9B). Previously, it was shown that PKP2 (but not PKP1 or PKP3) ectopic expression in Cos cells induced a branching cell morphology change, similar to that observed during $p120^{ctn}$ overexpression (unpublished data, Xinyu Chen). Cells overexpressing PKP2 generated numerous membrane protrusions, became polarized (in 2D) and exhibited cell body retraction ($p120^{ctn}$: data not shown; PKP2: Figure 4.9A, X. Chen).

In SCC9 cells transfected with PKP2, the actin cytoskeleton was completely abolished and actin filaments were rearranged into aggregates whereas neighboring untransfected cells exhibited normal actin morphology (Fig 4.8). These aster-like actin aggregates are likely due to contraction of the actin fibers that have been observed in cytochalasin-treated fibroblasts and are thought to be driven by myosin II-actin interactions (Verkhovsky et al., 1997). The degree to which the cells extended membrane protrusions appeared to depend on ectopic protein expression level and thus broad variability in morphology was observed in PKP2-transfected cells. Interestingly, PKP1 arm domain overexpression in a number of epithelial cell types induces a similar dendritic morphology (Hatzfeld et al., 2000), but full length PKP1 overexpression cannot mimic PKP2 or $p120^{ctn}$ expression (X. Chen unpublished observations), suggesting that PKP2 and PKP1 have differential functions or specificity in their ability to modulate actin organization processes.

These results suggest that PKP2 may interact with actin (directly or indirectly), and that PKP2 has the ability to functionally impact actin organization. Whether the interaction of PKP2 with actin is related to its ability to modulate actin organization is a question that remains to be determined. However, PKP2 could serve as a potential scaffold between plaque precursors and the actin cytoskeleton, a close association that has been described

previously in cells undergoing junction assembly (Green et al., 1987). Local actin contraction regulated by PKP2 may promote the translocation of these actin-associated precursors.

A conserved glycine in the PKP2 armadillo repeat domain is required for cell shape change, and suggests modulation of RhoA

As previously mentioned, the central armadillo domain of PKP2 is homologous to that of $p120^{ctn}$. Several groups have shown that $p120^{ctn}$ can inhibit RhoA signaling; however, multiple different mechanisms have been described: $p120^{ctn}$ can act as a Rho GTP dissociation inhibitor (GDI) and sequester Rho from certain GTP exchange factors (GEFs) (Anastasiadis et al., 2000); $p120^{ctn}$ can bind to RhoGEFs and activate Rac1 and Cdc42, which also inhibit Rho activation (Noren et al., 2000). $p120^{\Delta 622\text{-}628}$, which contains a deletion of 6 AA within the arm domain, loses its ability to induce the branching phenotype and abolishes its ability to bind to and inhibit the activity of RhoA small GTPase by blocking dissociation of the GDP from the GTPase (Anastasiadis et al., 2000). It was observed that when a PKP2 cDNA containing a random point mutation G629V was transfected into Cos cells, this mutation of PKP2 at Gly629 abrogated its ability to induce the morphology change whereas mutation of a more distal residue ($PKP2^{I722V}$) was not able to block the morphology change (Fig 4.9A, X. Chen). Although this G629V point mutation inhibited the PKP2 induced morphology change, it could still associate with actin in fibroblasts (Figure 4.9B).

Interestingly, Gly629 in PKP2 is conserved among all $p120^{ctn}$ family members. The homologous mutation of this residue to Ala or Val in ectopically expressed $mp120^{ctn}$ had the same inhibitory effect on the dendritic phenotype (data not shown, X. Chen). While this residue at position 629 is not within the Rho-interacting domain of $p120^{ctn}$, its proximity to this domain suggests that mutation of specific residues in the region may alter the conformation of the Rho binding domain. These results are consistent with the idea that this arm domain in PKP2 might also be involved in modulation of Rho GTPase signaling.

To test whether Rho activity is involved in mediating the PKP2-induced cell shape change, Cos cells were co-transfected with PKP2-FLAG and myc-RhoAV14 (constitutively active) or myc-RhoAN17 (dominant negative). Cells were stained for FLAG and myc and percentage of transfected cells was quantified. Expression of constitutively-active RhoA reduced the fraction of transfected cells undergoing morphology change by nearly 40% (Figure 4.10A). This suggests that RhoA signaling may be downstream of PKP2-induced actin re-organization and overall cell morphology change.

Interestingly, when SCC9 cells expressing PKP2- or control-siRNA were examined for RhoA activity during calcium switch, it was observed that in PKP2-deficient cells, RhoA activity was decreased over the course of the switch relative to control cells (data not shown), but an elevation in RhoA was observed in PKP2-deficient cells at steady state (Figure 4.10B). Taken together, these results suggest that RhoA signaling can be modulated by PKP2 and that this signaling leads to changes in actin organization. These results do not rule out the possibility that other Rho-family GTPases may be involved in this actin

reorganization. Rac and Cdc42 activity should be evaluated in PKP2-deficient cells in the future.

PKP2 regulates actomyosin contractility by inhibiting RhoA activity

A major pathway in RhoA signaling towards actin reorganization occurs through activation of Rho Kinase (ROCK), which activates myosin in several ways leading to actomyosin contractility and dynamic actin reorganization. ROCK can phosphorylate myosin directly (Amano et al., 1996) and can phosphorylate myosin phosphatase (MYPT). MYPT phosphorylation of Thr696 inactivates its phosphatase activity, which leads to enhanced myosin phosphorylation and therefore activation (Kimura et al., 1996). We demonstrated earlier that PKP2-deficiency leads to enhanced myosin activation and therefore asked whether MYPT phosphorylation was also upregulated in these cells. To address this question, cell lysates from control- or PKP2-siRNA transfected SCC9 cells were probed with phospho-specific antibodies against MYPT. Indeed, MYPT phosphorylation at was enhanced in PKP2 knockdown cells compared with control siRNA transfected cells (Figure 4.10C), consistent with enhanced myosin phosphorylation (Figure 4.10D).

RhoA-ROCK signaling also can activate other downstream effectors that are important in modulating actin organization. Ezrin/Radixin/Moesin (ERM) proteins, which tether actin to the plasma membrane (Niggli and Rossy, 2008), are phosphorylated by ROCK (Matsui et al., 1998). LIM kinase is another ROCK target, which phosphorylates and inactivates cofilin, an actin severing protein that is involved in actin filament formation (reviewed in (Huang et al., 2006)). To test whether PKP2-deficiency leads to the alteration

in these Rho-ROCK targets, phospho-specific antibodies were used to probe lysates from PKP2 siRNA or control siRNA transfected cells. In cells expressing PKP2siRNA, both ERM and cofilin phosphorylation levels were unchanged compared with control cells (Figure 4.10D, data not shown). These data demonstrate that PKP2-deficiency leads to increased myosin activity, but not increased phosphorylation of ERM or cofilin proteins, even though all are downstream targets of Rho-ROCK signaling. This differential phospho-regulation could be explained several ways including but not limited to the following: 1) PKP2 could regulate the activity or specificity of other signaling proteins that can phosphorylate myosin, or 2) PKP2-dependent regulation of Rho is due to its sequestration in a specific subcellular localization leading to differential effects on its downstream targets.

RhoA membrane localization is delayed in PKP2-deficient cells

Previously we demonstrated that PKP2 acts as a scaffold that recruits PKCα to DP (Bass-Zubek et al., 2008). It is possible that PKP2 could regulate Rho activity by 1) acting as a RhoGDI in a manner similar to p120ctn, or 2) serving as a scaffold that can regulate the subcellular localization of Rho thereby making it more or less available to signal to different effector proteins. To address these possibilities, SCC9 cells expressing PKP2 or NT siRNA were subjected to calcium switch followed by TCA fixation and immunostained against endogenous RhoA. In control cells, Rho localized to cell-cell borders. Early (15min), RhoA exhibited a diffuse ruffled-membrane-like pattern but became more concentrated in a linear membrane pattern later (45min) during junction assembly (Figure 4.11A, top panels). Quantification of RhoA membrane fluorescence intensity reflects these differences (Figure

4.11B). In cells transfected with PKP2 siRNA, Rho membrane localization was impaired both early and late, although enrichment of Rho could be seen at lamellipodial ruffles, which were also more prevalent during PKP2 knockdown (Figure 4.11A, bottom panels). Therefore when PKP2 is not present, Rho membrane recruitment is impaired. These results challenge the theory that PKP2 could be acting as a GDI. If PKP2 was a GDI, then loss of PKP2 would likely lead to enhanced Rho membrane recruitment rather than reduction in Rho membrane localization as was observed here.

Rho activation mimics the perturbed cell-cell junction maturation and stimulates early assembly

Junction assembly is accompanied by a reorganization of actin likely due to RhoA signaling. Effects of PKP2 overexpression on cell morphology can be mitigated by CA Rho expression, and PKP2 KD leads to modulation of Rho activity and localization. Based on these observations, we hypothesized that Rho-dependent changes in actin organization could stimulate desmosome assembly. To test the role of Rho activation during desmosome assembly, SCC9 cells undergoing calcium switch were treated with lysophosphatidic acid (LPA) or DMSO vehicle and immunostained for DP. LPA activates the LPA receptor, which is a G-protein coupled receptor that leads to Rho activation via Gα association with a RhoGEF (reviewed in (Moolenaar et al., 2004)). LPA-induced Rho activation in cells cultured in NCM (steady state calcium concentration) led to a very severe disruption of DP fluorescence at cell-cell contacts (Figure 4.12, NCM panel). Although the images shown represent wide field microscopy, it appears that the enhanced Rho activity led to dramatic

cell-cell overlap. However, at 15' in NCM, LPA-treatment led to enhanced DP border accumulation compared with control treated cells, but by 60 min, Rho stimulated cell-cell borders appeared somewhat less robust than control (Figure 4.12, 15min and 60min panels). This sequence of DP border accumulation supports the notion that Rho activation early is important for initial DP assembly, but later downregulation is important for junction linearization and maturation (see Figure 4.14 for diagram of temporal sequence of signaling events during desmosome assembly).

ROCK inhibition impairs DP assembly but enhances DP at mature contacts

Constitutive RhoA activation by LPA treatment leads to early desmosome assembly enhancement but impairs maturation of preformed junctions. PKP2 deficiency leads to downregulation of RhoA activity and border localization during junction assembly but enhances RhoA activity in unswitched (steady state) cells. Defects in DP assembly are observed at all time points during PKP2 knockdown, although very early DP membrane accumulation can occur (Bass-Zubek et al., 2008) (see Figure 4.14B for a diagram of the effect of PKP2 knockdown on the sequence of signaling events and DP assembly). To test if ROCK signaling is responsible for early desmosome assembly, control siRNA-transfected cells were treated with LY27632 over the course of a calcium switch. LY27632 is a specific inhibitor of several ROCK isoforms (Ishazaki et al., 2000). Consistent with the effects of LPA treatment (Rho stimulation), ROCK inhibition resulted in the opposite outcome on DP assembly. LY27632 treatment led to impairment of early desmosome assembly and

stabilization or maturation of preformed (steady state) junctions (Figure 4.13). However, ROCK inhibition did not reverse the effect of PKP2 knockdown on DP border accumulation in cells cultured at steady state (Figure 4.13), when PKP2-deficiency leads to an upregulation of Rho activity relative to control. This suggests that although Rho activity may be potentiated in PKP2-deficient cells compared to control cells at steady state, it is not responsible for the PKP2-knockdown induced disruption in desmosome maturation.

Discussion

Taken together, the data presented in this report support a role for PKP2 in modulating actomyosin contractility during desmosome plaque assembly. We demonstrated that actin reorganization correlates with the previously-described assembly of DP-containing plaque precursors. Our data indicate that actomyosin contraction is essential for proper desmosome plaque assembly, but this contractility requires PKP2-dependent spatial and temporal regulation of upstream signaling pathways including, but not limited to, RhoA GTPase signaling.

Myosin-dependent actin reorganization is essential for desmosome assembly

The role of the actin cytoskeleton is well characterized in the assembly of adherens junction proteins, logically because actin is the main cytoskeletal component of the adherens junction. Adherens junctions and desmosomes appear to be tightly linked in their assembly and stability, which makes deciphering specific signals that regulate assembly of one junction without having an effect on the other junction challenging. Likewise, IF attachment to desmosomes is essential for their proper formation and adhesive strength. However, both the actin IF cytoskeletal networks are critical for desmosome assembly (Godsel et al., 2005) and cooperate to support desmosome-mediated adhesive strength (Huen et al., 2002).

It was our goal in this study to further elucidate the mechanism of dynamic regulation of the actin cytoskeleton and desmosome plaque component DP during junction assembly and the role that armadillo protein PKP2 plays in this process. Pharmacologic inhibition of

actomyosin contraction led to impairment of desmosomes but not adherens junctions in our cell culture system, indicating that myosin II mediated actin contraction is required for proper desmosome assembly. Myosin activation was localized primarily to the cell cortex, and was slowly upregulated over the course of junction assembly, suggesting that localized actin contraction may be temporally and spatially regulated during desmosome assembly. Although actin is essential for the formation of adherens junctions in epithelial cells, adherens junction components appear to assemble normally in PKP2-deficient cells while exhibiting a minor maturation defect. This suggests that PKP2-dependent actin reorganization may occur later during overall junction assembly after most adherens junctions have accumulated at cell-cell borders but during the critical desmosome assembly time.

RhoA activation is important for early desmosome assembly but is later downregulated

The small GTPase RhoA is best known in its role as upstream regulator of actomyosin contractility. In this pathway, Rho activates ROCK, which phosphorylates myosin II leading to actomyosin contraction. In our studies, pharmacologic Rho activation stimulated early desmosome assembly (15min) but was detrimental to the later stages (1h) and to the stability of mature desmosomes (steady state). Conversely, pharmacologic ROCK inhibition led to a linearization of preformed desmosomal contacts but inhibited early DP assembly. These results suggest that Rho activation must be initiated early but then must be downregulated over the course of junction assembly and maturation (Figure 4.14A). Preliminary Rho activity assays suggest that Rho is activated early during calcium-switch

induced assembly (5-15min) but is downregulated thereafter. We also demonstrated that Rho is recruited to the plasma membrane over time in a calcium switch experiment, suggesting that its activity may be localized to regions of cell-cell contact.

Previous groups have shown that Rho activity localizes early to sites of cell-cell contact and then moves to the periphery as the cell-cell border elongates (Yamada and Nelson, 2007). Dynamic actin reorganization during cell-cell contact and epithelial sheet formation requires Rho-ROCK signaling (Vaezi et al., 2002); however, increased contractility distorts mature classical-cadherin contacts leading to increased cell-overlap thereby and preventing linearization and maturation of adhesion contacts (Brevier et al., 2008).

Putting it together: temporal relationship of actomyosin contractility signaling and desmosome assembly

Based on the studies presented in this chapter, we can propose a model for the temporal relationship of actomyosin contractility signaling and desmosome assembly. Early stages of cell-cell adhesion formation are characterized by the assembly of radially oriented actin bundles, the activation and membrane accumulation of Rho, and initial myosin phosphorylation and cortical accumulation (Figure 4.15A). Cytoplasmic DP particles form within the region of the circumferential actin bundle network, and initial DP accumulate at cell-cell contact begins (Godsel et al., 2005).

Later during assembly and maturation of the junction, a disappearance of radial actin fibers and a contraction of the circumferential actin cytoskeleton occurs correlating with the

timing of membrane-translocation of DP-containing particles (Godsel et al., 2005) and with the subsequent downregulation of Rho activity (Figure 4.15B). Figure 4.14A depicts the temporal relationship of actin reorganization- and DP assembly-events with the dynamics of myosin and Rho activation.

PKP2, a critical regulator of DP assembly, modulates actomyosin contractility signaling

PKP2, a desmosomal member of the armadillo-related protein family, is required for proper DP assembly (Bass-Zubek et al., 2008). Based on the homology between PKP and $p120^{ctn}$ central armadillo-repeat domains, we hypothesized that PKP2 could be modulating the actin cytoskeleton through related mechanisms. Using siRNA-mediated knockdown and overexpression studies, we demonstrated that PKP2 indeed has the ability to dramatically alter the organization of the actin cytoskeleton by modulation of actomyosin contractility.

Reduction in PKP2 levels by siRNA-mediated knockdown led to dramatic reorganization of the actin cytoskeleton that correlated temporally and spatially with blockade of DP assembly and cytoplasmic sequestration of DP particles. PKP2-knockdown also led to potentiation of myosin activation and enrichment in the submembranous cytoplasmic domain. PKP2 knockdown led to reduction of RhoA membrane recruitment and blunting of RhoA activation during calcium switch-mediated desmosome assembly but enhanced RhoA activity in cells cultured in steady state calcium levels (Figure 4.14B). In the absence of PKP2, circumferential actin contractility takes these actin rings farther from rather than closer to the membrane as normally occurs during contact maturation (Zhang et al., 2005). During this time, radial actin fiber disappearance is blocked, suggesting persistence

of Rho-ROCK activity (Vaezi et al., 2002). These abnormal actin morphologies lead to a stunted or immature desmosome assembly state in which proper modulation of actin organization is prevented (Figure 4.16A). In this state, DP particles are often found corralled behind the tightened circumferential actin cytoskeleton (Figure 4.4) and DP assembly is dramatically impaired (Figure 3.6). Although actin contraction is necessary for desmosome assembly, too much contraction or loss of dynamic reorganization appears to be detrimental to desmosome formation (Figure 4.16B).

PKP2 as a potential scaffold for Rho actomyosin contractility signaling elements

PKP2 appears at cell-cell borders very early during contact assembly where it colocalizes closely with E-cadherin based contacts and junctional actin. Indeed, PKP2 has been shown to interact with E-cadherin (Chen et al., 2002) and in reconstitution studies, PKP2 can even drive other desmosomal proteins into adherens junctions (Koeser et al., 2003). It is possible that this early adherens junction-localized pool of PKP2 could recruit Rho to the membrane where it would become activated by a membrane-tethered GEF. Rho membrane association is thought to be important for its ability to interact with GEFs and therefore become activated (Ridley, 2001); Alternatively, cytoplasmic PKP2 (associated with DP-precursors perhaps) could also compete with Rho GDIs for Rho binding thereby allowing for normal membrane recruitment and activation of Rho.

Potential Rho-independent signaling consequences of PKP2 deficiency

Previously we demonstrated that PKP2 serves as a scaffold and recruits PKC to DP during assembly where it regulates DP-IF interactions. In that report, we observed that other PKC substrates were hyper-phosphorylated when PKP2 was knocked down and concluded that PKP2-recruitment of PKC modulates the specificity and localization of its activity (Bass-Zubek et al., 2008).

PKC is known to be a major regulator of myosin and MLCK (Nishikawa et al., 1985) and can phosphorylate MLC directly (Bogatcheva et al., 2003; Turbedsky et al., 1997). Myosin activation by MLCK modulates the organization of the perijunctional circumferential actin cytoskeleton (Shen et al., 2006). It is possible that the increased circumferential actomyosin contractility in PKP2-deficient cells is due to mislocalized PKC signaling. This could potentially explain why ROCK inhibition did not rescue the effects of PKP2 knockdown. To further complicate the model, PKC-and Rho pathways intersect at many different points and in different cellular contexts. PKC can activate Rho and vice versa (Dovas et al., 2006; Hippenstiel et al., 1998; Mehta et al., 2001; Slater et al., 2001). As mentioned previously, PKC can phosphorylate Rho-ROCK target MLC but also lead to phosphorylation of MYPT (Patil and Bitar, 2006) and some ERM proteins (Ng et al., 2001). Preliminary data from our recent work suggests that both genetic and pharmacologic modulation of PKC leads to changes in actin contractility and organization (A. Bass-Zubek and R. Hobbs, unpublished observations). It is our working hypothesis that dysregulation of PKC activity and/or localization due to PKP2-deficiency is responsible for the enhanced actomyosin contractile signaling and absence of normal actin reorganization during junction

assembly. Mislocalized PKC activity could also be responsible for later Rho upregulation in PKP2-deficient cells.

Future Directions

Although it is clear that Rho signaling and actomyosin contraction are essential for proper desmosome assembly, and that PKP2 is essential for maintaining the subcellular homeostasis of signaling pathways that feed into this process, many questions remain. How, does PKP2 regulate Rho signaling? Evidence for specific subcellular regulation of Rho activity has been demonstrated by studies of p0071 in cytokinesis (Keil et al., 2007; Wolf et al., 2006). Future research directions include *in vitro* assays of Rho GTP hydrolysis in the presence or absence of PKP2, identification of potential GEFs, GDIs or GTPase activating proteins (GAPs) that might interact with PKP2, and biochemical studies to determine if PKP2 interacts directly with these proteins.

Current ongoing and future studies focus on uncovering the role of PKP2 in the dynamic co-regulation of desmosomal proteins (DP) and their associated cytoskeletal components (actin and IF) and Rho signaling using live-cell imaging techniques. These studies will demonstrate whether DP particle translocation described in (Godsel et al., 2005) occurs in association with circumferential actin contraction. Rhotekin Rho Binding Domain (RBD)/Rho fluorescence resonance energy transfer (FRET) or RBD overlay studies will be used to determine the effect of PKP2 knockdown on the subcellular localization of Rho activity in the context of DP assembly.

Another point that will need to be addressed in the future is the role of PKP2 on modulation of Rac and Cdc42 in the assembly process. Rac has emerged as a major regulator of adherens junction assembly, cadherin clustering and stabilization (Kraemer et al., 2007; Kuroda et al., 1998) and is activated by $p120^{ctn}$, further inhibiting Rho activity (Noren et al., 2000). It is possible that PKP2 could modulate Rac and Cdc42 signaling or localization, which could have direct implications for Rho modulation and in the radial actin bundle- and lamellipodial actin-dynamics during initiation of desmosome assembly.

Actin polymerization is important for formation of junctional actin (Zhang et al., 2005) and radial actin cables (Kobielak et al., 2004). Mena/VASP proteins regulate actin organization and are recruited to sites of E-cadherin ligation (Scott et al., 2006). These actin regulatory proteins all have the potential to be affected by signaling downstream of PKP2-deficiency. We have initiated studies to determine if some of these actin reorganization proteins are phosphorylated in PKP2-knockdown lysates or cells and have not observed any changes in protein localization or phosphorylation (ERM: see above; VASP: unpublished observations). Additionally, PKC knockdown or activation will be utilized to determine the role of PKC in the regulation of GTPases and in the stimulation of localized actin contraction and/or polymerization.

The studies that are presented in this report strongly indicate a specific role for the desmosomal armadillo protein, PKP2, in the regulation of multiple signaling pathways that are critical for actin cytoskeleton remodeling and desmosome plaque assembly independent of adherens junction assembly. Outside of playing a role in normal desmosome assembly,

the pathways regulating actomyosin contractility could play key roles in the pathogenesis of desmosome-based diseases. Rho activation has been implicated in the desmosomal autoimmune diseases pemphigus foliaceous and vulgaris. In one study, pemphigus autoantibodies (IgG) interfered with RhoA signaling and led to skin blistering, whereas stimulation of Rho activity prevented the IgG-induced blistering (Waschke et al., 2006).

Loss of functional PKP2 in patients with PKP2 mutations lead to congenital cardiac arrhythmias and often are fatal when undiagnosed (see chapter I). PKP2 deficiency in these patients could potentially lead to inappropriate activation of multiple signaling pathways, either independently or in a domino-effect, dramatically altering cell adhesion, cellular morphology or other cellular functions. Rho-ROCK-myosin activation also induces actin reorganization and actin contractility in cardiac myocytes (Brown et al., 2006), and ROCK inhibitors are being tested as potential therapeutics in coronary artery disease and cardiac hypertrophy (Lai and Frishman, 2005). Elucidation of the molecular mechanism by which these signaling pathways are regulated would provide potential therapeutic targets for diseases in which desmosomes are compromised. Modulation of signaling pathways disturbed by loss of PKP2 could serve as a potential future therapeutic approach.

FIGURES

Figure 4.1 DP precursor assembly coordinates with dynamic actomyosin activity

A. Analysis of F-actin remodeling during calcium switch in SCC9 cells. NCM: cells cultured in steady state normal calcium medium; 15': cells that were cultured in LCM and then switched to NCM for 15'; 3h: see 15'. Note numerous radially-oriented actin fibers at 15 min (green arrowheads), which mostly disappear by 3h in NCM. Red arrows depict siGlo particles. These images were part of a control set of cells from a separate PKP2 knockdown experiment. Lower panels: magnified images of boxed areas in upper panels.

B. DP remodeling during a calcium switch in SCC9 cells. Note the numerous cytoplasmic particles at 15 min which clear out by 3h as cell-cell borders mature. Lower panels: magnified images of boxed areas in upper panels.

C. Diagram of radial actin fibers (red) and concentric actin rings (blue) in epithelial cells.

Figure 4.1

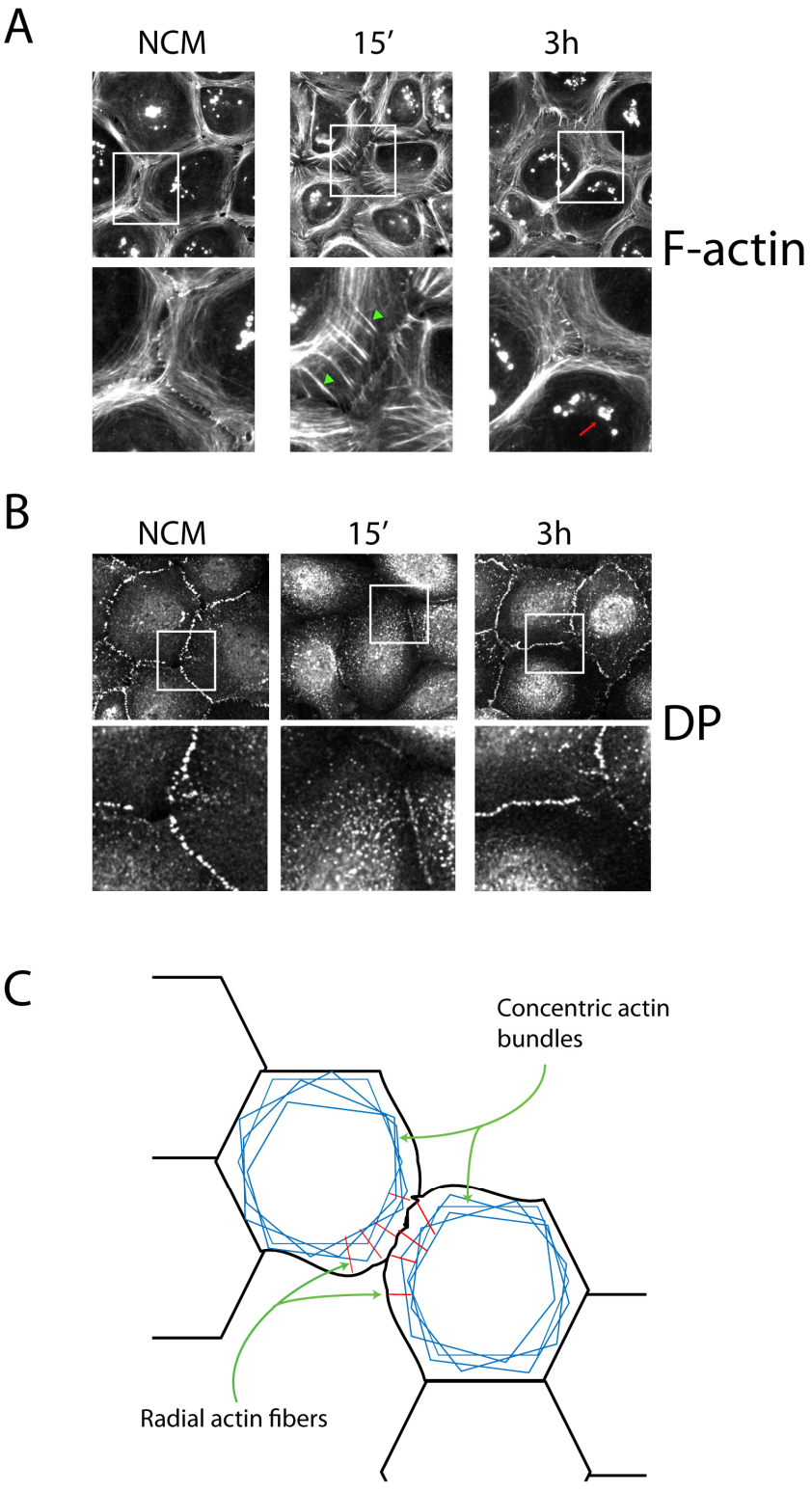

A
NCM
15′
3h
F-actin
B
NCM
15′
3h
DP
C
Concentric actin bundles
Radial actin fibers

Figure 4.2 DP particles are enmeshed in network of circumferential actin bundles

Confocal analysis of DP-GFP, keratin and F-actin in SCC9 cells reveals DP containing cytoplasmic dots, which line up along keratin IF are interspersed within the circumferential actin network. Tricolor image to the right of the "merge" image represents magnification of white boxed area.

Figure 4.2

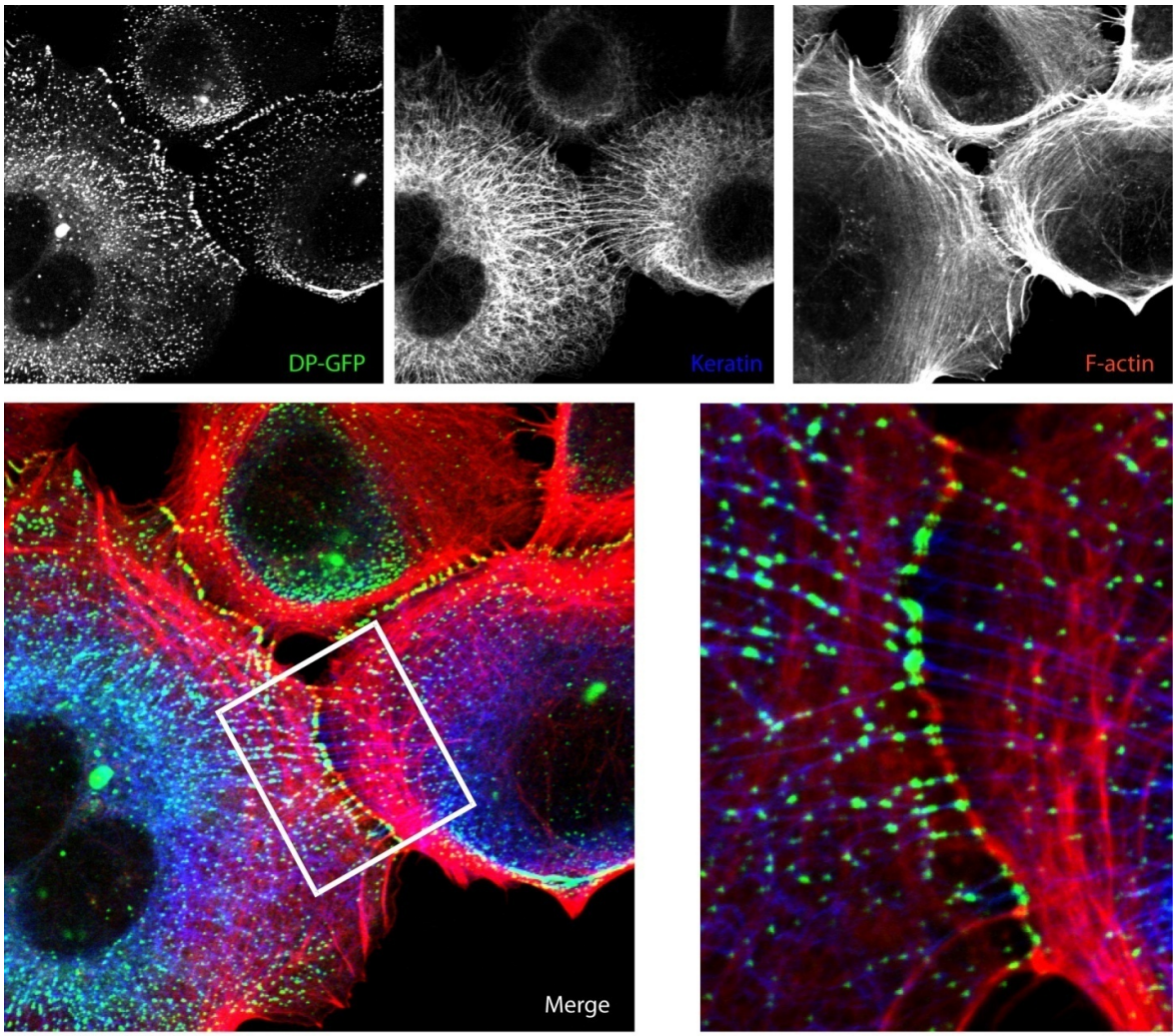


Fig 4.3 Myosin inhibition impairs DP but not E-cadherin border accumulation

A. DP-GFP and E-cadherin immunostaining of SCC9 cells cultured in NCM that were treated with 50uM Blebbistatin or DMSO for 15min.

B. Border fluorescence intensity quantitation of panel A.

C. Endogenous DP in SCC9 cells treated with 50uM Blebbistatin or DMSO at the beginning of calcium switch experiment. DP border accumulation is impaired at all time points.

Lisa Godsel performed this experiment.

Figure 4.3

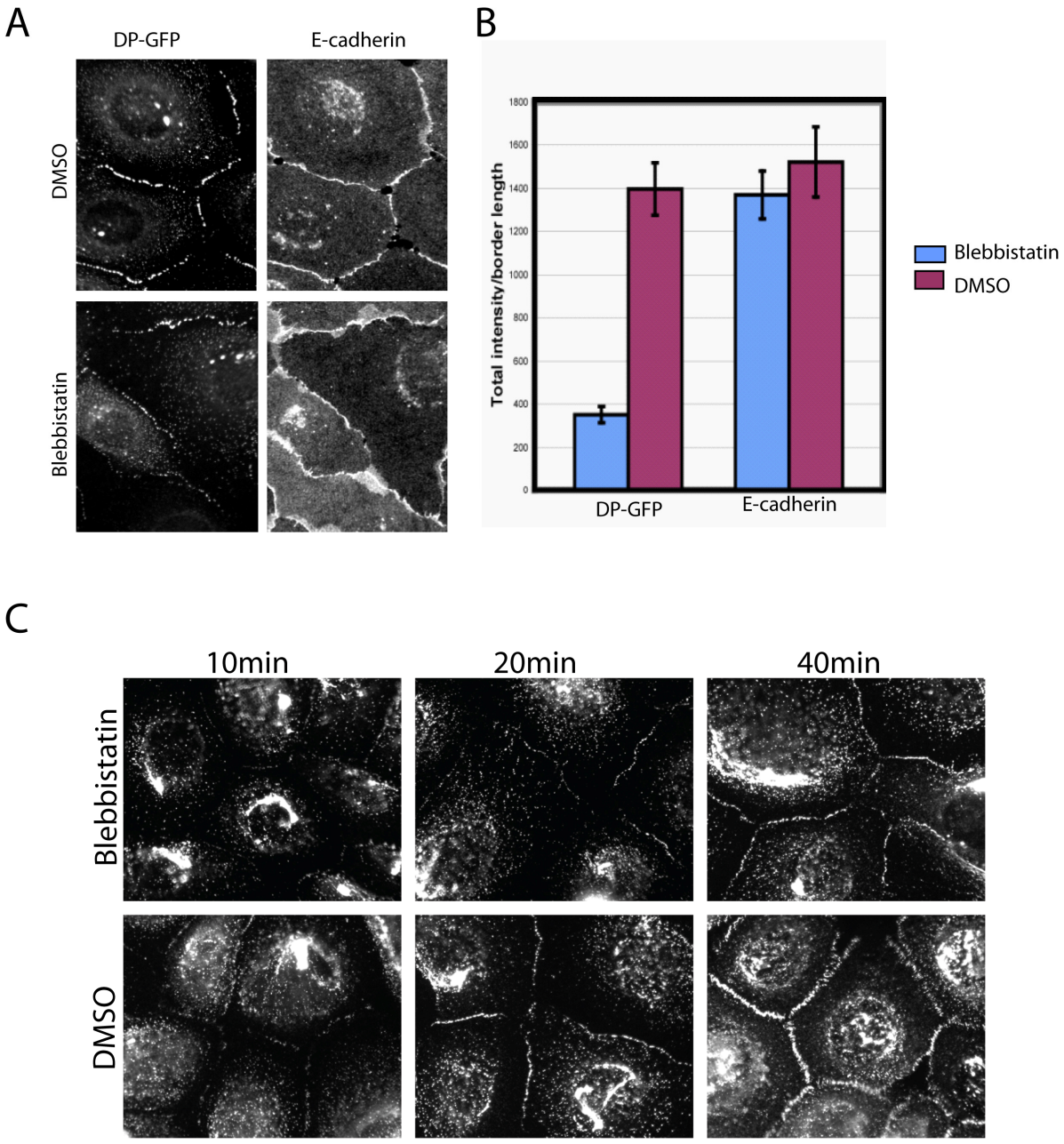

A
DP-GFP
E-cadherin
DMSO
Blebbistatin
B
Total intensity/border length
1800
1600
1400
1200
1000
800
600
400
200
0
DP-GFP
E-cadherin
Blebbistatin
DMSO
C
10min
20min
40min
Blebbistatin
DMSO

Figure 4.4 PKP2 modulates actin organization during cell-cell junction formation

A. SCC9 cells expressing control NT + siGlo or PKP2 siRNA undergoing calcium switch were stained for F-actin. Red arrow depicts siGlo particles.

B. Quantification of maximal circumferential actin fluorescence intensity in control or PKP2 siRNA transfected cells (separate experiment from that shown in Figure 3.4A). DP and F-actin immunofluorescence staining of control siRNA- or PKP2 siRNA transfected cells cultured in steady state calcium levels. White arrows indicate DP particles encircled by circumferential actin bundles in PKP2-deficient cells. Intensity was measured across a line drawn through circumferential actin of many cells per field of view (yellow line examples on left panels). An average of the maximal intensity values was obtained and plotted for each condition (right). Nicholas Garcia assisted in this quantification.

Figure 4.4

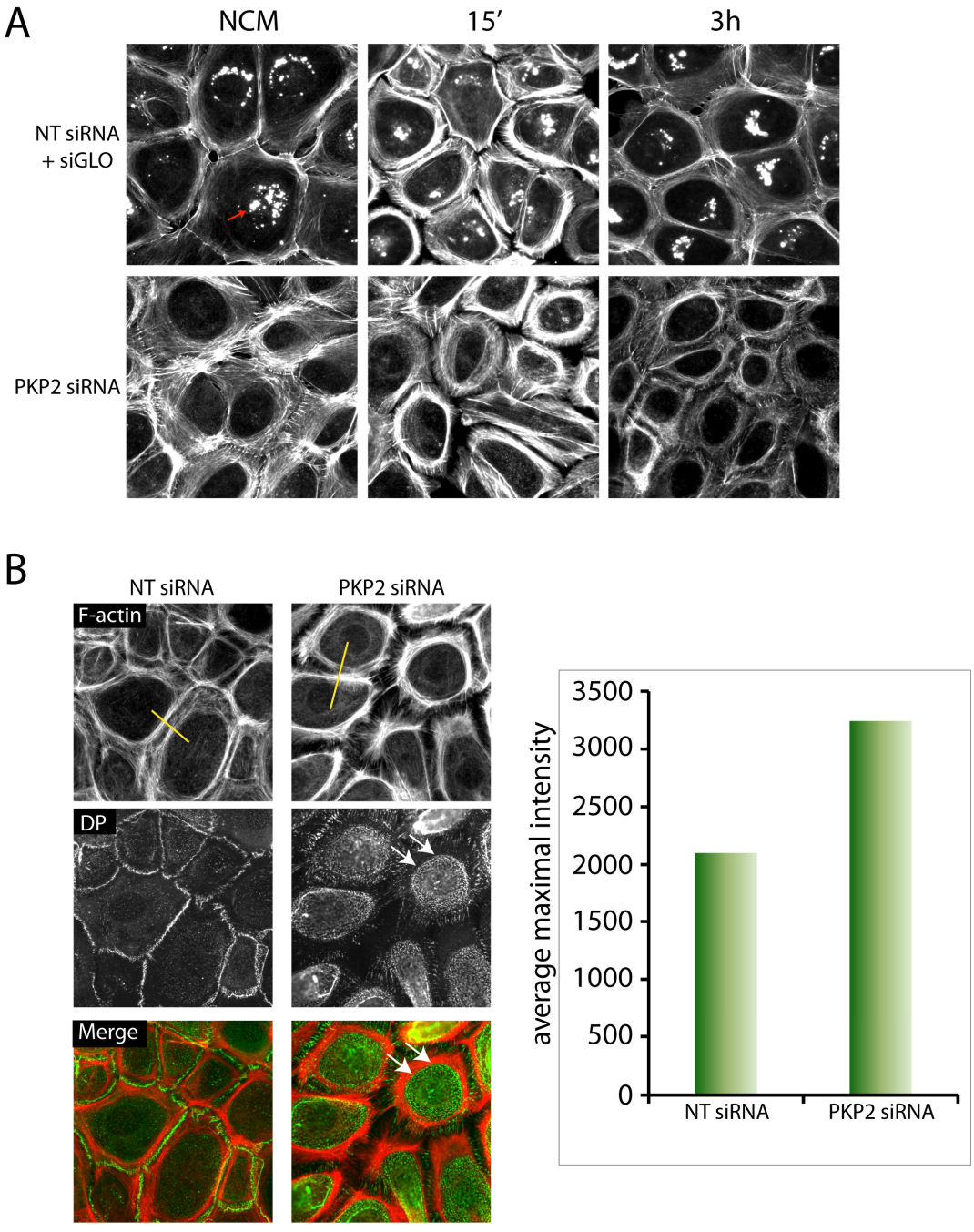

A
NCM
15′
3h
NT siRNA
+ siGLO
PKP2 siRNA
B
NT siRNA
PKP2 siRNA
F-actin
DP
Merge
average maximal intensity
3500
3000
2500
2000
1500
1000
500
0
NT siRNA
PKP2 siRNA

Figure 4.5 PKP2 knockdown has minimal effect on E-cadherin assembly

SCC9 cells transfected with control NT (+siGlo, red arrow) siRNA or PKP2 siRNA were subjected to calcium switch and probed for E-cadherin. E-cadherin border localization is largely unperturbed in cells lacking PKP2.

Figure 4.5

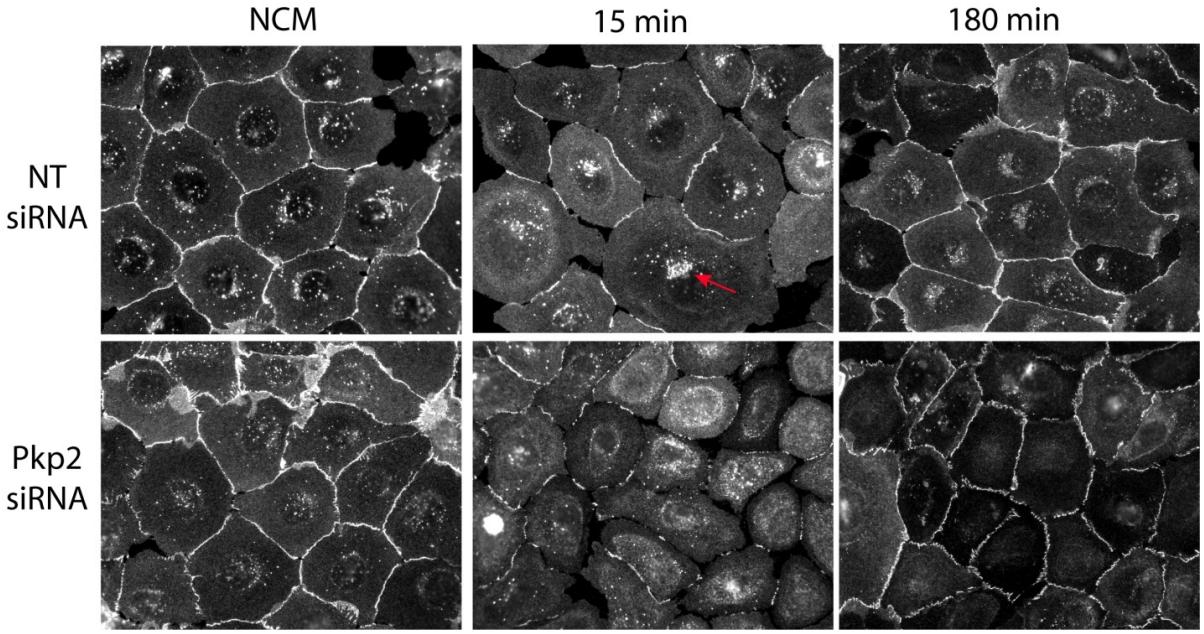


Figure 4.6 PKP2 regulates myosin II localization and activity

A. Western blot analysis of MLC phosphorylation (single pSer19) on SCC9 cell lysates from PKP2-siRNA or NT control siRNA transfected cells that were subjected to calcium switch. Myosin activation is transient over course of calcium switch but enhanced in PKP2 siRNA transfected cells at all time points.

B. Densitometry of p-myosin immunoblot in A. Values represent ratio of p-myosin intensity to GAPDH intensity.

C. pMLC cortical localization enriched in PKP2 siRNA transfected cells. SCC9 cells transfected with control NT or PKP2 siRNA undergoing calcium switch. Images are taken from 15' after switch to NCM, at which time myosin cortical accumulation is maximal. Yellow arrowheads point to cortical enrichment of pMLC. DP is shown to depict PKP2 knockdown (disruption of DP border accumulation).

D. Myosin subcellular pattern is altered in PKP2 knockdown cells. As in C, SCC9 cells transfected with siRNA at 15min in NCM. Total myosin accumulation in the cortex is enriched in PKP2 siRNA transfected cells whereas it is diffuse across the cytoplasm in NT siRNA transfected cells. Yellow arrowheads depict cortical accumulation of total myosin.

Figure 4.6

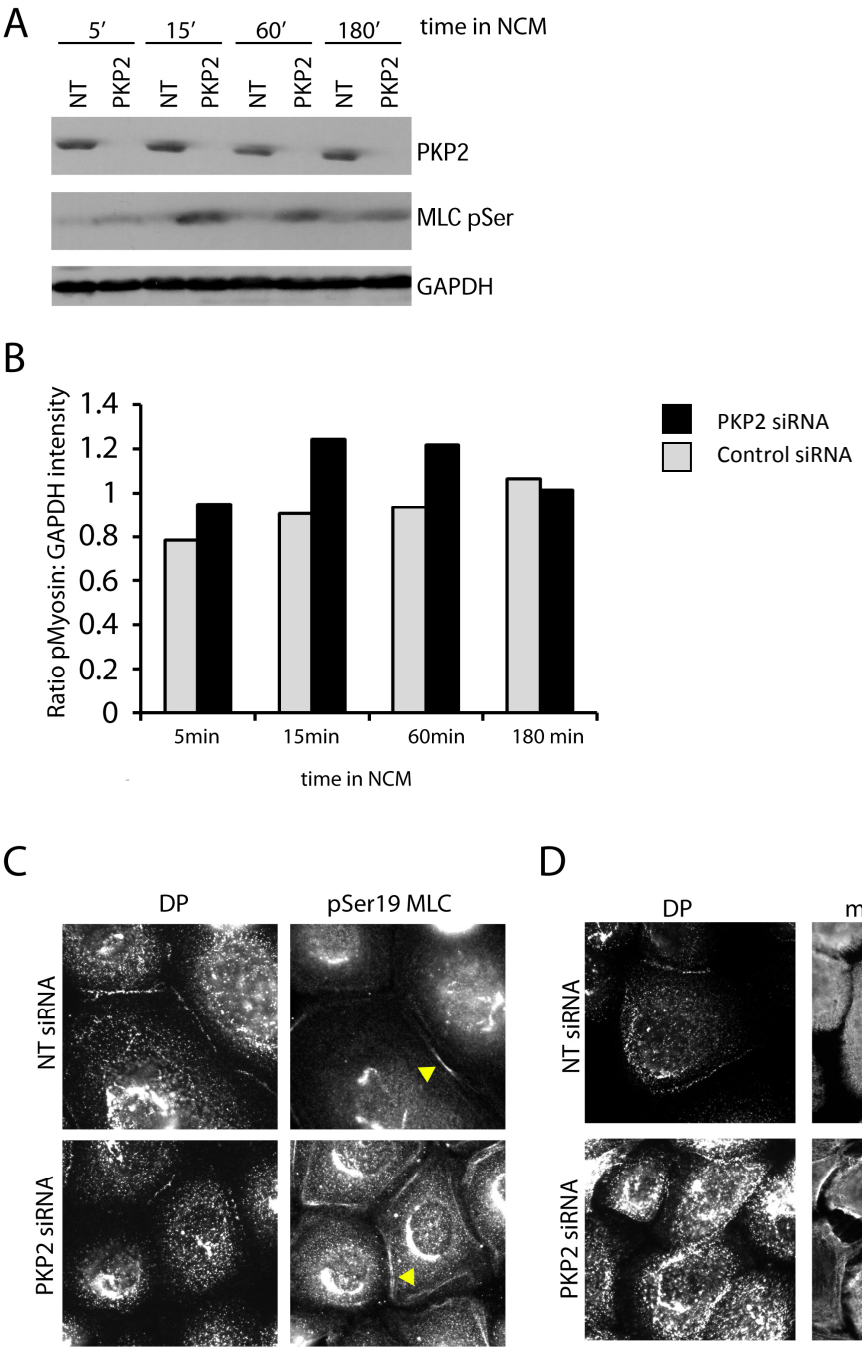

A
5′
15′
60′
180′
time in NCM
NT
PKP2
PKP2
MLC pSer
GAPDH
B
Ratio pMyosin: GAPDH intensity
1.4
1.2
1
0.8
0.6
0.4
0.2
0
5min
15min
60min
180 min
time in NCM
PKP2 siRNA
Control siRNA
C
DP
pSer19 MLC
NT siRNA
PKP2 siRNA
D
DP
myosin BT1
NT siRNA
PKP2 siRNA

Figure 4.7 PKP2 overexpression induces dramatic cell morphology change and actin disruption

SCC9 cells expressing PKP2-FLAG were stained with anti-FLAG antibody and phalloidin (F-actin). Stars represent untransfected cells with normal actin morphology.

Figure 4.7

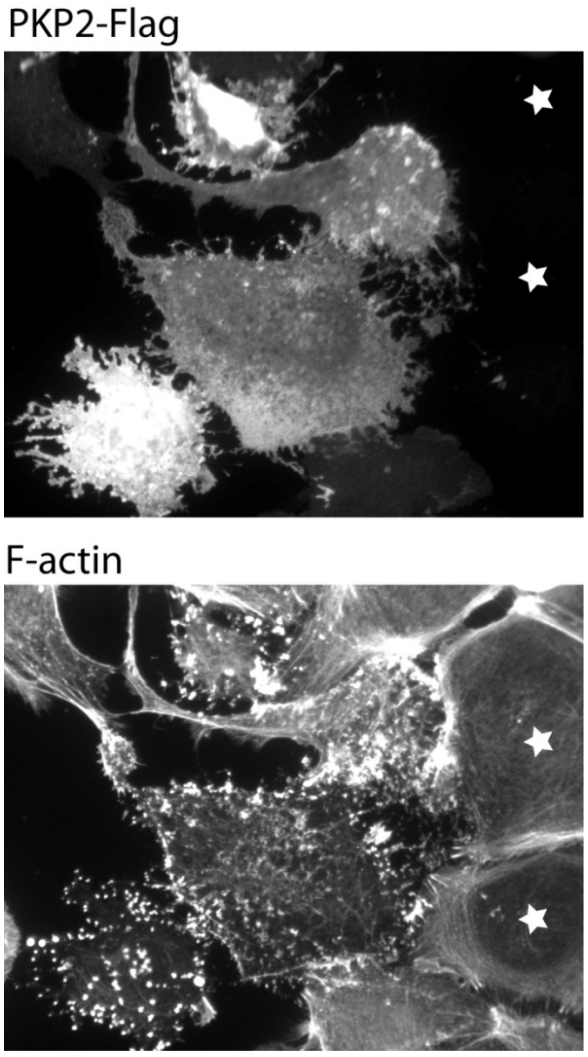


Figure 4.8 PKP2 colocalizes with actin and E-cadherin at cell-cell junctions

SCC9 cells undergoing calcium switch (steady state (NCM), 10 min or 60 min in NCM) and immunostained for PKP2, E-cadherin and F-actin. Triple colocalization at cell-cell junctions can be observed at all time points (white arrows).

Figure 4.8

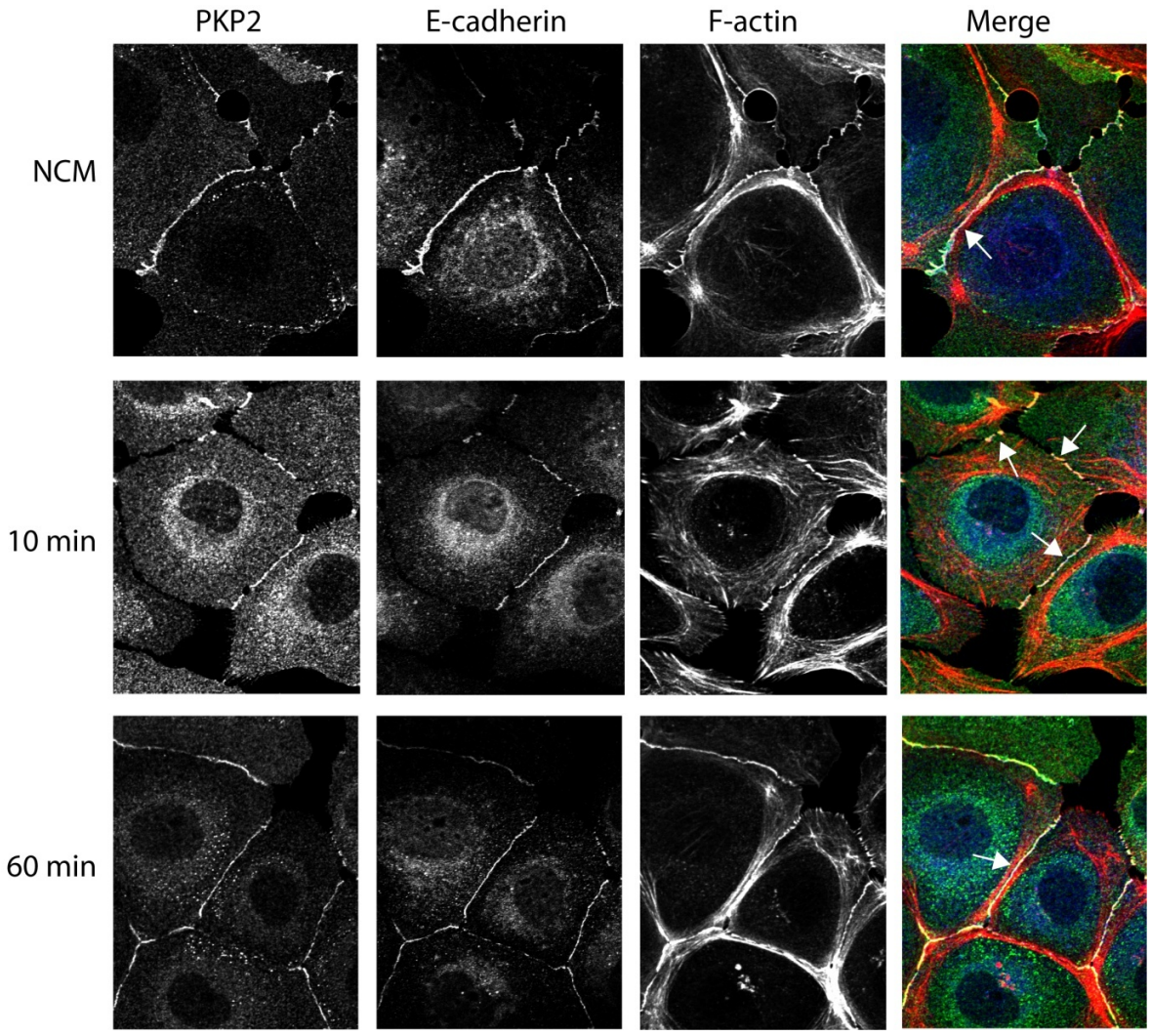


Figure 4.9 A point mutation abrogates PKP2-induced cell morphology change but does not disrupt PKP2-actin interaction

A. Cos cells transfected with PKP2-FLAG, PKP2^{G629V}-FLAG or PKP2^{I722G}-FLAG (experiment performed by X. Chen).

B. Confocal images of REF52 cells expressing PKP2-GFP and mRFP-actin or GFP-PKP2^{G629V} and mRFP-actin. Arrows indicate PKP2 alignment along actin filaments. Right panels are magnifications of the boxed area on the left panels.

Figure 4.9

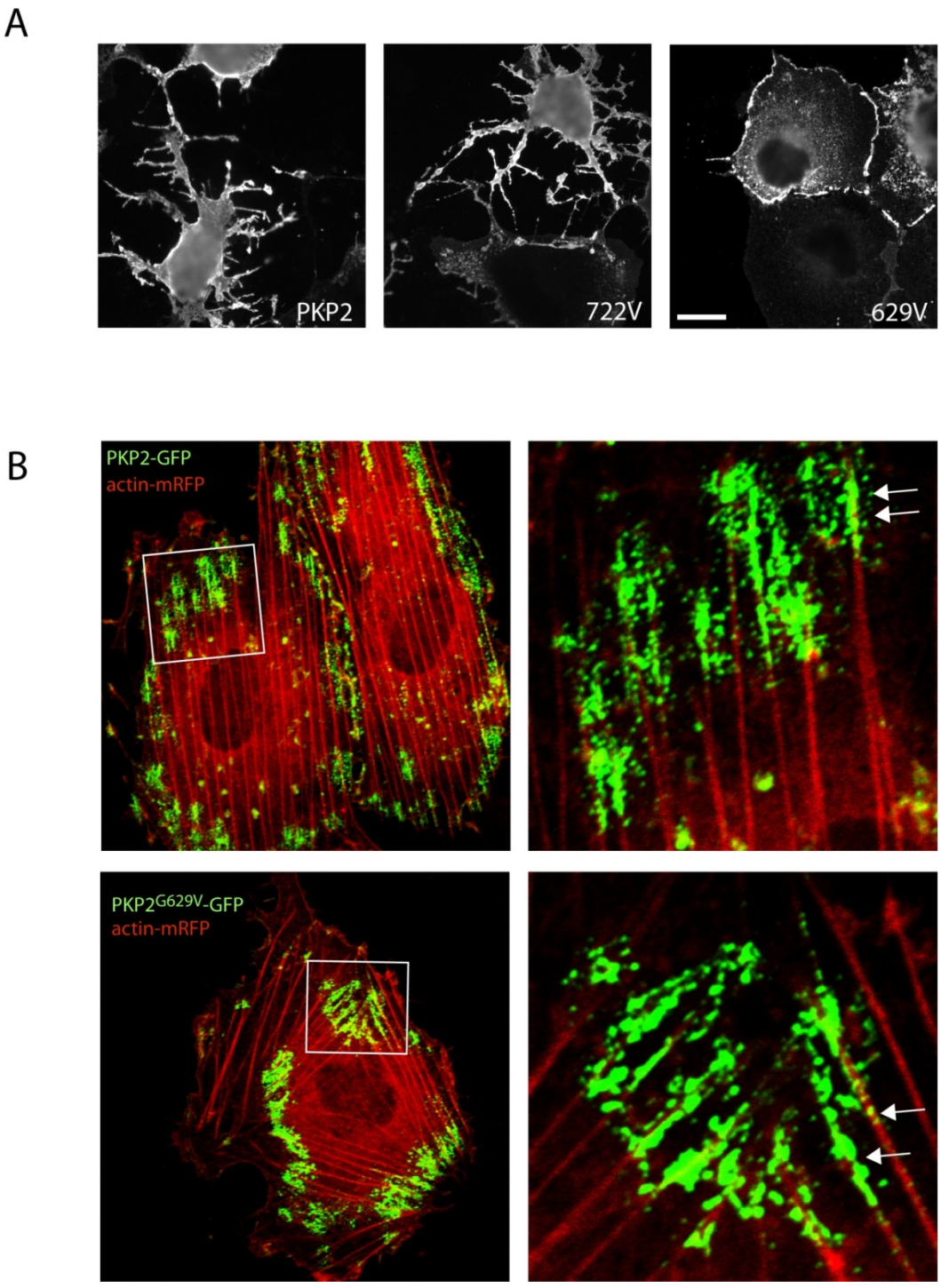

A
PKP2
722V
629V
B
PKP2-GFP
actin-mRFP
PKP2G629V-GFP
actin-mRFP

Figure 4.10 PKP2 expression alters Rho-dependent actin remodeling

A. Constitutively active RhoA can partially impair PKP2-overexpression induced cell morphology change. Cos20 cells were cotransfected with PKP2-FLAG and V14RhoA-myc or N17RhoA-myc. Transfected cells exhibiting 5 or more membrane protrusions were counted as undergoing morphology change. Quantification of percent of double-transfected cells undergoing morphology change is shown.

B. Steady state Rho activity is enhanced in SCC9 cells transfected with PKP2 siRNA compared to control siRNA transfected cells. RBD pulldown assay in untreated, LPA treated (5mM for 30min), NT siRNA- or PKP2 siRNA-transfected SCC9 cells (top panel). Densitometry of immunoblot was performed. Values represent active RhoA relative to total RhoA.

C. Steady state MYPT phosphorylation is enhanced in PKP2-deficient cells. Western blot analysis of lysates from SCC9 cells transfected with control or PKP2 siRNAs. PKP2, phospho-specific MYPT, total MYPT, or α-tubulin antibodies were used.

D. ERM phosphorylation is not altered in PKP2-deficient cells. Western blot analysis of lysates from steady state SCC9 cells transfected with control NT or PKP2 siRNAs. PKP2, phospho-specific MLC, phospho-specific ERM, or α-tubulin antibodies were used.

Lisa Godsel assisted with the experiments in C and D.

Figure 4.10

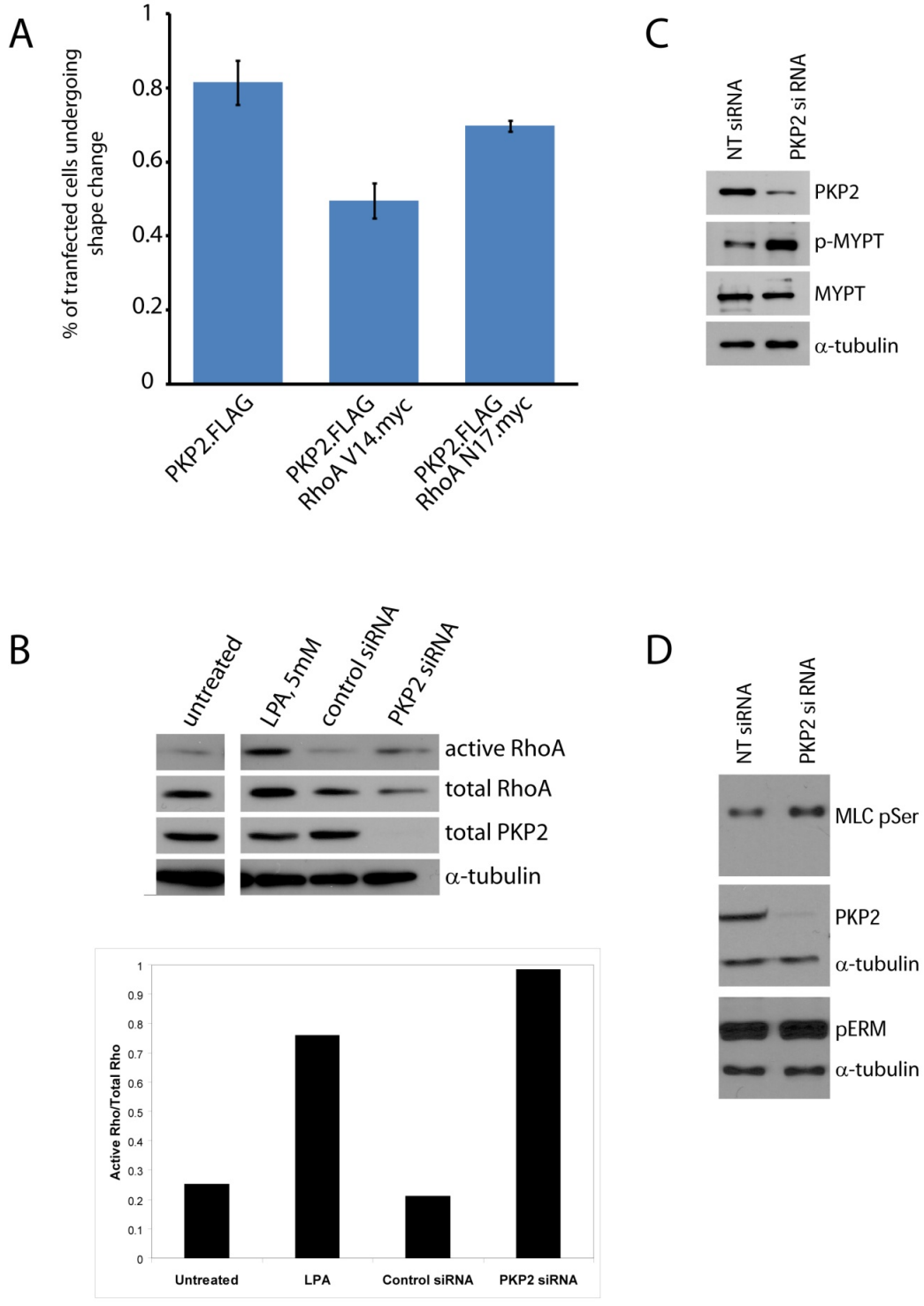

A
% of tranfected cells undergoing shape change
1
0.8
0.6
0.4
0.2
0
PKP2.FLAG
PKP2.FLAG RhoA V14.myc
PKP2.FLAG RhoA N17.myc
C
NT siRNA
PKP2 si RNA
PKP2
p-MYPT
MYPT
α-tubulin
B
untreated
LPA, 5mM
control siRNA
PKP2 siRNA
active RhoA
total RhoA
total PKP2
α-tubulin
Active Rho/Total Rho
Untreated
LPA
Control siRNA
PKP2 siRNA
D
NT siRNA
PKP2 si RNA
MLC pSer
PKP2
α-tubulin
pERM
α-tubulin

Figure 4.11 Rho activity and border localization during calcium switch is impaired during PKP2 knockdown

A. Rho membrane localization is impaired in PKP2 knockdown cells. Calcium switch of SCC9 cells transfected with PKP2 siRNA or control NT siRNAs. RhoA localization was visualized by TCA fixation followed by 0.2% Triton X-100 extraction and anti-RhoA immunofluorescence.

B. Normalized fluorescence intensity measurement of RhoA at cell-cell contacts. Lisa Godsel and Amanda Bass-Zubek planned this experiment, Lisa Godsel performed this experiment.

Figure 4.11

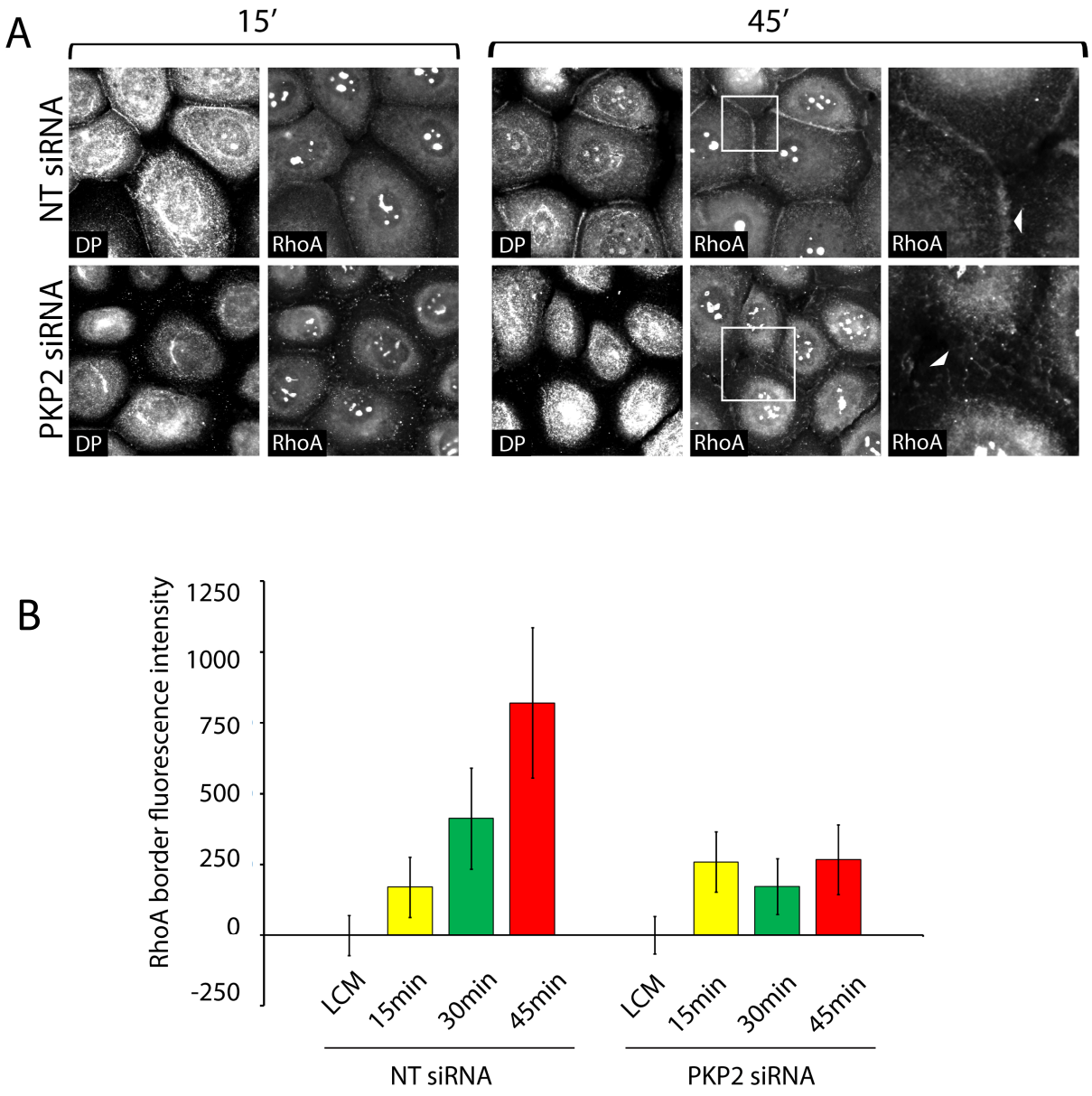

A
15′
45′
NT siRNA
PKP2 siRNA
DP
RhoA
B
RhoA border fluorescence intensity
1250
1000
750
500
250
0
-250
LCM
15min
30min
45min
NT siRNA
PKP2 siRNA

Figure 4.12 Rho activation perturbs mature cell-cell junctions but stimulates early desmosome formation.

DP staining in SCC9 cells treated with LPA (5uM) or DMSO vehicle during calcium switch. NCM cells were treated with drug for 30 min. DP accumulation at borders is disrupted at steady state (NCM) likely due to excess actomyosin contraction. At 15 min, DP accumulation is enhanced by LPA treatment, which is partially reversed by 60min.

Figure 4.12

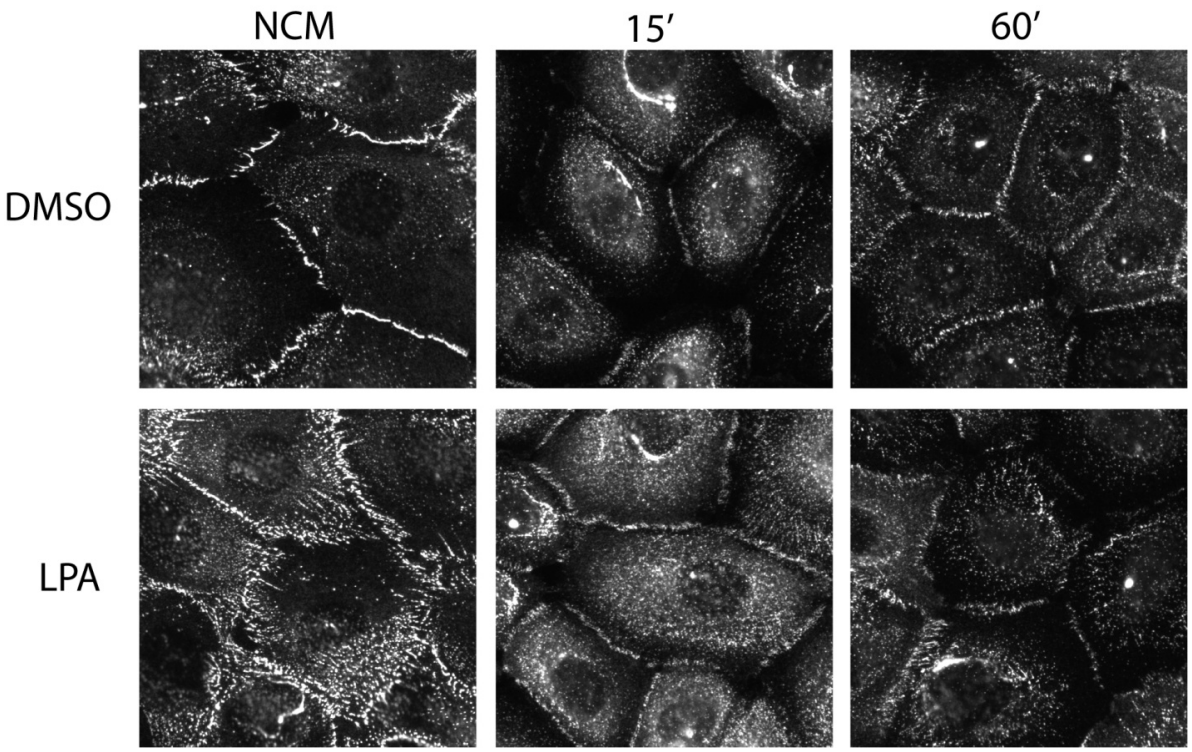


Figure 4.13 ROCK inhibition blocks DP assembly but enhances maturation of preexisting junctions and cannot rescue PKP2-knockdown impairment of DP assembly.

DP immunofluorescence staining of SCC9 cells transfected with NT control or PKP2 siRNAs undergoing calcium switch that were treated with ROCK inhibitor (LY27632) or no drug at the beginning of the switch. Steady state cells were treated with drug for 30'. Insets in NCM panels (no drug) depict PKP2 staining to show efficacy of PKP2-knockdown. Lisa Godsel and Amanda Bass-Zubek planned this experiment, Lisa Godsel performed this experiment.

Figure 4.13

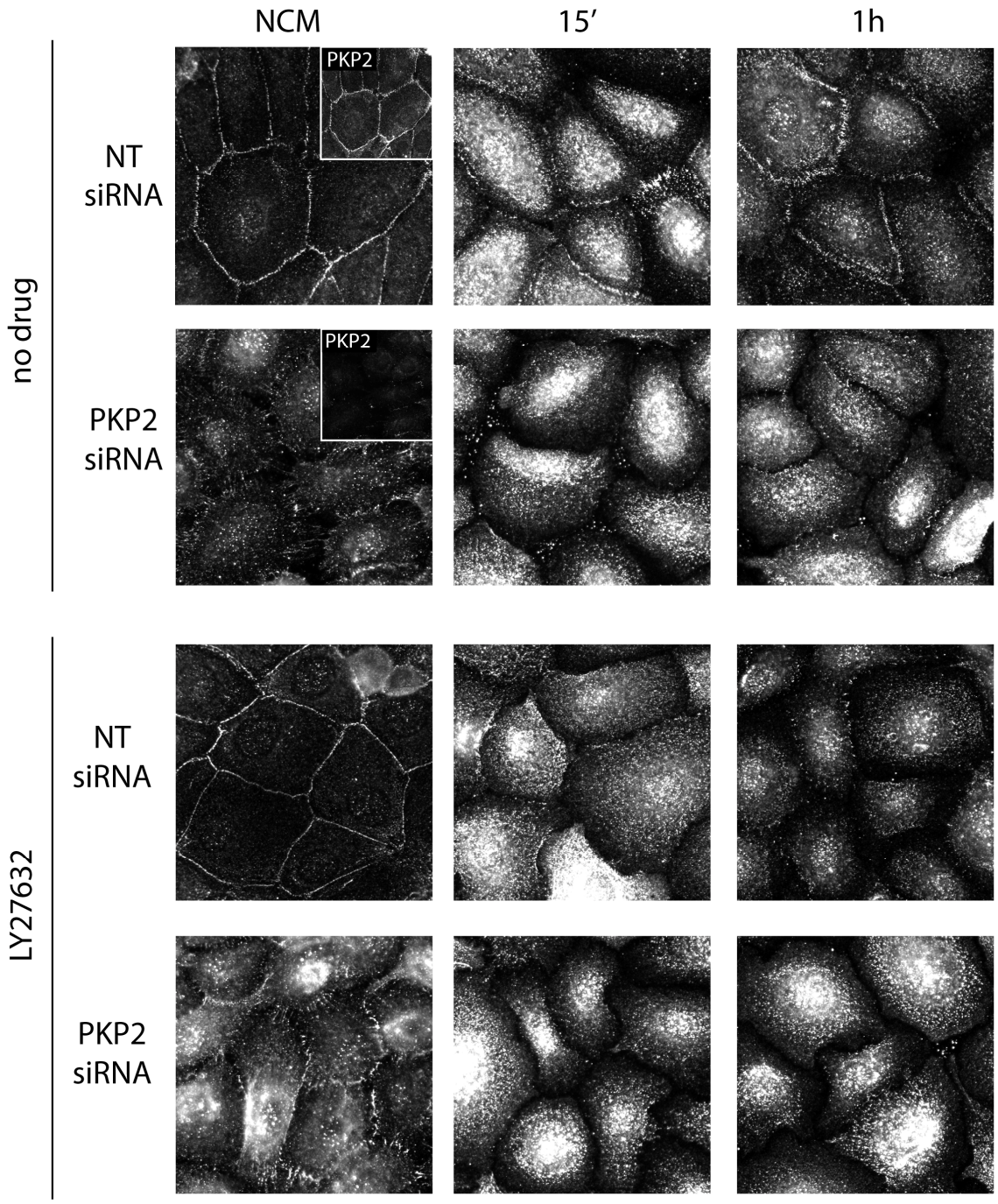

NCM
15′
1h
no drug
NT siRNA
PKP2 siRNA
PKP2
PKP2
LY27632
NT siRNA
PKP2 siRNA

Figure 4.14 Temporal sequence of signaling events during assembly. *I. Control vs. PKP2 knockdown*

In the control situation (dotted lines), our data demonstrate that Rho activation (middle graph) is induced 5 min after cell-cell contact, peaks at 15min and is downregulated. Myosin activity (bottom graph) is induced and becomes level around 30min-1h. Previously it was shown that DP border accumulation (top) exhibits biphasic pattern correlating to initial membrane localization and then a second wave due to translocation of precursors to nascent junctions (Godsel et al., 2005). When PKP2 is deficient (solid lines), Rho activation is blunted relative to control but is upregulated again at steady state conditions. Myosin activity is potentiated and DP assembly is impaired.

Figure 4.14

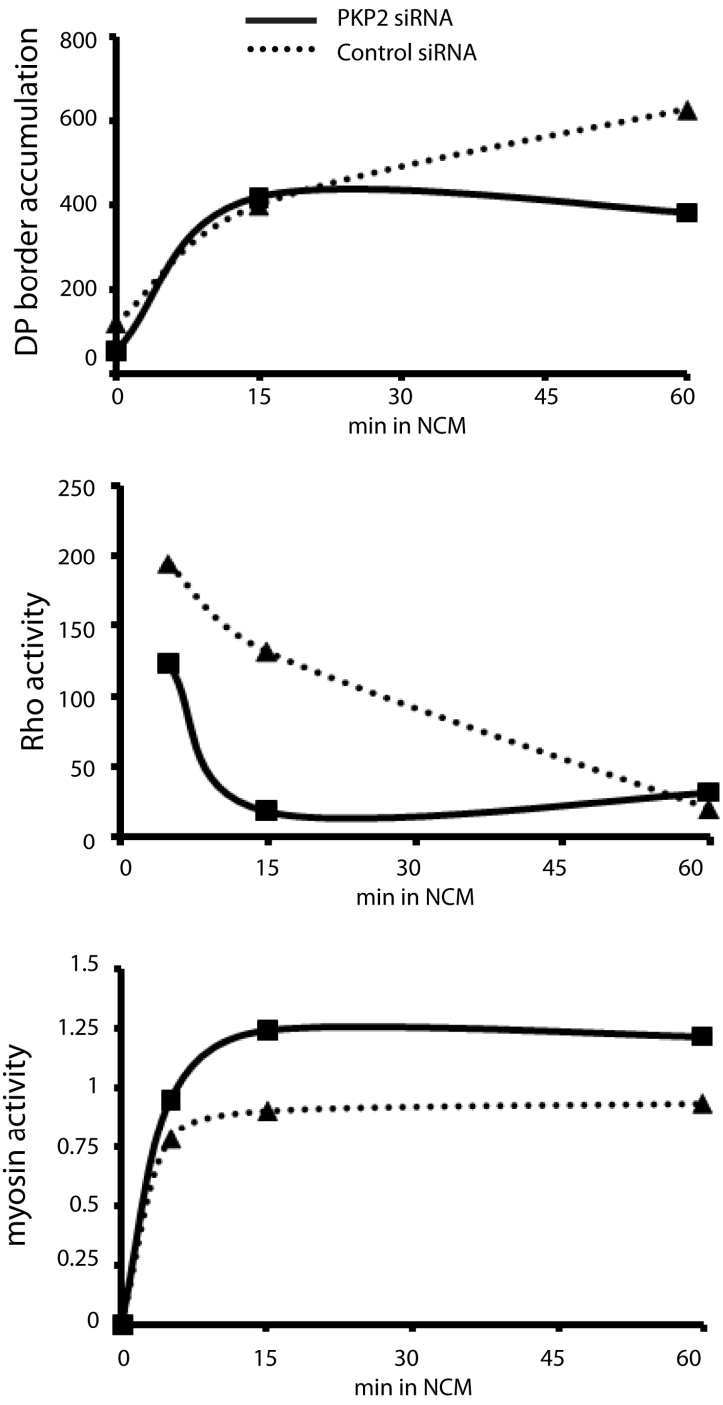

PKP2 siRNA
Control siRNA
DP border accumulation
800
600
400
200
0
0
15
30
45
60
min in NCM
Rho activity
250
200
150
100
50
0
0
15
30
45
60
min in NCM
myosin activity
1.5
1.25
1
0.75
0.5
0.25
0
0
15
30
45
60
min in NCM

Figure 4.15 Temporal sequence of signaling events during assembly. *II. Temporal comparison*

These graphs are hand drawn based on the data presented in Figure 4.14 to better demonstrate temporal sequence of events comparing Rho, myosin activation and DP assembly.

A. In the control situation (dotted lines), our data demonstrate that Rho activation (green line) is induced 5 min after cell-cell contact, peaks at 15min and is downregulated. Myosin activity (red line) is induced and becomes level around 30min-1h. Previously it was shown that DP border accumulation (black line) exhibits biphasic pattern correlating to initial membrane localization and then a second wave due to translocation of precursors to nascent junctions (Godsel et al., 2005)

B. When PKP2 is deficient (solid lines), Rho activation (green) is blunted relative to control but is upregulated again at steady state conditions. Myosin activity is potentiated (red) and DP assembly is impaired (black).

Figure 4.15

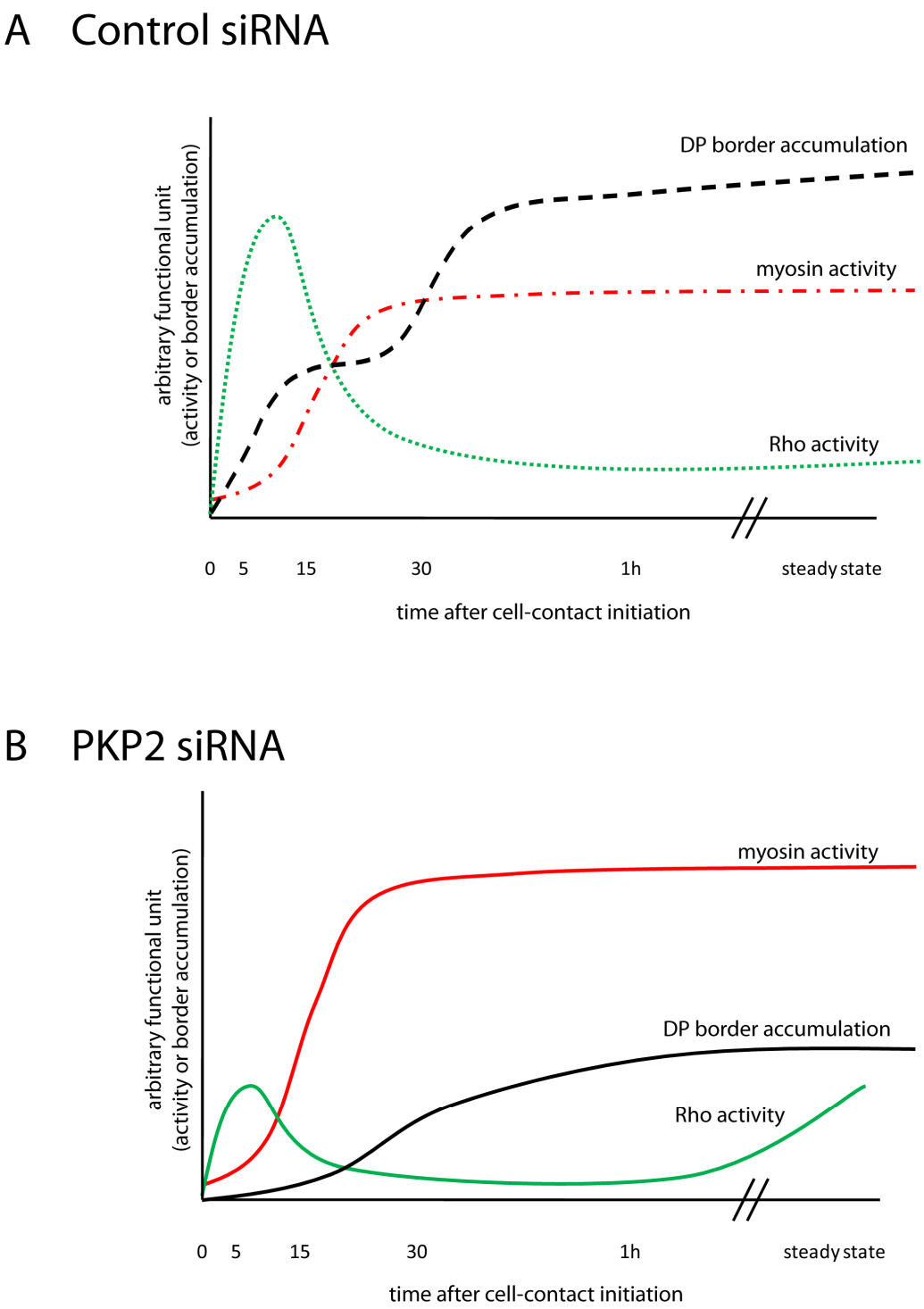

A Control siRNA
DP border accumulation
myosin activity
Rho activity
arbitrary functional unit
(activity or border accumulation)
0 5 15 30 1h steady state
time after cell-contact initiation
B PKP2 siRNA
myosin activity
DP border accumulation
Rho activity
arbitrary functional unit
(activity or border accumulation)
0 5 15 30 1h steady state
time after cell-contact initiation

Figure 4.16 Potential model of the role of PKP2 in actin and DP dynamics during junction assembly

A. Early during junction assembly (15min) PKP2 localizes both to cell-cell contacts where it colocalizes with cadherins already present and to DP particles in the cytoplasm. Radial actin bundles are present and Rho localizes to the membrane. Rho and myosin are maximally activated by this point which stimulates the breakdown of radial actin fibers and the contraction of the circumferential actin ring. PKC recruited to DP precursors by PKP2 may also stimulate myosin activation and contraction of the actin ring.

B. Later during junction assembly (45min-1h), circumferential actin is contracted towards the membrane, radial actin fibers are lost and Rho activity is then downregulated. DP accumulates at borders and the cytoplasm is relatively clear of DP particles.

Figure 4.16 (A)

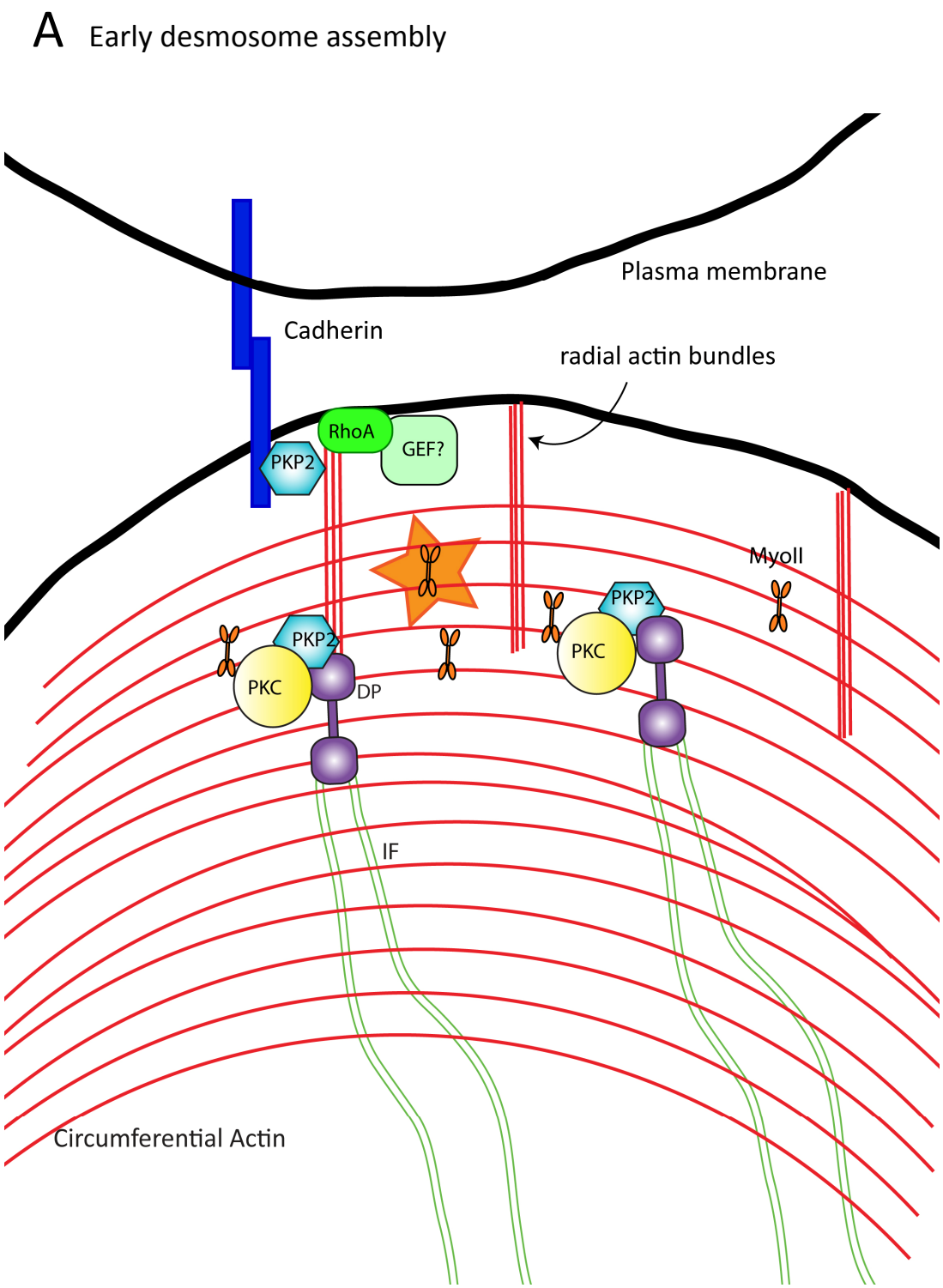

A Early desmosome assembly
Plasma membrane
Cadherin
radial actin bundles
RhoA
GEF?
PKP2
MyoII
PKP2
PKC
PKP2
PKC
DP
IF
Circumferential Actin

Figure 4.16 (B)

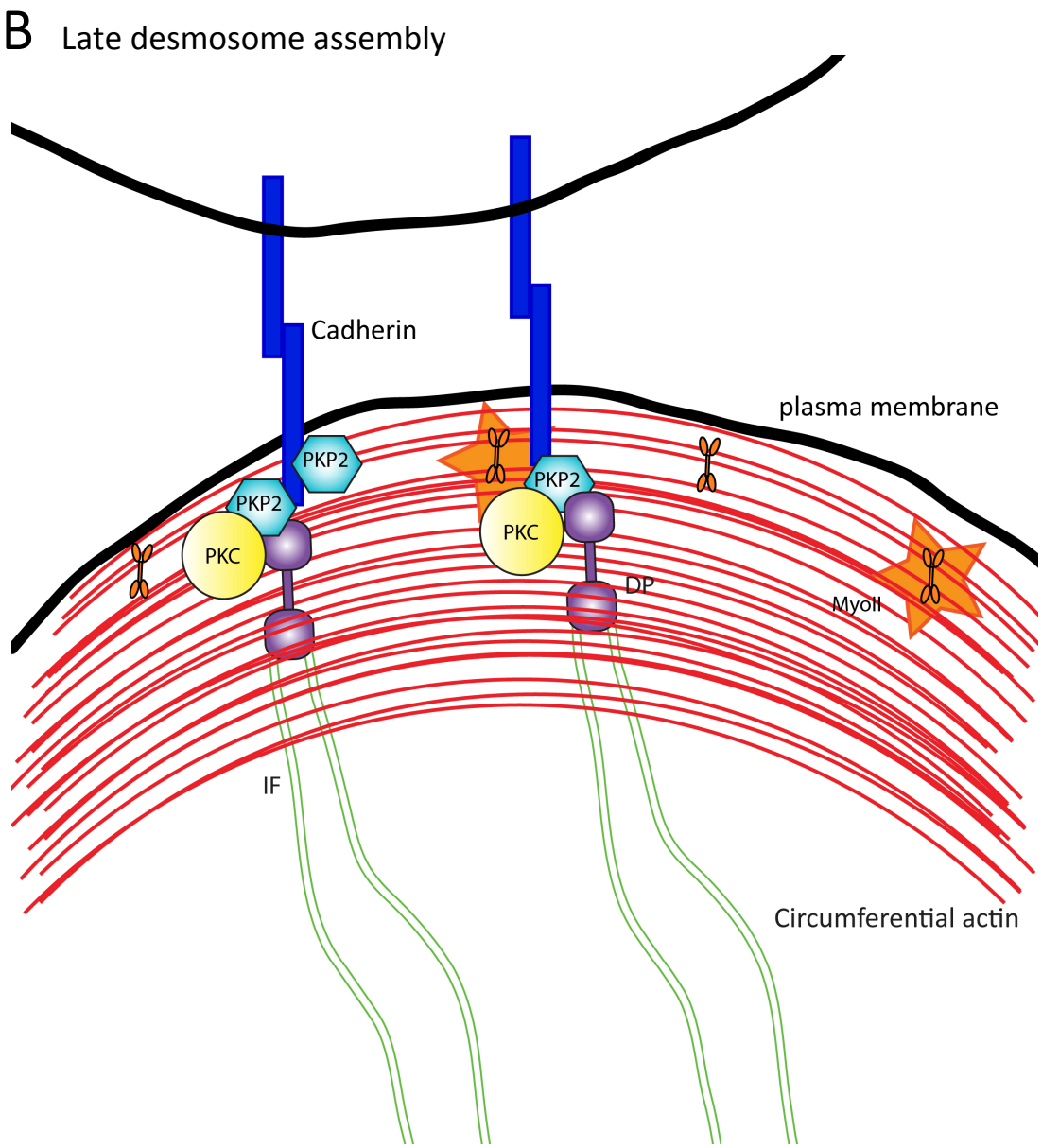

B Late desmosome assembly
Cadherin
plasma membrane
PKP2
PKP2
PKP2
PKC
PKC
DP
MyoII
IF
Circumferential actin

Figure 4.17 Potential model of cellular events during PKP2 deficiency: inappropriate actin contraction and immature junction assembly

A (early assembly); B (late assembly). Loss of PKP2 leads to aberrant PKC signaling, loss of Rho signaling and membrane localization, hyper-contraction of circumferential actin but no loss of radial actin fibers. DP-IF binding is enhanced and DP particles are sequestered within the cytoplasm behind the circumferential actin ring.

Figure 4.17 (A)

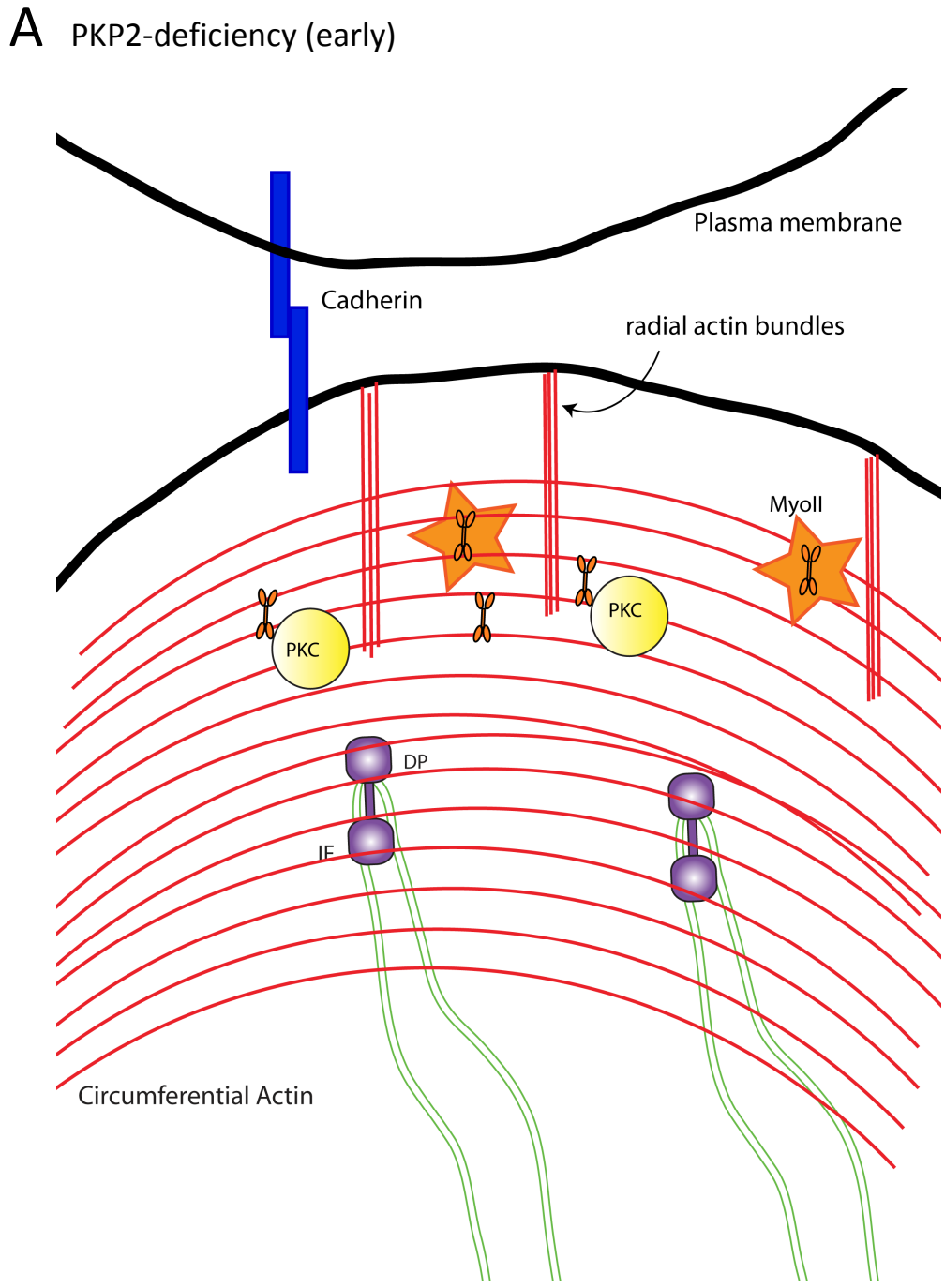

A PKP2-deficiency (early)
Plasma membrane
Cadherin
radial actin bundles
MyoII
PKC
PKC
DP
IF
Circumferential Actin

Figure 4.17 (B)

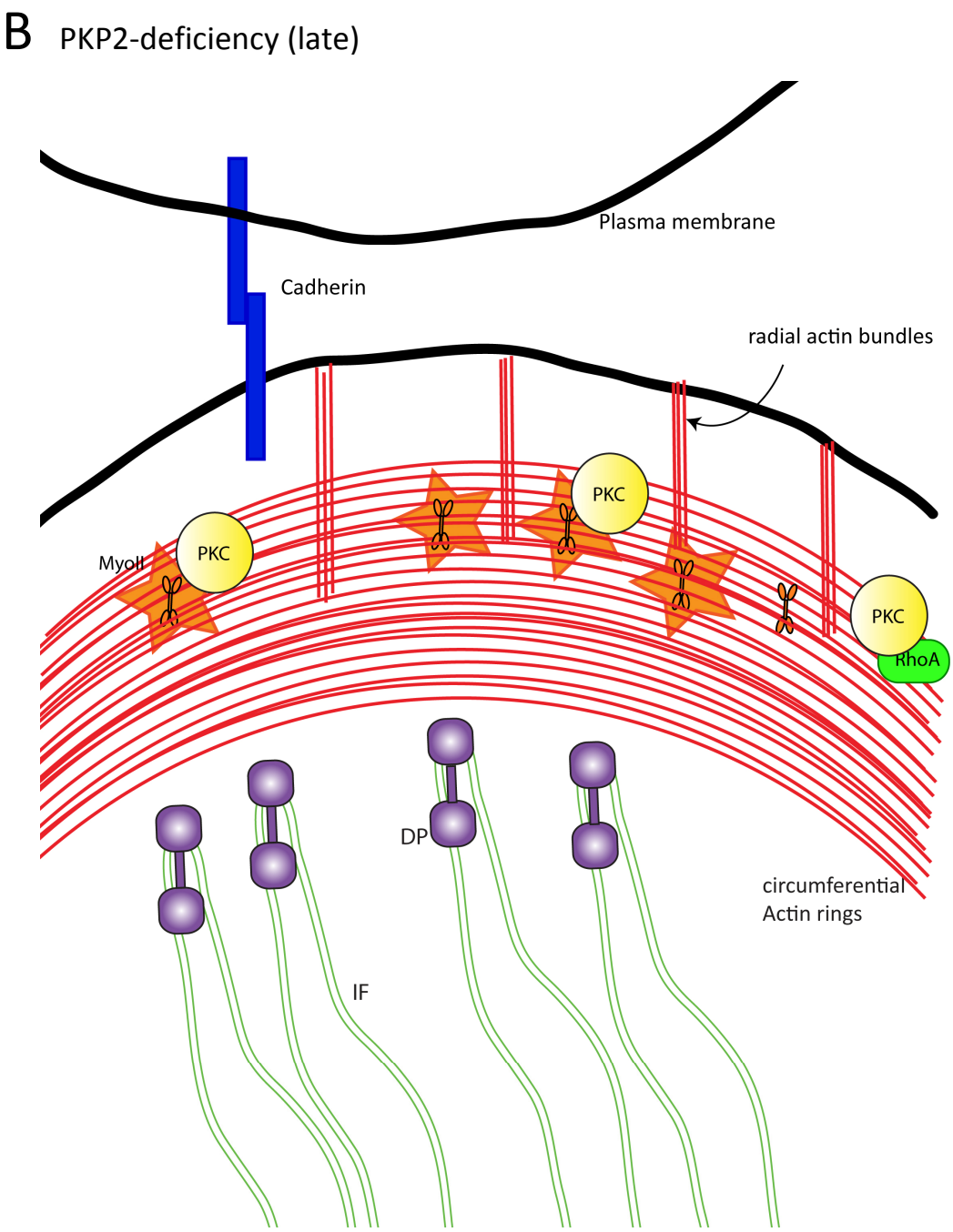
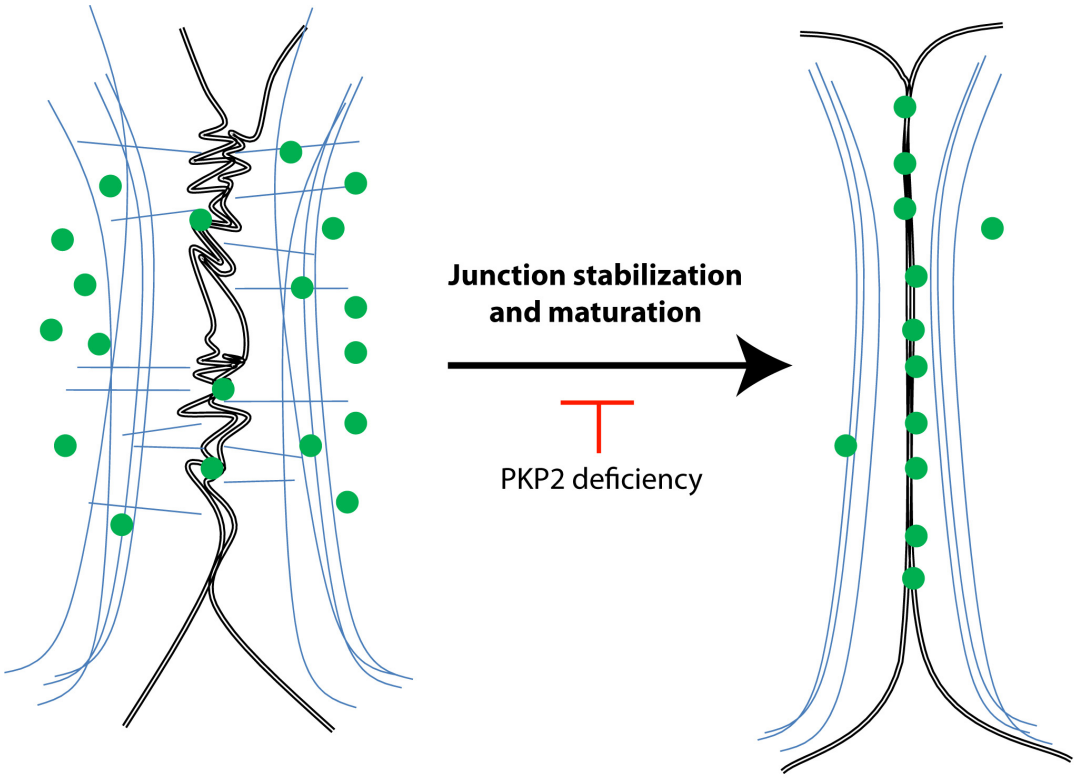

B PKP2-deficiency (late)
Plasma membrane
Cadherin
radial actin bundles
PKC
PKC
MyoII
PKC
RhoA
DP
IF
circumferential
Actin rings

Figure 4.18. General role of PKP2 on junction assembly and maturation

Loss of PKP2 impairs junction assembly and maturation by prohibiting proper actin dynamics.

Figure 4.18



Nascent/ immature junctions:
-radial actin fibers
-broad circumferential actin rings
- wavy borders
-membrane projections
-mostly cytoplasmic DP particles

Mature junctions:
-loss of radial fibers
-contracted circumferential actin
(towards membrane)
-smoother membrane contacts
-fewer DP cytoplasmic particles
-DP membrane accumulation

CHAPTER V

Plakophilin 2 coordinates desmosomal plaque and membrane components during junction assembly

Introduction

The assembly of desmosome membrane components versus that of plaque components is thought to occur in separate subcellular compartments. In previous chapters I have demonstrated a role for PKP2 in the regulation of the desmosomal plaque compartment by serving as a scaffold for modulating signaling pathways involved in this process. This chapter focuses on the role of PKP2 in the regulation of desmosomal cadherin assembly and stabilization.

Very little is known about the role of desmosomal armadillo proteins in junction assembly. In reconstitution studies, cells that do not express all desmosome components can be induced to form desmosomes if the correct components are transiently transfected. Both PKP2 and PG are required for proper desmosome reconstitution when co-transfected with DP in HT1080 fibrosarcoma cells that express endogenous DSG. PG and DP transfection without PKP2 led to formation of occasional desmosomes that appeared to contain small interruptions within the desmosome structure (Koeser et al., 2003). Desmosome reconstitution studies have also been performed in Cos cells to examine the assembly properties of co-transfected PKP1, PG and DP. In these studies, it was demonstrated biochemically that PKP1 competed with PG for DP binding; however, both armadillo proteins were necessary for proper junction accumulation of DP and formation of the characteristic desmosome-like ultrastructure (Bornslaeger et al., 2001). PKPs and PG are important for the final accumulation of DP and cadherins at cell-cell borders, but the

dynamic trajectories of these two compartments (plaque and membrane-associated) prior to reaching the membrane are not well understood.

Previous studies suggest that the desmosomal membrane compartment and plaque compartment assemble via separate mechanisms but both components require stable cell contact for surface stabilization (Pasdar et al., 1991; Pasdar and Nelson, 1988a; Pasdar and Nelson, 1988b; Pasdar and Nelson, 1989; Penn et al., 1987a; Penn et al., 1989). Desmosomal cadherins are trans-membrane proteins whose translocation to the cell surface can be blocked by 19°C incubation (Pasdar and Nelson, 1989), which halts membrane transport of glycoproteins (Matlin and Simons, 1983). Preliminary data demonstrate that DSG is translocated to the membrane along microtubule tracks (E. Amargo and K. Green). However, members of the desmosome core, DP and PKP2, form assembly-competent precursors in the cytoplasm that are not membrane-associated (Godsel et al., 2005). These plaque precursors translocate to cell-cell junctions in an actin-dependent manner (Godsel et al., 2005), and PKP2 and PKC-dependent modulation of their affinity for IF determines their efficiency of assembly (Bass-Zubek et al., 2008; Godsel et al., 2005). These two desmosomal components (core and membrane) do not appear to come together until they are stabilized at the plasma membrane (Pasdar et al., 1991). However, particles that contain both membrane and plaque components together have been observed in the cytoplasm in our studies, but these particles move in a retrograde manner and are likely engulfed desmosomes rather than desmosomal precursors (Godsel et al., 2005).

While characterizing the effect of siRNA-mediated knockdown of PKP2 on desmosomal plaque assembly (described in chapters III and IV), I observed a defect in

desmosomal cadherin assembly. I noted an expansion in the number of cytoplasmic particles that contained both DP and DSG together when PKP2 was knocked down; however, whether these particles are due to new biosynthesis or from internalized desmosomes is not yet clear. The following experimental results are very preliminary but support a potential role for PKP2 in the segregation of desmosomal components during their assembly.

Results

Desmosomal cadherin assembly is dramatically impaired in PKP2-deficient cells

Desmosomal cadherins require cell-cell contact and high extracellular calcium concentration in order to be stabilized and incorporated into junctions ((Pasdar and Nelson, 1989; Penn et al., 1987a)). If cadherins are not stabilized at the cell surface, they will be internalized and subsequently degraded (Kartenbeck et al., 1991; Kartenbeck et al., 1982; Mattey and Garrod, 1986b). Since PKP2 is required for desmosome plaque assembly, and the plaque is critical for IF-linkage to the complex and stabilization of the junction, it was hypothesized that PKP2 is also required for desmosome cadherin assembly and stabilization.

To test whether cadherins assemble properly in the absence of PKP2, siRNAs against PKP2 or nontargeting control (NT) were transfected into SCC9 cells that were then subjected to calcium switch. Assembly of endogenous DSC2 (Figure 5.1), DSG2 (Figure 5.2), and GFP-DSG2 (data not shown) was impaired at all time points. However, total levels of DSG and DSCs were not reduced in PKP2 siRNA transfected cells (Chapter III, Fig 3.4). Based on these data, it was postulated that loss of desmosomal cadherin localization at borders during PKP2-knockdown is not due to simple destabilization and degradation but rather to a defect in cadherin assembly.

Unlike DSG/DSC assembly, PG junction assembly is minimally affected by loss of PKP2

It has been suggested that PG is trafficked with DSG during junction assembly (Burdett and Sullivan, 2002). Since such a severe defect in DSC/DSG assembly is observed

in PKP2-deficient cells, it was hypothesized that PG would also be impaired under the same conditions. To test whether PG assembly is affected by PKP2 knockdown, SCC9 cells transfected with NT or PKP2 siRNA were subjected to calcium switch and immunostained for PKP2 and PG. PG accumulation at junctions was very minimally impaired in PKP2-deficient cells. Robust PG border accumulation occurred in PKP2-deficient cells, although some PG-containing cell-cell borders were more convoluted and less linear in the PKP2 knockdown condition (Figure 5.3). These borders are probably not as mature as in the control cells and exhibit fingerlike-projections on top of adjacent cells; however, the defect in PG assembly was not as severe as the cadherin assembly defect (Figures 5.1 and 5.2). PG is known to bind to classic cadherins (Hinck et al., 1994; Piepenhagen and Nelson, 1993) and could be trafficked to or stabilized with E-cadherin at adherens junctions during PKP2 knockdown. The fact that PG membrane pattern mirrors that of E-cadherin (See chapter III) during PKP2 knockdown is consistent with the idea that PG is recruited to cell-cell borders by associating with adherens junctions, which are minimally affected by loss of PKP2.

Mild microtubule paucity in PKP2-deficient cells

Recent studies on desmosomal cadherin trafficking suggest that desmosomal cadherins translocate to nascent junctions in a microtubule (MT)-dependent manner (E. Amargo, K. Green, unpublished observations; (Pasdar et al., 1991)) although desmosomal plaque assembly is MT independent (Godsel et al., 2005). Classical cadherin assembly occurs by MT-based translocation via motors such as kinesin (Chen et al., 2003; Ligon and Holzbaur, 2007; Mary et al., 2002; Stehbens et al., 2006). One possible mechanism for

desmosomal cadherin assembly impairment is the absence of functional microtubule tracks, defective MT dynamics, altered post translational MT modification, or defective MT-membrane anchoring. Therefore, SCC9 cells expressing PKP2 or control siRNAs were stained for tubulin. MTs were not disassembled but there was a relative paucity of cortical MTs and reduced MT-membrane attachment in cells deficient in PKP2 (Figure 5.4). Interestingly, DP can regulate microtubule border anchoring by recruitment of a MT organizing protein, ninein (Lechler and Fuchs, 2007). It is possible that since PKP2 is required for proper DP assembly (See chapter III), defective DP border accumulation results in impaired MT membrane anchorage. These data do not rule out the potential uncoupling of desmosomal cadherins from their microtubule-based motor or inappropriate MT activation, and future studies would include live imaging of DSG trafficking along MTs during PKP2 knockdown.

PKP3 cannot compensate for loss of PKP2 and subcellular localization mirrors that of DP

To further characterize the effect on desmosomal plaque proteins, cells expressing PKP2 siRNA or control were stained for DP and PKP3. Previously, we showed that PKP3 is not extensively colocalized with DP in assembly-competent plaque precursors (Godsel et al., 2005). Although PKP3 protein expression was not upregulated during PKP2 knockdown (Chapter III, Figure 3.4), PKP3 border accumulation was impaired in PKP2-deficient cells (Figure 5.5). Moreover, PKP3 and DP exhibited extensive colocalization at remaining borders during PKP2 knockdown (Figure 5.5B, arrows), and in cytoplasmic particles during PKP2 knockdown (Figure 5.5Bb'). These data taken together led to the hypothesis that

PKP2 could be playing a role in junction component segregation during assembly. In the condition of PKP2-deficiency, DP and other components that do not normally colocalize during assembly are allowed to come together prematurely either in the cytoplasm, at remaining junctions or at other non-junctional ectopic locations.

PKP2 as potential regulator of desmosomal protein segregation during assembly?

Desmosomal cadherins and plaque components exhibit differential assembly kinetics based on biochemical studies performed in the late 1980's-early 1990's (Pasdar et al., 1991; Pasdar and Nelson, 1988a; Pasdar and Nelson, 1988b; Pasdar and Nelson, 1989; Penn et al., 1987a; Penn et al., 1987b). Our recent work demonstrated that like PG and PKP3, desmosomal cadherins do not colocalize with DP in cytoplasmic plaque precursors while PKP2 is highly concentrated in nearly all plaque precursor particles in both SCC9 and A431 cells (Godsel et al., 2005). Since I observed PKP3 and DP colocalized both at remaining sites of cell-cell contact and in the cytoplasm in Figure 5.5, I sought to determine if colocalization of desmosomal cadherins and DP is enhanced in PKP2-deficient cells. DP-GFP expressing SCC9 and A431 cells (not shown) or parental SCC68 cells (not shown) were transfected with siRNA and subjected to calcium switch. Confocal microscopic analysis revealed that DSG2 and DP-GFP (or endogenous DP and DSG2) colocalized in cytoplasmic particles more often when PKP2 was knocked down than in control cells (Figure 5.6). These colocalized particles nearly always aligned with keratin IF, whereas only a subpopulation of DSG particles (without DP-GFP) aligned with keratin IF. Similarly, enhancement of PG colocalization with DG2 and DP-GFP in cytoplasmic particles was also observed in PKP2

deficient cells (Figure 5.7). These results suggest that there may is a merging of previously-separate plaque and membrane compartments in the absence of PKP2. One possible explanation for this merging could be that PKP2 deficiency induces premature junction component interaction at ectopic locations. In other words, PKP2 deficiency could lead to the formation of half desmosomes at the dorsal membrane surface. Another possible explanation could be that these particles represent engulfed desmosomes due to reduced stability in the absence of PKP2.

DSG internalization is enhanced during PKP2 knockdown

In our previous studies, retrospective analysis of DP-GFP particles during live cell imaging demonstrated that DG2 and PG sometimes do colocalize with DP-GFP particles, but often these particles were moving in a retrograde fashion or were pre-existing (prior to cell-cell contact initiation) (Godsel et al., 2005). These inward-moving particles were likely internalized whole or half-desmosomes rather than newly synthesized assembling precursors. Internalization of half desmosomes or desmosome components is a continual process until junctions are stabilized by cell-cell contact (Demlehner et al., 1995; Mattey and Garrod, 1986b).

Although desmosomal protein levels are not reduced during PKP2 knockdown, it is possible that desmosomes are destabilized by PKP2 knockdown which leads to their internalization. Results from a preliminary biotinylation internalization experiment in which SCC68 cells expressing PKP2- or control siRNA were assessed for internalized levels of DSG2 suggest that internalization of this cadherin is slightly enhanced during PKP2

knockdown (B. Desai, data not shown). Whether this increase in cadherin internalization is due to normal turnover from reduced junction stabilization or due to an "active" internalization process stimulated by some signal downstream of PKP2 remains to be determined. However, this biotinylated pool of cadherins represents the detergent soluble compartment and is not likely to represent a pool of engulfed junctions.

E-cadherin does not colocalize with DP and DG2 in cytoplasmic particles during PKP2 knockdown

While E-cadherin and DSG exhibit very different membrane patterns in PKP2 deficient cells (DSG membrane localization is dramatically impaired; E-cadherin membrane localization is minimally affected), neither E-cadherin nor DSG protein levels change under these conditions (Chapter III, data not shown). If generalized junction engulfment was occurring, one would expect to see some colocalization of E-cadherin with these DSG and DP containing particles. To determine if E-cadherin and DSG2 co-internalization during PKP2 knockdown occurs, a colocalization experiment was performed. SCC9 cells expressing DP-GFP were transfected with PKP2 or control siRNAs and stained for E-cadherin and DSG2 or E-cadherin and a-catenin. E-cadherin was not present in any DP-DG2 cytoplasmic particles (Figure 5.8) and α-catenin mirrors E-cadherin patterns (data not shown). These results support the idea that PKP2-deficiency leads to the formation of DP-DSG complexes that exclude E-cadherin and therefore are probably not due to nonspecific engulfment of junctions.

In an experiment in which only extracellular DSG2 was stained (cells were fixed in paraformaldehyde and then stained with an extracellular DSG2 antibody without prior permeabilization), these particles were still observed to contain colocalized DP-GFP and DSG2 (data not shown). This supports the possibility that these particles are probably not internalized desmosomes and that they may represent half desmosomes localized at the dorsal cell surface.

Discussion

In this chapter, I presented preliminary experiments demonstrating that DP, DG and Keratin, PG and PKP3 containing particles are more abundant in PKP2-deficient cells relative to controls. These particles do not contain α-catenin or E-cadherin and appear to be localized to the dorsal cell surface, whether these particles are biosynthetically derived or derived from existing junctions remains to be determined. EM studies are currently underway to begin to characterize their location and to ultrastructure. Live-cell imaging using multiple-fluorophore tags for DP and DSG during PKP2 KD will be necessary to determine when and where these complexes come together or whether they were pre-existing internalized desmosomes. Metabolic labeling and/or live SNAP-tag-based expression studies or fluorophore-conjugated antibody microinjection studies will be necessary to determine if there is an effect of PKP2-deifciency on biosynthetic processing and trafficking or turnover of DSGs during assembly.

Hypothesis 1: Role of PKP2 in desmosome component segregation

Here we present data that support a potential role for PKP2 in regulating junction component segregation during their assembly. Loss of PKP2 leads to enhanced colocalization of DP with DSG, IF, PG, and/or PKP3. In this scenario, perhaps PKP2 normally interacts with DP in non-membrane associated particles and competes with PG for DP binding in the cytoplasm. Although a competition between PG and PKP2 for DP association has not been demonstrated, PKP1 has been previously shown to compete with PG

for DP binding (Bornslaeger et al., 2001). In this scenario, when PKP2 is deficient, PG (associated with DSG) can then interact with DP that is stuck on IF (as demonstrated in Chapter III) due to loss of PKC recruitment. The IF bundles run along the plasma membrane and throughout the cell, thereby serving as ectopic sites of premature desmosome formation prior to arrival at the sites of cell-cell contact.

Hypothesis 2: PKP2 knockdown leads to internalized desmosomes and/or defective recycling

It is known that when desmosomes are not stabilized by cell-cell contact, their components are turned over (Mattey and Garrod, 1986b; Pasdar and Nelson, 1988a; Pasdar and Nelson, 1989; Penn et al., 1987a; Penn et al., 1989). Cells cultured in LCM for several days contain dots that have DP, Dg and colocalize with keratin IF. By EM, these are half desmosomes in vesicles that contain DP and DSG2 (Demlehner et al., 1995; Duden and Franke, 1988). It is possible that the particles I observed in PKP2-deficient cells are similar to those seen in cells cultured in LCM and are endocytosed half desmosomes that are stuck along IF due to the enhanced DP-IF association seen during PKP2 knockdown (Chapter III).

PKP2 serves as a scaffold for signaling proteins such as PKC and perhaps Rho GTPase signaling components (Chapters III and IV). Outside of a direct structural stabilization of desmosomes, PKP2 could also be modulating other signaling pathways that alter the stability of these junctional components. Generalized phosphorylation and EGFR-dependent signaling is known to lead to defective desmosome adhesion and enhanced desmosome component internalization (Amino et al., 1996; Gaudry et al., 2001; Lorch et al., 2004; Miravet et al., 2003; Yin et al., 2005a). PKC activation has been shown to enhance E-

cadherin endocytosis and impair recycling in polarized epithelial cells (Le et al., 2002). We previously observed that PKC hyperphosphorylated some of its substrates when PKP2 was knocked down. It is possible that aberrant PKC signaling due to PKP2 deficiency could regulate the trafficking of desmosomal proteins. Identifying new kinase or scaffold binding partners for PKP2 may provide insight into novel roles of this armadillo protein in modulating desmosome stability and internalization.

Hypothesis 3: PKP2 deficiency impairs biosynthetic trafficking of cadherins

MT density was reduced in PKP2-deficient cells. If MTs are required for the anterograde trafficking of newly synthesized cadherins or for the internalization of surface desmosomes, the dynamics of the MTs may be altered such that these normal functions would be impaired. Interestingly, Rho small GTPases have been shown to modulate and be modulated by MT dynamics (Liu et al., 1998; Wittmann and Waterman-Storer, 2001). If PKP2-deficiency dramatically alters Rho subcellular localization and activity (Chapter IV), then this could play a role in the modulation of MT dynamics. Future studies focusing on obtaining live MT dynamics relative to desmosomal proteins during assembly as well as investigations of the effect of PKP2-deficiency on MT post-translational modification may be beneficial.

Taken together, these preliminary results suggest a potentially important role that PKP2 may play in the coordination of desmosomal cadherin and plaque compartments during junction assembly. Whether PKP2 determines segregation of these desmosomal components,

controls active endocytic or recycling dynamics, mediates anterograde biosynthetic cadherin trafficking, or structurally stabilizes desmosomes at junctions remains to be determined. This chapter provides further evidence that PKP2 may have the power to influence broad signaling pathways and cellular functions during junction assembly and cell homeostasis.

Figures

Figure 5.1 DSC2 assembly is impaired in PKP2-deficient cells.

Calcium switch of SCC9 cells expressing control NT or PKP2 siRNAs and immunostained for DP and DSC2. At 6h and even at steady state (NCM), DSC2 border accumulation is dramatically reduced in PKP2 knockdown cells compared to control cells.

Figure 5.1

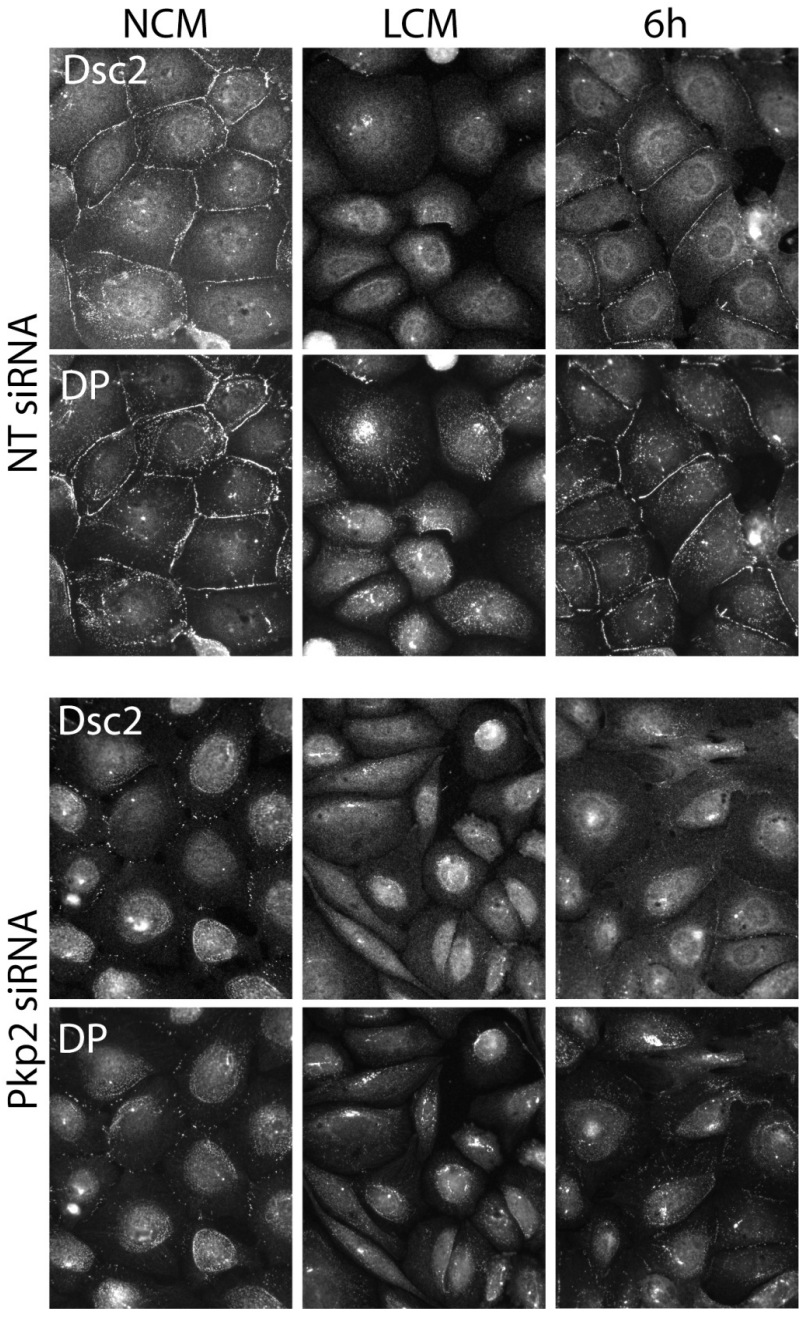


Figure 5.2 DSG2 border accumulation is impaired in PKP2 knockdown

Calcium switch of SCC9 cells expressing control NT or PKP2 siRNAs and immunostained for DP and DSG2. At 6h and even at steady state (NCM), DSG2 border accumulation is dramatically reduced in PKP2 knockdown cells compared to control cells. Arrowheads depict regions of cell-cell contact.

Figure 5.2

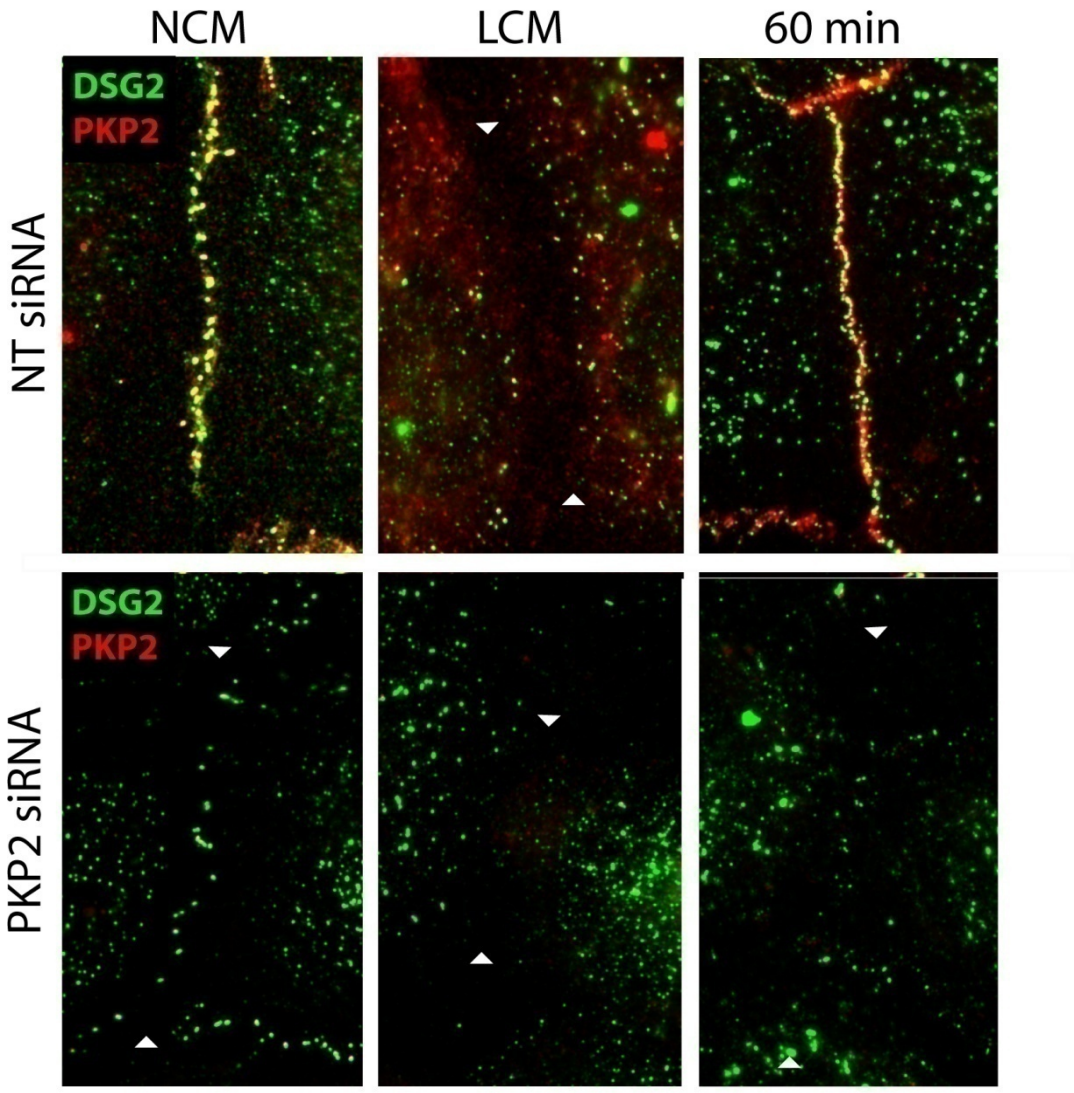


Figure 5.3 PG border accumulation is minimally affected by PKP2 knockdown

Calcium switch of SCC9 cells expressing control NT or PKP2 siRNAs and immunostained for PKP2 and PG. At all time points and at steady state (NCM), PG border accumulation in PKP2 siRNA transfected cells is similar to that in control cells.

Figure 5.3

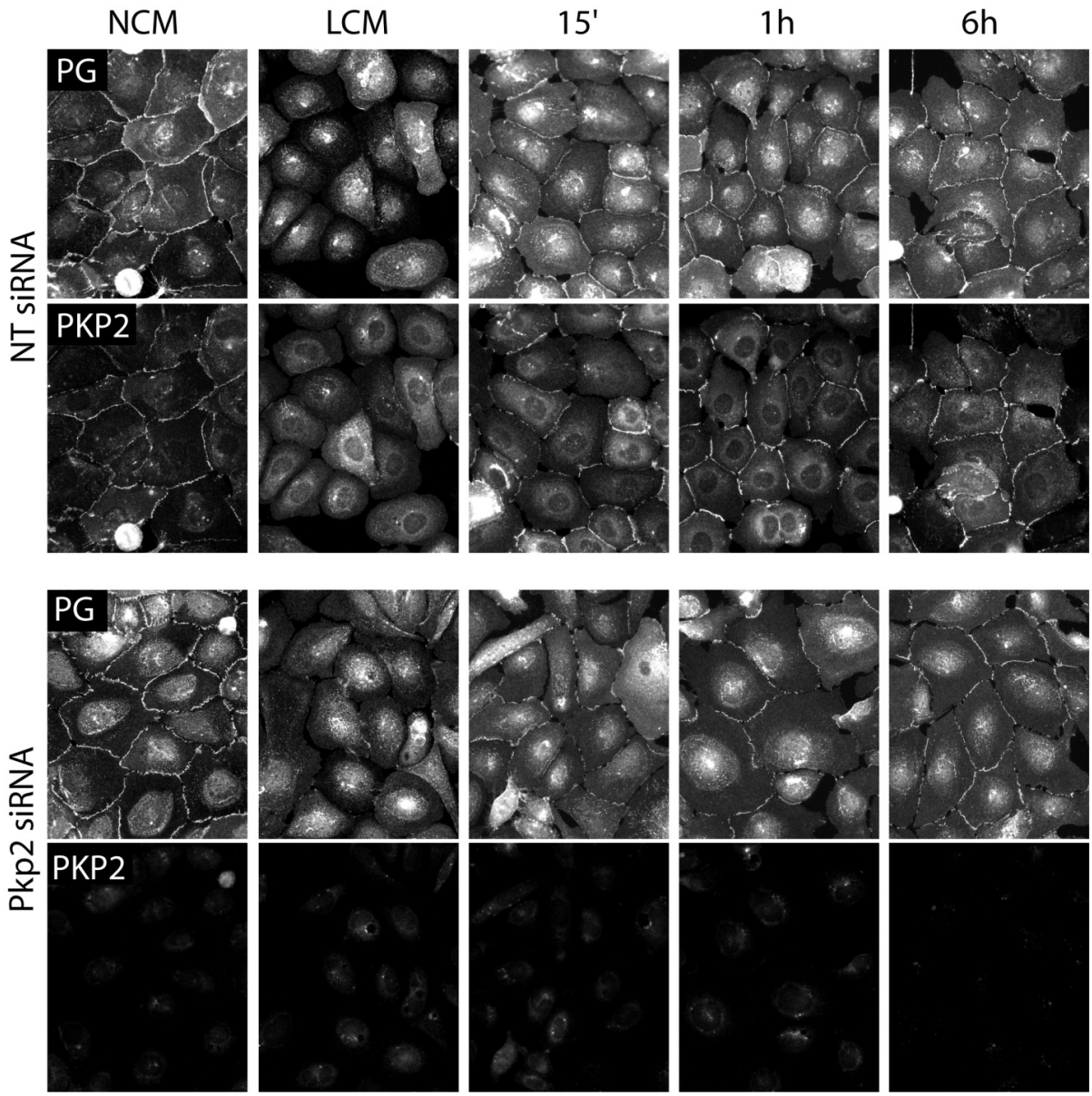


Figure 5.4 MT density is reduced by PKP2 deficiency

SCC9 cells transfected with control siRNA or PKP2 siRNA immunostained for tubulin and DP. MT density appears to be reduced but structure is not affected.

Figure 5.4

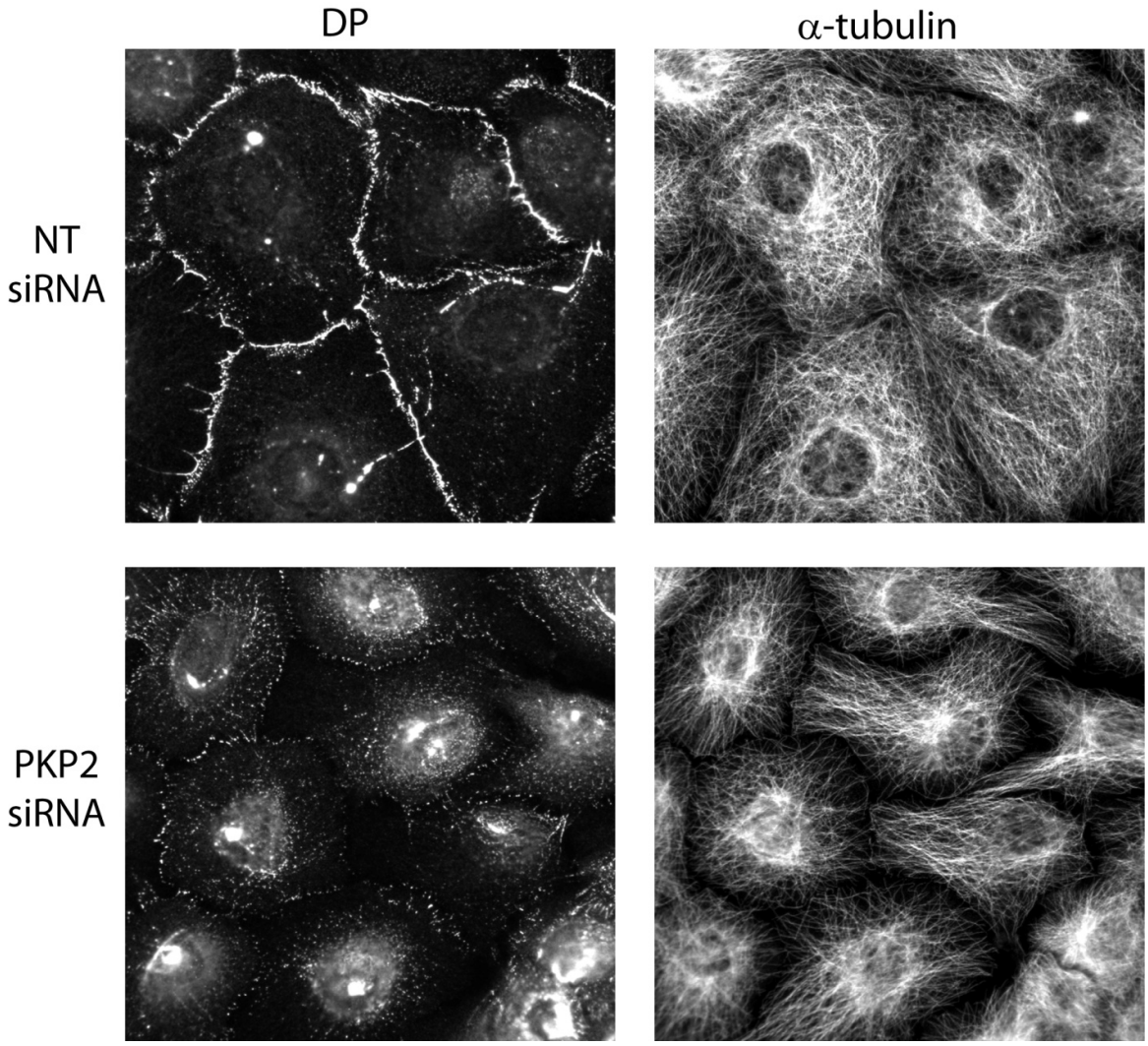


Figure 5.5 PKP3 assembly is impaired in PKP2 deficient cells

SCC9 cells transfected with NT control or PKP2 siRNA were subjected to calcium switch and immunostained for DP and PKP3. PKP3 border accumulation was impaired in PKP2 knockdown cells; however, wherever DP remained localized to borders, PKP3 was also present. A, control siRNA transfected cells at 1h post switch to NCM. Ba' PKP2siRNA transfected cells, 1h. Bb' high magnification of boxed area in Ba'. Remaining DP colocalizes with PKP3.

Figure 5.5

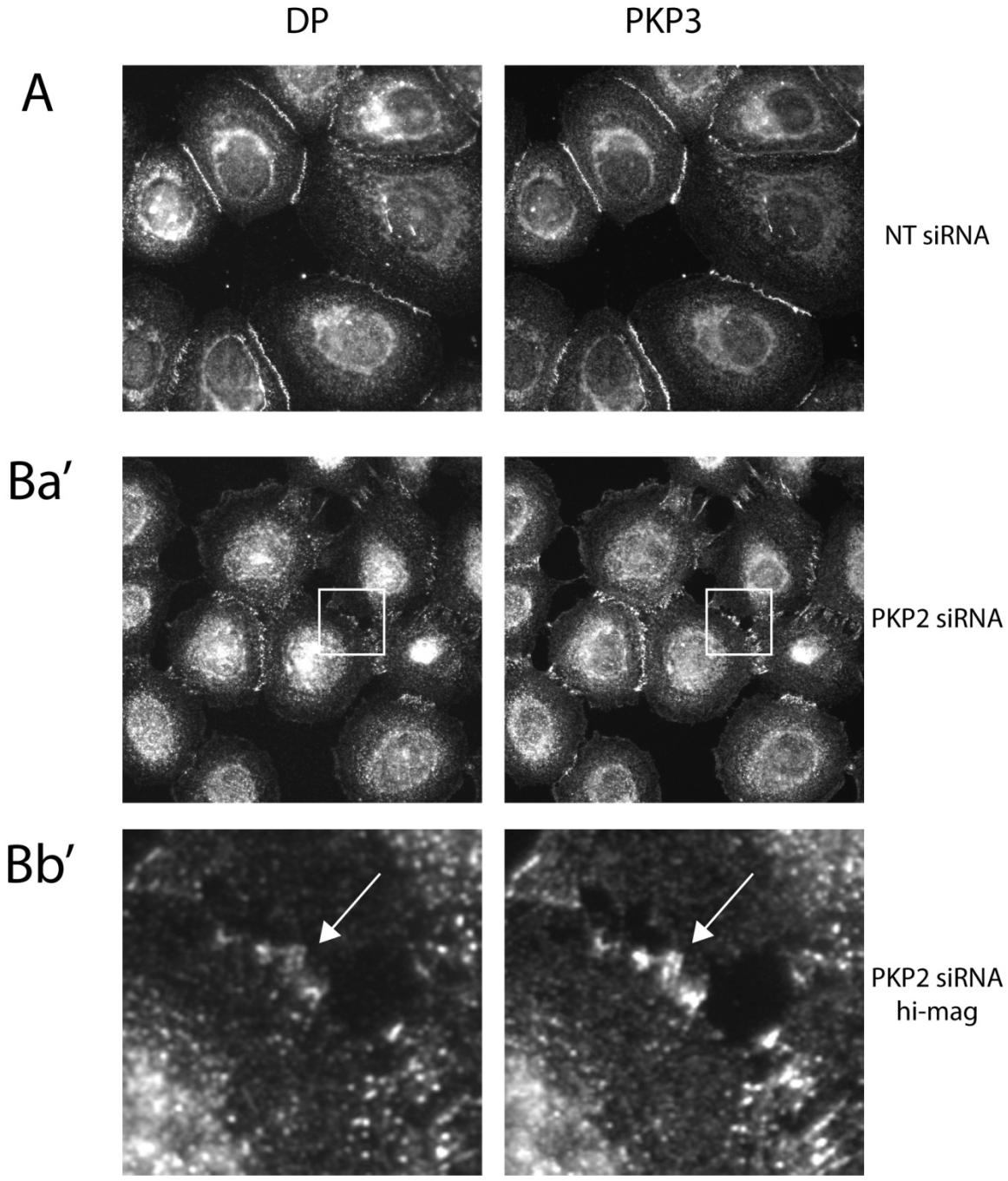


Figure 5.6 DP and Dg2 exhibit enhanced colocalization with keratin in PKP2-deficient cells

A. Triple label confocal micrograph of control NT siRNA-transfected SCC9 cells. Yellow circles indicate examples of dots in which DP and DSG2 are colocalized, which tend to align along keratin. Red circles are examples of DSG2 particles that do not colocalize with DP-GFP. These are not always aligned with keratin IF.

B. PKP2 siRNA transfected cells, as above. In control cells there are more non-colocalized particles than in PKP2 knockdown. Blue circles indicate DP-GFP particles that do not colocalize with DG2. Yellow circles indicate examples of colocalized particles. Nearly all colocalized particles align with keratin.

Bottom panel: percentage of DP particles that also contained DSG2.

Figure 5.6

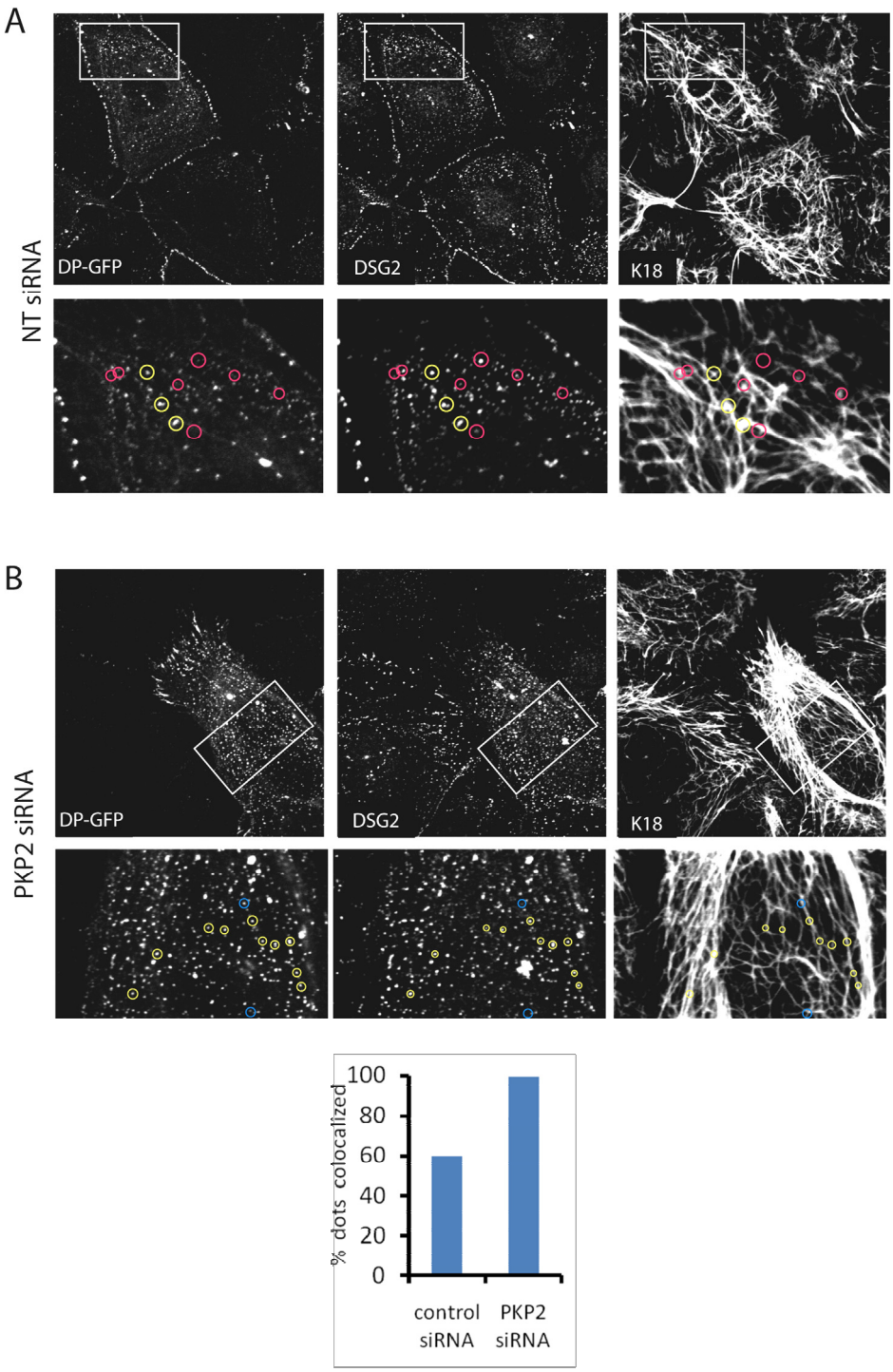

A
NT siRNA
DP-GFP
DSG2
K18
B
PKP2 siRNA
DP-GFP
DSG2
K18
% dots colocalized
100
80
60
40
20
0
control siRNA
PKP2 siRNA

Figure 5.7 Triple colocalization of DP, DG2 and PG is enhanced in PKP2 knockdown cells

SCC9 cells transfected with PKP2 or NT control siRNAs were stained for endogenous DG2, DP and PG. White circles indicate examples of particles positive for all three proteins.

Figure 5.7

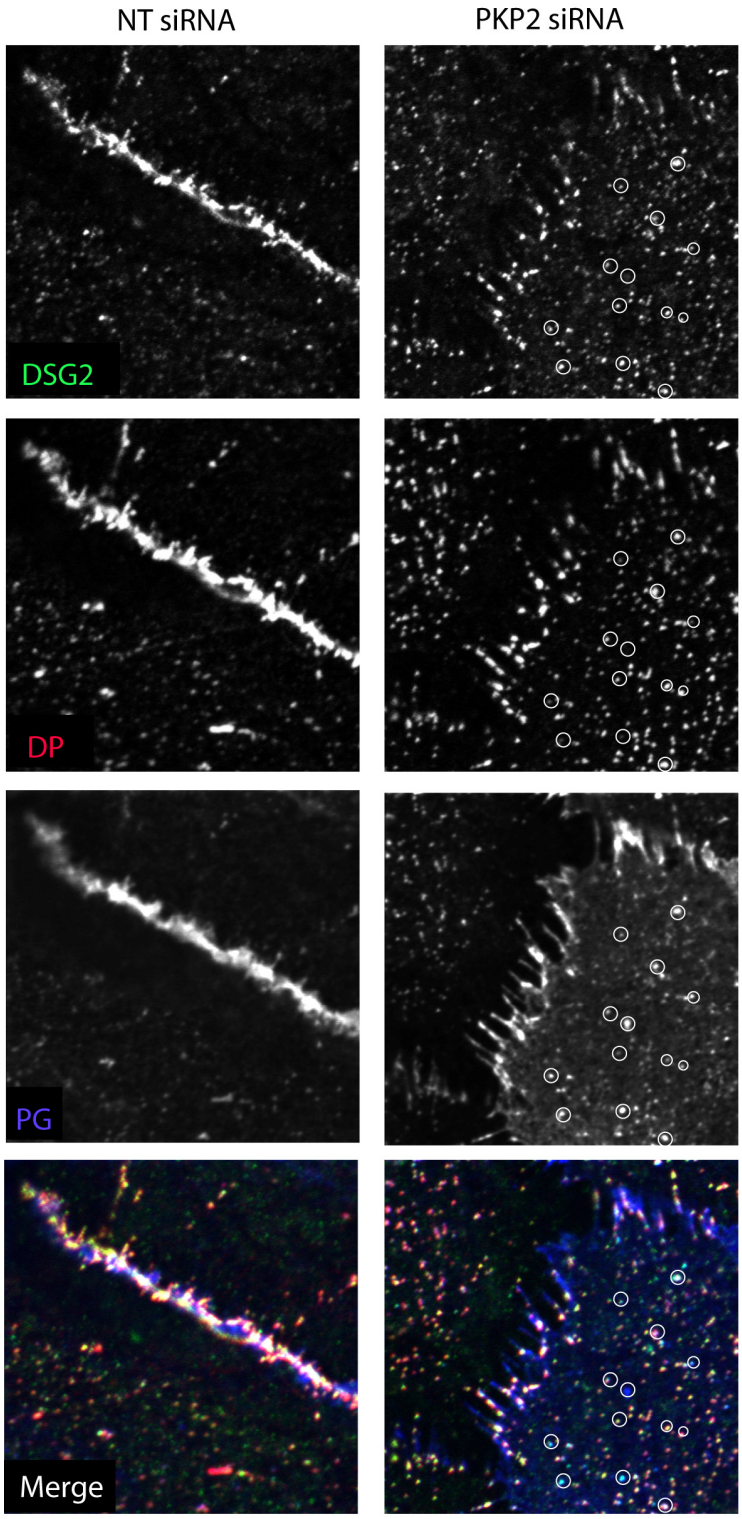
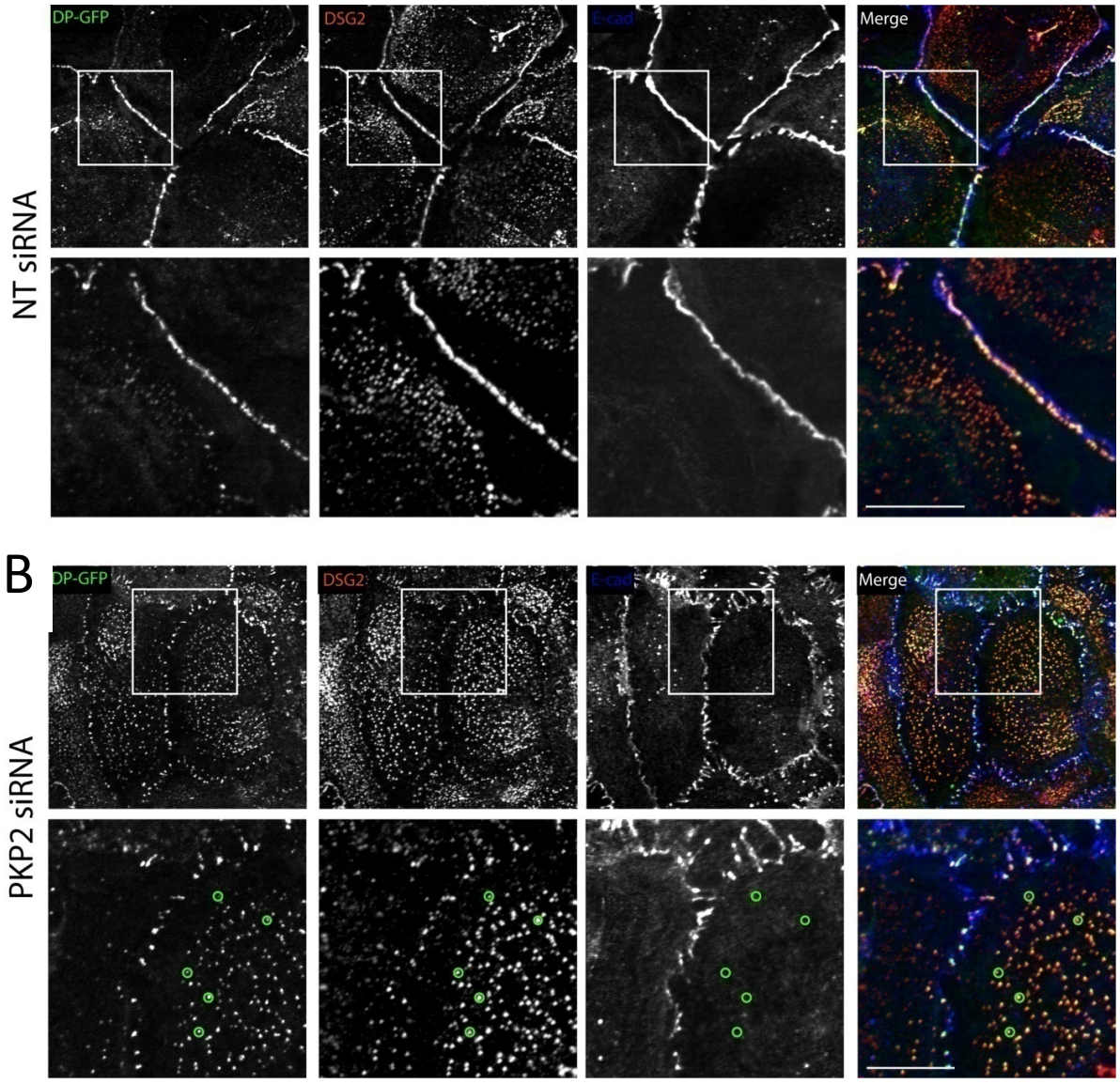


Figure 5.8 DSG/DP particles exclude E-cadherin

Confocal images of DP-GFP SCC9 cells transfected with control NT (A) or PKP2 siRNA (B). Lower panels depict magnification of boxed areas above. Green circles highlight examples of DP and Dg2 containing particles that exclude E-cadherin.

Figure 5.8


DP-GFP
DSG2
E-cad
Merge
NT siRNA
B
DP-GFP
DSG2
E-cad
Merge
PKP2 siRNA

CHAPTER VI

Conclusions and Future Directions

In this dissertation I have discussed several novel actions of PKP2 in the coordination of multiple events that are essential for different aspects of desmosome assembly. This research opens new avenues for our desmosome assembly research program including 1) novel roles for an armadillo family member in the control of serine/threonine kinase specificity for desmosome components with broad cellular consequences, 2) unique functions of PKP2 in the temporal and spatial regulation of actin contractile signaling molecules, and 3) potential function of PKP2 in managing the segregation of two normally separate junction components or in the control of cadherin biosynthesis, trafficking or internalization during desmosome assembly.

PKP2-dependent regulation of PKC signaling is required for DP assembly

In chapter III, I examined the role of PKP2 in the regulation of plaque assembly via PKC signaling. We had previously shown that DP is present in macromolecular cytoplasmic complexes that form and translocate to junctions during cell-contact induced desmosome assembly. The behavior of these precursors depended on the ability of DP to be able to bind to IFs but maintenance of a dynamic IF-interaction was important for its junction assembly. Enhanced binding of DP to IF by mutation of a single serine in the C-terminus retarded its assembly efficiency, suggesting that phosphorylation of this site allows for transient dissociation from IF (Godsel et al., 2005). To understand the underlying mechanisms directing the assembly of these precursors, I sought to characterize their molecular makeup.

It was demonstrated here that PKP2 was the only desmosomal protein that colocalized with nearly 100% of membrane-directed DP particles and that PKP2 was

required for proper DP assembly. Loss of PKP2 unexpectedly led to aberrant accumulation of DP along IF in several cell types, suggesting that PKP2 may be involved in phospho-regulation of DP-IF interactions. I further showed that PKP2 is essential for recruiting PKC to DP complexes and that this recruitment leads to changes in PKC substrate specificity. PKC activation or expression was able to rescue DP assembly in PKP2-deficient cells, supporting a pathway in which PKP2 is an essential upstream regulator of desmosome specific-PKC activity during assembly.

In the future, it will be interesting to determine with high spatial resolution where PKC activity is localized relative to desmosome precursor assembly trajectories. Future studies further characterizing the specific role of PKC (or potentially other ser/thr kinases or phosphatases) in phosphorylation of the DP c-terminus will be critical for understanding the pathogenesis of certain cardiac and skin congenital diseases in which DP-IF interactions are disrupted. Live imaging studies comparing the dynamics of PKP2 and DP during assembly will provide a clearer understanding of how and where PKP2 and DP interact during this process. Finally, the initiation of cell-cell contact induces the formation of these precursors within the cytoplasm, but the nature of the communication between this contact event and the instruction of plaque formation and assembly is unknown. Future studies combining live imaging of PKC activity or other signal-specific indicators with manipulation of cell contact conditions will be crucial for the elucidation of these very early signaling events that drive desmosome assembly.

PKP2 dependent modulation of actin contractility is essential for desmosome assembly

In Chapter IV, the role of actin reorganization and signals involved in the regulation of actin were investigated. Earlier studies showed that actin and IF closely associate during junction assembly (Green et al., 1987) and that actin was required for proper precursor assembly efficiency (Godsel et al., 2005). Since PKPs are related to $p120^{ctn}$ and can induce cell shape change when overexpressed, it was hypothesized that PKP2-dependent modulation of actin organization plays a role in actin-dependent DP assembly.

We demonstrated that actomyosin contractility was necessary for DP assembly, that PKP2 deficiency led to upregulation of myosin activity and impaired the necessary actin reorganization for junction assembly and maturation. Our results suggest a potential role for PKP2 in the recruitment of Rho to the membrane and the regulation of Rho activity. We showed that early Rho activation is required for initiation of assembly but later downregulation is required for proper maturation. While maturation of AJ may be impaired in PKP2-decifienct cells, the assembly defect observed was desmosome-specific. This result is consistent with the notion that PKP2 regulates these actin contractile signaling pathways in a temporally- or spatially- restricted manner. These studies provide evidence supporting a mechanistic basis for the role of PKP2 in actin reorganization and in the management of signaling proteins that are involved in controlling necessary actin reorganization during junction assembly.

Future directions include identifying the contribution of other signaling pathways that contribute to altered actomyosin contractility in response to PKP2 knockdown. Live imaging using Rho activity probes will be used to determine where and when Rho is activated normally, and how Rho activity dynamics change when PKP2 is deficient. Live imaging of

actin and DP will be necessary to study their co-dynamics and the effect of PKP2-deficiency on this process. Biochemical studies on the interaction of PKP2 with Rho or Rho regulatory proteins as well as studies focusing on the role of PKP2 in stimulating or inhibiting actin polymerization dynamics should also be considered.

Chapter V: Role of PKP2 in desmosomal cadherin assembly

In Chapter V, I initiated studies focusing on the role of PKP2 in the coordination of desmosome components. Based on previous studies from our lab as well as other groups, the two major desmosome compartments (membrane and plaque) undergo distinct means of assembly (Godsel et al., 2005; Pasdar et al., 1991). While characterizing the effect of PKP2 siRNA-mediated knockdown on the assembly of the desmosomal plaque, I observed a dramatic defect in desmosomal cadherin assembly (but not adherens junction assembly). I further demonstrated an enhancement in the colocalization of desmosomal plaque component, DP, with desmosomal cadherins and other arm proteins. Normally , these components colocalize only at the membrane and in few retrograde moving particles (Godsel et al., 2005).

These results bring forward new directions for investigating the role of PKP2 in the regulation of cadherin trafficking, endocytosis and coordination with plaque components during assembly. In the future, more in depth studies towards elucidating the biosynthetic course of desmosomal cadherins during PKP2 knockdown, the fate of internalized junctions and the dynamics of these processes will be essential.

In conclusion, these studies suggest that PKP2 plays a central regulatory role in multiple facets of desmosome assembly. PKP2 serves as a scaffold protein to recruit essential signaling proteins to junctional precursors in order to modulate their interactions with IF and promote their assembly. PKP2 modifies actin contractility signaling and organization, which is crucial for desmosome plaque assembly and junction maturation. Finally, PKP2 coordinates the interaction of different junctional components during assembly. These results set the stage for exciting future studies focusing on the role of PKP2 in regulation of desmosome dynamics and disease.

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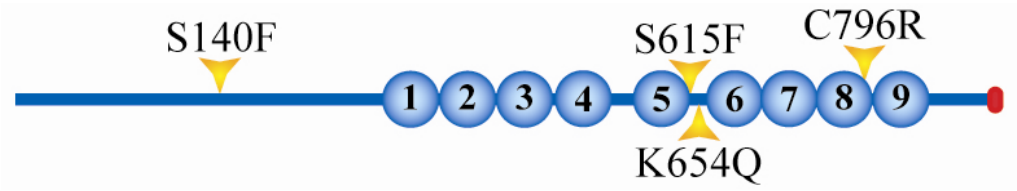
Appendix

1. PKP2 ARVC mutations

In the effort to begin to understand the mechanism by which congenital PKP2-mutations may affect DP assembly, a series of PKP2 constructs containing point substitutions were generated with the intent of examining the effect of their expression on DP assembly. These mutations were described in patients with PKP2-related ARVC (Gerull et al., 2004) and were chosen because they were the few mutations that resulted in amino acid substitution rather than early stop or alternate splicing. Even though I was not able to fully initiate the studies using these constructs, I feel that it is appropriate to document their generation here. These mutants were made with the help of Todd Gocken.

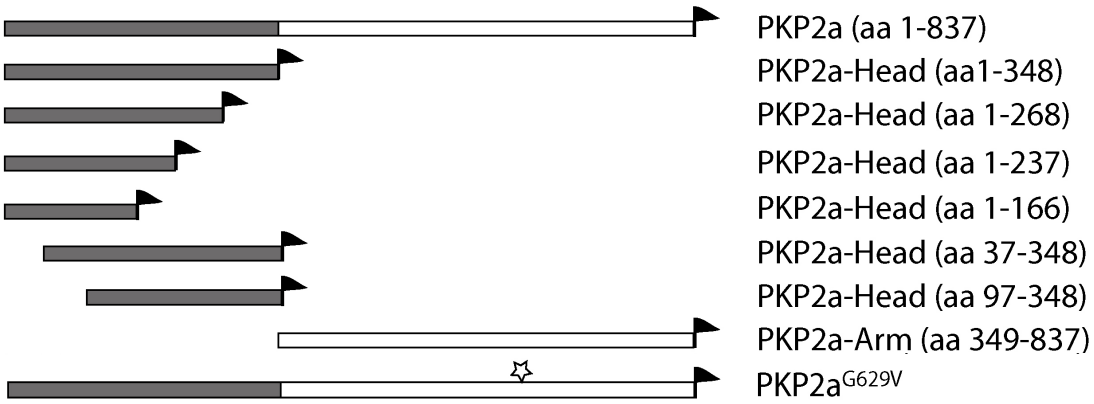


$PKP2^{S140F}$
$PKP2^{S615F}$
$PKP2^{K654Q}$
$PKP2^{C796R}$

2. PKP2 deletion constructs

I generated a number of siRNA refractory silent mutations in PKP2 head deletion constructs with the intention of using these to examine which PKP2 domains are important for modulating DP assembly. These mutations destroy PKP2-siRNA target sites without altering the protein sequence thereby allowing the expression of these constructs on the

background of endogenous PKP2 knockdown. The following constructs all contain two nucleotide pair mutations at all 4 target sites (Nicholas J. Garcia helped with sequencing these constructs):



PKP2a full length pkp2siRNA refractory
p1376 (PKP2a-FLAG with siRNA refractory silent mutations)
p1377 (GFP-PKP2a with siRNA refractory silent mutations)

PKP2^{G629V} full length constructs:
p1312 (eGFP.PKP2^{G629V})

PKP2 Head domain
p1379 (PKP2-Head with siRNA refractory silent mutations)
p1380 (PKP2-H aa 1-268 siRNA refractory)
p1385 (PKP2-H aa 1-237siRNA refractory)
p1386 (PKP2-H aa1-166 siRNA refractory)
p1387 (PKP2-H aa37-348 siRNA refractory)

PKP2 Arm and c-tail
p1388 (PKP2-arm.FLAG pkp2siRNA refractory)

3. Other PKP constructs generated

p1312 GFP-PKP2^{G629V}
PKP2^{G629V} in pAdTrack
PKP2 and PKP2^{G629V} in Ad intermediate vector
PKP2 and PKP2^{G629V} in pECE (intermediate cloning vector)

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EDUCATION

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Northwestern University, Chicago IL	Ph.D.	2008
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SCIENTIFIC EXPERIENCE

1997-1999 Project Aide
Advisor: Dr. Karina F. Meiri, PhD.
Department of Pharmacology, SUNY Upstate Medical University, Syracuse, NY
Cell biology of membrane microdomain associations of Growth Associated Protein-43 (GAP-43) in neuronal growth cones

2000-2001 Undergraduate Senior Independent Researc
Advisor: Dr. Dean R. Tolan, PhD
National Science Foundation–Research Experience for Undergraduates
Department of Biology, Boston University, Boston, MA
Evolution of the vertebrate genome using sequence comparison of Carassius auratus fructose 1,6 bisphosphate aldolase family genes as a model.

2001-2010 N.I.H. Medical Scientist Training Program
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2000 National Science Foundation-Research Experience for Undergraduates award
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2001 Phi Beta Kappa, Boston University Chapter, Boston, MA

2005 American Society for Cell Biology Worthington Pre-doctoral Travel Award -A.B.-Z. was one of seven top-ranked pre-doctoral ASCB travel award recipients

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Robert H. Lurie Comprehensive Cancer Center of Northwestern University, Chicago IL

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SOCIETIES AND MEMBERSHIPS

American Society for Cell Biology

PRESENTATIONS

Poster Presentations

Oct 2004	"Novel Functions of Plakophilin 2 in Regulating Cell Junctions and Morphology," Poster session, Northwestern University Medical Scientist Training Program Annual Retreat
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Oct 2006	"Role of Plakophilin 2 in Protein Kinase C-regulated Desmoplakin Assembly into Desmosomes," Cancer Biology Chairs and Program Directors Meeting, Asilomar, CA

Oral Presentations

Aug 2000	"Characterizing the Evolution of the vertebrate genome by Analysis of Vertebrate Aldolase Genes," *NSF-REU Biology Symposium*, Boston University, Boston, MA.
May 2001	"Characterization of the Evolution of the Vertebrate Genome by Sequence Analysis of the Vertebrate Aldolase Gene Family," *Senior Work for Distinction in Biochemistry and Molecular Biology Symposium*, Boston University, Boston, MA.
Jul 2004	"Spatial and Temporal Regulation of Desmosome Assembly: Role of Plakophilin 2," *Integrated Graduate Program Qualifying Exam*, Northwestern University Feinberg School of Medicine, Chicago, IL
Mar 2005	"The Role of Plakophilin 2 in Regulating Epithelial Junction Dynamics," *Calandra Seminar Series*, Northwestern University Feinberg School of Medicine, Chicago, IL
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TEACHING EXPERIENCE

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2005-2006 Teaching Assistant. Medical Histology Laboratory (Structure-Function Course). Dr. Larry Cochard, Course Director, Department of Cell and Molecular Biology, Northwestern University Feinberg School of Medicine, Chicago, IL

NATIONAL SCIENTIFIC MEETINGS

American Society for Cell Biology Annual Meeting, San Francisco, CA, 2005
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