DIPA IS A NOVEL, ISOFORM-SPECIFIC BINDING PARTNER OF THE TUMOR-ASSOCIATED p120 ISOFORM 1

By

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CHAPTER 1

ISOFORMS OF p120

Introduction

p120-catenin (hereafter referred to as p120) is an Armadillo repeat domain protein that regulates cadherin-catenin complex stability at cell-cell junctions. In addition to its structural role, p120 is a signaling molecule that mediates actin cytoskeletal dynamics through interactions with small Rho GTPases and it intercedes with the canonical Wnt pathway through binding to the transcriptional repressor Kaiso. It is the prototypic member of a protein family that includes Armadillo Repeat deleted in Velo-Cardio Facial syndrom (ARVCF), δ-catenin, p0071, and plakophilins 1-3 (Reynolds, 2007; Carnahan et al., 2010). The p120 gene (*CTNND1*) encodes multiple isoforms with cell type-specific expression and dramatically different capabilities. The aforementioned roles have been the subject of recent reviews (Hartsock and Nelson, 2008; Pieters et al., 2012; Menke and Giehl, 2012). This introduction will address these topics with a focus on the specific involvement of different p120 isoforms and family members. The final portion of the chapter will discuss emerging functions of p120 in neural development and function.

CTNND1 hypothetically encodes 48 alternatively spliced isoforms that range in size from about 75 – 120 kDa. These isoforms comprise combinations of 4 different ATG start sites (1-4) and 4 alternatively spliced exons (A-D) (Keirsebilck et al., 1998; Mo and Reynolds, 1996; Aho et al., 1999) (Fig. 1). The longest isoforms contain an N-terminal head domain with a coiled-coil motif and a regulatory region that contains most, but not

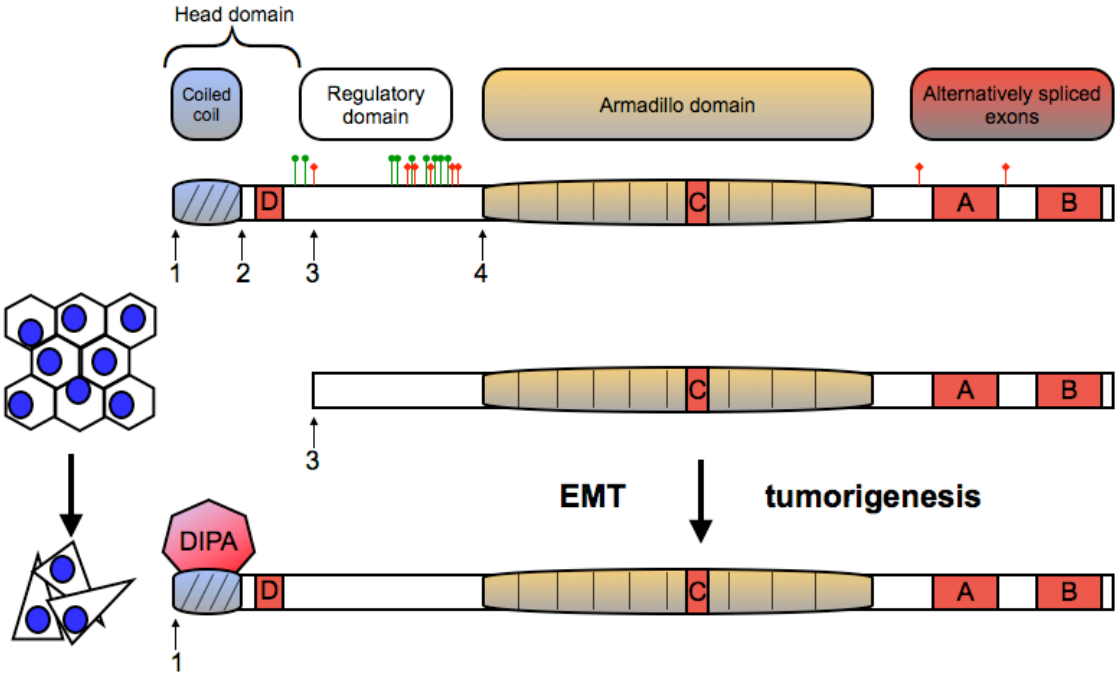

Figure 1. Schematic of p120 catenin isoforms and conserved domains. Full-length p120 is shown at the top with the conserved head domain, alternative translational start codons 1-4, and alternatively spliced exons A-D. Tyrosine phosphorylation sites are indicated with green markers and serine/threonine sites with red markers. Shorter isoforms starting at site 3 are associated with epithelial cells (shown on left as hexagons). EMT or tumorigenesis can induce a switch to increased relative amounts of longer p120-1 isoforms in mesenchymal cells (triangles). DIPA binds to the N-terminal coiled-coil of p120-1.

all, of the p120 tyrosine and serine/threonine phosphorylation sites. The central Armadillo repeat domain is required for binding to cadherins, regulating small Rho GTPases, and mediating Kaiso transcriptional repression (Ishiyama et al., 2010; Anastasiadis et al., 2000; Daniel and Reynolds, 1999). The C-terminal tail of p120 has a poorly understood function confounded by the largely unknown functions of alternatively spliced exons A and B.

p120 is expressed almost ubiquitously. However, it is predominately located at the baso-lateral plasma membrane of epithelial cells and also enriched at synaptic densities in neurons and intercalated discs in cardiomyoctyes. Less abundant p120 protein is diffusely cytoplasmic in some epithelial and other cell types, including fibroblasts and adipocytes (Golenhofen and Drenckhahn, 2000). Mo and Reynolds were the first to show that the two major p120 isoforms are generated by alternative translation initiation codons 1 or 3. They showed that fibroblasts and macrophages preferentially express the longer p120 isoform 1, whereas epithelial cells tend to have higher expression of the shorter p120 isoform 3. They also observed that MDCK epithelial cells up-regulate expression of long isoforms when transformed with *v-src* (Mo and Reynolds, 1996). Isoforms beginning with start codon 2 are rarely detected, and those beginning with codon 4 are only detected in low abundance (Keirsebilck et al., 1998). Exon C is expressed at low levels except in brain tissues, the same location where isoforms lacking exon D are observed (Keirsebilck et al., 1998; Aho et al., 1999).

The two major isoforms have different affects on cellular motility. One hypothesis predicts that p120 isoform 1 (long) is predominant in mesenchymal cells because it influences adherens junctions to permit or promote migration, while p120 isoform 3

(short) is predominant in epithelial cells to regulate adhesion of non-migrating cells. Importantly, both of these major isoforms are equally capable of binding to E-cadherin (Reynolds et al., 1996), so their differences are not based simply on affinity. In the mouse, p120 isoform 3 is enriched at cell junctions of glandular, mucosal, and epidermal cells, which require adherens junction stability to maintain tissue integrity and apico-basolateral polarity. In the same study, p120 isoform 1 staining is abundant in the endothelium, myocardium, serosal epithelia, and choroid plexus (Montonen et al., 2001). Unlike macrophages, these cells do not rely on motility for their function, but they may require p120 isoform 1 for highly specialized functions.

The most well studied function of p120 is its regulation of classical cadherins. Cadherins are single transmembrane proteins that homodimerize via extracellular domains to create calcium-dependent intercellular contacts (Fig. 2). Type I and II cadherins are the core structure of adherens junctions, which initiate and maintain cell-cell adhesion while establishing apico-basolateral polarity (Harris and Tepass, 2010; Hartsock and Nelson, 2008; McNeill et al., 1993). Vertebrates have 26 cadherins that are defined by their five extracellular cadherin (EC) domains and conserved cytoplasmic tail (Gallin, 1998; Hulpiau and Roy, 2009). p120 belongs to a family of intracellular proteins called catenins (from the Latin *catena*, meaning "chain") that link cadherins to the actin cytoskeleton for establishing and strengthening cell-cell contacts (Harris and Tepass, 2010; Hatsell et al., 2003; Ozawa et al., 1989). Catenins are also critical for morphogenic and mitogenic signaling pathways, of which β-catenin is the most well characterized. It is the key signaling molecule in the canonical Wnt pathway, which is important for normal development and cancer, reviewed in (Polakis, 2012).

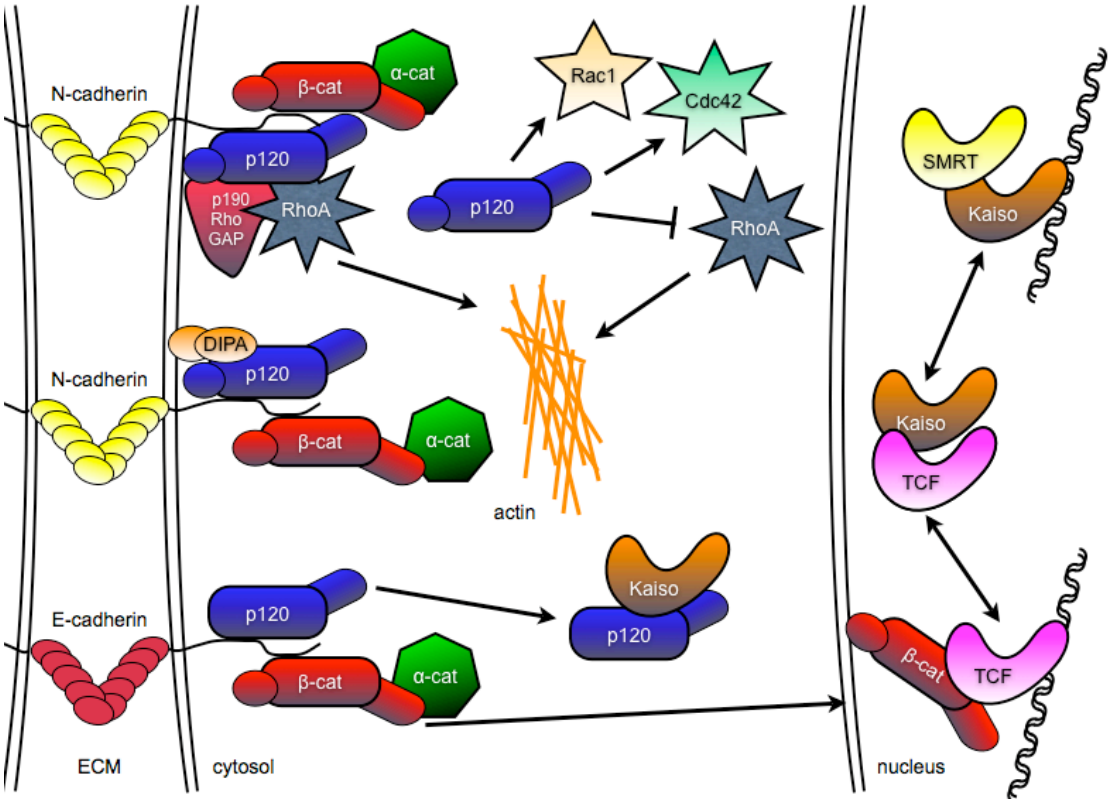

Figure 2. p120 is a master regulator of cadherin stability and adherens junctions. p120-1 typically associates with N-cadherin and p120-3 with E-cadherin. DIPA co-localizes with p120-1 and N-cadherin. β-catenin and α-catenin interact with cadherins and stabilize the actin cytoskeleton. p120 can regulate RhoA through p190RhoGAP at adherens junctions or directly in the cytoskeleton. p120 sequesters Kaiso in the cytoplasm from its dual role as a transcriptional repressor with SMRT and a Wnt pathway inhibitor by blocking TCF/β-catenin binding.

p120, α-catenin, β-catenin, and plakoglobin all associate with the cytoplasmic domain of classical cadherins (Fig 1). Specifically, p120 binds to the juxtamembrane domain (JMD) of the cadherin cytoplasmic tail via its conserved Arm-repeat domain (Ishiyama et al., 2010; Ohkubo and Ozawa, 1999; Reynolds et al., 1994; Shibamoto et al., 1995; Yap et al., 1998). β-catenin and plakoglobin can compete for binding to the more C-terminal catenin-binding domain (CBD) of cadherins (Adams et al., 1996). Monomeric α-catenin binds directly to β-catenin and functionally links the cadherin complex to the actin cytoskeleton (Drees et al., 2005; Yamada et al., 2005).

Different p120 isoforms have variable, and sometimes opposing, effects on cadherin stabilization. Over-expression studies have produced results suggesting that the phosphorylated N-terminal regulatory domain, which is missing in p120 isoform 4, inhibits junctional stability (Aono et al., 1999; Ireton et al., 2002; Ozawa and Ohkubo, 2001). More recent reports contend that phosphorylation status of the regulatory domain is responsible for this effect. For example, serine and threonine phosphorylation enhances efficient junction recovery during calcium switch assays (Fukumoto et al., 2008). Although p120 was initially described as an efficient Src substrate, the mechanisms of adherens junction phospho-regulation remain unclear. Unlike other Src substrates, p120 tyrosine phosphorylation occurred only with transformation-inducing Src mutations (Reynolds et al., 1989), suggesting that p120 phosphorylation is an integral part of *v-src*-induced transformation. With respect to isoforms, the major phosphorylated sites are present in all p120 isoforms except those beginning at start site 4, which are not commonly expressed *in vivo*. A detailed discussion of p120 phosphorylation is beyond the scope of this review, but the relationships between p120 isoforms and cadherins will

be addressed in a subsequent section.

The p120 subfamily

p120 is the prototypic member of a subfamily of catenin proteins that also interact with the JMD of classical cadherins. p120 and the six other subfamily members in vertebrates: ARVCF, δ-catenin, p0071, and plakophilins 1-3 are evolutionarily derived from a single δ-catenin-like ancestor (Carnahan et al., 2010). ARVCF (Armadillo Repeat gene deleted in Velo-Cardio-Facial Syndrome), δ-catenin, and p0071 all contain a central armadillo repeat domain, an N-terminal regulatory region with a coiled-coil domain, and a C-terminal region with a PDZ ligand domain. Notably, p120 lacks the C-terminal PDZ, which may be a key structural trait that has allowed p120 to become the dominant subfamily member in vertebrates (Carnahan et al., 2010). Another feature is the ability of p120 to undergo alternative splicing, a trait shared in the mammalian subfamily only by ARVCF (Waibler et al., 2001). *Xenopus* δ-catenin reportedly has three alternative translation initiation codons and three alternatively spliced exons (Gu et al., 2009). ARVCF localizes to adherens junctions and binds to the JMD of classical cadherins via its armadillo repeat domain in a manner mutually exclusive to p120 (Kaufmann et al., 2000; Paulson et al., 2000). It can also bind to ZO-1 and ZO-2 proteins at tight junctions through its PDZ ligand domain, which has also been implicated in ARCFV nuclear translocation (Kausalya et al., 2004; Mariner et al., 2000). Like ARVCF, δ-catenin and p0071 can compete with p120 for E-cadherin binding (Hatzfeld et al., 2003; Lu et al., 1999; Yang et al., 2010). The mechanism of ARVCF activity in development and neuronal function is unclear, but it is genetically associated with Velo-Cardio-Facial Syndrome, DiGeorge Syndrome, and schizophrenia (Mas et al., 2010; Sirotkin et al.,

1997). Similarly, δ-catenin deletion is associated with mental retardation and adult facial dysmorphism in Cri-du-chat syndrome (Church et al., 1995; Medina et al., 2000). The complicated relationship between p120 subfamily members and neuro-developmental disorders will be examined in more detail at the end of this chapter.

The plakophilins 1-3 are more distantly related to p120, and while they do have a conserved armadillo repeat domain, they do not interact with classical cadherins. They do, however, bind to desmosomal cadherins via their N-terminal head domain (Chen et al., 2002; Hatzfeld et al., 2000; Kowalczyk et al., 1999).

p120 and its subfamily members have overlapping expression patterns throughout mammalian tissue, but they do not always have redundant functions. For example, δ-catenin, ARVCF, and p0071 are all expressed in mammary tissue. But in mice with p120 genetically deleted, these family members do not restore cadherin stability (Kurley et al., 2012). Despite the structural similarities among the p120 subfamily members, they are not always capable of functional redundancy.

Epithelial-to-Mesenchymal Transition

Epithelial-to-mesenchymal transition (EMT) is a set of cellular programs that confer on epithelial cells the ability to disassemble cell-cell adhesions, obtain mesenchymal characteristics, re-establish plasticity, and migrate through the extra-cellular matrix (Acloque et al., 2009). These changes within the cell are mediated by EMT transcription factors, like Snail, Slug, Twist, and Zeb (Thiery et al., 2009). The molecular mechanisms of EMT generally include reprogramming of epigenetic regulation, suppression of E-cadherin and tight junction proteins, and up-regulation of certain mesenchymal proteins,

such as N-cadherin, Vimentin, and Smooth Muscle Actin (Peinado et al., 2004). Physiologic EMT programs are essential for embryogenesis. In this case, the expression of EMT transcription factors is regulated by secreated factors such as TGFβ, Wnt, and Notch ligands (Thiery et al., 2009). EMT is also required for maintaining homeostasis during wound healing, in which TGFβ signaling is particularly important (Weber et al., 2012). In metastasis, cancer cells hijack EMT mechansims in order to invade local tissue, resist apoptosis, and colonize distant sites. The main goal of migrating to a distant site and establishing new cells is common among these processes. The major transcription factors that regulate physiologic and pathologic EMT are the same, too. Also, the microenvironment-dependent initiation of EMT by growth factors and morphogenes is similar. Key differences such as the capability to self-renew, suppress apoptosis, and induce immunosuppression separate metastatic EMT from normal physiologic processes (Scheel and Weinberg, 2012).

Its function in EMT programs may be unclear, but p120-1 is clearly associated with metastasis in human cancer. The association between abnormal p120 expression and cancer has been known for a decade (Thoreson and Reynolds, 2002). Recently, studies have distinguished the prevalence of specific p120 isoforms in metastatic disease. In breast, lung, and renal cell cancers, p120-1 expression is positively correlated with metastasis and is an independent predictor of poor prognosis (Miao et al., 2010; Talvinen et al., 2010; Yanagisawa et al., 2008). In contrast, a study of lung squamous cell cancer and adencocarcinoma reports that p120-1 negatively correlates with lymph node metastasis, whereas p120-3 exhibits a positive correlation (Liu et al., 2007).

The phenomenon of p120 isoform switching in metastatic disease is supported by

mechanistic studies in tissue culture. The p120-3 to p120-1 switch is observed in epithelial cells with the exogenous expression of EMT transcription factors SIP1/ZEB2, Snail, E47, or Slug (Ohkubo and Ozawa, 2004; Sarrió et al., 2004; Vandewalle et al., 2005). In addition, the novel metastasis promoter, Zeppo1, confers an invasive phenotype on mammary epithelial cells and induces a p120 isoform switch (Slorach et al., 2011). Potentially, p120-1 and p120-3 differentially regulate the Rho family of small GTPases, which function in EMT-associated cytoskeletal remodeling, cellular protrusion formation, and motility (Aono et al., 1999; Ohkubo and Ozawa, 1999; Ozawa and Kemler, 1998).

Regulation of Rho GTPase Activity

Rho, Rac, and Cdc42 are the principal members of a GTPase subfamily that controls cytoskeletal formation, motility, polarity, and proliferation. p120 inhibits RhoA via interaction with the RhoGEF Vav2 and p190RhoGAP (Noren et al., 2000; Wildenberg et al., 2006). In fact, p120 recruitment of p190RhoGAP to the cell membrane is required for Rac-dependent inhibition of Rho in fibroblasts (Wildenberg et al., 2006). p120 can also directly bind to Rho and act as a GDI (guanine-nucleotide dissociation inhibitor) (Anastasiadis et al., 2000) (Fig. 2). *In vivo*, p120 inhibition of RhoA in the skin is required to depress NFκB-mediated inflammation (Perez-Moreno et al., 2006). p120 isoform 1A has an increased ability to bind inactivated RhoA compared to shorter isoforms, which may explain its ability to promote invasion in breast and lung cancer cells (Liu et al., 2009; Yanagisawa et al., 2008). However, exogenous p120-1 *and* p120-3, but not the shorter p120-4, can inhibit RhoA as is evident by their induction of a

"dendritic branching" cellular morphology (Aho et al., 2002).

The widely accepted effect of p120 on Rac1 and Cdc42 is to induce their activation (Grosheva et al., 2001; Johnson et al., 2010) (Fig. 2). However, studies in pancreatic cancer cells lacking PPARγ suggest that p120 has the opposite effect (Nakajima et al., 2008). With regard to specific isoforms, Liu *et al.* show that p120-1A over-expressed in A549 cells can deactivate Rac1. Similarly, exogenous p120-3A down-regulates Cdc42 but up-regulates RhoA (Liu et al., 2009). The context of each system clearly contributes to the observed effect of p120. It must also be appreciated that Rac1 and Cdc42 facilitate the trafficking of E-cadherin and the formation of adherens junctions (Wang et al., 2005), so opposing data may reflect GTPase activity at different points within a feedback loop.

p120 interaction with Rho GTPases is also important for tumorigenesis. In Rac- and Src-transformed MDCK cells, p120 inhibition of RhoA is required for growth in soft agar media (Dohn and Reynolds, 2009). Unpublished data from our lab show that exogenous p120-1A, but not p120-3A, can rescue this anchorage-independent growth in the absence of endogenous p120. Since both isoforms can equally stabilize E-cadherin, their differential interaction with RhoA, or some other binding partner, is likely responsible for the isoform-specific rescue. Independently of Rho activity, p120 is required for Rac1 activation and transformed cell growth in E-cadherin-deficient cells (Soto et al., 2008). There is no significantly different impact on Rac1 activity between p120 isoforms (Yanagisawa et al., 2008), but this area has not been extensively studied.

p120 subfamily members also regulate Rho GTPases in a context-dependent manner that is essential for normal development. For example, δ-catenin can induce dendrite-like branching in NIH3T3 cells similarly to p120. This effect is reproducible in primary

hippocampal neurons where neurite branching and δ-catenin are abundant *in vivo*. Unlike p120, the branching phenotype is inhibited by δ-catenin C-terminal truncation mutants (Kim et al., 2002), suggesting that the C-terminal PDZ domain interacts with an essential co-factor for RhoA inhibition. Other binding partners may mediate a balance between neurite branching and elongation by inhibiting RhoA through a complex that involves phosphorylation-dependent binding of δ-catenin to cortactin, an actin cytoskeleton linker protein (Martinez et al., 2003). Morpholino knockdown experiments in *Xenopus* show that ARVCF and p120 are required to inhibit RhoA and activate Rac for normal early development (Fang et al., 2004). The same results were observed with δ-catenin knockdown in *Xenopus* (Gu et al., 2009). ARVCF inhibition of RhoA activity may depend on its interaction with the cytoskeletal regulators KazrinA and p190RhoGAP. δ-catenin and p0071, but not p120, can bind to KazrinA as well (Cho et al., 2010). p0071 regulates RhoA specifically during cytokinesis. It binds to Ect2, a Rho GEF, to activate RhoA at the cleavage furrow. Over-expression or knockdown of p0071 causes a failure of cytokinesis leading to multinucleated cells and apoptosis (Keil et al., 2007; Wolf et al., 2006). The p120 subfamily members appear to have some redundant function, but there are distinct differences that have evolved to regulate GTPase activation either directly or indirectly. It appears that these functional differences are emerging not as the exception but as the rule.

p120, Kaiso, and Wnt signaling

Kaiso was initially described as a novel p120 binding partner through yeast two-hybrid screening (Daniel et al., 1999). Kaiso is a transcriptional regulator containing an

N-terminal BTB/POZ domain (*B*road complex, *T*ramtrak, *B*ric à brac/*Po*x Virus and *Z*inc finger) and a C-terminal zinc finger domain. It belongs to a family of BTB/POZ-zinc finger proteins that includes ZBTB4, BCL-6, PLZF, and others implicated in cancer and development (Carnahan et al., 2010; Daniel, 2007). The BTB/POZ domain is a protein-protein interaction motif that is required for Kaiso homodimerization and binding to CTCF and NCoR, a vertebrate insulator protein and histone deacetylase, respectively (Defossez et al., 2005; Yoon et al., 2003). The zinc finger region contains three C_2H_2 fingers and is necessary for binding directly to p120 and DNA (Daniel and Reynolds, 1999). Kaiso and p120 interact in an isoform-dependent manner in which only p120-3 can directly bind to Kaiso (Dai et al., 2009; Jiang et al., 2012; Zhang et al., 2011).

Kaiso mediates transcriptional repression of Wnt target genes through binding to either a specific consensus sequence or methylated CpG pairs (Buck-Koehntop et al., 2012; Daniel et al., 2002). *Wnt-11*, *siamois*, *matrilysin*, *MTA2*, and *cyclinD1* are among the putative Kaiso target genes (Kim et al., 2004; Park et al., 2005; Spring et al., 2005; Yoon et al., 2003). More recent data suggest that Kaiso does not require DNA binding for its transcriptionally repressive function. Instead, Kaiso binds directly to TCF family members and blocks their interaction with DNA, thus inhibiting the canonical Wnt pathway (Ruzov et al., 2009; Ruzov et al., 2009) (Fig. 2). High-throughput ChIP-seq data suggest that Kaiso is not specifically a Wnt-related protein (Raghav et al., 2012). Prior to terminal differentiation into adipocytes, mesenchymal cells express Kaiso, which guides the transcriptional co-repressor SMRT to DNA promoter elements of key adipogenic genes (Raghav et al., 2012) (Fig. 2).

The interaction between p120 and Kaiso has been extensively studied in examination

of the canonical Wnt pathway. In a subcloned SW480 colorectal cancer cell line, p120 constitutively binds to CK1ε, and upon Wnt3a treatment, p120 Ser268 is phosphorylated by CK1ε, which activates p120-3 to form an exclusively cytoplasmic complex with Kaiso (Casagolda et al., 2010; Valle-Perez et al., 2011) (Fig. 2). These data support earlier findings that reveal a positive-feedback loop in which Wnt-induced stabilization of p120 sequesters Kaiso to relieve inhibition of Wnt signaling (Park et al., 2005). In support of this model, p120-1A over-expression during *Xenopus* development results in similar defects as Kaiso null mutants (Paulson et al., 1999). Kaiso ablation results in multiple developmental defects, consistent with its inhibition of Wnt signaling (Kim et al., 2004). In *Xenopus* embryos and HEK293T cells, the dual-specificity kinase Dyrk1A positively stabilizes p120-1 in the nucleus, which leads to Kaiso sequestration and de-repression of the Wnt target genes *Siamois* and *Wnt11* (Hong et al., 2012). p120-mediated de-repression of Kaiso target genes has also been shown in breast, gastric, and endothelial cells (Ogden et al., 2008; Vermeulen et al., 2012; Zhang et al., 2010). Considering earlier work that showed only p120-3 can bind Kaiso directly, perhaps there is an indirect effect of p120-1 on Kaiso function mediated through p120-3.

In mammalian cancer models, Kaiso may actually increase the effect of Wnt signaling. Kaiso knockout mice are viable and have no specific phenotype. These mice also have no alteration in transcription of the Kaiso target genes *Wnt11*, *S100A4*, *MTA2*, or *Rapsyn*, suggesting a potential redundant function for Kaiso-like proteins in mammals (Prokhortchouk et al., 2006). However, Kaiso knockout combined with Apc^{min} mice, a Wnt pathway-driven colorectal cancer mouse model, results in smaller intestinal tumors and longer survival than Apc^{min} mutation alone (Prokhortchouk et al., 2006). In

colorectal cancer cell lines, Kaiso can bind to the methylated promoters of the tumor suppressors *CDKN2A*, *HIC1*, and *MGMT* in Colo320 and Hct116 cells. When Kaiso is depleted, these genes are up-regulated and contribute to enhanced etoposide cytotoxicity (Lopes et al., 2008).

δ-catenin may also act similarly to p120 in that it can bind directly to Kaiso causing de-repression of *MTA2*, *MMP7,* and *CCND1* transcription in A549 lung cancer cells (Dai et al., 2010). However, no specific mention was made as to whether full-length δ-catenin (resembling p120-1) or an N-terminal truncation (resembling p120-3) interacts with Kaiso. Both *MMP7* and *CCND1* are well-characterized targets of the canonical Wnt pathway (Crawford et al., 1999; Polakis, 2012). Another link to the role of p120 and δ-catenin in Wnt signaling is that, like β-catenin, they have both been reported as targets of GSK3-beta phosphorylation (Hong et al., 2010; Oh et al., 2009). Hong *et al.* show that p120-1 is serially phosphorylated by the β-catenin destruction complex and ubiquitylated by the β^{TrCP} E3-ligase. However, the GSK3β phosphorylation sites reported by Oh *et al*. are not analogus to those on p120-1. The p120 interation with Kaiso is an important example of p120-mediated transcriptional control. Regarding p120 isoforms, Kaiso specifically binds p120-3, Dyrk1A stabilizes p120-1, and Wnt mediates p120-1 degradation. These data imply that p120 isoforms are specifically involved in Wnt signaling through different but potentially related mechanisms.

p120 and family in neural development

This dissertation characterizes the novel and specific interaction between p120-1 and Delta-Interacting Protein A (DIPA), encoded by the *CCDC85B* gene. As described in

Chapter IV, DIPA also binds to ARVCF, p0071, and δ-catenin. Because DIPA and p120-1 are highly expressed in brain tissue, I began searching for their physiologic function in the central nervous system. This section is included to reveal relationships from the literature that have influenced my on-going efforts.

p120 isoform 1 and δ-catenin are abundantly expressed in the brain and play a role in normal cortical development. The highest p120-1 expression is at the plasma membrane of choroid plexus and ependymal cells that lie within and surround the ventricles, which is consistent with its role in maintaining effective cellular adhesion (Chauvet et al., 2004). Neuroblasts that arise within the proliferative ventricular zone of the forebrain are also enriched with p120-1 (Chauvet et al., 2003). These cells are highly motile and require rapid recycling of N-cadherin for both tangential and radial migration (Shikanai et al., 2011). Radial migration is a scaffold-cell dependent process that requires radial glial cells to form a track onto which migrating neurons adhere as they move distally (Kawauchi, 2012) (Fig. 3A). Defects in radial migration result in ectopic accumulations of neurons in the deep layers of the neocortex, which are referred to as Periventricular Heterotopia (PVH) or Subcortical Band Heterotopia (SBH) depending on location. Respectively, these abnormalities reflect the failure of immature neurons either to initiate or continue migration outward from the proliferative ventricular zone to their position of function in the outer cortex. Such malformations of cortical development can manifest as mental retardation, epilepsy, and microcephaly (Pang et al., 2008).

Genetic studies in humans and rodents have revealed surprisingly few genes that are mutated in abnormal neuronal migration syndromes. The two most commonly mutated

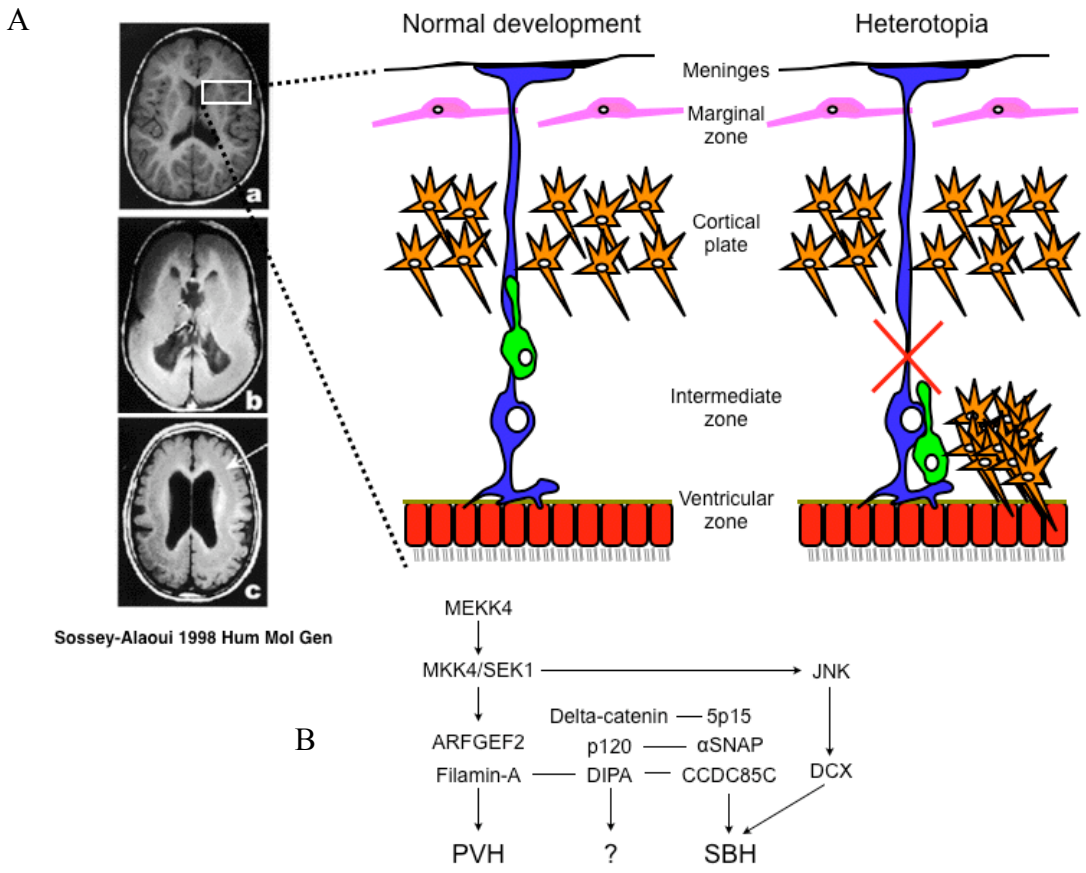

Figure 3. p120 and abnormal cortical development. A) Coronal sections of human brains: a) normal, b) lissencephaly, and c) SBH (arrow). Schematics showing immature neurons (green) normally migrate from the ventricular zone along radial glial cells (blue) into the cortical plate, becoming mature neurons (orange). Any defect in migration, glial formation, or ependymal cell (red) integrity will cause neurons to mature in the ventricular region or below the cortical plate, causing PVH or SBH respectively. B) Diagram of genes related to and mutated in PVH and SBH.

genes in human PVH are *FLNA*, which encodes the actin-cytoskeletal protein Filamin-A (Sarkisian et al., 2008), and *ARFGEF2*, which regulates the molecular trafficking of Filamin-A (Kawauchi, 2012) (Fig. 3B). Filamin-A was identified in a protein complex that was immunoprecipitated with anti-DIPA antibodies (see Chapter IV). Factors other than neuronal migration are likely to contribute to PVH. In the *MEKK4*$^{-/-}$ mouse model of PVH, ependymal cells along the ventricular surface have disrupted adherens junctions and basement membranes (Sarkisian et al., 2006). Mechanistically, this defect is mediated in part by MEKK4 (a MAP3K) phosphorylation and activation of MKK4/SEK1 (a MAP2K), which in-turn phosphorylates and stabilizes an excess of Filamin-A, but the physiologic consequence of ependymal dysfunction is a failure of radial glial cells to anchor properly. Thus, the scaffold itself fails and neurons differentiate without leaving the ventricular zone (Sarkisian et al., 2008). Clinical genetic data show that duplication and trisomy involving chromosome regions 5p15.1 – 5p15.33 are associated with PVH (Sheen et al., 2003). Although there are multiple genes in this 20Mb region, it does contain the δ-catenin (*CTNND2*) locus at 5p15.2. This connection to PVH is entirely speculative, and δ-catenin deletion alone does not cause heterotopia, but the association may be informative as more data are obtained. The larger implication is that adherens junctional proteins may participate in cortical malformation pathogenesis through dysregulation of cell-cell contacts and/or Rho GTPases.

Genetic causes of the second malformation, SBH, have a more direct link to p120 and DIPA. Human SBH is often associated with mutations in *DCX*, which encodes the microtubule-associated protein Doublecortin. The c-Jun NH_2-terminal Kinase (JNK, a MAPK) phosphorylates Doublecortin at neuron growth cones to stabilize microtubules

during migration (Gdalyahu et al., 2004). JNK is activated by MKK4/SEK1, thus MEKK4 may be simultaneously affecting Filamin-A and DCX to regulate migration (Moriguchi et al., 1995) (Fig. 3B). Another mutation, in the membrane fusion protein Soluble N-ethylmaleimide sensitive factor (NSF) attachment protein alpha (αSNAP), is the genetic error in the hydrocephalus with hop-gait (*hyh*) mouse, which also exhibits SBH (Chae et al., 2004). αSNAP is a mediator of exocytosis and regulator of epithelial cell polarity that is required for p120 expression and adherens junction formation (Naydenov et al., 2012). Another mutant mouse, hemorrhagic hydrocelphalus (*hhy*) exhibits SBH and is caused by a mutation in the *CCDC85C* gene, which encodes a poorly characterized protein containing two coiled-coil domains with 54% identity and 73% similarity to DIPA. Because of p120-1 and δ-catenin function in cellular adhesion and migration during brain development, they are likely candidates as effectors in a neocortical patterning pathway, which may also involve DIPA and CCDC85C.

Studies examining the direct role of p120 in neural development do not show heterotopia or migratory failure, but p120 loss does result in synapse structure and function defects associated with mental retardation and neurodegeneration. Floxed-p120 gene deletion in mouse neuroepithelial precursors driven by an *emx1*-IRES-Cre recombinase results in normal cortical morphology (Elia et al., 2006). These data are surprising given the critical role of p120 in regulating cadherin function and cytoskeletal dynamics. However, the high expression of δ-catenin in these neurons suggests that it can compensate for the p120 deficiency (Kawamura et al., 1999; Ho et al., 2000). The selective deletion of p120 in neuroblasts does not test the requirement of radial glial cells to express and maintain N-cadherin. Global knockout of p120 causes embryonic lethality

due to a failure of allantoic-chorionic membrane fusion, which precludes neural development studies (Birchmeier, 2012).

During neuronal migration, p120-1 is distributed in neural axons and growth cones but it is redistributed to dendrites and synapse-containing spines in mature neurons (Chauvet et al., 2003). Although p120 knockout in neuroblasts does not disrupt gross brain architecture, individual pyramidal neurons in the hippocampus have reduced dentritic tree complexity, spine density, and synapse density. Hippocampal extracts from p120 knockout mice also have decreased Rac1 and increased RhoA activity (Elia et al., 2006). N-cadherin and p120-1 interaction in neurons is required for inhibiting RhoA, which allows for proper voltage-activated calcium channel function at synaptic junctions (Marrs et al., 2008).

Another neuronal function of p120 is to inhibit Amyloid β (Aβ) peptide formation by binding to Presenilin-1 (Kouchi et al., 2009). Presenilin-1 is the catalytic component of the γ-secretase complex, which cleaves transmembrane proteins, including E-cadherin, N-cadherin, receptor tyrosine kinases, and Amyloid Precursor Protein (APP). Presenilins are the most commonly mutated genes in familial Alzheimer's Disease (De Strooper et al., 1998). Alzheimer's Disease and other neurodegenerative diseases have a characteristic accumulation of Aβ plaques, derived from APP cleavage (Cairns, 2009). *In vitro* p120 binding to E-cadherin decreases the Presenilin-1-mediated processing of APP and the amount of secreted Aβ (Kouchi et al., 2009). p120-binding partner Dyrk1A (see above in *p120, Kaiso, and Wnt signaling*) is encoded by one of about 20 genes on chromosome 21 that are critical for Down's Syndrome (trisomy 21) (Park et al., 2009). Dyrk1A phosphorylates Presenilin-1 causing increased γ-secretase activation, suggesting

a molecular pathway for the high incidence of early onset Alzheimer's Disease in patients with Down's Syndrome (Ryu et al., 2010). p120 function in synapse maintenance and plaque formation is emerging as a potentially important factor in neurodegerative disease.

The p120 subfamily members are also implicated in neurodegeneration and other brain disorders. δ-catenin and p0071 directly interact with Presenilin-1 (Stahl et al., 1999; Zhou et al., 1997). The interaction between δ-catenin and Presenilin-1 is essential for dendritic spine and synapse morphogenesis and function (Arikkath et al., 2009). δ-catenin deficient mice are viable and fertile but exhibit retracted dendrites, reduced spinal density, and decreased cortical responsiveness after 5 weeks of life (Matter et al., 2009). These morphologic and physiologic abnormalities contribute to cognitive and motor deficits, such as inability to navigate a water maze, impaired reaction to fearful stimuli, and decreased balance on a rotating rod (Israely et al., 2004).

Summary and Significance

p120 mediates multiple cellular functions, such as cell adhesion, cytoskeletal arrangement, and transcriptional regulation. Its specific relationships with cadherin stability and Rho inhibition suggest mechanisms in which p120 downregulation contributes to EMT and migration in development and disease. p120 isoforms are largely under-studied and historically considered to have significantly overlapping functions. However, the data reviewed here suggest that different isoforms have specialized functions and p120-1 specifically is implicated in neuro-developmental and neurodegenerative diseases.

CHAPTER II

MATERIALS AND METHODS

Production of antigen

DIPA was cloned in to pBG100 (Center for Structural Biology, Vanderbilt University). The sequence verified clone was transformed into *Rosetta* (DE3) *E. coli* cells. Large-scale expression was carried out in 2 L of autoinduction media (Studier, 2005) at 37°C overnight. Cell pellet was resuspended in 25 mM NaH_2PO_4, 500 mM NaCl, 10% Glycerol, (pH 8.0) with a total volume of 50 ml. Resuspended cells were lysed during two passages under 15k to 20k psi using an Emulsiflex C3 (Avestin, Ottawa, ON). Lysate was spun down at 130000 x g for two hours. The clarified lysate was discarded and the pellet was resuspended in 25 mM NaH_2PO_4, 500 mM NaCl, 8 M Urea, (pH 8.0) and incubated at room temperature (RT) for one hour. Cells were pelleted and the supernatant was added to Cobalt resin (Pierce, Rockford, IL) pre-equilibrated with 25 mM NaH_2PO_4, 500 mM NaCl, 8 M Urea, (pH 8.0) and rotated overnight at RT. The resin was separated with centrifugation, compacted in a disposable column, and washed with 10 column volumes of 25 mM NaH_2PO_4, 500 mM NaCl, 6 M Urea (pH 8.0). The column was then washed with the same buffer with an additional imidazole gradient from 0 mM to 250 mM. Twenty-five 2 mL fractions were collected at a flow rate of approximately 1 mL/min. Fractions were then analyzed by dot blot to ascertain the location of His-tagged protein using an Anti-6xHis antibody (Roche, Indianapolis, IN). Fractions containing 6xHis-tagged DIPA were subjected to SDS-PAGE and the cleanest

fractions were pooled for immunization, while fractions containing non-specific bands were pooled to screen immune sera.

Immunization and hybridoma preparation

Four A/J mice (Stock #000646, Jackson Laboratory, Bar Harbor, ME) were injected both sub-dermally and intramuscularly in the thigh with a total of 50 μg of 6xHis-tagged full-length DIPA protein in Freud's complete adjuvant. At the same time, the mice were bled via the submandibular face vein to obtain a pre-bleed. Sera were extracted by centrifugation using BD Microtainer tubes and evaluated for antigen-specific antibody titers using enzyme-linked immunosorbent assay (ELISA) as described below. Four weeks after the initial immunization, the mice were boosted with the same dose of protein but utilizing incomplete adjuvant (also used in subsequent boosts). After a two-week interval, the mice were again bled, and antibody titers were assessed by ELISA. Additional boosts were given at eight and 12 weeks after the initial immunization. In each case, antibody sera titers were similarly evaluated two weeks after each immunization. A single A/J mouse showing the most selective and concentrated anti-DIPA titers was chosen for a final boost (50 μg) via an intraperitoneal injection without adjuvant. Four days after this final boost, spleen cells were harvested and electro-fused (Yu et al., 2008) with Sp/20 (from Dr. William Sutherland, University of Virginia) or NS1 (from Dr. Robert Jeffery Hogan, University of Georgia) murine myeloma cells. The products of the fusion were plated into methylcellulose-based semi-solid media (ClonaCell, Stemcell Technologies, Vancouver, BC) containing the selective reagents hypoxanthine, aminopterin, and thymidine (HAT). After approximately 10 days, colonies

of interest were picked and distributed individually into 96-well plates based on *in situ* interaction with fluorescently labeled mouse IgG-Fc 488 DyLight (Jackson Laboratory, catalog # 515-485-062) utilizing the ClonePix instrument (Genetix, Sunnyvale, CA). Individual clones were expanded in liquid media containing serum, hypoxanthine, and thymidine (Medium E, Stemcell Technologies, Vancouver) and maintained at a density of $5x10^5$–$1x10^6$ cells/ml for generating antibody-rich supernatants. Supernatants from hybridomas were assayed for antigen-specific antibodies by solid-phase ELISA, using full-length DIPA in sodium dodecyl sulfate (SDS) buffer as bait. Positively scoring hybridomas were re-screened for performance by Western blotting, immunofluorescence, and immunoprecipitation. The most promising anti-DIPA clones were selected, extensively subcloned to ensure monoclonality, and cryopreserved.

ELISA procedure

The following solutions were prepared as follows from chemicals obtained from commercial sources; Carbonate-bicarbonate coating buffer (pH 9.6) was prepared from Na_2CO_3 (1.59 g/L), $NaHCO_3$ (2.39 g/L), and thimerosal (0.10 g/L); PBS-Tween (pH 7.4) was prepared from NaCl (8.00 g/L), KH_2PO_4 (0.20 g/L), Na_2HPO_4 (1.15 g/L), KCl (0.20 g/L), Tween 20 (1.00 mL/L), and thimerosal (0.10 g/L); BSA layered (5.0 g) on PBS-Tween (500 mL); 1 nM ABTS solution in 70 mM citrate-phosphate buffer (pH 4.2) was prepared from citric acid (5.64 g/L), Na_2HPO_4 (5.84 g/L), and AzBTS-$(NH_4)_2$ (0.548 g). For ELISA experiments, ELISA quality plates (Immulon 2HB flat bottom microtiter plates 96 well or 4HB flat bottom 384 well plates) (Nunc, Rochester, NY) were coated with 10 mL per plate of a 5 μg/mL solution of DIPA antigen in carbonate bicarbonate

coating buffer and incubated at 4°C overnight. Plates were washed three times with 100 μL of PBS-Tween with a Bio-Tek ELx 405 automatic microplate washer (Winooski, VT) and incubated with 100 μL of PBS-Tween for 30 minutes at 37°C, after which the PBS contents were discarded. Aliquots of murine sera dilutions or hybridoma supernatants (depending on the stage of the antibody development process) were incubated in the coated wells in a final volume of 100 μL in PBS for 60 minutes at 37°C. The plates were then washed three times with PBS-Tween, followed by the addition of aliquots of diluted horse radish peroxidase (HRP)-conjugated affinipure goat anti-mouse IgG Fc region specific secondary antibody diluted in PBS-Tween/BSA at 1:5000 incubated at 37°C for 60 minutes. After washing the plates three times, ABTS solution was prepared (1.8 mL of H_2O_2 was added per 1 mL of ABTS) and immediately added to each well in 100 μL aliquots. To determine peroxidase activity, absorbance at 414 nm was measured after 15 and 30 minutes from each well using a Bio-Tek Powerwave HT 340 plate reader with Gen5 software (Winooski, VT). Isotyping was done via ELISA per manufacturer's protocol (mouse isotyping kit, catalog # 37503) (Pierce, Rockford, IL).

Cells, tissue culture, and antibodies

All cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2% L-glutamine, penicillin (100 U/mL), and streptomycin (100 mg/mL), (Gibco BRL, Gaithersburg, MD). The cell lines used to assess immunofluorescence, immunoblotting, and species cross-reactivity were A431 epidermoid carcinoma cells (human), HCA7 colon carcinoma cells (human), HepG2 hepatocarcinoma cells (human), HT29 colon carcinoma cells (human), IEC6 intestinal

epithelial cells (rat), MDCK kidney epithelial cells (canine), and NIH3T3 fibroblasts (mouse). Anti-Flag MAb (M2 cat. #F1804) was purchased from Sigma-Aldrich (St. Louis, MO). Anti-MBP (12B12), anti-Tubulin (DM1α), and KT3 MAbs (Tomonari1988) were obtained from the Vanderbilt Antibody and Protein Resource (Nashville, TN). Anti-p120 monoclonal antibody pp120 was purchased from BD Transduction (San Jose, CA), and Anti-ZO-1 polyclonal antibody is from Santa Cruz Biotechnology (Santa Cruz, CA).

Immunoblotting, immunoprecipitation, and immunofluorescence

Procedures for immunoblot analysis, immunoprecipitation, and immunofluorescence have been described previously in detail (Daniel and Reynolds, 1999). Briefly, cells were lysed in a buffer containing 0.5% Nonidet P-40, 10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM sodium vanadate, 0.1 trypsin inhibitor units of aprotinin, and 5 mg of leupeptin per mL. Whole cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Blots were briefly blocked at 4°C with 5% non-fat dried milk in TBS pH 7.4 and incubated overnight at 4°C with hybridoma supernatant or primary antibody (0.2–2.0 mg/mL) in 3% milk/TBS. The membranes were then washed five times with TBS before incubation with the secondary anti-mouse 680 and/or anti-rabbit 800 antibody (Licor, Lincoln, NE) in Odyssey blocking buffer/TBS for 30 min at RT. Blots were finally washed three times with TBS plus 0.1% Tween-20 and three times with TBS and then processed with the Odyssey immunodetection system (Licor, Lincoln, NE)

according to the manufacturer's protocols. For immunoprecipitation, hybridoma supernatant or primary antibody was incubated with magnetic Dynabeads pre-conjugated to Protein G (Vanderbilt Antibody and Protein Resource, Nashville, TN) at 4°C for 2 hours. Dynabeads were then washed three times in NP-40 buffer and combined with whole cell lysates that were extracted as described above. Dynabeads and lysate were incubated at 4°C for two hours before being washed three times with NP-40 buffer and solublizied in Laemlli Sample Buffer (LSB) (Daniel1999). For immunofluorescent labeling, cells were plated and cultured for two days on glass coverslips before fixing with 3% paraformaldahyde (PFA) and permeabilization with 0.2% Triton X-100 in PBS (pH 7.4). Primary antibody and hybridoma supernatant incubations were performed at RT for 30 min in 3% milk/PBS at 0.5–1.0 mg/mL. After washing three times with PBS, the coverslips were incubated with Alexa fluor donkey-anti-mouse 594 and/or Alexa fluor goat-anti-rabbit 488 (Life Technologies) secondary antibodies in 3% milk/PBS at 1:800 dilution for 30 min at room temperature. The coverslips were finally washed 3 times with PBS, mounted on glass slides with Prolong Gold (Life Technologies), and visualized using a Zeiss Axioplan 2 microscope (Zeiss, Thornwood, NY).

DIPA polyclonal antibody production and purification

The full-length 6xHis-tagged DIPA protein described above was used to immunize two New Zealand white rabbits by Covance (Princeton, NJ). Sera was collected from both rabbits and tested by Western blot and immunofluorescence. Polyclonal antibodies (PAbs) were subsequently purified from anti-sera by affinity chromatography on Protein-G sepharose (AKTA Xpress, GE Life Sciences, Piscataway, NJ).

DNA constructs and recombinant protein production

Full-length human DIPA (encoded by the *CCDC85B* gene) was purchased from the Dana Farber/Harvard Cancer Center DNA Resource Core (Cambridge, MA) in the form of pENTR223-CCDC85B-fusion (clone #HsCD00288507). To create 3xFlag-DIPA, CCDC85B was recombined into the LZRS-GW-IRES-Neo^R using LR recombinase from Life Technologies (Grand Island, NY). Viral production and cell transduction protocols are described elsewhere (Ireton2002, Davis2003). Maltose-binding protein (MBP)-tagged DIPA protein and fragments were created by PCR cloning the CCDC85B gene into the pMal c2 vector (New England BioLabs, Ipswitch, MA). To knockdown canine DIPA, shRNA constructs were ordered as oligomers, annealed, and cloned into pLentiLox3.7-Puro. The specific targets are 1) 5'-GGGAGAACCTGGCGCTTAA-3', 2) 5'-GACTGAGGCTCATCTTCCT-3', and 3) 5'-GCCTGGCTCTGGGTGAGGA-3'. The p120 knockdown and add-back constructs are described in (Dohn and Reynolds, 2009). To create C-terminally tagged GFP-fusion proteins, p120-1A, p120-3, δ-catenin, and p0071 were PCR amplified, cloned pENTR2B, and recombined into pLentiLox3.7-Gateway-GFP with LR recombinase from Life Technologies (Grand Island, NY).

CHAPTER III

MONOCLONAL ANTIBODIES TO DIPA: A NOVEL BINDING PARTNER OF p120-CATENIN ISOFORM 1

Introduction

p120 is a master regulator of classical cadherin stability and is important for epithelial homeostasis, development, tumorigenesis, and metastasis. It is the prototypic member of a family that includes other Armadillo repeat-containing proteins ARVCF, γ-catenin, p0071, and plakophilins 1-3 (Reynolds, 2007). In addition to, but not exclusive from, its regulation of cadherin turnover, p120 modulates activity of the Rho family small GTPases (Anastasiadis et al., 2000; Dohn and Reynolds, 2009; Noren et al., 2000; Wildenberg et al., 2006). The human p120 gene (*CTNND1*) encodes multiple spliced isoforms that range in size from about 75–120 kDa. These isoforms comprise combinations of four different ATG start sites (1-4) and three alternatively spliced exons (A-C). Although most cell types contain multiple isoforms, the long p120 isoform 1 and shorter isoform 3 are predominant in mesenchymal and epithelial cells, respectively (Keirsebilck et al., 1998). Snail, Slug, and Twist transcription factors are embryonic Epithelial-Mesenchymal Transition (EMT) inducers that cause isoform switching from p120 isoform 3 to isoform 1 via the Epithelial Splicing Regulatory Proteins 1 and 2 (Ohkubo and Ozawa, 2004; Sarrió et al., 2004; Warzecha et al., 2009)

During validation of a yeast two-hybrid screen using human p120 isoform 1AB as bait, I detected a p120 isoform 1-specific interaction with Delta-Interacting Protein A (DIPA). DIPA was originally thought to bind the Hepatitis Delta Antigen (Brazas and

Ganem, 1996) but has since been found to have no relationship to Hepatitis Delta (Taylor, 2009) . DIPA is a 202-amino acid protein that contains two coiled-coil domains and no other identifiable domains. At the protein level, it maintains a high degree of conservation with greater than 97% identity among mammals (Fig. 4). Exogenous DIPA co-localizes with p78/MCRS1/MSP58 to centrosomes, and its over-expression can repress SRF and AP-1 signaling (Du et al., 2006). Endogenous DIPA can regulate adipocyte differentiation by inhibiting C/EBP-β and –γ (Bezy et al., 2005). When induced by p53 activation, DIPA can compete with β-catenin for binding to the TCF4 transcription factor, which effectively down-regulates Wnt target gene expression (Iwai et al., 2008). Thus, DIPA is mostly found in the nucleus and appears to function primarily in transcriptional regulation.

The DIPA interaction with p120 is interesting in part because p120 is predominantly membrane-associated and functions in cadherin stability and cytoskeletal rearrangement. As an analogy, the well-known association of another cadherin binding partner β-catenin with TCF/LEF family members is critical for canonical Wnt signaling, which drives normal development and colorectal cancer. TCF proteins can bind directly to β-catenin, DIPA, and the transcription factor Kaiso, a BTB/POZ zinc-finger protein first discovered as a p120 binding partner (Daniel and Reynolds, 1999; Iwai et al., 2008; Ruzov et al., 2009). Notably, the β-catenin destruction complex, which is inhibited by Wnt stimulation and mutated in most colorectal cancer, appears to selectively ubiquitylate and degrade p120 isoform 1 (Hong et al., 2010). Thus, several lines of evidence suggest that p120 isoform 1, and by association DIPA, is functionally distinct, perhaps analogous in some respects to the cytoplasmic pool of β-catenin involved in canonical Wnt signaling.

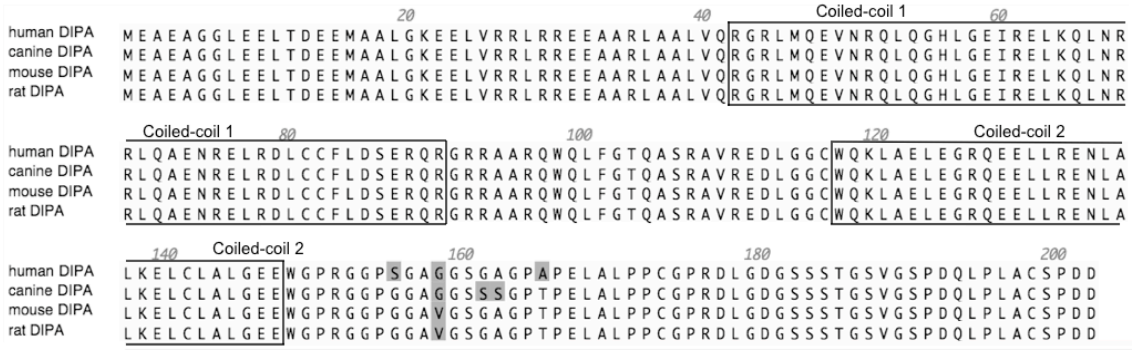

Figure 4. Alignment of human, canine, mouse, and rat DIPA proteins. Reference sequences are from the NCBI Protein database, and ClustalW alignment was performed with MacVector software. The non-identical amino acids are shaded, and the coiled-coil 1 (42-90aa) and coiled-coil 2 (117-147aa) domains are boxed.

To determine the function of DIPA and its relationship to p120, I have generated and characterized the first DIPA-specific monoclonal antibodies (MAbs).

Results

Initial characterization

Four mice were immunized with full-length 6xHis-tagged DIPA protein solubilized in 6 M urea, and one mouse was picked based on anti-sera immunofluorescence and Western blotting. Hybridoma generation yielded 423 ELISA-positive clones, but only 42 clones were effective in Western blotting and immunofluorescence. Further screening that included immunoprecipitation yielded five MAbs that were chosen for detailed characterization: 2G7, 3E3, 5E11, 8E11, and 15F11. The results of these studies are presented in Figures 2 through 6, and their characteristics are summarized in Table 1.

Immunoprecipitation and immunoblotting

To test the ability of these MAbs to detect denatured DIPA in an immunoblot assay, over-expressed 3xFlag-tagged DIPA was immunoprecipitated from transduced MDCK cells and subjected to Western blot analysis (Fig. 5A). The membrane was cut into strips and probed by each of the MAbs. The strip probed with an anti-Flag MAb was used as a positive control, and a DIPA rabbit polyclonal antibody (PAb) is shown for comparison. With the exception of MAb 2G7, all MAbs recognized two bands: a monomeric 3xFlag-

Table 1. Summary of anti-DIPA monclonal antibody characteristics
IP, immunoprecipitation; WB, Western blotting; IF, immunofluorescence
(-), no detection; (+), minimal detection; (++), good sensitivity or specificity; (+++), good sensitivity and specificity
Epitopes refer to recombinant DIPA fragments described in Fig. 6B.

Clone	epitope	IP	WB	IF	isotype
2G7	CC2	-	-	+	IgG1
3E3	C-term	++	+++	+++	IgG2b
5G11	C-term	+	-	+	IgG1/IgG2a
8E11	C-term	+	-	+	IgG2b
15F11	CC1	+	+	-	IgG1

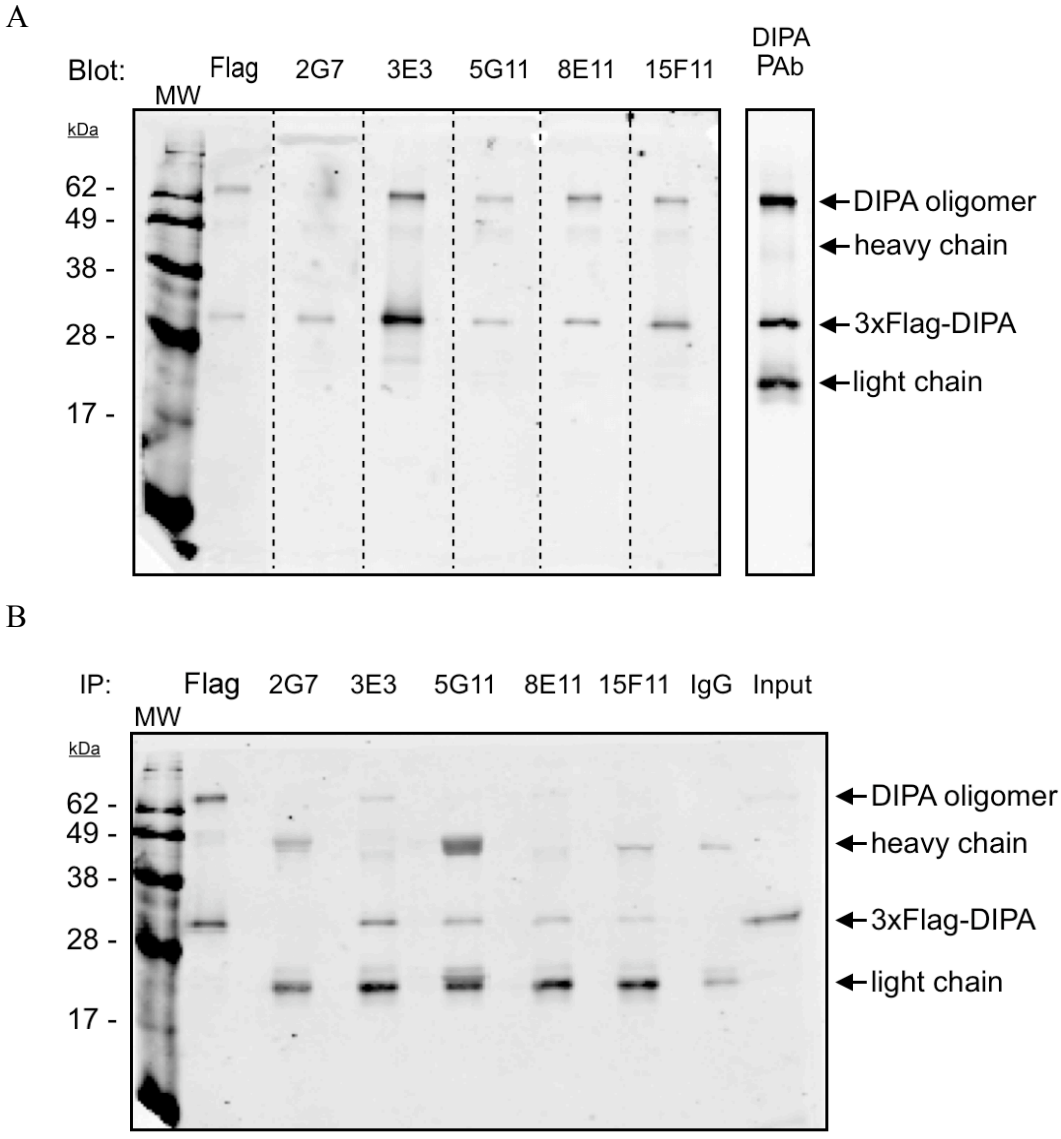

Figure 5. Reciprocal immunoprecipitations of exogenous DIPA. (A) Anti-Flag MAb was used to immunoprecipitate 3xFlag-DIPA from NP-40-detergent cell lysates of transduced MDCK cells, and immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose. Dashed lines represent where individual strips of the nitrocellulose membrane were realigned after being probed separately. Anti-Flag and DIPA PAb were used as controls. The DIPA PAb was probed on the same strip as MAb 15F11 but detected on a separate channel using the Odyssey imaging system. (B) A portion of the same MDCK lysate from (A) was immunoprecipitated with each MAb, anti-Flag, or an irrelevant IgG control. Precipitates were subjected to Western blotting as in (A) but probed with anti-Flag MAb. MW, molecular weight marker.

tagged DIPA of about 30 kDa and a higher molecular-weight species of about 75 kDa, which I believe may be a highly insoluble oligomer.

To identify the MAbs that recognize and immunoprecipitate 3xFlag-DIPA, I bound each MAb or an irrelevant mouse IgG MAb (KT3) to magnetic Dynabeads and subsequently incubated them with cleared lysate from MDCK cells expressing 3xFlag-DIPA. These lysates were then separated by SDS-PAGE, transferred to nitrocellulose, and probed by Western blotting with the anti-Flag MAb (Fig. 5B). Again, MAb 3E3 detected the DIPA oligomer, whereas the other MAbs could not. 2G7 was unable to immunoprecipitate 3xFlag-DIPA.

To determine the ability of these MAbs and the DIPA PAb to detect endogenous DIPA, I immunoblotted equal amounts of lysate from wild-type cell lines of rat, mouse, canine, or human origin (Fig. 6). Cell lysate from MDCK cells over-expressing 3xFlag-tagged DIPA was used as a positive control. Lysate from MDCK cells with stably expressed shRNA against canine DIPA was used to test for specificity and to determine the migration of endogenous DIPA. Endogenous monomeric DIPA (approximately 25 kDa) migrates faster through the gel presumably because it lacks the 3xFlag epitope and amino acid linker in the exogenous DIPA. MAb 3E3 does not react with a number of faint "background" bands recognized by the DIPA PAb (Fig. 6B and F). MAb 3E3 was not able to detect DIPA from IEC6 or NIH3T3 cells, suggesting that either these cells express low levels of DIPA or MAb 3E3 may not cross-react with rodent DIPA. MAb 3E3 detected a faster migrating band that may represent a post-translationally modified DIPA, as previously reported (Du et al., 2006). Notably, the 75 kDa DIPA oligomer was

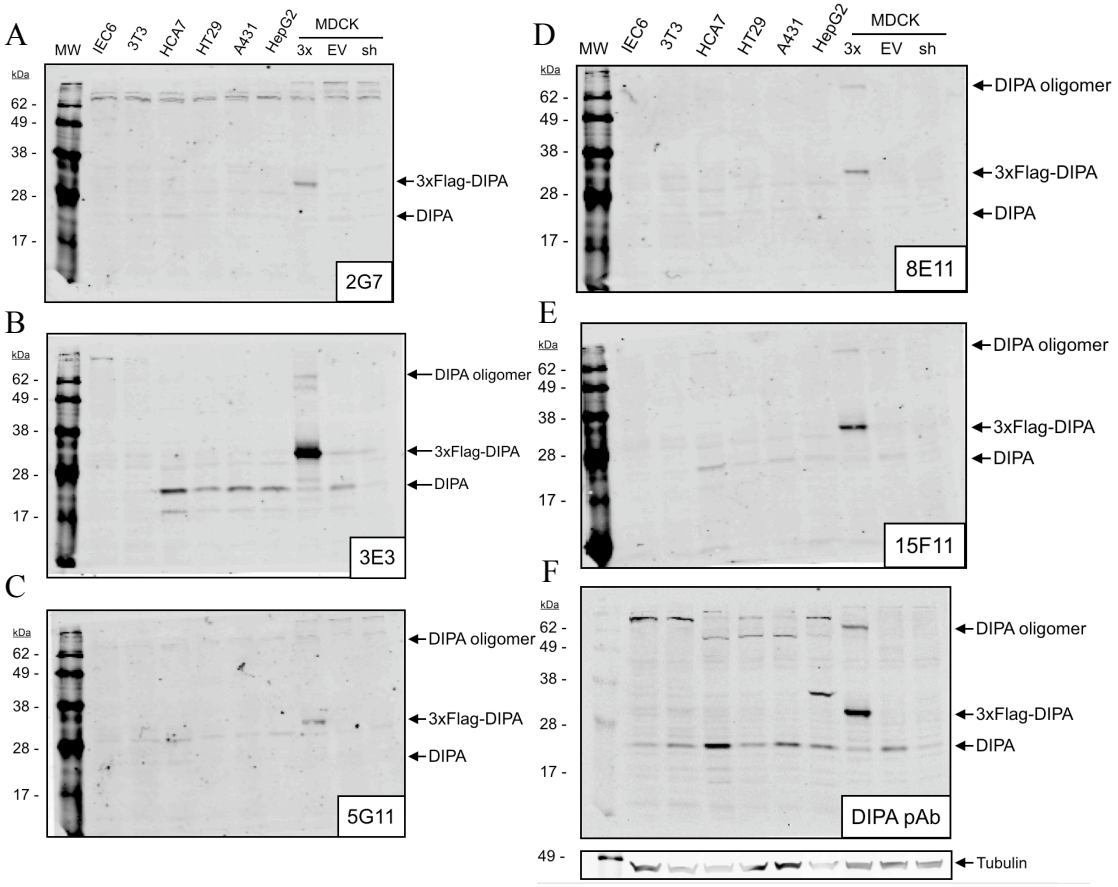

Figure 6. Detection of endogenous DIPA by MAbs. Cell lysates were isolated from IEC6 (rat), NIH3T3 (mouse), HCA7 (human), HT29 (human), A431 (human), HepG2 (human), and three transduced MDCK (canine) cell lines with RIPA detergent. Lysates were separated by SDS-PAGE (60 μg per lane) and transfered to nitrocellulose. Membranes in panels A-E were probed with MAbs 2G7, 3E3, 5G11, 8E11, and 15F11 respectively. Panel F represents the same membrane used in panel E that was probed with DIPA PAb and anti-Tubulin but detected on a separate channel using the Odyssey imaging system.
MDCK Labels: 3x, lysate with over-expressing 3xFlag-DIPA; EV, lysate with empty vector; sh, lysate with stable expression of shRNA against canine DIPA.

not detectable in wild-type lysates. It is apparent from panels B, E, and F that HCA7 human colorectal cancer cells express a relatively large amount of DIPA.

Immunofluorescence

To test the efficacy of the DIPA MAbs in immunofluorescence staining, I incubated fixed and permeabilized cells with the five different MAb hybridoma supernatants (Fig. 7). The MAbs showed predominantly nuclear and cytoplasmic staining in most cell lines, which is consistent with previous reports (Bezy et al., 2005; Du et al., 2006; Iwai et al., 2008). Surprisingly, some antibodies recognized DIPA at cell-cell junctions, which is unreported in the literature. MAbs 2G7 and 3E3 produced junctional staining patterns in MDCK cells. MAbs 2G7 and 5G11 staining appeared at junctions in A431 cells, but 3E3 did not. Interestingly, 3E3 did not detect a robust nuclear pattern in NIH3T3 cells, but rather a low-intensity diffuse cytoplasmic staining with occasional nuclear accumulations. 15F11 produced low-intensity staining in all tested cell lines and had little nuclear staining.

I further characterized the MAb 3E3 by immunofluorescence because of its junctional staining pattern in MDCK cells, which is similar to that of p120 (Dohn and Reynolds, 2009). I co-stained control and DIPA-knockdown MDCK cells with MAb 3E3 and the DIPA PAb (Fig. 8). As I expected, the polyclonal has high sensitivity but poor specificity evident by the persistent nuclear and Golgi-like staining in the DIPA-knockdown cells. However, the DIPA PAb junctional staining does not appear in the MDCK shDIPA cells. The MAb 3E3 staining in all cell compartments almost completely

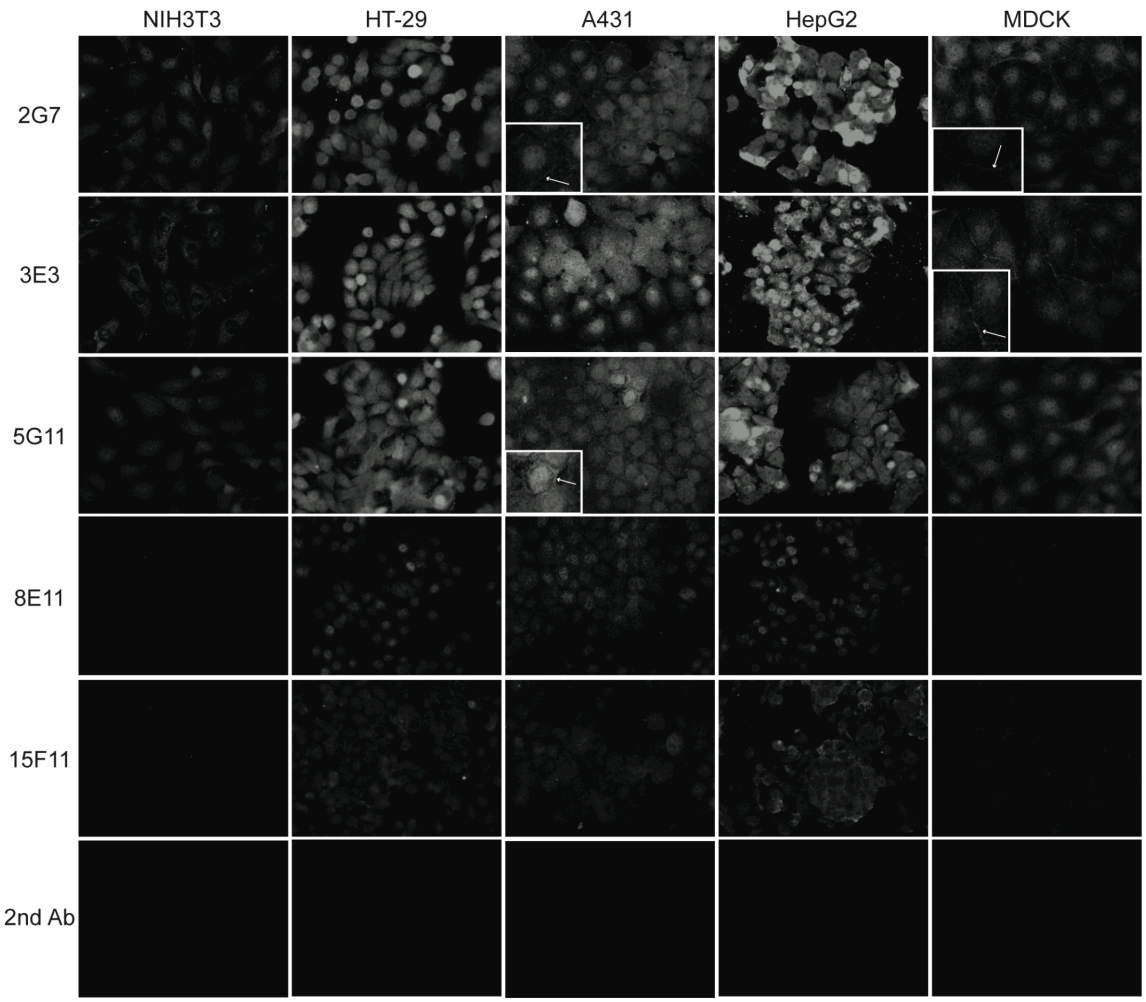

Figure 7. Performance of anti-DIPA MAbs in immunofluorescence assays. DIPA MAbs were incubated with PFA-fixed and TritonX-100-permeabilized cells as labeled. Goat-anti-mouse Alexa fluor 594 secondary antibodies were used to detect DIPA MAbs in all panels. No primary antibodies were used in the bottom panels (2° only). Insets show magnifications to better visualize DIPA junctional staining (arrows). Bar = 50 μm

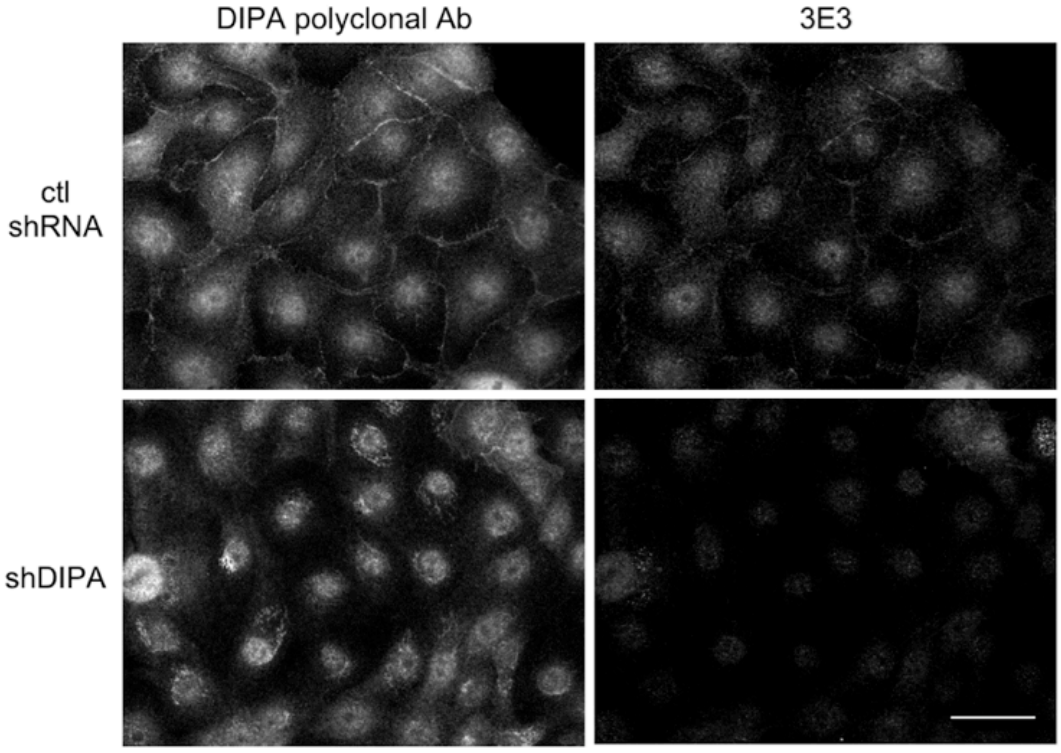

Figure 8. Comparison of MAb 3E3 and DIPA PAb by immunofluoresce. MDCK cells stably expressing either non-silencing shRNA control (top) or canine DIPA-directed shRNA (bottom) were fixed, permeabilized, and probed with either DIPA PAb or the 3E3 MAb. Bar = 50 μm

disappears in the DIPA-knockdown cells (Fig. 8). Thus, I believe that both the nuclear and the cell-cell junctional staining represent endogenous DIPA.

Epitope mapping

To determine the epitopes to which the DIPA MAbs bind, MBP-tagged full-length recombinant DIPA and five fragments (Fig. 9A) were produced using *Rosetta E. coli*. The tagged proteins were purified from bacterial lysates with maltose resin extraction, separated by SDS-PAGE, and detected by Western blotting using each of the five MAbs and an anti-MBP MAb (Fig. 9B). Because the MBP tag is 42 kDa, all proteins migrated slower than endogenous DIPA. All five MAb were able to detect the full-length DIPA. MAb 2G7 only detected the ΔC-term, CC2, and ΔCC1 fragments, and therefore its epitope lies between amino acids 118 and 149. MAbs 3E3, 5G11, and 8E11 only detected ΔCC1 and C-term fragments, so they bind to DIPA between amino acids 142 and 195. MAb 15F11 detected the CC1 and ΔC-term fragments, therefore its epitope must be within the first 118 amino acids. Some amount of protein degradation is evident in all lanes. The higher molecular weight DIPA oligomer is appreciable in lanes 1, 4, and 5 of the anti-MBP blot, which suggests that this species requires the CC2 domain for oligomerization. Thus, these five DIPA MAbs recognize a diversity of epitopes that span the whole protein.

Discussion

To better understand the physiologic function of DIPA and its relationship to p120 isoform 1, I have generated a novel panel of MAbs against human full-length DIPA. Of

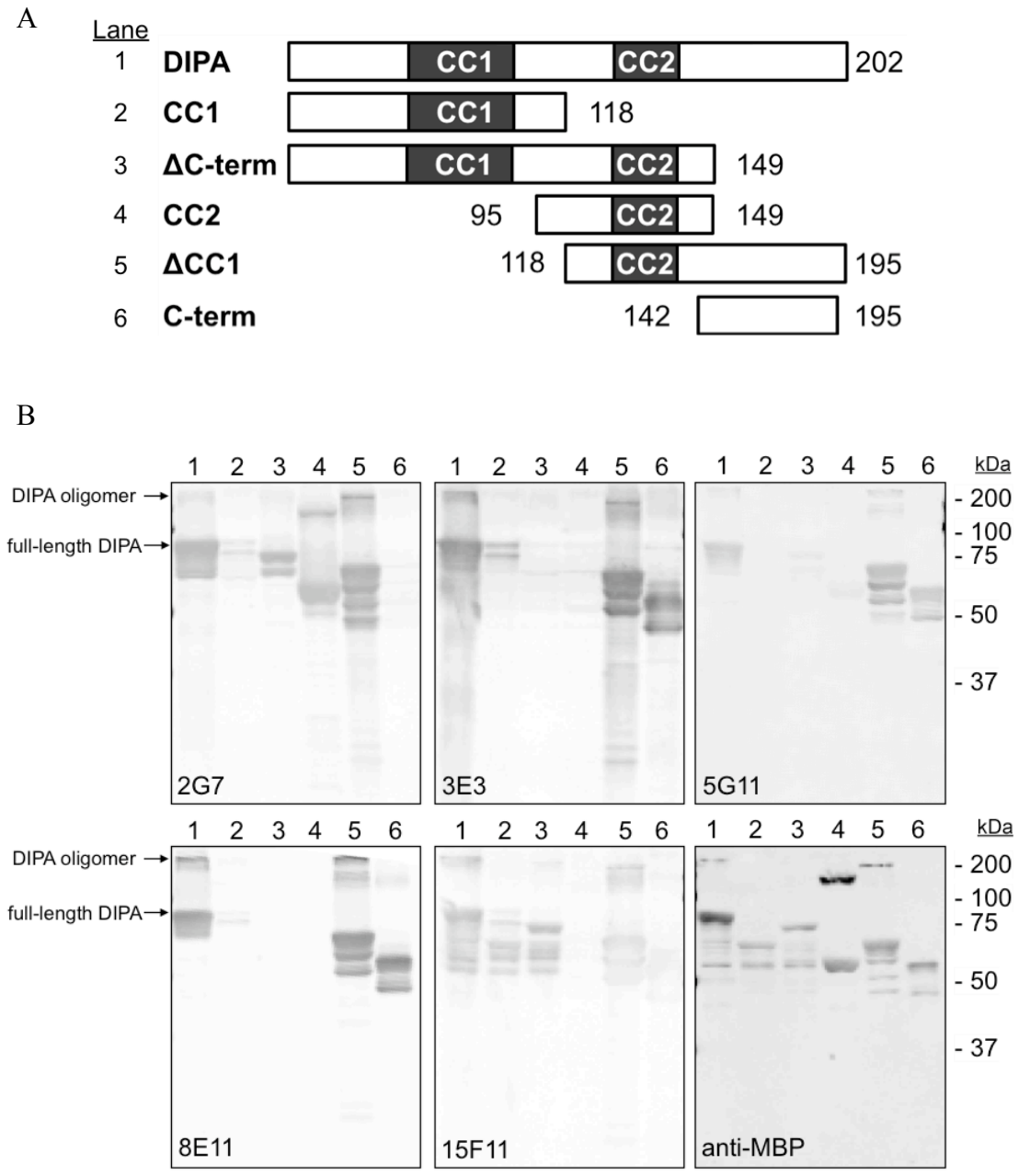

Figure 9. Epitope mapping of the DIPA MAbs. (A) Schematic of full-length DIPA and five overlapping fragments. The coiled-coil regions are labeled as "CC1" or "CC2," and the terminal amino acid residues are indicated next to each fragment. (B) Purified lysates from *Rosetta* BL-21 cells were separated by SDS-PAGE, transferred to nitrocellulose, and probed with each of the DIPA MAbs or anti-MBP as control. Lanes 1-6 were loaded with lysates containing MBP-tagged full-length, CC1, ΔC-term, CC2, ΔCC2, and C-term DIPA respectively.

the five MAbs characterized here, MAb 3E3 appears to be the most sensitive and specific by Western blotting and immunoprecipitation. Although the data shown were generated with antibody supernatants, I have validated the results with purified MAb 3E3 and determined an optimal working concentration of 0.25 ug/mL for immunofluorescence and 1.0 ug/mL for Western blotting. Interestingly, 3E3 was the only MAb that could immunoprecipitate the DIPA oligomer from MDCK cells over-expressing 3xFlag-DIPA (Fig. 5B). Whether this higher molecular weight species exists endogenously is unknown, but it clearly forms when DIPA is over-expressed, because it is recognized by three distinct antibodies (*i.e.* anti-Flag, DIPA PAb, and anti-MBP) (Figs 5, 6F, and 9A). Once formed, the DIPA oligomer is highly insoluble, as it is unaffected by boiling in LSB containing β-mercaptoethanol and isolation by SDS-PAGE.

MAb 3E3 performed well in nearly all assays but does not appear to detect endogenous DIPA from the IEC6 (rat) or NIH3T3 (mouse) cell lines by Western blot. The MAb 3E3 epitope lies within the C-terminal region of DIPA (amino acids 142-195), and V158 is the only non-identical residue in rodent DIPA compared to human and canine. It is possible that the 3E3 MAb requires a glycine at position 158 for binding. On the other hand, the abundance of DIPA in IEC6 and NIH3T3 cells may be below the threshold for immunoblot detection, because MAb 3E3 does detect an immunofluorescence signal in NIH3T3 cells (Fig. 7). Further studies with shRNA and a DIPA point mutant will be required to answer this question. Experimentally, it would be an advantage if MAb 3E3 recognizes only human and canine DIPA while the PAb detects all mammalian DIPA.

The faster migrating band detected by MAb 3E3 (Fig. 6B) is likely to reflect phosphorylation of DIPA at T12. Exogenous DIPA with a T12A point mutant has been detected as a single band by Western blot (Du et al., 2006). The T12 residue is predicted to be a casein kinase I and II substrate by NetPhos 2.0 (University of Denmark) and PhosphoMotif Finder (Human Protein Reference Database, Johns Hopkins University).

MAbs 2G7, 3E3, and 5G11 behaved similarly in immunofluorescence applications. In addition to the more prominent nuclear staining, all three detected DIPA at cell-cell junctions. Although DIPA was not readily detected at junctions in all cell types, the result was clear in A431 and MDCK cells. As noted, shRNA controls revealed MAb 3E3 to be highly specific when compared to the DIPA PAb (Fig. 8). The differential junctional staining among MAbs 2G7, 3E3, and 15F11 in A431 and MDCK cells may reflect cell compartment-specific modifications or binding partners that differentially obscure epitopes.

In summary, I have generated five DIPA MAbs and characterized their performance in commonly used antibody-based assays. They comprise diverse isotypes and interact with at least three distinct epitopes. Of these, MAb 3E3 appears to be the best overall with respect to both specificity and performance in a variety of methods.

Chapter IV

DIPA IS A NOVEL, ISOFORM-SPECIFIC BINDING PARTNER OF THE TUMORIGENESIS-ASSOCIATED p120 ISOFORM 1

Introduction

Although p120 is well-known for its role in cadherin-mediated adherens junctions, it interacts with a number of novel binding partners in the cytoplasm and nucleus whose roles are, at best, indirectly related to adhesion. Such recently discovered binding partners include Dyrk1A, RPTPμ tyrosine phosphatase, and CKIε (Hong et al., 2012; Kim, 2011; Valle-Perez et al., 2011). These findings have revealed new mechanisms for functions of p120 in development and cancer.

In addition, p120 is expressed as multiple isoforms, but the significance of this characteristic is not well understood. p120-1 and p120-3 are the two major isoforms and their relative abundance is dependent on cell type and context. p120-1 is particularly interesting because it is the only isoform that can rescue anchorage-independent growth in p120-knockdown MDCK cells transformed with dominant-active Rac1 (unpublished data; Dohn and Reynolds, 2009). In lung cancer xengraphic mouse models, p120-1 over-expression is permissive for tumorigenesis, but p120-3 is inhibitory (Liu et al., 2009). p120-1 is up-regulated at the expense of p120-3 during EMT (Ohkubo and Ozawa, 2004; Sarrió et al., 2004; Vandewalle et al., 2005). Zeppo1, a novel metastasis-promoting transcriptional repressor, also induces p120 isoform switching from p120-3 to p120-1. Re-introducing p120-3 can repress the invasive phenotype bestowed on breast cancer cells by Zeppo1 (Slorach et al., 2011). Up-regulation of p120-1 is closely correlated with

mesenchymal and metastatic behavior, suggesting an important, but so far unknown, function for the isoform-1 specific head domain. As one approach to filling this gap, I conducted a yeast two-hybrid-based screening strategy to identify isoform-1 specific binding partners. Of the potential candidates, DIPA was chosen for further characterization and functional analysis because of its relationship to other cancer-related proteins and pathways.

As described in chapter II, DIPA is a 202 amino acid protein with 2 internal coiled-coil domains and no other conserved motifs. It is predominantly a nuclear protein with transcription-repressing function through interactions with TCF4 and C/EBPβ (Iwai et al., 2008; Bezy et al., 2005). DIPA itself is transcriptionally up-regulated by both Heregulin and Hepatocyte Growth Factor (HGF) (Hellman et al., 2008; Saeki et al., 2009). Thus, DIPA is physically and functionally linked to a number of proteins with critical roles in cancer, but its function is not known.

Here, I show that DIPA and p120-1 interact specifically and that their functional disruption potentially underlies a genetic condition with abnormal brain development. First, the interaction is fine mapped in yeast two-hybrid assays. Next, Madin-Darby Canine Kidney (MDCK) cells are used to show that endogenous DIPA is expressed at adherens junctions, which is dependent on p120. Finally, δ-catenin and ARVCF are capable of rescuing junctional DIPA localization in the absence of p120.

Results

In collaboration with Hybrigenics, I screened p120-1AB against a library comprised of cDNA from 4 breast cancer epithelial cell lines: T47D, MDA-MB468, MCF7, and

BT20. To identify isoform-specific binding partners, I used direct yeast two-hybrid assays with readily available cDNAs to validate 23 of the 52 independent hits. Candidate binding partners with a reproducible interaction to p120-1AB were then re-screened to test binding to p120-1A and 3A. Secondary screening of isoform-specific binding partners by immunofluorescence and immunoprecipitation verified these interactions in mammalian cells. Finally, to determine potential functional relevance, I tested whether the absence of p120 had an impact on the abundance or localization of a given hit.

The head domain of p120-1AB interacts with both DIPA coiled-coil domains. To fine map their interaction, truncation mutants of p120 and DIPA were tested in direct yeast two-hybrid assays (Fig. 10). Both DIPA coiled-coils are required for full-strength binding to p120-1AB, however, the N-terminal half of DIPA is sufficient for a weaker interaction. Reciprocally, DIPA binds to p120-1AB and p120-1A, but not p120-3A. The p120 N-terminal coiled-coil and regulatory domains together are sufficient for binding DIPA, but not the p120 coiled-coil by itself. The p120 subfamily members ARVCF, p0071, and δ-catenin contain highly conserved N-terminal coiled-coil domains. Both ARVCF and p0071 proteins interact with DIPA by direct yeast two-hybrid. δ-catenin was also tested but could not be interpreted because of high auto-activity in the GAL4-DNA-binding domain fusion construct. These direct yeast-two hybrid assays confirm that DIPA is a specific binding partner to the conserved head domain of p120-1 and p120 subfamily members.

To validate the yeast two-hybrid data in mammalian cells, I conducted co-expression

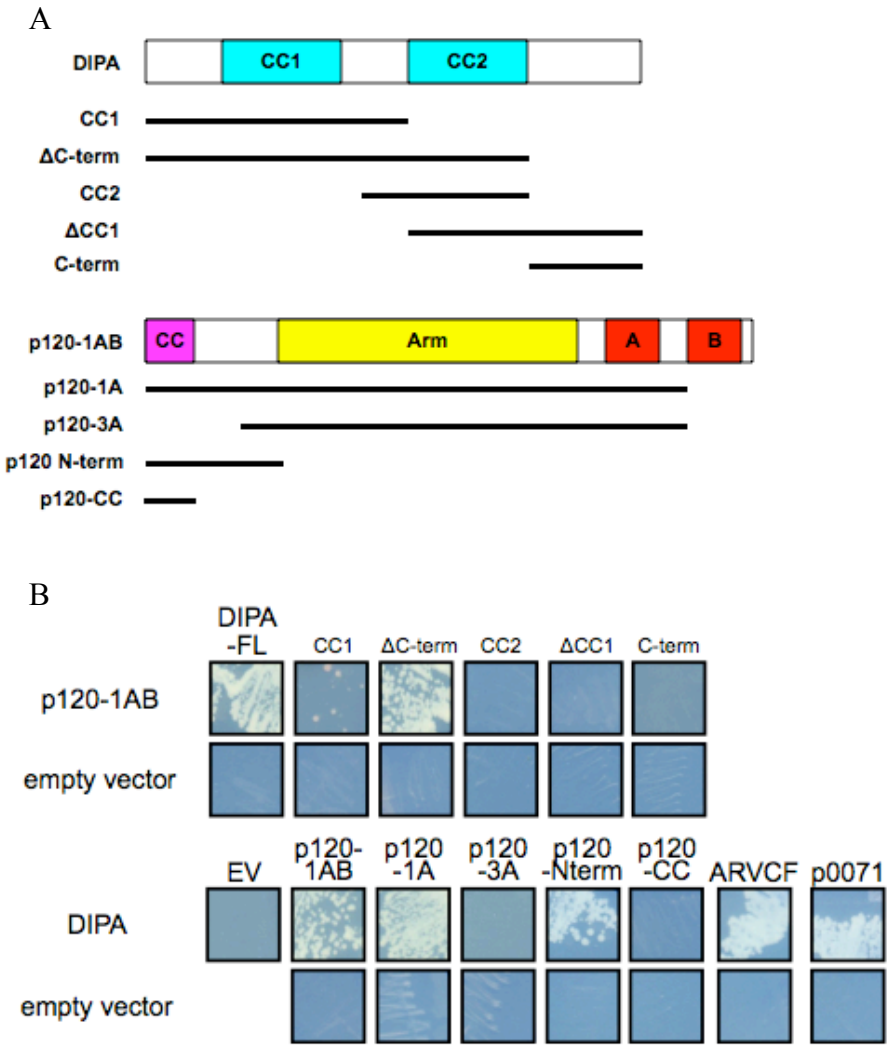

Figure 10. Schematic and yeast two-hybrid for p120-1 and DIPA interaction. A) DIPA is shown at the top with coiled-coil domains 1 and 2 (CC1, CC2), and p120 is at the bottom with coiled-coil (CC), armadillo repeat (Arm), and alternatively spliced exons A and B. Truncation fragments are represented by lines under each schematic. B) Direct yeast two-hybrid assays were used with p120 genes fused to the GAL4-DNA binding domain and DIPA genes fused to the GAL4-activation domain.

with knockdown and shRNA rescue experiments in a MDCK cell model. MDCK cells exhibit classic epithelial morphology and form well defined cell-cell junctions that are ideal for high resolution co-localization studies. Figure 11 shows that exogenous DIPA co-immunofluoresces (Fig. 11A) and co-immunoprecipitates (Fig. 11B) with p120-1A, but not p120-3A. For Figure 11A, 3xFlag-tagged DIPA was introduced into MDCK cells by retroviral infection and then cells were stained with anti-Flag and anti-p120 antibodies. Note that 3xFlag-DIPA is expressed throughout wild type MDCK cells but predominantly in the cytoplasm and nucleus (Fig. 11A top row). There is no dramatic change to the exogenous DIPA localization in p120 knockdown cells, but p120-1A rescue causes a striking recruitment of 3xFlag-DIPA to the cell membrane. Rescue with p120-3A does not have this effect. The same two cell lines from the bottom two rows of Figure 11A were used to co-immunoprecipitate 3xFlag-DIPA with the p120 monoclonal antibody pp120 (Fig. 11B). Reciprocally, p120 was co-immunoprecipitated with DIPA using the anti-Flag monoclonal antibody. Note that p120-3A does not immunoprecipitate with DIPA. These data show that the isoform-specific interaction between DIPA and p120-1A can be recapitulated in mammalian cells.

To examine whether endogenous DIPA localizes to cell-cell junctions, a polyclonal antibody against full-length human DIPA was used to stain MDCK cells. Figure 12A shows endogenous DIPA localization in wild type and control cells (top row), DIPA knockdown cells (middle row), and shRNA rescue cells (bottom row). Note that the junctional, cytoplasmic, and nuclear staining patterns produced by this antibody are the same as observed with over-expressed 3xFlag-DIPA (compare Fig. 11 and Fig. 12). MDCK cell lines with stable DIPA knockdown and shRNA rescue show that the

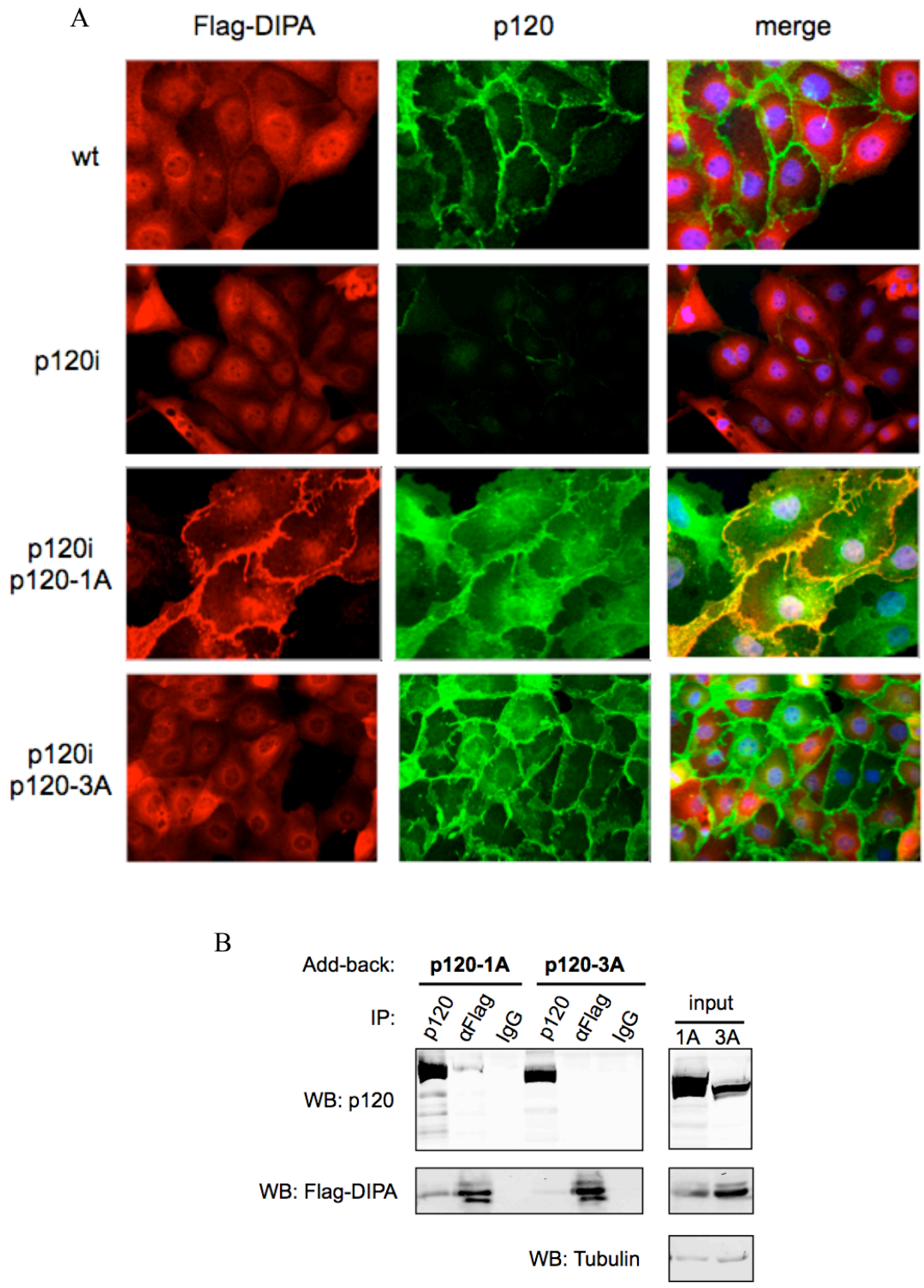

Figure 11. Co-immunofluorescence and co-immunoprecipitation of p120-1 and DIPA. A) Immunofluorescence in MDCK cells with anti-Flag and F1aSH (anti-p120 polyclonal) antibodies. B) pp120 antibody immunoprecipitated exogenous p120-1, but not p120-3A, with 3xFlag-DIPA from MDCK lysates.

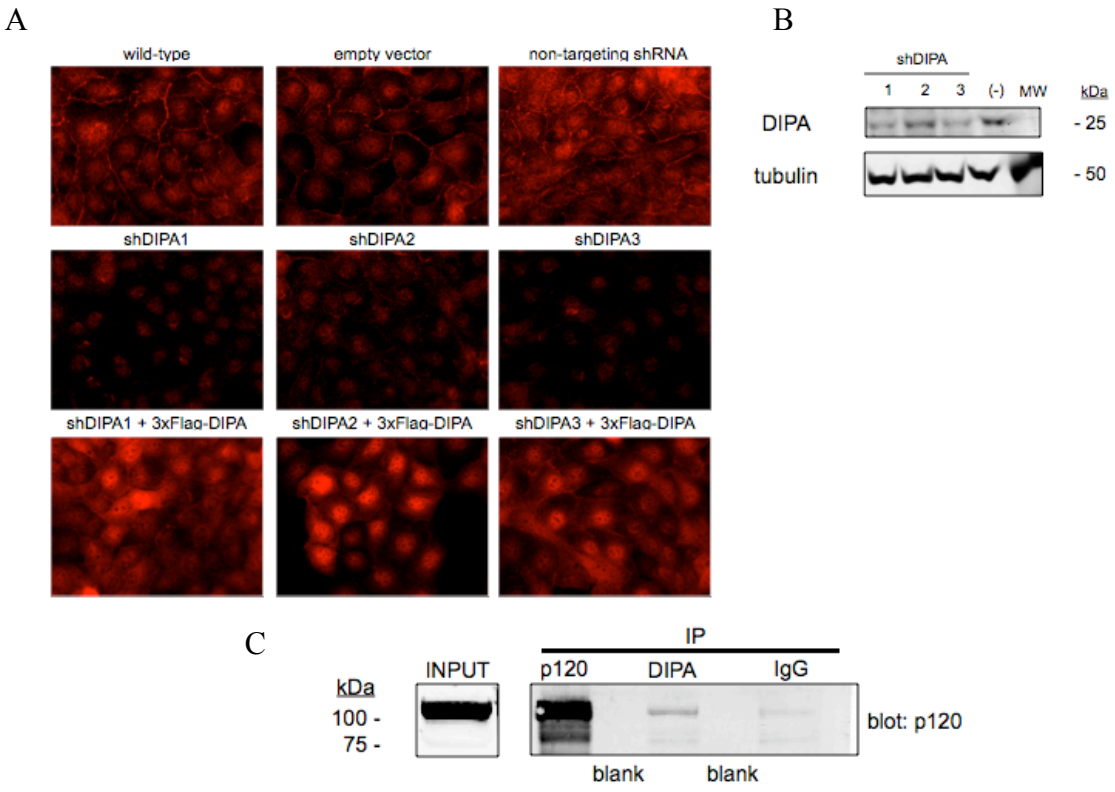

Figure 12. Endogenous DIPA subcellular localization and co-immunoprecipitation with p120-1. A) Specific DIPA junctional staining with the 337 polyclonal antibody in MDCK cells , which is absent in three different knockdown cell lines and rescued by human 3xFlag-DIPA expression. B) Western blot of whole cell lysates from MDCK cells with shDIPA 1, 2, and 3. (-) is a non-targeting shRNA control, and MW is the molecular weight marker. C) Immunoprecipitation of p120-1 with DIPA 3E3 monoclonal antibody. The blot was probed with pp120 monoclonal antibody.

junctional staining is specific with the known caveat that this antibody detects a non-specific golgi-like antigen. By immunoblotting, all three cell lines expressing DIPA knockdown constructs have reduced DIPA protein, but shDIPA1 and shDIPA3 are the most efficient (Fig. 12B). To determine if endogenous DIPA and p120 interact, 3E3 DIPA monoclonal antibody (described in Chapter III) was used to co-immunoprecipitate p120-1 in wild type MDCK cells (Fig. 12C). Because DIPA co-migrates on SDS-PAGE with the 3E3 antibody light chain, I was unable to visualize endogenous DIPA in the p120 immunoprecipitate (data not shown). These results show that endogenous DIPA localizes to the cell membrane and co-immunoprecipitates with p120-1.

The junctional localization of endogenous DIPA is dependent on p120-1 expression. MDCK cells expressing non-targeting control shRNA or p120 knockdown were stained with DIPA and either p120 polyclonal antibody or ZO-1 polyclonal antibody (Fig. 13). Control cells exhibit co-localization of endogenous DIPA and p120 (Fig. 13A, top row). But, cells with stable p120 knockdown fail to maintain adherens junctions and have reduced DIPA junctional staining (Fig. 13A, bottom row). Tight junctions, however, are retained as represented by ZO-1 staining (Fig. 13B). p120 and DIPA co-localize perfectly in adherens junctions, which is better visualized in the enlarged images in Figure 13C (top). The tight junction marker ZO-1 does not co-localize with DIPA (Fig. 13C, bottom). These data provide further evidence for a model in which p120-1 is required to recruit DIPA to adherens junctions.

Yeast two-hybrid experiments showed that p120-1, ARVCF, and p0071 can all bind to DIPA. These proteins, along with δ-catenin, all share a conserved head domain with a coiled-coil motif. To identify p120 isoforms and/or family members capable of directly

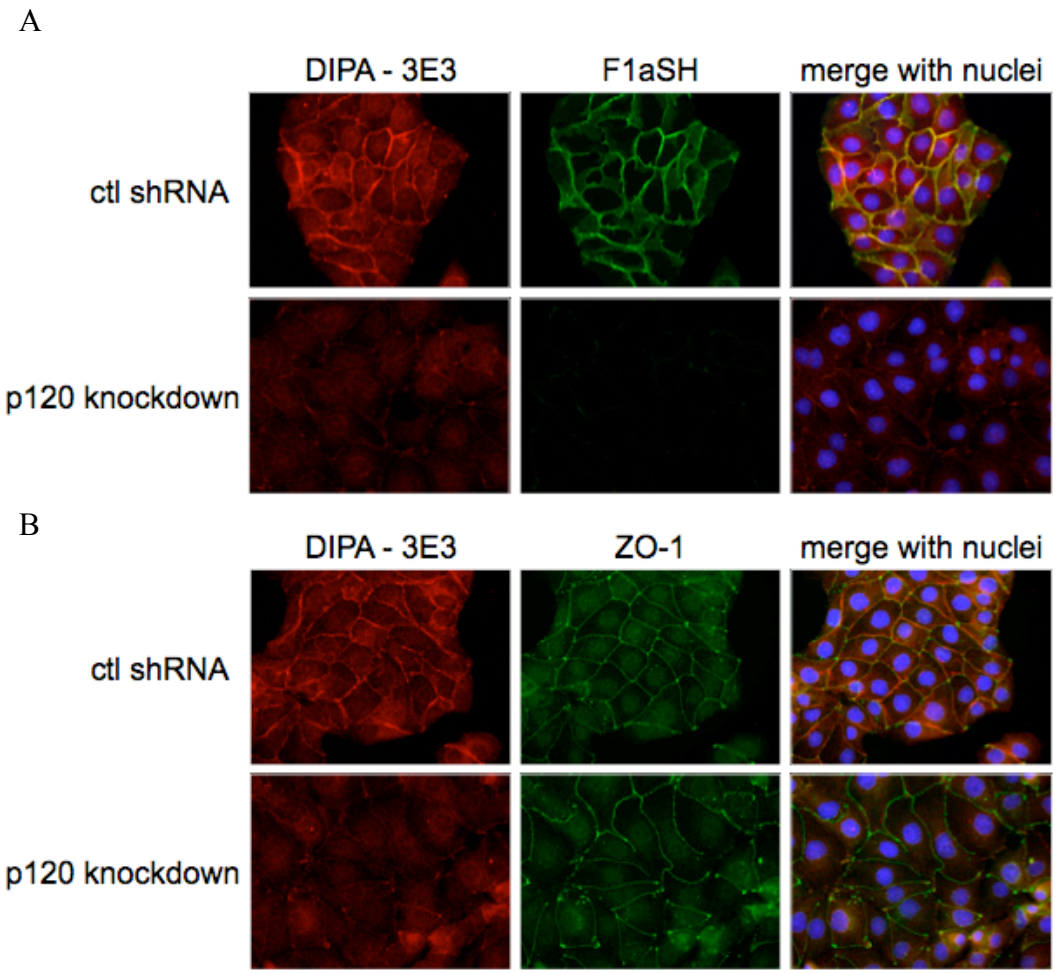

Figure 13A and B. p120 is required for DIPA localization to adherens junctions. Non-targeting control shRNA or p120-knockdown shRNA were stably expressed in MDCK cells. DIPA monoclonal antibody 3E3 was used to co-stain cells with either p120 (F1aSH polyclonal antibody) or ZO-1 (polyclonal antibody).

C

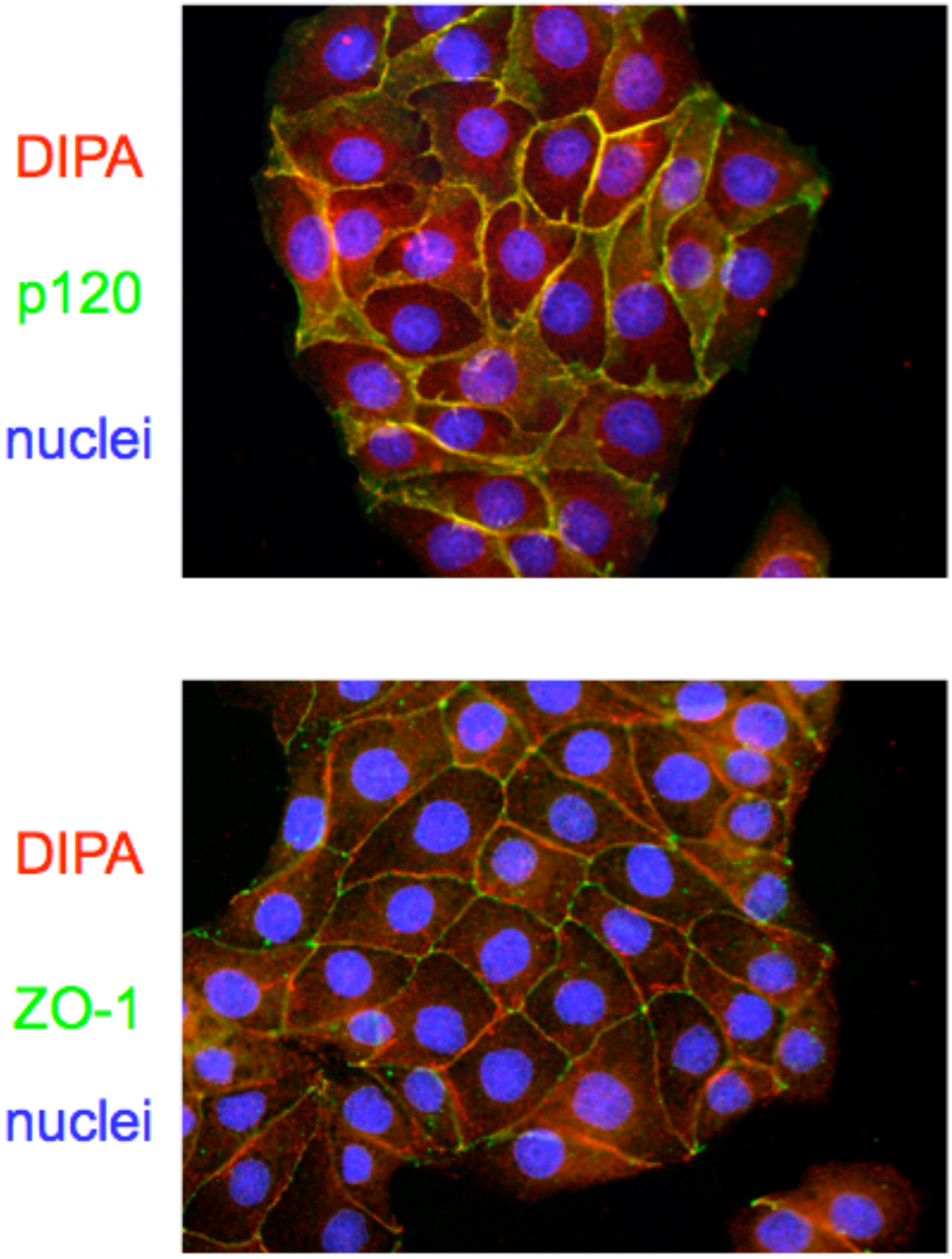

Figure 13C. DIPA co-localizes with p120 at adherens junctions and not tight junctions. C) Enlarged images of the merged panels from MDCK shRNA control cells, 13A (top) and 13B (bottom).

recruiting DIPA to adherens junctions, MDCK cells with stable p120 knockdown were infected with retroviruses containing C-terminal GFP fusion proteins. Figure 14 shows clearly that p120-1A-GFP, δ-catenin-GFP, and p0071-GFP can recruit endogenous DIPA to the cell membrane. Neither p120-3A-GFP nor GFP alone can engage DIPA. These results reveal that DIPA interacts with the p120 head domain and its subfamily members for junctional localization. This finding potentially represents an evolutionarily conserved function for DIPA at adherens junctions.

Discussion

The selective expression of p120-1 in mesenchymal cell types suggests a distinct role in mesenchymal functions, most likely mediated by its unique head domain. One possibility is that the p120 head domain recruits to the adherens junction binding partners whose activities are essential for tumorigenesis, EMT, and/or metastasis. Alternatively, the head domain itself has an unidentified activity though induction of structural, and thus functional, changes in adherens junctions.

DIPA is the first ever identified isoform-1 specific binding partner. The yeast two-hybrid data strongly suggest that the interaction is direct, although the unlikely possibility of an undetected bridge protein has yet to be ruled out. Coiled-coil domains commonly participate in protein-protein interactions (Burkhard et al., 2001), and the p120 subfamily members have highly conserved N-terminal coiled-coils which interact with DIPA either by yeast two-hybrid or in MDCK cells. These data suggest that the function of DIPA itself is highly conserved and might be effectively studied in the context of development. Indeed, DIPA proteins from all mammalian species share over 99% identity at the amino

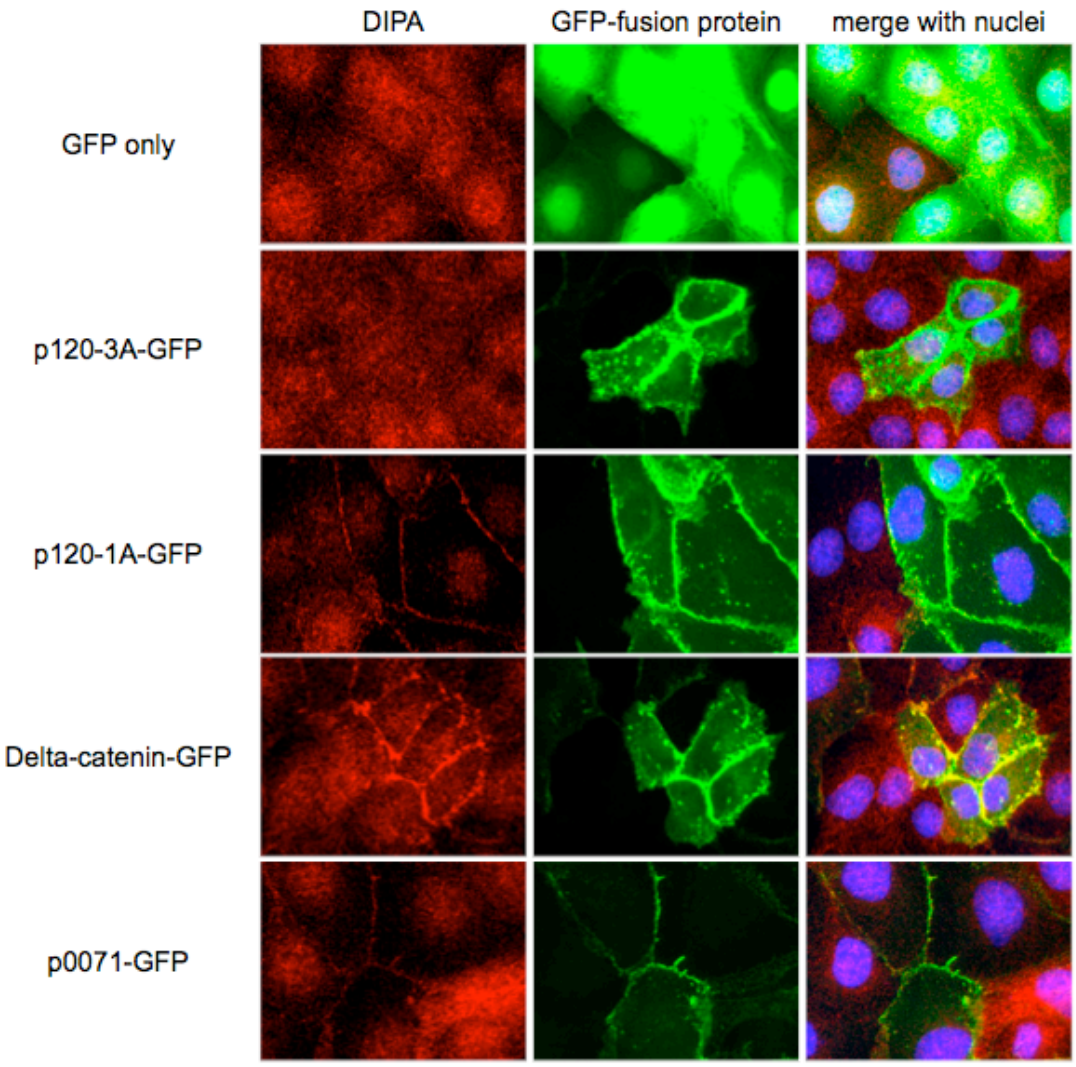

Figure 14. p120-1A, Delta-catenin, and p0071 can rescue junctional DIPA expression in the absence of endogenous p120. MDCK cells stably expressing p120-knockdown shRNA were infected with virus expressing GFP fusion proteins or GFP control. DIPA was detected with the 337 polyclonal antibody.

acid level, and homologous DIPA proteins are found in reptiles and flies.

Our data show clearly that DIPA and p120-1 interact at adherens junctions. Moreover, membrane localization of DIPA is selectively dependent on p120-1 (Fig. 13 & 14). DIPA may influence cell-cell adhesion or cytoskeletal remodeling, however, experiments based on over-expression and/or knockdown of DIPA in MDCK cells reveal no changes in trans-epithelial resistance, adherens junction formation, or actin stress fiber formation (data not shown). It is possible that DIPA does not have an active role at the membrane. For example, p120-1 and its subfamily members may simply sequester DIPA from the nucleus where it presumably functions as a transcriptional repressor (Du et al., 2006). Though not well characterized, DIPA appears to function in a cascade of transcriptional events associated with adipogenesis. Through its direct interaction with C/EBP-β and -γ, DIPA can repress differentiation of 3T3-L1 cells into adipocytes (Bezy et al., 2005). Interestingly, Kaiso was recently identified as an essential factor for the localization of SMRT, a transcriptional co-repressor, to the promotors of adipogenic genes (Raghav et al., 2012). Exogenous expression of p120 itself in the cytoplasm of 3T3-L1 can down-regulate adipogenesis (Kim, 2011). The mechanisms of their interactions have not been elucidated, but p120 and its binding partners are emerging as important modulators of adipogenesis and, perhaps, mesenchymal fate determination.

In support of its role as a transcriptional regulator, p120 has previously been linked to TCF4, a Wnt pathway transcription factor inhibited by DIPA (Iwai et al., 2008). In some model systems, Kaiso competes with TCF/LEF factors for access to target gene promoters, and p120-3 can promote Wnt signaling by sequestering Kaiso out of the nucleus (Park et al., 2005; Hong et al., 2012). It is possible by similarity that the p120-1

interaction with DIPA functions to relieve DIPA-mediated repression of TCF4 and promote Wnt pathway activity.

One potential role for DIPA is in regulation of adherens junctions during motility. HGF (also known as scatter factor) induces migration in epithelial cells through its receptor c-Met. p120-1 promotes this motile phenotype and p120 mutants lacking the N-terminal 78 amino acids prevent HGF-induced scattering (Cozzolino et al., 2003; Yanagisawa et al., 2008). HGF also up-regulates DIPA transcription (Saeki et al., 2009). p120-1 or one of its subfamily members is clearly required for DIPA localization to cell junctions in MDCK cells (Fig. 12A, B). One possibility is that DIPA is up-regulated by HGF to interact with p120-1 and regulate migration.

The identification of the DIPA and p120-1 interaction opens a window into mechanisms that regulate neuronal development and diseases of cortical malformation. Amino acids spanning the DIPA coiled-coil domains are 54% identical and 73% similar to a region within the CCDC85C protein. The gene encoding CCDC85C has a pathogenic mutation in the recently described hemorrhagic hydrocephalus (*hhy*) mouse (Mori et al., 2011). These mice develop Subcortical Band Heterotopia (SBH) stemming from a failure of neurons to migrate normally through the neocortex. Consistent with SBH, the ependymal cells lining the lateral ventricals are dysmorphic, which contributes to the development of hydrocephalus. Another p120-related protein, Soluble N-ethylmaleimide sensitive factor (NSF) attachment protein alpha (αSNAP), is mutated in the hydrocephalus with hop-gait (*hyh*) mouse, which also has a SBH (Chae et al., 2004). The αSNAP protein is a vesicle trafficking regulator that is required for p120-1 stability specifically and membrane localization of all p120 isoforms (Naydenov et al., 2012).

Additionally, exogenous DIPA immunoprecipitates in a complex with the actin-cytoskeletal protein Filamin-A, which is the most commonly dysregulated protein in human periventricular heterotopias (data not shown; Sarkisian et al., 2008). It is unknown whether CCDC85C interacts directly with p120 or any of its subfamily members, or whether DIPA mutation causes developmental defects. However, these results suggest a potential role for p120-1 and DIPA or CCDC85C in the molecular mechanisms of cortical development.

CHAPTER V

SUMMARY AND FUTURE DIRECTIONS

Summary

This dissertation describes a project that began with an interest in understanding the role of specific p120 isoforms in cancer. Through yeast two-hybrid screens and validation, DIPA was identified as the first ever p120-1 specific binding partner. Because of its relationships to cancer-related genes, I investigated DIPA as a likely key to unlocking the function of p120-1 in tumorigenesis.

The first half of this project comprised the building of novel reagents for studying DIPA and p120. A panel of polyclonal and monoclonal antibodies were generated with specific affinity to DIPA, a series of DIPA knockdown and shRNA rescue cell lines were created, truncation mutants of DIPA and p120 were cloned, and fusion proteins of p120 isoforms and family members were produced. These tools continue to be critical to the project and have been helpful to other lab members.

The second half of the project focused on the characterization of the interaction between DIPA and p120. First, DIPA was observed for the first time as a cell-cell junction-associated protein. Secondly, precise co-localization and specific co-immunoprecipitation was shown for DIPA and p120-1. Finally, it was determined that p120-1 is required for DIPA junctional localization, and that p120 subfamily members can sufficiently rescue this phenotype in the absence of p120.

Behind the figures lie countless attempts to link the DIPA/p120 interaction to known

p120 functions, such as adherens junction formation and maintenance, RhoGTPase regulation, and Wnt signaling. Other, more objective, assays were performed to determine the effect of DIPA over-expression or knockdown on cell proliferation, apoptosis, differentiation, EMT, and migration. Not a single experiment showed that DIPA affected these processes.

Mere months before my scheduled defense date, a paper was published describing the pathogenic mutation in CCDC85C, which causes severe hemmorhagic hydrocephalus and subcortical band heterotopia (Mori et al., 2011). Subsequent connections to DIPA and p120 biology were identified and the focus of my project shifted dramatically toward neural development.

Future Directions

The significance of DIPA binding to p120 subfamily members is that the function of DIPA is likely conserved throughout evolution. Also, the complexity of neural development may preclude tissue culture for phenotypic experiments. Therefore, it is possible that a specific phenotype will be apparent if DIPA is manipulated in an experimentally favorable animal model. To this end, I am currently collaborating with Caleb Doll and Josh Gamse, Ph.D. in the Vanderbilt Department of Biological Sciences to study the effects of DIPA over-expression and depletion in zebrafish neural development. I have begun experiments with injection of DIPA mRNA or morpholinos into one-cell stage zebrafish embryos. The aim of these studies is to determine if DIPA affects the migration and patterning of neurons in the cortex. Initial results suggest a potential role for DIPA in neural tube development, but more studies are necessary for

validation.

DIPA and p120 may interact to affect specific cellular processes required for neuron migration and ependymal cell function that are perturbed in the *hyh* and *hhy* mutant mice. One such process is the regulation of cilia, which has not been tested with respect to DIPA function. Primary cilia are essential for sonic hedgehog signaling, motility, and other aspects of brain development. Motile cilia are closely related structures that line ependymal cells and function to maintain hydrostatic pressure within the ventricles. Failure of either primary cilia or motile cilia, as is seen in the *hhy* mouse, is causally associated with cortical malformation and/or hydrocephalus (Mori et al., 2011). To determine the role of DIPA in primary cilia formation and maintanence, I have performed knockdown and shRNA rescue experiments in polarized MDCK cells. The initial results suggest that DIPA is important for normal primary cilia formation, but further verification is required.

The data presented here also suggest that neural development may be regulated by DIPA and a p120 subfamily member, like δ-catenin. Alternatively, the DIPA-like protein CCDC85C could bind to p120-1 or subfamily member and regulate a developmental function. My future directions also include testing these interactions and investigating the possibility that the DIPA/p120 relationship is representative of a larger family of interacting proteins.

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