The Role and Mechanisms of Dlg5 in the Regulation of the Hippo Signaling Pathway

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ABSTRACT

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The Hippo signal transduction pathway plays a pivotal role in regulation of normal development, adult organ homeostasis, and cancer. At the core of the Hippo pathway, MST1/2 kinases phosphorylate and activate LATS1/2 kinases, which in turn phosphorylate and inactivate transcriptional co-activators YAP1 and TAZ. Phosphorylated YAP1 and TAZ are inhibited from entering the nucleus and are instead cytoplasmically retained or degraded. Cells monitor their microenvironment, by sensing local cell density and cellular polarity, and use Hippo signaling to regulate the maintenance of normal cell numbers necessary for tissue function. The mechanisms responsible for the connection between the cellular microenvironment and Hippo pathway are poorly understood. In this study, in collaboration with Dr. Andrew Emili's laboratory in the University of Toronto, I reveal a novel mechanistic connection between the Hippo signaling

pathway and the apical-basal cell polarity protein DLG5. I found that DLG5 binds to MST1/2 kinases of the core Hippo pathway. Inactivation of *Dlg5* in mice *in vivo* and in primary cells *ex vivo* results in increased Hippo pathway activity and decreased levels and activity of YAP1 and TAZ. Overexpression of DLG5 inhibits Hippo signaling and promotes the expression of Hippo pathway target genes. In genetic epistasis experiments in mice, *Dlg5* shows a strong genetic interaction with both *Yap1* and *Taz (Wwtr1)*. Mechanistically, I found that DLG5 negatively regulates the Hippo pathway by inhibiting the binding between MST1/2 and LATS1/2. This study increases the understanding of the connection between apical-basal polarity and the Hippo pathway and identifies apical-basal polarity family protein DLG5 as a novel interactor and regulator of the Hippo signal transduction pathway.

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CHAPTER 1: INTRODUCTION

Apical Basal Cell Polarity

Introduction

In multicellular organisms, cells have to come together to form complex structures such as tissues and organs. In order for development to occur correctly and for tissue homeostasis to be maintained, cells need to orient themselves properly relative to neighboring cells and adhere to them. When these processes aren't able to proceed normally, either developmental defects or cancer can result.

Core Apical Basal Polarity Proteins

There are three main protein complexes that are important for the establishment and maintenance of apical basal polarity. aPKC-Par3 (Bazooka)-Par6 and Crumbs-Pals1(Stardust)-Patj localize to and help define the apical domain [1-4]. On the other side of the cell, the Dlg-Lgl-Scrib (Discs large-Lethal giant larval-Scribble) proteins promote the establishment of a basolateral domain identity while antagonizing the function of the apical complexes [3, 5] (Figure 1). The apical complexes in turn antagonize the basolateral polarity complexes, and through this mutual inhibition the apical and basolateral domains are defined and apical junctional complexes (AJCs) are established. AJCs consist of tight junctions (TJs) and adherens junctions (AJs) [4]. AJCs are not only important for the separation of the apical and basolateral domains, but also intercellular adhesion and 3D organization of tissues and organs.

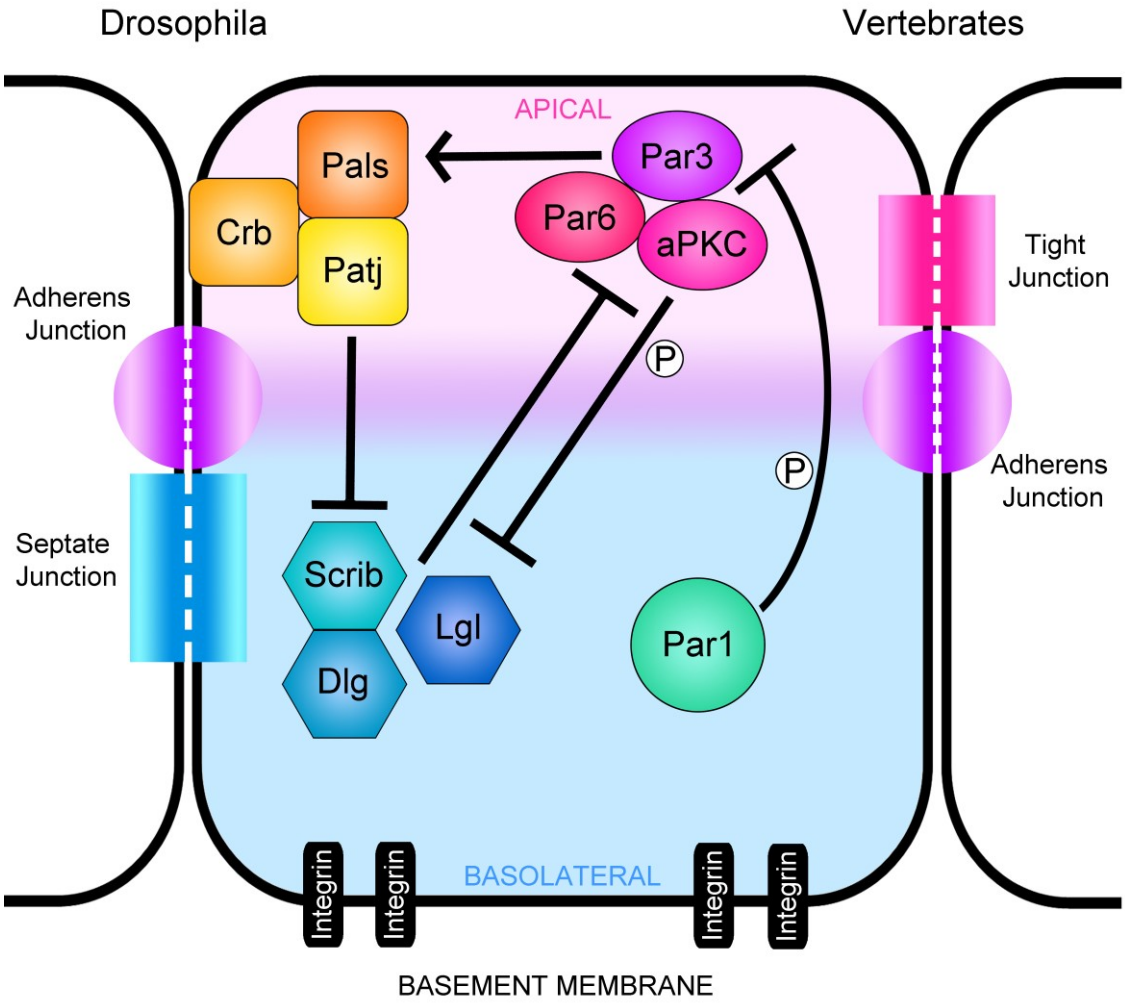


Figure 1: **Core apical-basal polarity proteins.**
The apical-basal polarity proteins mutually phosphorylate and antagonize one another, thereby defining the apical and basolateral domains. Source: Modified from [4] and [6].

The precise molecular mechanism by which the Dlg-Lgl-Scrib proteins inhibit the apical proteins is not known, however, Dlg-Lgl-Scrib proteins are known to antagonize the apical aPKC-Par3-Par6 and Crumbs-Pals1-Patj complexes [7, 8]. While there is still more to learn, we do have a basic understanding of the mechanisms involved in the establishment of apical basal polarity; aPKC functionally inactivates Lgl by phosphorylating Lgl when it is in the apical domain [9, 10]. Lgl in turn inhibits the basal localization of aPKC, restricting aPKC to the apical domain [11]. In addition, during the initial stages of polarization, Lgl competes with Par3 for binding to aPKC and Par6, thereby inhibiting tight junction formation [12]. Impaired activity of the basolateral proteins results in mislocalization of apical markers and epithelial overgrowth [5]; Par1 is another basolateral polarity protein that is able to inhibit the apical aPKC-Par3-Par6 complex. Par1 inhibits Par3 function and promotes its dissociation from Par6 and aPKC. Another protein, cell division cycle 42 (Cdc42), also needs to be mentioned here as it binds to members of the aPKC-Par3-Par6 polarity complex and regulates their function. Par6 interacts with Cdc42 via its Cdc42/Rac interaction binding (CRIB) motif and this enhances the kinase activity of Par6-bound aPKC [13]. The binding of Cdc42:GTP to Par6 causes Par6 to unfold and reveal a Pals1 binding site [14]. A key function of Par6 is to facilitate the interaction between aPKC and Par3 and Lgl [12]. Mutant screens in *Drosophila* suggest that there is also negative regulatory feedback between the Crb-Pals1-Patj and Dlg-Lgl-Scrib complexes, although the precise mechanism is not understood [7, 8]. Thus, while it is clear that the apical and basal polarity complexes antagonize one another's functions and localization, all of the mechanistic details are not yet known.

Discovery of Apical Basal Polarity Proteins

aPKC-Par3 (Bazooka)-Par6 complex

The first polarity proteins were discovered in *C. elegans* in 1988 as part of a genetic screen aimed at identifying the genes that disrupt the asymmetries normally present in the *C. elegans* zygote [15]. After fertilization, the zygote undergoes a series of asymmetric divisions, which creates daughter cells of different sizes and cell fates. Ribonucleoprotein particles called P granules asymmetrically localize to one pole prior to cell division and segregate with the germline daughter P-cell [3, 16, 17]. The genetic screens, which were aimed at identifying genes that caused abnormalities in the cleavage pattern, timing of cleavage, or P-granule distribution, were able to identify six *par* genes (short for partition defective).

Sequence analysis revealed that Par1 and Par4 are protein kinases [18, 19], Par2 contains a ring finger zinc-binding domain and an ATP-binding motif [20], Par3 and Par6 both contain PDZ domains (PSD-95, Discs large, ZO-1) [21, 22], and Par5 is a 14.3.3 protein, which binds to target proteins in a phosphorylation-dependent manner [23, 24] (Figure 2). Several years after first six *pars* were identified, a seventh *par* protein, atypical protein kinase C (PKC3) was discovered [25]. In wild type zygotes, aPKC, Par3, and Par6 localize to the anterior cortex, while Par1 and Par2 localize to the posterior cortex [19, 21, 22, 25, 26]. It was determined that PAR-3, PKC-3, and PAR-6 act together and that removing any of these proteins results in mislocalization of the other two [22, 25]. Hence the notion of the aPKC - Par3 - Par6 apical polarity complex was born.

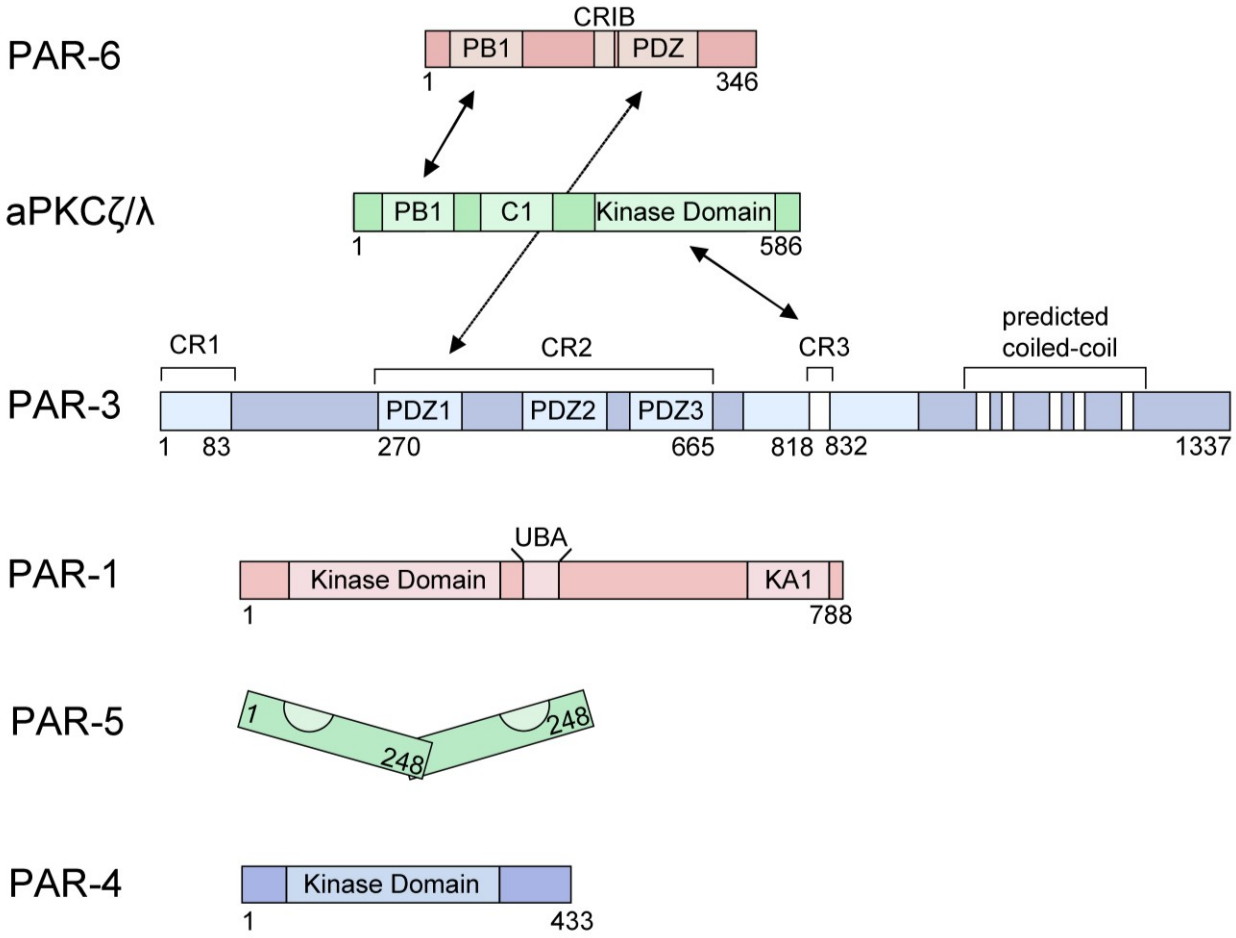


Figure 2: The PAR family of proteins, with relevant domains.
PAR-6 contains three functional domains: a Phox and Bem1p (PB1) domain that mediates dimerization/oligomerization, a Cdc42/Rac1 interactive binding (CRIB) domain that binds to active small GTPases, and a PSD-95, Dlg1, ZO-1 (PDZ) domain. aPKC contains a PB1 domain, a C1 domain that mediates the binding of diacylglycerol and phorbolesters, and a kinase domain. PAR-3 contains three PDZ domains and several predicted coiled-coil domains. The conserved regions (CR) 1 to 3 of PAR-3 are conserved between different species. aPKC and PAR-6 interact via their PB1 domains and the interaction between PAR-3 and aPKC is mediated by the kinase domain of aPKC and the CR3 of PAR-3. PAR-6 and PAR-3 directly interact via the PDZ domain of PAR-6 and first PDZ domain of PAR-3. PAR-1 is a Ser/Thr kinase with an N-terminal kinase domain, an ubiquitin-associated (UBA) domain, and a kinase-associated domain 1 (KA1) domain. PAR-5 is a member of the 14-3-3 family of proteins, which form dimers and bind to target proteins in a phosphorylation-dependent manner. PAR-4 is a Ser/Thr kinase without typical domains. In contrast to the apical PAR-3 - aPKC - PAR-6 proteins, basolateral PAR-1, PAR-4 and PAR-5 are not though to form a complex. Source: Modified from [27].

PAR-1 and the PAR-3 – aPKC – PAR-6 protein complex are part of the *par* family and mutually inhibit one another by phosphorylation. PAR-1 phosphorylates PAR-3 and inactivates the PAR-3 – aPKC – PAR-6. PAR-1 phosphorylates PAR-3 at two serine residues, S144 and S889, and this primes PAR-3 for binding to PAR-5 (14-3-3). 14-3-3 proteins can bind to a variety of signaling molecules, including kinases, phosphatases, and transmembrane receptors [28]. 14-3-3 proteins have been shown to function in several distinct ways including changing the conformation of bound proteins, masking specific sequences or structural features, and facilitating the interaction between two simultaneously bound proteins by serving as a scaffold [29]. This binding of PAR-5 to phosphorylated PAR-3 functionally inactivates PAR-3 by inhibiting its association with aPKC and PAR-3 oligomerization [30]. aPKC, in turn, inactivates PAR-1, by phosphorylating PAR-1 at threonine residue T595 which leads to the binding of PAR-5 (14-3-3) to phosphorylated PAR-1. As a consequence, PAR-1 is excluded from the membrane and its kinase activity is strongly attenuated. These mutually inhibitory phosphorylations cause the PAR-3 – aPKC – PAR-6 complex to be excluded from the basolateral membrane domain and PAR-1 to be excluded from the TJ region, respectively. These two functions together contribute to the establishment and maintenance of distinct apical and basolateral membrane domain identities.

Crumbs-Sdt-Dpatj Complex

Crumbs (crb) was first identified in *Drosophila* by Jürgens et al. and named after the severe disruption of the cuticle that is observed in *crb* mutants [31]. Normally, in epithelial cells, crumbs localizes to the apical membrane and cell borders. *Crumbs* is a transmembrane protein with a long extracellular component composed of 29 EGF-like repeats and a short intracellular

region [32] (Figure 3). It was Tepass et al. who first recognized the connection between *crumbs* and epithelial polarity [32]. The zonula adherens is a large circumferential cell-cell adhesion structure located at the apical lateral cell surface, which is a junctional complex component in both *Drosophila* and mammals. Embryos which lack *crumbs* are unable to assemble or maintain zonula adherens and this leads to a loss of cell polarity and cell-cell adhesion [32, 33].

The Crumbs binding partner and *Drosophila* Pals1 homologue, *Stardust (sdt),* was discovered when Tepass and Knust observed that the defects in *Drosophila sdt* mutant embryos were very similar to the *crb* mutant phenotype [34]. Their work indicated that *sdt* and *crb* are in the same pathway and that *sdt* operates downstream of *crb,* which is thought to activate *sdt* [35]. Stardust has a PDZ domain, which it uses to bind to Crumbs, as well as a SH3 (Src homology region 3) domain and GUK (Guanylate kinase – thought to be inactive because it is missing the P-Loop that binds ATP) domain [36, 37]. The presence of PDZ, SH3, and GUK domains identifies stardust as a MAGUK (membrane associated guanylate kinase) family protein.

The third component of this polarity complex was discovered by Bhat et al. and named *Discs Lost (dlt)* [38]. *Dlt* was shown to interact with Crb, have four PDZ domains, and be required for epithelial cell polarity. Later studies revealed that the *dlt* gene locus in fact contained several genes and the protein discovered by Bhat et al. was subsequently renamed *Dpatj* (the phenotype attributed to *dlt* actually corresponded to a cytoplasmic Codanin-1-like protein) [39]. While Pielage et al. reported that *Dpatj* mutations did not disrupt cell polarity, Nam et al. did find that *Dpatj* was required for AJ and apical domain morphogenesis of photoreceptor cells [40]. Shortly after, several other studies observed that Dpatj forms a complex with Crb in *Drosophila* [41, 42].

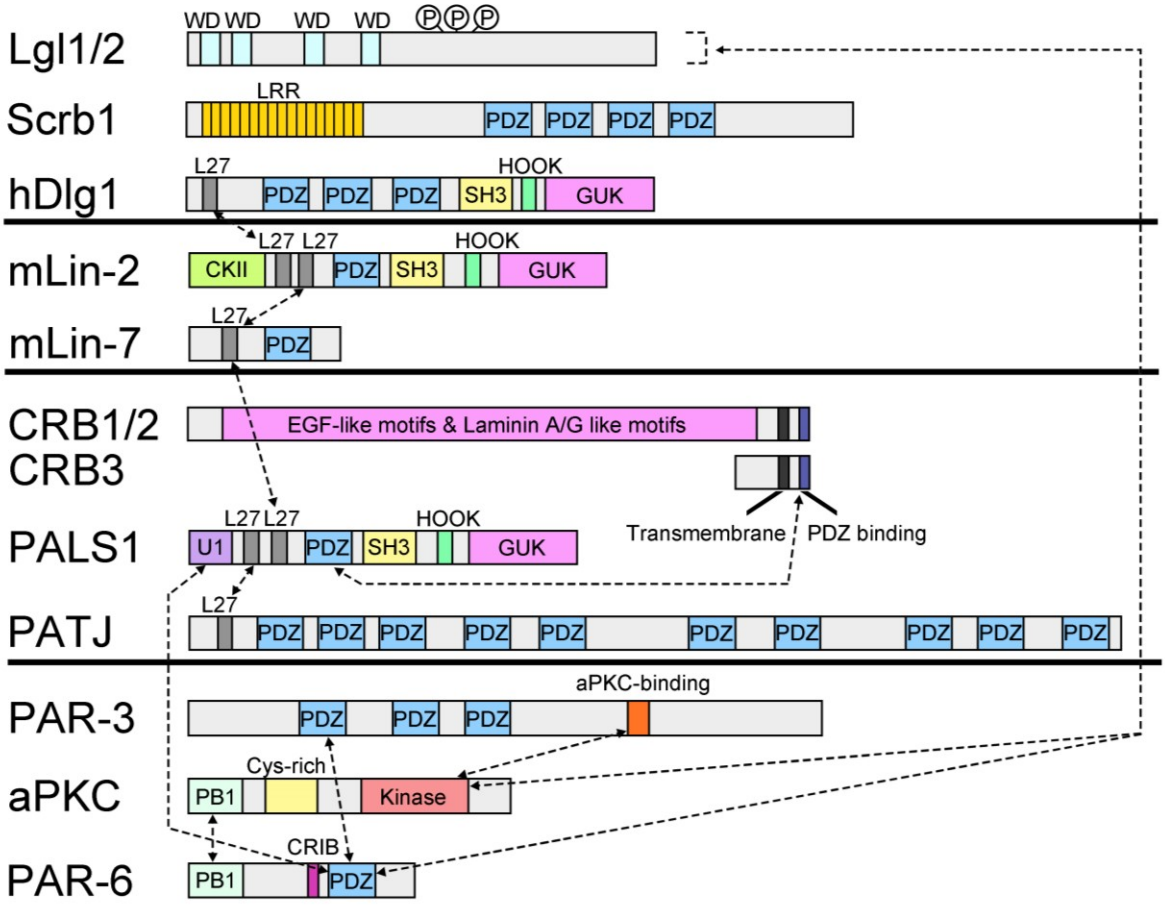


Figure 3: Domain structures of core mammalian polarity proteins and their interactions. The phosphate groups in Lgl1/2 are Ser residues phosphorylated by aPKC. CKII, CaM kinase-like domain; GUK, guanylate kinase domain; KA1, kinase-associated 1 domain; L27, Lin-2 Lin-7 domain; LRR, leucine-rich repeat, PB1, Phox and Bem1p domain; PDZ, postsynaptic density 95/Discs Large/Zonula Occludens 1 domain; SH3, Src homology 3 domain; U1, unknown interaction domain 1; WD, WD40 repeat; Protein interactions are shown by dotted lines with arrows. Source: Modified from [43].

Dlg-Lgl-Scrib Complex

The *scribble* gene *(scrib)* was identified as part of a screen to identify genes that were important for epithelial morphogenesis in *Drosophila*. The cuticle surface of *scrib* mutants was riddled with holes, hence inspiring the name *scribble* [44]. The Scrib protein has 16 leucine-rich repeats (LRR) as well as four PDZ domains, which makes it part of the LAP (LRR and PDZ) family of proteins [45]. Localization of Scrib protein changes over the course of development; Early in development, Scrib localizes to basal membranes, then the zona adherens, and eventually the septate junctions. *Scrib* mutants exhibit a mislocalization of apical proteins such as Crb and DPatj to the basolateral membrane domain, which suggests that an important function of *Scrib* is to keep apical polarity proteins away from the basolateral membrane [44].

The correlation between *scribble* and *discs large (dlg)* and *lethal giant larvae (lgl*) was first noted by Bilder et al., who observed that *dlg* and *lgl* mutants had similar phenotypes and that Dlg, Lgl, and Scrib localization overlaps in epithelial cells [46]. *Lgl* and *dlg* were identified as tumor suppressors because their mutation resulted in tumor formation and death in *Drosophila* larva [46]. Mutations in *dlg* result in neoplastic overgrowth of the imaginal discs in *Drosophila* [47]. Bilder et al. also demonstrated that *dlg* and *lgl* genetically interact. Dlg is a MAGUK family protein which contains three PDZ domains, an SH3 domain, and GUK domain, as well as a L27 (Lin2 Lin7 binding) domain [47]. Lgl mutations resulted in tumors in the larval brain and imaginal discs [48], suggesting the *lgl* may play a role in cell proliferation and post-mitotic differentiation. Lützelschwab et al. analyzed the protein sequence of *lgl* and hypothesized that it may be important for cell adhesion, based on its structural homology to other proteins [49].

Lützelschwab also identified a number of short repeating motifs in the protein, which were later identified as WD repeats (short amino acid sequences ending in Trp (W)-Asp (D)).

Mammalian Apical Basal Polarity Complexes

While there are significant similarities between *C. elegans*, *Drosophila*, and mammalian polarity mechanisms, the mammalian apical-basal polarity complexes are more complex, due in part to the large expansion of the number of corresponding orthologous genes, their homologues, and various differential splicing isoforms (Table 1).

The aPKC Family

In mammals there are two aPKC genes: *PRKCI* and *PRKCZ*, which I will refer to here as aPKCλ/ι and aPKCζ [50, 51]. Both aPKCλ/ι and aPKCζ appear to be highly expressed in the lung and brain, while aPKCζ is also expressed in the testis and kidney. Structurally, aPKCλ/ι and aPKCζ differ from conventional PKCs because they have N-terminal Phox and Bem1 (PB1) domains, which allow them to interact with PAR6. Additionally, each of these kinases has an incomplete C1 domain and is missing a C2 domain. Therefore, unlike conventional PKCs, they cannot be activated by Ca^{2+} [52].

Differences in expression patterns may be responsible for the differences in aPKCλ/ι and aPKCζ mouse mutant phenotypes; however, there isn't sufficient data to suggest differing functions for these two aPKC isoforms. Analyses of aPKC knockout mice have provided some insight about their function *in vivo*. aPKCζ knockout mice are grossly normal, but show defects in NF-κB signaling [53]. By contrast, full knockout of aPKCλ results in lethality by embryonic day 9 [54]. Loss of aPKCλ in mouse podocytes (glomerular visceral epithelial cells) results in

Table 1: Polarity complex proteins in *Drosophila melanogaster, Caenorhabditis elegans,* and mammals.
Gene symbols and aliases for core polarity proteins. Modified from source: [3].

Table 1
Polarity complex proteins in *Drosophila melanogaster*, *Caenorhabditis elegans* and mammals

	Drosophila melanogaster	*Caenorhabditis elegans (1)*	Mammals	
			Gene symbols (2)	Human (2,3)
				Aliases and previous symbols
Par6/Par3/aPkc complex	DmPar6	–	*PARD6A*	PAR6; PAR6A; TIP40; PAR6α; PAR6C
		–	*PARD6B*	PAR6B
		Par-6	*PARD6G*	PAR6G; Par6γ; PAR6D
	Bazooka	–	*PARD3*	PAR3A; PAR3; PAR3α; ASIP; CTCL Tumor antigen se 2–5
		Par-3	*PARD3B*	PAR3β; PAR3L; ALS 2CR 19
	DmaPkc	PKC-3	*PRKCi*	PKCI; nPKCι; aPKCι/λ; PRKCι/λ
		–	*PRKCz*	PKC2; nPKCζ
Crb/Pals/Patj complex	Crb	Crb1	*CRB1*	RP12; LCA8
		Eat-20	*CRB2*	
		–	*CRB3*	
		–	*MPP1*	EMP55
		–	*MPP2*	DLG2
		–	*MPP3*	DLG3
		–	*MPP4*	DLG6
	Sdt	TAG-117	*MPP5*	Pals1
		C50F2.8	*MPP6*	Pals2; VAM-1; p55T
		Y55B1BR.4	*MPP7*	
	Dpatj*	–	*INADL*	PATJ
		MPZ-1	*MPDZ*	MUPP1
Scrib/Dlg/Lgl complex	Scrib	LET-413	*SCRIB*	SCRB1; CRIB1; Vartul; LAP4
	Dlg	dlg-1	*DLG1*	SAP97; hDlg
		–	*DLG2*	PSD-93; Chapsyn110
		–	*DLG3*	SAP102; NE-DLG
		–	*DLG4*	PSD95; SAP90
		F44D12.1	*DLG5*	P-DLG
	Dlgl	– (5)	*LLGL1*	L2GL1; DLG4; HUGL1; LGL1
		–	*LLGL2*	L2GL2; HGL; LGL2

disrupted apical basal cell polarity, focal segmental glomerulosclerosis, and a disassembly of slit diaphragms (interdigitated processes that form specialized intercellular junctions) [55]. Conditional depletion of aPKCλ in the eye lens results in cataracts, fibre disorganization, and disruption of apical junctions [56]. Brain specific knockout of aPKCλ results in loss of adherens junctions, retraction of apical processes, impaired interkinetic nuclear migration, and disordered neuroepithelial tissue architecture [57]. By contrast, intestine specific knockout of aPKCλ causes no obvious detrimental consequences to intestinal epithelial tissue architecture, function, or polarity [58].

The Par Family

PAR3

There are two Par3 genes in mammals: *PARD3* and *PARD3B*. PAR3A (coded by PARD3) interacts with PAR6 via its first PDZ domain [59]. While PAR3B has three PDZ domains as well as a region that is homologous to an aPKC binding domain, it does not appear to be able to bind to aPKC [60, 61]. PAR3A is expressed at high levels in brain, heart, and kidney, while PAR3B is expressed at high levels in the kidney, lung, and skeletal muscle [59]. Knocking out *PAR3* in mice causes embryonic lethality, due to defects in epicardium development [62]. Cells of *PAR3*-deficient epicardium show defects in the localization of PAR-6 and aPKC to the apical domains. Since the cell-substratum and cell-cell interactions provide the spatial cues for epithelial cell polarity, the authors reasoned that PAR3 interprets the spatial cell polarity cues from integrins and cell-cell contacts to assist in the establishment of the apical cortical domain.

PAR6

In mammals there are three PAR6 genes: *PARD6A, PAR6B,* and *PAR6G.* All three have an N-terminal PB1 domain, which allows them to bind to aPKC, as well as a Cdc41/Rac interaction binding (CRIB) motif, which allows the PAR6 proteins to bind GTP-bound and activated Cdc42 or Rac GTPases [63]. In addition, PAR6A/C (coded by the *PARD6A* gene), PAR6B, and PAR6D/G (coded by the *PARD6G* gene) all have PDZ domains that allow them to bind to PAR3 and CRB3 [22, 59, 64, 65].

PAR1

Mammalian Par-1 is excluded from the apical membrane domain and tight junctions and instead localizes to basolateral membranes [66]. There are four mammalian PAR1 family members: mPar-1c (MARK1), mPar-1b (EMK/MARK2), mPar-1a (C-TAK1/MARK3), and mPar-1d (MARKL1, MARK 4) [67-71]. The microtubule-affinity regulating kinases (MARK/partition-defective (Par-1)) kinases are part of the adenosine monophosphate-activated protein kinases (AMPK)/sucrose non-fermenting 1 (Snf1) subfamily of the Ca^{2+}-calmodulin-dependent protein kinases (CaMK) group [72]. Mammalian aPKCζ is able to interact with mPar-1b (EMK/MARK2) and phosphorylates it on T595 [73]. Phosphorylation of mPar-1b by aPKC negatively affects mPar1-b kinase activity and membrane localization. A number of studies have examined the effect of knocking out *MARK* genes *in vivo* to better understand their function. *Mark1* knockout mice have not been reported in the literature. *Mark2* null mice are smaller than littermates and demonstrate reduced fertility [74]. Segu et al. observed that these mutants have impaired spatial learning and memory [75]. *Mark2* knockout mice also display autoimmune

disease and immune system dysfunction as well as increased metabolic rate, decreased adiposity, and insulin sensitivity [76, 77]. *Mark3* knockout mice demonstrate reduced adiposity in addition to resistance to hepatic steatosis and defective gluconeogenesis [78]. *Mark4*$^{-/-}$ are born at the expected Mendelian ratios and show no overt abnormalities; however, *Mark4*$^{-/-}$ male (but not female) mice are insulin hypersensitive and resistant to diet-induced obesity [79].

The CRB Family

Three genes homologous to *Drosophila* crumbs have been identified in mammals: *CRB1, CRB2,* and *CRB3*. CRB1 is present in the brain and retina and CRB2 is present in the brain, retina, and kidney [80, 81]. CRB3 is predominantly expressed in epithelial tissues and skeletal muscle [65, 82, 83]. The extracellular domain of CRB3 is much shorter than that of either CRB1 or CRB2. [84] In addition, CRB3 has a SH3 binding site, which is missing in other CRB proteins [65]. Otherwise, the cytoplasmic domains of CRB1, CRB2, and CRB3 are fairly similar and each of them contains a PDZ domain-binding motif [65] and FERM (band 4.1-ezrin-radixin-moesin) protein binding domain [42, 85].

Knockout mice of all three CRB genes have been generated and analyzed. *CRB1*$^{-/-}$ knock-out mice develop focal retinal disorganization of the photoreceptor layer and experience progressive retinal degeneration after prolonged exposure to light [86]. *CRB2* is required for normal gastrulation and loss of *CRB2* causes embryonic lethality in mice [87]. Conditional loss of *CRB2* in the mouse retina results in progressive disorganization and thinning of the photoreceptor layer [88]. *CRB3*$^{-/-}$ mice die shortly after birth and have cystic kidneys as well as abnormal lungs. Their intestines display villus fusion, apical membrane blebs, and disrupted microvilli [89].

PALS Mammalian Homologue

PALS1 (also called membrane associated palmitoylated protein 5, MPP5) is the mammalian homologue of *Drosophila* Sdt protein. PALS1 was first identified as a Lin7 binding partner [90]. Lin-7 is known to form a complex with Stardust family member MPP7 and Dlg1 that regulates the stability of DLG1 and directs it to cell junctions [91]. PALS1 is expressed at high levels in the placenta and kidney and at lower levels in the brain, heart, and skeletal muscle [90]. Similar to *Drosophila* Sdt, PALS1 is a MAGUK family protein, which contains PDZ, SH3, hook, and GUK domain as well as two L27 domains [36, 37, 90]. PALS1 functions as an adapter protein, which links CRB and PATJ [92]. PDZ domains of PALS1 bind to both CRB1 and CRB3 proteins via their PDZ domain-binding motifs [83, 92]. The second L27 domain of PALS1 binds to Lin7 [83], while the first L27 domain binds to Patj [92]. Conditional loss of Pals1 in the mouse retina results in reduced levels of Crumbs complex proteins adjacent to adherens junctions and retinal disorganization and degeneration [93]. Complete loss of PALS1 is embryonic lethal, while conditional knockdown of PALS1 in the brain disrupts progenitor cell renewal and induces widespread apoptotic cell death [94].

PATJ Mammalian Homologue

The mammalian homologue of *Drosophila Dpatj* is Pals1-Associated Tight Junction Protein (PATJ). It is also called human Inactivation No After-Potential D-Like (hINADL). PATJ has multiple PDZ domains and localizes to the tight junctions. PATJ is predominantly expressed in epithelial tissues, particularly small intestine, colon, bladder, testis, ovary, pancreas,

heart, kidney, lung as well as brain and skeletal muscle [95, 96]. PALS1 binds to the L27 (also called MAGUK Recruitment or MRE) domain of PATJ [92].

LGL

Two different *LGL* genes have been identified in mammals: *Llgl1* and *Llgl2*. LGL1 protein (encoded by *Llgl1*) is expressed in most tissues, while LGL2 protein (encoded by *Llgl1*) is absent in the developing brain, but present in the majority of other tissues [3]. LGL1 and LGL2 contain multiple WD40 protein repeats, which assemble into a propeller-like structure, which is often involved in protein-protein interactions. Overexpression of LGL1/2 during polarity establishment disrupts formation of junctional complexes in Madin-Darby canine kidney (MDCK) epithelial cells, but this effect is not seen in cells that are already polarized [97]. In order to become basolaterally localized, LGL needs to be phosphorylated by aPKC during the polarity establishment phase [97, 98]. Mutant LGL1 that cannot be phosphorylated by aPKC abnormally localizes to the apical membrane domain of MDCK cells [99].

Llgl1 and *Llgl2* knockout mice were generated and analyzed in the Vasioukhin laboratory [100, 101]. *LLGL1* knockout mice display a perinatal lethal phenotype. They exhibit disorganization of neural progenitor cells, brain hyperplasia, apoptosis, and prominent hydrocephalus [100]. Loss of polarity and subsequent loss of asymmetric cell divisions of neural progenitor cells cause defects in cell cycle exit and differentiation, which causes an increased number of proliferating cells that form structures that resemble primitive neuroectodermal tumors. *Llgl2*$^{-/-}$ pups are smaller than their littermates at birth. This is due to the defects in placental branching morphogenesis. However, after birth, *Llgl2*$^{-/-}$ pups quickly catch up in size and display no obvious abnormalities later in life [101].

SCRIB

There is only one mammalian homologue of *Drosophila scrib*: SCRIB (sometimes called VARTUL). It was isolated in a screen aimed at identifying proteins targeted by the E6 oncoprotein of the human papilloma virus (HPV) [102]. The human SCRIB protein is found in most cell types and is expressed at high levels in the breast, intestine, skin, and placenta [103]. SCRIB is a large cytoplasmic protein with many domains; at its N-terminus SCRIB has 16 LRR, which are followed by 2 LAP-specific domains (LAPSD), and 4 PDZ domains [104]. The LRR domain targets SCRIB to basolateral membrane [103], while its PDZ domains are responsible for SCRIB's interaction with ZO2 (Zona Occludens 2), also known as Tight Junction Protein 2 (TJP2). While ZO2 interacts with a number of tight junction proteins like ZO-1, Caludin, and Occludin, the SCRIB/ZO-2 interaction most likely occurs at the cell junctions instead of the tight junctions [105-107]. When expression of SCRIB is transiently reduced in MDCK cells, they show abnormalities in cell morphology and a delay in tight junction formation [108].

Knockout of SCRIB in mice results in neonatal lethality. $SCRIB^{-/-}$ mice have defects in neural tube closure and show disrupted polarity of stereocilia in the cochlea [109, 110]. Interestingly, Scrib mutant mice do not show obvious apical-basal polarity defects, but display a prominent planar cell polarity phenotype, indicating that mammalian Scrib may have at least partially diverged functionally from its *Drosophila* counterpart [110].

The DLG Family

There are five mammalian Discs Large (DLG) proteins (Figure 4). They contain an SH3 domain, a hook, a GUK domain, and multiple PDZ domains. These domain structures identify them as part of a large membrane associated guanylate kinase (MAGUK) family of proteins, which are known to play a significant role in cell adhesion and establishment of tight junctions [111].

In addition to PDZ, SH3 and GUK domains, DLG1 contains an N-terminal L27 domain [3, 112]. This L27 domain allows DLG1 to bind to Lin7 and Lin2 (also called calcium/calmodulin-associated serine protein kinase, hCASK) [91, 113, 114]. Binding of Lin2 to DLG1 is necessary for lateral membrane domain localization of DLG1 in MDCK cells. Lin7 helps stabilize DLG1 and along with MPP7 helps localize it to cell junctions [91]. Based on phylogenetic analysis it is known that the C-terminal PDZs of DLG5, DLG1-3, and ZO1-3 subfamily are very closely related. In addition, the fourth PDZ domain in DLG5 appears to have arisen from a duplication of the third PDZ domain [112].

The knockout of Dlg1 in mice results in neonatal lethality [115, 116]. *Dlg1*$^{-/-}$ mice have cleft palates, kidney and ureter hypoplasia, hydronephrosis, and strongly impaired peristalsis of the ureter as well as absence of a vagina and seminal vesicle [115, 116]. Mahoney et al. did not find widespread defects in epithelial polarity and cell proliferation. The authors speculated that the absence of defects in epithelial polarity and proliferation *in vivo* may be explained by the presence of functionally redundant DLG family members. By contrast, Iizuka-kogo et al. found that loss of Dlg1 function did not disrupt cell-cell junctional complexes in the developing ureters but did impair epithelial cell proliferation. *Dlg2*, *3* and *4* mutant mice have somewhat milder

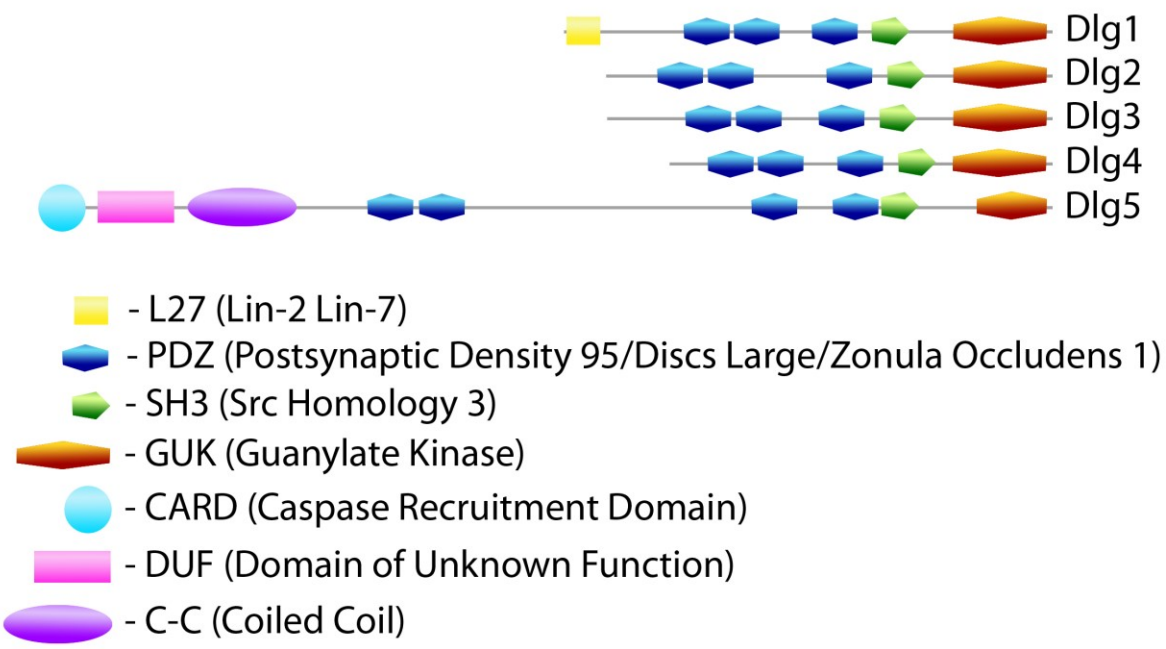


Figure 4: **Discs Large (DLG) Family of Proteins.**
The domains within the five members of Discs Large family of proteins as determined by http://prosite.expasy.org/.

phenotypes, which may be due to their aforementioned potential redundant function. *Dlg2*$^{-/-}$ mutants display defects in NMDA receptor-mediated postsynaptic function [117]. *Dlg3*$^{-/-}$ mice have defects in synaptic plasticity as well as impaired spatial learning [118], while *Dlg4*$^{-/-}$ mice display enhanced long-term potentiation and impaired learning [119].

Significance and mechanisms of Dlg5 function

This thesis work focuses on the investigation of the role and mechanisms of mammalian Dlg5, which is discussed in more detail here. In contrast to other DLG family proteins, Dlg5 is much larger than other Dlg proteins (Figure 4). It contains 4 PDZ domains, as well as an additional domain of unknown function (DUF), caspase activation and recruitment (CARD) domain, and coil-coiled domain at the N-terminus [120]. Dlg5 is expressed at high levels in the kidney and brain and at more moderate levels in the heart, liver, lung, and skin [120].

Dlg5$^{-/-}$ mice were generated and analyzed in the Vasioukhin laboratory [120, 121]. *Dlg5*-null pups are smaller than their littermates and exhibit hydrocephalus, kidney cysts, and an emphysema-like lung phenotype [120]. The hydrocephalus in *Dlg5*$^{-/-}$ mice is caused by a buildup of cerebrospinal fluid in the lateral ventricles, which results from a fusion of the Aqueduct of Sylvius that connects the third and fourth ventricles in the brain. The neural progenitor cells that normally line the developing Aqueduct of Sylvius remain intact in Dlg5-mutants; however, their organization is severely disrupted. The mutant cells fail to maintain proper apical basal cell polarity, which is necessary for the maintenance of the tubular morphology of the aqueduct. Staining for markers of apical-basal cell polarity reveals loss of apical staining for aPKC and mislocalization of AJC proteins N-cadherin, β-catenin, and ZO-1 in Dlg5$^{-/-}$ neural progenitor cells. N-cadherin, which typically localizes to AJC at the ventricular

surface, instead displays a punctate cytoplasmic staining in mutant cells. Multiple follow up experiments determined that Dlg5 is important for cadherin-catenin complex stabilization and cell surface delivery of N-cadherin. Normal cadherin delivery to the AJCs is tightly regulated by post-Golgi vesicle trafficking machinery and the fusion between cadherin-bearing vesicles and the plasma membrane is controlled by the SNARE protein complexes [120]. Nechiporuk et al. demonstrated that Dlg5 binds to Syntaxin-4, a principal basolateral plasma membrane t-SNARE protein, and facilitates targeted delivery of cadherin by linking vesicles with cadherin-catenin cargo with t-SNARE complexes at the membrane [120]. A follow up paper demonstrated an important role of Dlg5 in dendritic spine formation and synaptogenesis in the developing mouse brain; a defect in N-cadherin delivery to the plasma membrane was primarily responsible for the observed phenotype [122].

Disruption of apical basal polarity in *Dlg5*$^{-/-}$ mice is also apparent in organs other than the developing brain. *Dlg5*$^{-/-}$ mice develop renal cysts and their blood urea nitrogen (BUN) levels are abnormally high, which is indicative of impaired kidney function. Analysis of developing collecting ducts in Dlg5$^{-/-}$ kidneys reveals mislocalization of aPKC, which is consistent with defects in the maintenance of apical-basal cell polarity. A similar mislocalization of aPKC was also observed at early time points in developing *Dlg5*$^{-/-}$ lungs. This was followed by a distention of distal air sacs, defective lung branching morphogenesis and impaired differentiation of the lung progenitor cells [120, 121].

Two additional studies, which used truncated forms of Dlg5, identified additional roles for Dlg5. Wakabayashi et al. used yeast two-hybrid screening to identify vinexin as a novel binding partner for Dlg5 [123]. Vinexin is a cytoskeletal protein and participates in the regulation of cytoskeletal organization and signal transduction. Vinexin localizes to sites of cell-

extracellular matrix adhesion and cell-cell contact. Vinexin binds to vinculin, which also localizes to cell-cell and cell-matrix junctions and is a part of the cadherin-catenin junctional complex. Wakabayashi found that Dlg5 colocalizes with β-catenin, suggesting that Dlg5 can link the vinexin-vinculin complex and β-catenin at sites of cell-cell contact [123]. Nakamura et al. also identified p55/MPP1, a palmitoylated membrane MAGUK family protein, as a Dlg5 binding partner by yeast two-hybrid screening. MPP1 is a component of the ternary complex that attaches the spectrin-based skeleton to the plasma membrane in erythrocytes [124].

Additional information implicating Dlg5 in the regulation of self-renewal and differentiation of neural progenitor cells was generated in the laboratory of Dr. LoTurco [125]. Neurogenic cell divisions in the brain occur either in a ventricular or abventricular orientation. Ventricular divisions result in a radial progenitor that stays at the ventricular surface and a neuronal precursor that migrates away from the ventricular surface. Abventricular divisions instead result in two neuronal precursors. [126-128]. Citron kinase (CitK) is a serine/threonine kinase that acts downstream of RhoA to activate myosin-mediated cytokinesis. CitK regulates neurogenic cell division and localizes to the apical membrane domain of neural progenitor cells at the ventricular surface of the developing brain [125]. Chang et al. demonstrated that Dlg5 physically interacts with CitK and CitK fails to apically polarize in *Dlg5*$^{-/-}$ neural progenitors cells. Neural progenitors in the *Dlg5*$^{-/-}$ neuroepithelium undergo an increased number of abventricular divisions and decreased number of ventricular divisions, suggesting that Dlg5 is necessary for proper orientation of cell divisions relative to the lateral ventricle surface in the embryonic brain [125].

Dlg5 also appears to play a role in migration in mammalian cell models. Tomiyama al. identified that the loss of Dlg5 in prostate cancer cells results in increased invasion and

migration. Girdin is a key regulator of migration and localizes to the leading edge (while in quiescent cells Girdin localizes to the cytoplasm around the nucleus) [129, 130]. At the leading edge, Girdin interacts with Akt and participates in actin remodeling. Tomiyama et al. found that Dlg5 depletion results in increased Akt-mediated phosphorylation of Girdin at Ser-1416 [131]. The authors went on to show that Dlg5 interacts with and inhibits the activity of Girdin, which suppress the migration of prostate cancer cells. A genome wide RNAi screen conducted by Smolen et al. validates that Dlg5 has a role in cell motility. Smolen et al. found that the loss of Dlg5 results in induction of cell migration of MCF10A mammary epithelial cells [132].

Sezaki et al. examined the role of Dlg5 in the epithelial-to-mesenchymal transition (EMT) of porcine renal epithelial cells [133]. EMT is the process by which epithelial cells can be transformed into mesenchymal cells and is thought to play a role in conversion of early stage tumors into invasive malignancies [134]. EMT is also associated with dramatic changes in gene expression, including a loss of epithelial markers, such as E-cadherin, and a concomitant increase in mesenchymal markers, such as a-smooth muscle actin (SMA) and fibronectin [135-137]. Sezaki et al. found that RNAi-mediated knockdown of Dlg5 suppresses the expression of E-cadherin and promotes the expression fibronectin and SMA. The induction of SMA expression by Dlg5 depletion depends on TGF-β receptor (TbR)-mediated signaling. These observations suggest that Dlg5 plays a role in TGF-β receptor-dependent signaling and EMT. Additional work by Sezaki et al. found that Dlg5 interacts and colocalizes with both TGF-β type I (TbRI) and type II (TbRII) receptors at the plasma membrane and that the overexpression of Dlg5 results in increased degradation of TbRI [138].

The results that connect Dlg5 to cell migration and EMT are consistent with other studies that suggest Dlg5 plays a role in pancreatic tumorigenesis [139, 140]. Nakamura et al. found that

loss of Dlg5 in pancreatic tumors was associated with increased lymph node metastasis [139], while Taniughi et al. found that down regulation of RAB6KIFL/KIF20A, a kinesin required for membrane trafficking of Dlg5, can attenuate the growth of pancreatic ductal adenocarcinoma PDAC cells [140].

In addition to its role in cell migration and cancer, Dlg5 is also thought to be associated with human inflammatory bowel disease (IBD) and Crohn's disease (CD). Stoll et al. identified two DLG5 haplotypes, R30Q and P1371Q, which are associated with IBD and CD. The R30Q variant is located at the N-terminal part of the Dlg5 protein [141, 142]. P1371Q is part of a conserved PxxP motif, located between the 3rd and 4th PDZ domains (PxxP motifs are thought to typically bind SH3 domain proteins or a group IV WW domain protein after phosphorylation of serine or threonine) [142].

Only some of studies that followed were able to confirm the association between DLG5 and IBD and DLG5 and CD, while others have suggested a gender specific effect: Newman et al. confirmed the association between P1371Q, but not R30Q, and IBD and CD in a Canadian cohort [143], while Lakatos et al. found that DLG5 R30Q is not associated with IBD in Hungarian IBD patients but predicts clinical response to steroids in Crohn's disease [144], Friedrichs et al. performed a multivariate logistic regression analyses on samples from Germany, Italy and Quebec and found that the R30Q variant of Dlg5 was associated with increased susceptibility to CD in men, but not women [145]. Biank et al. suggests that DLG5 R30Q variant may have a protective effect in CD susceptibility in female children [146]. Similarly, Browning et al. observed the R30Q allele to be associated with decreased risk of CD in women [147] while Lin et al. found the R30Q variant to be present at a higher frequency in male IBD

patients in Pennsylvania. Taken together these studies suggest that human Dlg5 may be associated with IBD and CD in certain populations.

Friedrichs et al. also took note of Dlg5's N-terminal caspase recruitment domain (CARD) and recognized that DLG5 shares this domain with nucleotide-binding oligomerization domain containing 2 (NOD2), the first susceptibility factor for CD identified in genetic studies. Friedrichs et al. performed a phylogenetic analysis of Dlg5 relative to other CARD family members and determined that DLG5 is much more closely related to CARD10, CARD11 and CARD14, which are CARD-containing proteins that initiate pro-inflammatory NFκB signaling, than to DLG1-4 [141]. The CARD family can be separated into four groups based on their domains: the nucleotide-binding-domain (NBD) CARDs, the coiled-coil CARDs (CC-CARDs), the bipartite CARDs, and the CARD-only proteins [148]. CC-CARDs are able to recruit bipartite-CARDs, via CARD–CARD interaction and these mediate the assembly of components into caspase and NFκB signaling pathways. The CARD of DLG5 is most similar to that of apoptotic protease activating factor 1 (APAF1), a CARD protein that initiates apoptosis. APAF1 is thought to function as sensor for cell stress and becomes activated by cytochrome c released by mitochondria. Upon oligomerization via its NBD domain, APAF1 recruits caspase 9 (CASP9) via CARD domain, which results in activation of CASP9 and leads to induction of apoptosis [149]. It is uncertain whether DLG5 functions in a similar manner to APAF1; however, based on phylogenetic analysis, it is possible that Dlg5 may regulate NFkB or caspase activation [141].

While there is extensive knowledge about mammalian Dlg5, not as much is known about the function of Dlg5 in *Drosophila*. Recent work by Aranjuez et al. demonstrated that Dlg5 is required for the migration of border cells in *Drosophila*. Border cells are 6–10 cells that migrate

in a highly regulated fashion during mid oogenesis [150]. Dlg5 was identified as one of a number of PDZ domain containing proteins that are required for normal migration of border cells. This function of Dlg5 in cell migration may be similar to its role in promoting cell migration in mammalian cell culture systems.

Apical Basal Polarity and Cancer

In recent years, a number of studies have revealed an intriguing connection between the disruption of the apical basal cell polarity and cancer. In their highly influential review in Cell, Drs. Hanahan and Weinberg identified six fundamental hallmarks of cancer: the maintenance of proliferative signaling, evasion of growth suppressors, resistance to cell death, initiation of replicative immortality, induction of angiogenesis, and activation of invasion and metastasis [151]. Two hallmarks were added a few years later: evasion of immune destruction and reprograming of energy metabolism [152]. The mechanisms of apical basal polarity and the disruption thereof are intimately connected to several hallmarks of cellular transformation and tumor cell dissemination during metastatic progression.

AJCs play a critical role in normal tissue organization. They physically separate the apical and basal cell membrane domains as well as allow cells to adhere to their neighbors. This adhesion is fundamental to the formation of complex structures, such as tissues and organs, and provides a link between the intracellular polarity and extracellular organization. In addition to their important mechanical function, AJCs are also signaling centers that can be thought of as biosensors which mediate communication between adjacent cells and assist in the assimilation of external signaling cues [153]. This is particularly relevant when considering tumor formation in

the context of the tissue microenvironment. The ability of the external microenvironment to influence oncogenic transformation is well evidenced in experiments in which highly malignant tumor cells were injected into mouse and chicken embryos, only to revert to a normal noncancerous phenotype [154, 155]. The tumor-suppressive nature of the normal tissue microenvironment is further evidenced by the difference in the transforming potential of powerful oncogenes between *in vivo* models and *in vitro* cell culture. For instance, chicken embryos injected with v-Src virus fail to develop tumors, however, cells isolated from infected tissues and cultured *in vitro* become transformed within 24 hours [156].

Perhaps the most direct link between apical basal cell proteins and cancer comes from studies in *Drosophila.* The basolateral polarity proteins Dlg, Lgl, and Scrib are neoplastic tumor suppressors, and loss of these proteins causes tumor-like growths in imaginal discs and brain [5, 46, 157]. Misregulation of mammalian Dlg, Lgl, and Scrib is also apparent in many human tumors [158-160]: Scrib has been shown to be downregulated and mislocalized in invasive cervical cancer [161], colon adenocarcinoma [162], endometrial cancer [163], and prostate cancer [164], while Dlg1 is downregulated and mislocalized in cervical, colon, and kidney carcinoma [165]. Lgl1 is downregulated in hmalignant melanoma [166] as well as lung, ovarian, prostate, breast, and colon carcinoma [167]. In addition, aPKC and Par6 are often overexpressed in human tumors [159, 160]. Yet, despite the numerous studies that indicate that apical basal polarity proteins are misregulated or mislocalized in cancer, the role of polarity proteins in the development in cancer in mammals remains contentious. It is unclear whether this loss of polarity is a cause of tumor formation or its consequence [168]. Another complicating factor in mammalian model systems is the large number of corresponding orthologs and splicing isoforms (Table 1).

One convincing link between apical basal polarity and cancer lies in the direct connection between tumor causing viruses and polarity proteins. There are about 150 subtypes of HPV and, of these, only about a dozen are cancer causing viruses [168]. HPV-16 and HPV-18 are two particularly oncogenic strains, and they together are thought to cause approximately 70% of all cervical cancers worldwide [169, 170]. Interestingly, the E6 viral oncoprotein in these viruses contains a C-terminal PDZ binding motif (PBM) [171]. This motif is not present in low risk HPV strains, indicating that this motif may play a direct role in the oncogenic nature of HPV-16 and HPV-18 viruses. The PBM motif allows E6 from HPV-16 and HPV-18 to bind to cellular proteins with PDZ domains, including a number of the core polarity proteins: DLG1, SCRIB, and PATJ. In each case, E6 recruits ubiquitin-protein ligases, which target the polarity proteins for proteasome-mediated degradation [102, 172, 173]. The substrate specificity of PBMs in E6 proteins is thought to be sequence dependent, since E6 PBM motifs of HPV-16 and HPV-18 have different sequences and preferentially bind different polarity proteins (SCRIB and DLG1 respectively) [174]. It should be noted that HPV isn't the only oncogenic virus containing proteins capable of binding and degrading cell polarity proteins. For example, human T Lymphotropic Virus 1 (HTLV-1) causes Adult T Cell Leukaemia (ATL) [175]. Like HPV E6, HTLV-1 Tax oncoprotein also possesses a PBM motif, which allows it to bind SCRIB, DLG1, and LIN-7 [171, 176, 177]. By contrast, HTLV-2, which is not oncogenic, lacks a PBM motif in its Tax protein, which suggests that the ability of the HTLV virus to bind PDZ proteins may be required for oncogenicity. HTLV-1 also encodes a second protein, Env, which contains a PBM motif that binds to DLG1. This interaction results in the localization of Dlg1 to the virological synapse between adjacent T cells and is thought to stabilize cell contacts and facilitate viral transmission [178].

Another way in which apical basal polarity proteins are thought to connect to cancer is through regulation of energy metabolism. Tumor development, which consists of chronic and increased cell proliferation, predictably necessitates a corresponding adjustment of energy metabolism in order to fuel cell growth and division [152]. Liver Kinase B1 (LKB1/STK11) is a mammalian homologue of *C. elegans* polarity protein Par4 that has been shown to play a role in both cell polarity and energy metabolism. LKB1 is a powerful tumor suppressor and mutations in LKB1 are responsible for Peutz-Jeghers syndrome (PJS) [179]. PJS patients display abnormal pigmentation, develop benign hamartomas in the gastrointestinal tract, and are predisposed to cancers in a number of other organs. The majority of people with PJS have germline abnormalities in LKB1 gene [179, 180]. LKB1 is a master serine/threonine kinase that, in response to changes in intracellular energy levels (particularly those related to decreasing ATP levels), phosphorylates AMP-activated protein kinase (AMPK), as well as at least 13 other downstream kinases [181]. The mTOR pathway, which is downstream of AMPK, is activated by energy stress and integrates nutrient and growth factor signaling [160]. The mTOR pathway is misregulated in lung tumors with LBK mutations and in hamartomas from patients with PJS and [182, 183]. In addition to its role in energy metabolism, LKB1 is also important for cell polarization [180]. *C. elegans* LKB1 homologue PAR4 is needed for proper cytoplasmic partitioning and asymmetric cell division [15], while *Drosophila* PAR4 is required for the establishment of epithelial apico-basal polarity in follicle cells and anterior-posterior polarity in oocytes [184]. Initially, it was thought that LKB1 acts on cell polarity through its phosphorylation of Par-1 [185], however more recent work suggests that LKB1 may predominantly control cell polarity through its action on AMPK [186-188].

Many epithelial tissues require the presence of stem and progenitor cells, which are necessary for the maintenance of normal tissue homeostasis [189]. Stem and progenitor cells are highly polarized and they can divide symmetrically for self-renewal, and asymmetrically to self-renew and generate differentiated daughter cells [190]. Interestingly, many of the genes that are responsible for apical-basal cell polarity also regulate mitotic spindle orientation and cell fate. When the normal localization or function of polarity proteins is disrupted, stem or progenitors may divide symmetrically rather than asymmetrically, which can result in the accumulation of dividing stem and progenitor cells with tumor like qualities [191]. *Drosophila* neuroblasts have distinct apical–basal cortical domains, a mitotic spindle that asymmetrically aligns along the apical–basal axis, and divide unequally to produce a large apical neuroblast and a small basal daughter cell (GMC). Dlg/Scrib/Lgl proteins are normally enriched at apical cortex during prophase/metaphase. *dlg, lgl,* and *scrib* mutant neuroblasts have defects in basal protein targeting, a reduced apical cortical domain and reduced apical spindle size. Defects in apical domain and spindle pole size can result in symmetric divisions, rather than the normal asymmetric divisions, resulting in abnormally small neuroblasts and large GMCs. [192]. A study by Wirtz-Peitz reveals another link between asymmetric division and apical basal cell polarity by looking at the role of Aurora-A in *Drosophila* [193]. Aurora-A (AUR-A) kinase, is required for the asymmetric segregation of the cell fate determinant Numb during cell division. Numb is uniformly cortical in interphase but becomes polarized during mitosis. Aurora-A phosphorylates *Drosophila* Par6, which activates aPKC, suggesting yet another connection between apical basal polarity and asymmetric cell division, and hence a possible role in tumor development [193].

Epithelial-Mesenchymal Transition (EMT) is thought to play an important role in the metastasis of epithelial cancers [194]. Epithelial cells have strong cell-cell adhesions, which are mediated by tight junctions, adherens junctions, and desmosomes. Epithelial cells exhibit apical basal cell polarity and have limited motility. By contrast, mesenchymal cells have increased motility and exhibit anterior-posterior polarity. The process of EMT involves the disruption of apical-basolateral polarity and tight junctions and results in increased motility [135-137]. EMT-associated transcriptional repressors Zinc finger E-box-binding homeobox 1 (ZEB1) and Snail Family Zinc Finger SNAI1/2 repress the mRNA expression of polarity proteins CRB2 and LGL2 by binding to their promoter elements [195]. Additionally ZEB1 has also been shown to promotes tumor cell dedifferentiation by repressing CRB3, LGL2, and PATJ [196, 197]. Transforming growth factor β (TGFβ) signaling promotes EMT during tumor progression and development [198, 199]. Par6 directly interacts with TGFβ receptors at the AJC and it is phosphorylated by TGFβRII. This phosphorylation of PAR6 promotes its interaction with E3 ubiquitin ligase Surf1, which promotes degradation of RhoA. [200]. RhoA is an actin cytoskeletal regulator important for stabilization of the AJC and degradation of RhoA at the AJCs promotes their disassembly and facilitates EMT. Taken together these data suggest a strong link between apical basal polarity and multiple mechanisms involved in tumor development and progression.

The Hippo Signal Transduction Pathway

Discovery of the Hippo Signaling Pathway

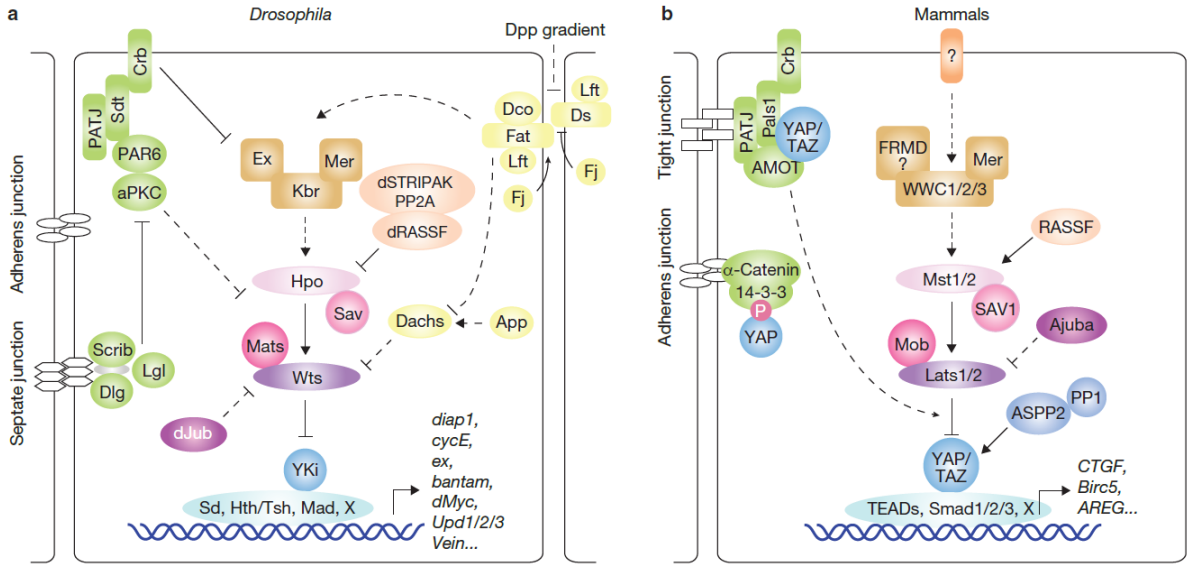
Many genes central to the Hippo pathway were discovered through genetic screens in *Drosophila* [201]. Dramatic overgrowth of imaginal discs was observed in *Drosophila* larvae mutant for Hippo (*Hpo*) or Warts (*Wts*) genes, which encode pivotal serine/threonine kinases of the Hippo pathway [202-207]. *Hippo* mutant flies have enlarged heads, with abnormally large eyes, and an overgrown, folded cuticle on their heads and antennae, thus inspiring the name 'hippo', short for hippopotamus [202]. In *Drosophila*, loss of the Hippo pathway kinases results in imaginal cell overgrowth, because mutant cells proliferate more quickly than normal and do not respond to pro-apoptotic signals [202-204, 206, 207]. Interestingly, the changes in the Hippo pathway primarily affect the number of cells produced, with minimal impact on overall tissue patterning. Overall, these pioneering studies in *Drosophila* identified the Hippo pathway as an important developmental regulator which controls apoptosis and restricts cell proliferation.

The mammalian orthologs of *Drosophila* Hippo are called Mst1 (Mammalian STE20-like Kinase 1, gene name *STK4*) and Mst2 (gene name *STK3*). They were discovered independently from studies on *Drosophila*, when researchers sought to identify new kinases homologous to yeast MAPKKK activating kinase Ste20 using degenerate PCR [208-210]. At the same time, another group independently identified Krs1 (Kinase Responsive to Stress 1, Mst2) and Krs2 (Mst1) when they purified two kinases activated by proapoptotic conditions through heat shock (55°C), m-arsenite, okadaic acid, and staurosporine [211]. Previously these same researchers had also observed that these particular kinases are activated by the oncogenic tyrosine kinase v-Src [212]. Phylogenetic analysis of Mst1 and Mst2 reveal homology with the Germinal Center Kinase (GCK) subgroup of the Ste20 kinase family. The GCKs contain a catalytic domain near

the N terminus and a highly variable C-terminal tail. Mst1 and Mst2 belong to group II of the eight GCK subfamilies [213]. Tao-1, another Sterile 20 family kinase, which directly phosphorylates Hippo at T195 in the activation loop, is a member of group VIII of the GCK family of proteins [214].

The Core Hippo pathway: *Drosophila*

Central to the Hippo signaling pathway are two kinases: Hippo (*Hpo*), a Ste20-like kinase, and Warts (*Wts*), a nuclear Dbf2-related (NDR) family kinase (Figure 5). Hippo and Warts [202-207], together with their adapter proteins Salvador (*Sav*) [215] and Mob As a Tumor Suppressor (*Mats*) [216], control the activity of the transcriptional co-activator Yorkie (*Yki*), which is at the heart of the Hippo signaling pathway [217]. When Hippo kinase is activated it binds to Salvador then phosphorylates and activates Warts and Mats [207, 218]. Warts kinase is known to preferentially target HX(R/H/K)XXS motifs for phosphorylation [219-221]. Activated Warts inhibits Yorkie's transcriptional activity by phosphorylating it at three sites (S111, S168, and S250) [217, 219-222]. Phosphorylation of Yorkie by Warts at S168 is particularly important as it causes Yorkie to bind to 14-3-3 proteins, which retain it in the cytoplasm away from the nucleus [219, 220, 222, 223]. Yorkie is a non-DNA binding transcriptional coactivator which is known to bind to several transcription factors, including the TEA-domain transcription factor Scalloped (*Sd*), the Zn-finger transcription factor Teashirt (*Tsh*), and the homeodomain transcription factor Homothorax (*Hth*). These transcription factors allow Yorkie to activate expression of downstream target genes including *cyclin E* (a cell cycle regulator), *diap1* (an inhibitor of apoptosis), *Myc* (a growth promoter), and *bantam* (a miRNA that promotes cell survival and proliferation), which together



Drosophila	Mammals
Hpo	Mst1/2
Sav	SAV1
Mats	Mob
Wts	Lats1/2
Yki	YAP/TAZ
Sd, Hth/Tsh, Mad	TEADs, Smad1/2/3

Figure 5: The Hippo pathway in *Drosophila* and in mammals.
Matching colors indicate corresponding proteins in *Drosophila* (a) and mammals (b). Arrowed or blunted ends indicate activation or inhibition, respectively. Dashed lines indicate unknown mechanisms. Core components of the Hippo pathway in *Drosophila* and mammals are listed below parts a) and b) and are color-coded to match the proteins shown above.
Source: [224]

promote cell proliferation and inhibit apoptosis [202-204, 206, 207, 215, 217, 225-227]. It is important to note that the Hippo pathway activation of downstream gene targets is cell type specific: Hippo signaling regulates *cyclin E, bantam,* and *diap* in the imaginal discs [202, 203, 206, 207, 215, 227], but it also regulates *vg* and *wg* in the wing discs (which are a subset of the imaginal discs) [228, 229] and *melt* in differentiating photoreceptor cells [230].

The Core Hippo pathway: Mammals

The central components of the *Drosophila* Hippo Signaling Pathway are well conserved in mammals (Figure 5), where they play an important role in growth regulation and tumor suppression [231]. MST1 and MST2 are Hippo orthologs. Activated MST1/2 auto-phosphorylate at T183/T180, which increases their kinase activity [232]. Similarly to Hippo signaling in *Drosophila*, MST1/2 activate LATS1/2 (orthologs of *Drosophila* Warts). MST1/2 phosphorylate adaptor proteins MOB1 (a homologue to Mats) and SAV (also known as WW45), which leads to increased binding of MOB1 to LATS1 and increased LATS1 activity. [220, 233-235]. In addition, like *Drosophila* Hippo kinase, MST1/2 can also activate LATS1/2 by direct phosphorylation of LATS1/2 in the activation loop (S909) and in the hydrophobic domain (Thr1079) [234]. LATS1 is also able to auto-phosphorylate at S909 [234].

The mammalian Hippo cascade leads to inhibition of two Yorkie orthologs, Yes-Associated Protein 1 (YAP1) and the Transcriptional Coactivator With PDZ-Binding Motif (TAZ), also known as WW Domain-Containing Transcription Regulator Protein 1 (WWTR1) [220, 221, 236-239]. LATS1/2 phosphorylates YAP1 at S127 and TAZ at S89 (which are equivalent to S168 phosphorylation on *Drosophila* Yorkie). When mammalian YAP1 and TAZ are phosphorylated at these sites, they bind to 14-3-3 proteins, which sequester them in the

cytoplasm, just like *Drosophila* 14-3-3 proteins sequester S168 phosphorylated Yorkie in the cytoplasm. In mammals, phosphorylation of S314 of TAZ and S381 of YAP1 by LATS1/2 can also prime YAP1 and TAZ for subsequent phosphorylation by casein kinase 1, ubiquitination, and proteasomal degradation [240, 241]. When YAP1 and TAZ are not phosphorylated, they are able to enter the nucleus and trigger activation of downstream targets by binding to transcription factors: Scalloped orthologues TEAD1-4, as well as several other transcription factors that have not been shown to mediate the growth-promoting function of YAP including Runt Related Transcription Factor (RUNX), ErbB4, and p73 [201, 242, 243]. Two downstream targets of TEAD transcription factors are *Connective Tissue Growth Factor (CTGF)* and *Cysteine-Rich Angiogenic Inducer 61* (*CYR61*) [244]. Expression levels of CTGF and CYR61 can be used as readouts for YAP1/TAZ mediated activation of the TEAD transcription factors.

YAP1 and TAZ: Similar, but distinct effectors of the Hippo pathway

Mammalian YAP1 and TAZ have many similarities, but also some differences (Figure 6). YAP1 has an N-terminal proline-rich domain, a TEAD-binding region, two WW domains (although some YAP1 variants only have one), an SH3-binding motif, a coiled-coil domain, a transcription activation domain, and a C-terminal PDZ-binding motif (Figure 7). TAZ has many of the same domains, but lacks the proline-rich domain and SH3-binding motif and has only one WW domain [245]. Genetic studies revealed different phenotypes in YAP1 and TAZ mutant

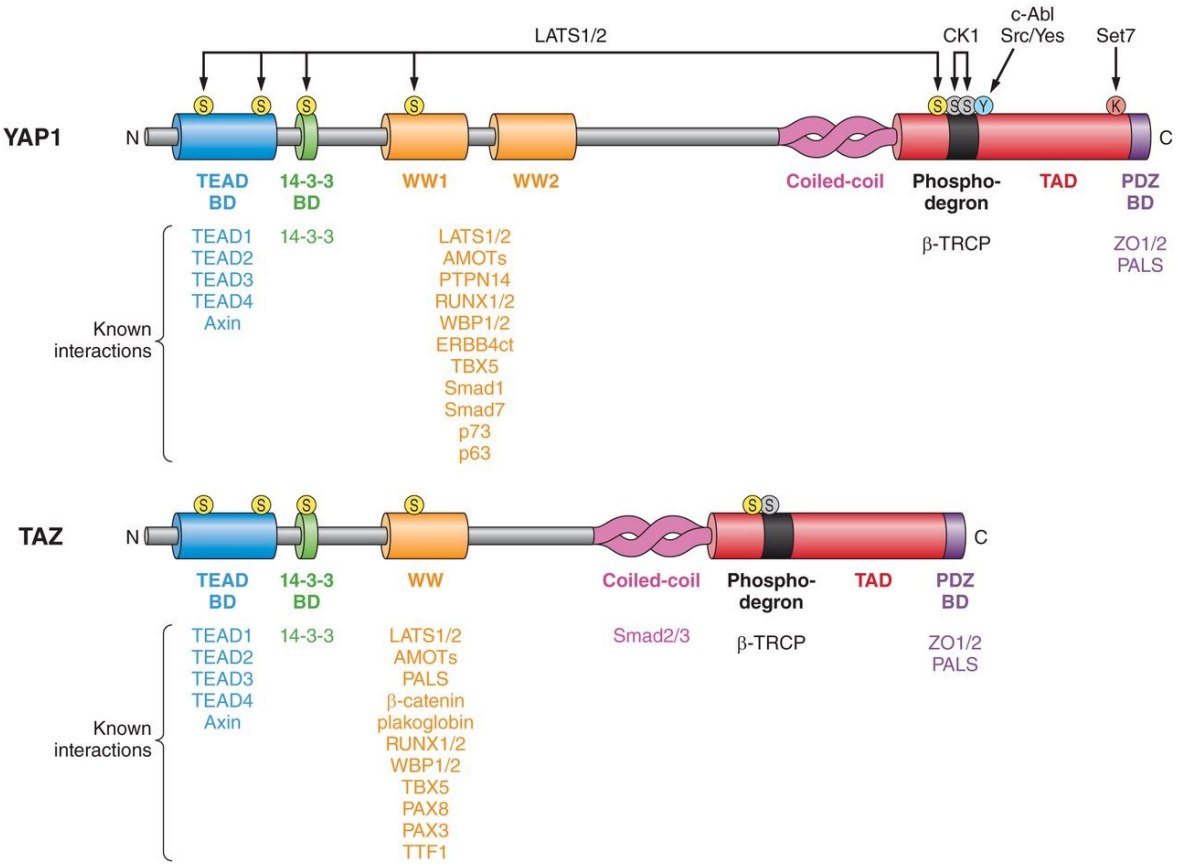


Figure 6: **Schematic representation of YAP and TAZ domains with mapped interactions with other proteins.**
The five serines of YAP and the corresponding four serines of TAZ that are targeted by LATS1/2 phosphorylation are indicated in yellow, the CK1 phosphorylation sites on both proteins are indicated in gray, and the c-Abl phosphorylation site on YAP is indicated in cyan. The TEAD binding domain (TEAD BD), the domain that binds 14–3-3 proteins (14-3-3 BD) upon phosphorylation by LATS1/2, the transcriptional activation domain (TAD), and the PDZ binding domain (PDZ BD) are indicated above. Source: [246].

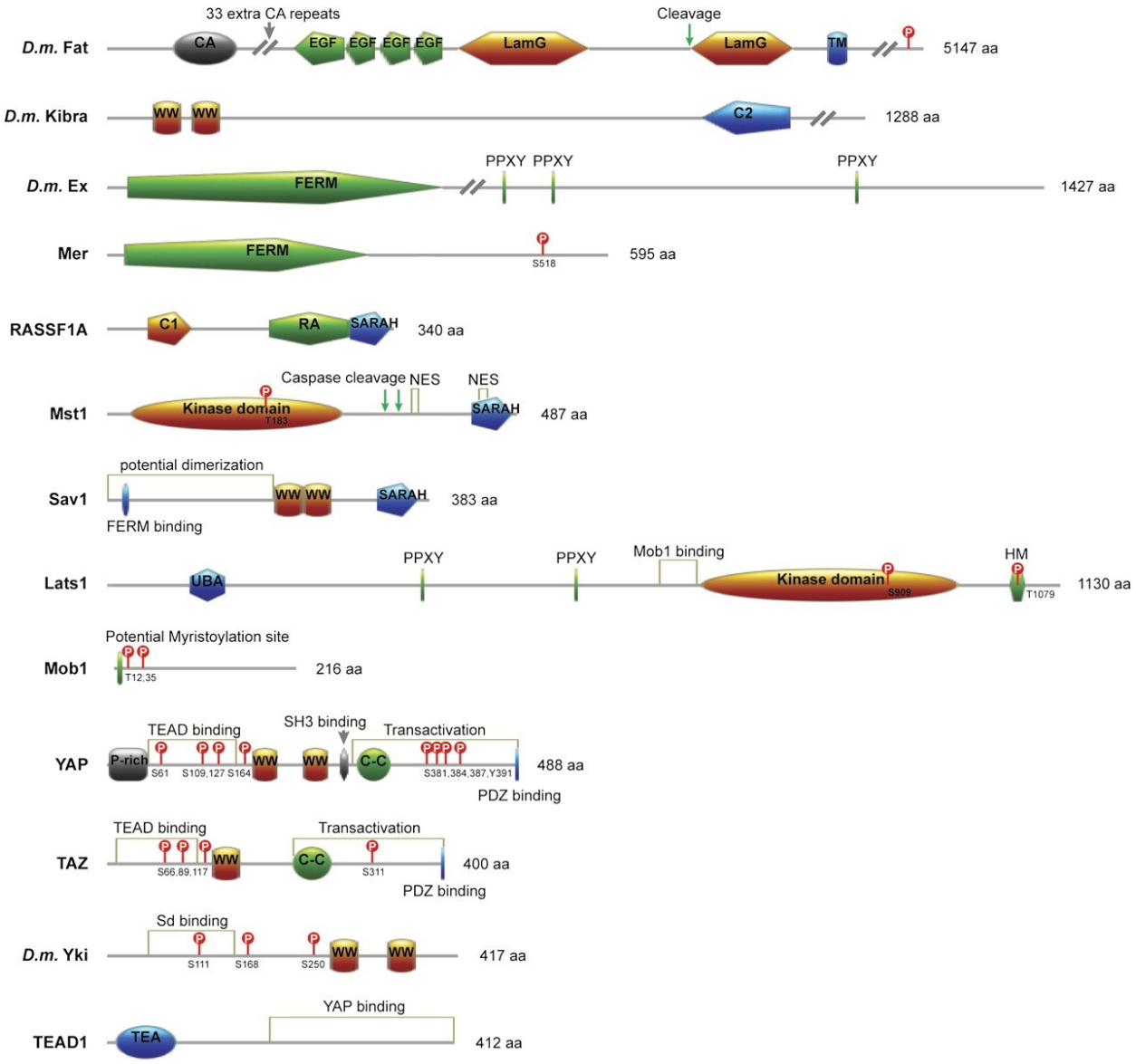


Figure 7: **Domain organization and key modifications of the Hippo pathway components.** Human sequences are drawn above unless designated by *D.m.*, which indicates *Drosophila* sequences. (C1) C1 domain; (C2) C2 domain; (CA) Cadherin repeats; (C-C) coiled-coil domain; (EGF) EGF-like domain; (FERM) FERM domain; (HM) hydrophobic motif; (LamG) Laminin G domain; (PPXY) PPXY motif; (P-rich) proline-rich domain; (RA) Ras association domain; (SARAH) SARAH domain; (TEA) TEA DNA-binding domain; (TM) transmembrane region; (UBA) ubiquitin-associated domain; (WW) WW domain. Source: [242].

mice. YAP1 knockout mice die by embryonic day E8.5 due to yolk sac defects [247] while TAZ knockout mice survive to adulthood [248]. $Taz^{-/-}$ mice are smaller than their wild type littermates, have polycystic kidneys, and an emphysema-like lung phenotype [248, 249]. YAP1 and TAZ double knockout embryos die during early embryogenesis before the morula stage [250].

Experiments in cell culture reveal significant differences between YAP1 and TAZ. Chan et al. observed that high levels of TAZ are associated with increased invasion of breast cancer cells while high levels of YAP are not [251]. Work by Rosenbluh et al. found that colorectal cancers driven by β-Catenin require YAP1, but not TAZ, for survival and tumorigenesis [252]. In experiments with tagged constructs in vitro, Hong et al. demonstrated that TAZ, but not YAP1 binds to PPARγ, a transcription factor known to be critical for adipocyte differentiation [253]. Qin et al. determined that in human embryonic stem cells (hESCs) knocking down TAZ but not YAP1 results in loss of self-renewal and differentiation into neuroectoderm and that LATS2 represses reprogramming in human cells by antagonizing TAZ but not YAP1 post-transcriptionally [254]. Another study found that YAP and TAZ are differentially expressed in neuronal and glial cells with distinct topographic distribution patterns and that peripheral nerve injury induces distinct spatial temporal changes in the spinal YAP and TAZ expression in rats [255]. Taken together, these studies demonstrate that YAP1 and TAZ have significant differences in their expression patterns and may also activate somewhat different downstream gene targets, hence resulting in functional differences.

Hippo pathway: Key differences between *Drosophila* and mammals

There are several key differences in the Hippo Pathway between *Drosophila* and mammals. Mammalian MST1 and MST2 contain cleavage sites for caspase 3 at $DEMD^{326}$ and $DELD^{321}$, respectively [256-258]. In addition, Mst2 can be cleaved by caspase 6/7 at $TMTD^{349}$ [259]. By contrast, *Drosophila* Hippo lacks a consensus caspase recognition sequence and is not cleaved [202-204, 206, 207]. In mammals, pro-apoptotic signals result in the formation of ~36kDa MST1/2 fragments that are extensively phosphorylated on their activation loops and are thought to be more active than full-length MST1/2. Distinct from the cytoplasmically localized full-length MST1/2, the proteolytically cleaved fragments are prominently nuclear and they demonstrate different substrate specificity [260].

In mammals, YAP1 and TAZ contain a C-terminal PDZ binding motif which is required for nuclear localization of YAP1 and pro-apoptotic signaling [237, 261]. *Drosophila* Yorkie lacks this PDZ binding motif. In addition, *Drosophila* Yorkie also lacks the C-terminal serine phosphorylation site that allows mammalian YAP1 and TAZ to be targeted for SCFβ-TrCP E3-mediated degradation [240, 241].

Hippo pathway and Organ Size in Mammals

The first studies to identify the importance of Hippo Pathway signaling in mammalian organ size control involved overexpression of YAP1 in adult mouse liver [220, 262]. YAP1 overexpression in livers results in a several fold increase in liver mass. Hepatocytes in the transgenic livers are also smaller and more densely packed than control livers, suggesting that

overexpression of YAP1 results in increased cell number. Interestingly, when YAP1 overexpression is halted, the liver quickly reverts to its normal size (presumable due to apoptotic culling of excess cells) [220]. Strikingly, the impact of Hippo pathway signaling on organ size may be tissue-specific [263]. Tamoxifen induced knockdown of Mst1/2 in all mouse organs, resulted in differential enlargement in different tissues. While liver and stomach were consistently enlarged in MST1/2 mutants, the size of lung, kidney, and intestine did not change. Spleen and heart also showed enlargement, but less frequently than liver or stomach [263].

Presently, it is not clear what is responsible for the tissue-specific differences in organ size in Hippo mutants. However, it is likely that the Hippo pathway may have different roles in different cellular contexts (i.e. growth control in one context, cell cycle exit in another) [201]. Yap phosphorylation is increased in *Mst1*$^{-/-}$ *Mst2*$^{-/-}$ livers, indicating that these kinases impact organ size through their actions on canonical Hippo pathway proteins [263-266]. However, it appears that an unknown kinase may be phosphorylating and activating LATS1/2 in liver cells [264]. Also, in skin epidermis *Mst1/2*$^{-/-}$ does not result in any abnormalities or hyperplasia and Mst1/Mst2 null keratinocytes show no changes in YAP1 S127 phosphorylation [267]. Additionally, a kinase other than LATS1/2 may be phosphorylating Yap1 in MEFs [264].

Pluripotency and the Hippo pathway

There is substantial evidence to suggest that Hippo signaling is important for regulating the balance between stem, progenitor, and differentiated cells in a variety of organs and organisms. In epidermis and intestine, YAP1 is prominently expressed and is nuclear in the basal skin layer and intestinal crypts, the tissue compartments harboring stem and progenitor cells [262, 268]. Activation of YAP1 signaling in mouse adult skin and intestinal epithelium,

results in an expansion of progenitor pool at the expense of differentiated cells [262, 269]. In mouse embryonic stem cells YAP1 promotes pluripotency and inhibits differentiation in a LATS1/2 dependent manner [270]. Hippo signaling also controls stem cell proliferation in the intestinal *Drosophila* epithelium [271]. NF2/Merlin is a positive regulator of the canonical Hippo signaling pathway. Deletion of NF2/Merlin in mouse liver results in increased liver to body weight ratios and expansion of "oval cells" (facultative liver progenitor cells derived from liver stem cells) [272, 273]. Inactivation of Sav1 or Mst1/2 in mouse hepatocytes also leads to accumulation of oval cells [265, 266]. Inhibition of MST1/2 or overexpression of YAP1 in the neuroepithelium of chick embryos results in expansion of neural progenitor cells in the spinal cord [274]. In the mouse brain, NF2 limits neural progenitor pool expansion by inhibiting Yap and Taz [275]. In mitotic mouse retinal progenitors YAP1 is downregulated during neuronal differentiation [276]; Forced expression of YAP1 prolongs proliferation in the postnatal mouse retina, while inhibition of YAP1 by RNA interference (RNAi) decreases proliferation and increases differentiation [276].

Negative regulation of the Hippo pathway

Thus far, several negative regulators of the Hippo pathway have been identified. One way that the Hippo pathway can be negatively regulated is by G-protein-coupled receptor (GPCR) signaling. Lysophosphatidic acid (LPA) and sphingosine 1-phosphophate (S1P) in serum can act through G12/13-coupled receptors to inhibit the Hippo pathway kinases Lats1/2, and thus activate YAP and TAZ transcription coactivators [277]. However, not all GPCR signaling negatively regulates the Hippo pathway. Glucagon and epinephrine stimulation of GPCRs activates Lats1/2 kinase activity, thereby inhibiting YAP function. Thus, depending on

the coupled G protein, GPCR signaling can either activate or inhibit the Hippo-YAP pathway [277].

In *Drosophila*, Hippo pathway signaling can be inhibited by dRASSF which competes with Salvador for binding to Hippo [278]. Like its mammalian counterparts, dRASSF contains an N-terminal Ras Association (RA) domain and an C-terminal SARAH domain, which allows dRASSF to associate with and inhibit Hippo. In mammals, RASSF1A and Nore1A have both been shown to interact with MST1 via their SARAH domains [279]. Overexpression of RASSF1A or Nore1A inhibits MST1 activation, however coexpression of Ras with these RASSF proteins enhances rather than inhibits MST1 activity [232].

dSTRIPAK (Drosophila Striatin-interacting phosphatase and kinase), a Drosophila PP2A phosphatase complex, can also negatively regulate Hippo signaling [280]. Inhibition of the phosphatase complex resulted in enhanced Hippo activation loop phosphorylation at Thr195 (equivalent to T183/T180 in mammalian MST1/2) and hyperactivation of the Hippo pathway in vivo. Moreover, dSTRIPAK was present in Hippo/dRASSF complexes, but absent in Hippo/Salvador complexes, suggesting that Hippo dephosphorylation occurs when Hippo binds to dRASSF instead of Salvador [280].

F-actin is another negative regulator of Hippo pathway signaling. Angiomotin proteins (AMOT, AMOTL1, AMOTL2) link F-actin to YAP regulation. AMOT130, like 14-3-3 protein, can bind to YAP and cytoplasmically retain it. F-actin can compete with YAP for binding to AMOT130, thus freeing YAP from being cytoplasmically retained by AMOT130 [281].

Lastly, Yorkie promotes expression of negative regulators of Hippo signaling Expanded (Ex), Merlin (Mer), Kibra (Kibra), and Dachsous (Ds), suggesting the existence of negative

feedback mechanisms [282-285]. These regulators of the Hippo pathway will be discussed in more detail in the next section of the introduction.

Crosstalk between cell polarity, the Hippo pathway, and other signaling pathways

Upstream regulation of the Hippo pathway

One notable regulatory module in the Drosophila Hippo pathway is the Expanded (Ex)/ Merlin (Mer)/ Kibra (Kibra) complex [283, 285-288]. It is thought that these proteins act as an apical scaffold that promotes Hippo pathway activity [285]. In contrast to *hpo* and *wts* mutants, the imaginal disc overgrowth phenotypes of *ex*, *mer*, and *kibra* flies are more subtle than those of *hpo* and *wts* mutants. Interestingly, double mutant combinations of *ex*, *mer*, and *kibra* are more severe than single mutants and resemble *hpo* mutants, suggesting these proteins function synergistically [283, 285, 286, 289]. Work by Pellock et al. demonstrates that Merlin and Expanded may be acting in somewhat different downstream pathways. Both *mer* and *ex* restrict cell and tissue growth in the fly eye; however, *ex* clones exhibit delayed cell cycle exit, while *mer* clones do not. Loss of *mer* greatly disrupts normal apoptosis, while loss of *ex* results in a much milder phenotype [287].

In the epithelial cells of *Drosophila* imaginal discs, Expanded, Merlin, and Kibra localize apically and are thought to act as adaptor proteins because they contain FERM (band 4.1-ezrin-radixin-moesin) protein binding domains [285, 286, 289]. These proteins form a complex and interact with several core components of the Hippo pathway; Yu et al. demonstrated multiple interactions between Kibra, Expanded, Merlin and the proteins of the Hippo pathway: Kibra interacts with Salvador and Hippo, Merlin binds to Salvador, and Expanded binds to Hippo

[289]. In tissue culture, Kibra is able to complex with Warts and loss of Kibra induces a marked reduction in Yorkie phosphorylation without affecting the interaction between Yorkie and Warts[285]. Initially, it was believed that Expanded, Merlin, and Kibra act only upstream of the Hippo pathway. However, recent studies suggest that Expanded/Merlin/Kibra-mediated regulation of the Hippo Pathway is more complex than originally thought. Expanded is able to directly interact with Yorkie, causing it to be sequestered from the nucleus [219, 290]. Expanded binds to the WW domain of Yorkie via its PPXY motif [291]. Interestingly, Expanded, Merlin, and Kibra are also transcriptional targets of Yorkie, indicating the presence of a negative feedback mechanism [283, 285, 292].

Merlin/NF2, Kibra, and Expanded are conserved in mammals, however thus far only Merlin/NF2 has been shown to directly regulate the Hippo pathway. Loss of NF2 in the mouse liver causes an overgrowth phenotype similar to that of *mst1*$^{-/-}$ *mst2* $^{-/-}$ mutants [272, 273]. Merlin is necessary for contact inhibition in cultured cells and promotes export of YAP1 from the nucleus [273, 293, 294]. Yin et al. demonstrated that Merlin is important for membrane localization of LATS [295]. In vitro experiments suggest mammalian Merlin is able to bind to Kibra and that their coexpression causes synergistic phosphorylation of Lats1/2 [273, 289].

Hippo pathway regulation by mechanical signals and cell architecture

Recent studies have discovered a connection between cell polarity, the actin cytoskeleton, cell architecture and the Hippo Pathway [296-298]. Interestingly, YAP1 and TAZ function is strongly affected by extracellular matrix rigidity and cell geometry [299, 300]. Taken together, these studies indicate that the Hippo Pathway is responsive to the mechanical cues exerted by the

neighboring cells. Such cues may be transduced through cell-cell and cell-substratum junctions, the cytoskeleton, and other cellular structures.

In multicellular organisms, cells are often in direct contact with the extracellular matrix (ECM), which consists of secreted extracellular molecules that provide structural support and important signaling molecules. The extracellular matrix plays an important role in regulation of cellular behavior. For example, cells proliferate more rapidly on a stiff rather than a flexible matrix [301]. Substrate rigidity can also affect cell fate decisions; mesenchymal stem cells (MSCs) can become adipocytes in soft environments, but differentiate into osteocytes on a stiff matrix [302, 303]. Interestingly, these differences in cell behavior are mediated by the Hippo pathway. YAP1 and TAZ localization is predominantly nuclear on stiff and cytoplasmic on flexible substrates [300]. Loss of YAP1 and TAZ inhibits osteogenic differentiation on stiff surfaces, indicating that YAP1 and TAZ mediate the cellular response to substrate stiffness [300]. Interestingly, the non-phosphorylatable form of YAP1 continues to be regulated by substrate stiffness, suggesting that this process is MST1/2 LATS1/2 independent [300]. Cell geometry has also been shown to regulate the Hippo pathway. Micropatterned substrates can be fabricated to induce cells to adopt a round and compact or flat and spread out morphologies [299, 300]. These surfaces contain prefabricated adhesive "islands" of different sizes and profiles, surrounded by non-adhesive areas, which forces the cells to assume either a compact or spread-out shapes. The localization of YAP1/TAZ shifts from predominantly nuclear in spread-out mesenchymal stem cells (MSCs) to predominantly cytoplasmic in compact MSCs. Each "island" contains only a single cell, ruling out that possibility that cell–cell contacts are responsible for this change in YAP1/TAZ localization [300]. Thus, taken together, these studies

indicate that YAP1 and TAZ localization and transcriptional activity is prominently affected by cell geometry.

Several studies have also observed a link between changes in the actin cytoskeleton and Hippo pathway signaling. Cytoskeletal assembly and disassembly is regulated by a number of actin binding proteins. Diaphanous (Dia) stimulates F-actin polymerization, while Cpa and Cpb are capping proteins which heterodimerize to prevent F-actin polymerization. Knockdown of Cpa and Cpb or overexpression of activated Dia results in increased proliferation and tissue overgrowth of *Drosophila* imaginal discs [299, 304, 305]. Overgrowth of these tissues is accompanied by and is dependent on over-activation of Yorkie [304, 305]. Interestingly, while this overgrowth phenotype is suppressed by the knockdown of Yorkie, or overexpression of Warts, it is not affected by overexpression of Hippo or Expanded. This indicates that F-actin-mediated regulation of YAP1 signaling acts upstream of Warts and downstream of Hippo in the canonical Hippo pathway [304]. Remodeling of the cytoskeleton can also occur in response to changes in cell density and shape. For example, F-actin stress fibers are abundant at low density, but uncommon at high density. Stress fibers are also more common in flat than in round cells [299, 300]. Nuclear localization of YAP1 and TAZ is positively associated with the presence of stress fibers and when stress fibers are disrupted, YAP1 and TAZ are localized away from the nucleus [299, 300, 304, 306]. Interestingly, the actin cytoskeleton is also necessary for inhibitory phosphorylation of YAP1 when cells detach from their neighbors and the ECM [306]. Cells undergo an apoptotic process called anoikis upon detachment from the extracellular matrix, and this apoptotic mechanism is suppressed by overexpression of YAP1 [306]. It is important to note that LATS1/2 levels are reduced in metastatic prostate cancers, which could be due to increased YAP1 protein levels preventing anoikis and encouraging survival and metastasis of

cancer cells [306]. Taken together these studies reveal several mechanisms by which mechanical stimuli and the cytoskeleton could regulate YAP1 and TAZ activities, through both LATS dependent and independent mechanisms.

In addition to cell-substratum adhesion, cell-cell adhesion structures also regulate the Hippo signaling pathway. Adherens Junctions (AJs) are cell-cell adhesion structures that contain cadherin and catenin molecules. In epithelial cells, the cytoplasmic domain of E-cadherin interacts with β -catenin, which links it to the cytoskeleton via actin-binding protein α-catenin [307]. When normal adherent cells come in contact with their neighbors, they cease to proliferate and this is referred to as contact inhibition. Cultured cells require E-cadherin and β-catenin to undergo contact inhibition [267, 308, 309]. Interestingly, when Kibra, Merlin, or LATS1/2 are knocked down, cells are no longer able to undergo contact inhibition, suggesting that Hippo signaling is necessary for the signal transduction pathway that regulates contact inhibition [221, 309-311]. When α-catenin is knocked out in keratinocytes, YAP1 is hyperactivated and cells are no longer able to undergo contact inhibition [311]. α-catenin binds to the 14-3-3-YAP1 protein complex in the cytoplasm and sequesters YAP1 from the nucleus [221, 267, 311]. Conditional deletion of α-catenin in the epidermal stem cells causes formation of squamous cell skin tumors and YAP1 is necessary for growth of these tumors [311]. Hyperactivation of YAP1 in epidermal keratinocytes results in a phenotype resembling loss of α-catenin. This includes an expansion of the epidermal stem cell compartment, thickening of the skin, and formation of skin lesions resembling squamous cell tumors [244, 267]. Interestingly, the functional interaction between YAP1 and α-catenin behaves differently in Drosophila. Yang et al. found that when the AJ components E-cadherin or α-catenin were knocked down in *Drosophila* tissues, Yorkie activity was non-cell-autonomously elevated but cell-autonomously

decreased [312]. Another study by Rauskolb et al. found that α-catenin increases the binding of AJs protein dJub to Warts, which leads to an inhibitory recruitment of Warts to apical junctions and subsequent increased Yorkie activity [313]. Thus it appears that, in certain contexts, α-catenin and E-cadherin can function as positive regulators of Hippo signaling in Drosophila.

Several other proteins that associate with the AJs also appear to be connected to the Hippo pathway. Zyxin and Ajuba (dJub in *Drosophila*), two proteins which localize to the AJs, have recently been implicated as regulators of the Hippo pathway. Zyxin and Ajuba are both LIM-domain containing proteins, which antagonize Warts [314, 315]. dJub was shown to co-immunoprecipitate with Warts in *Drosophila* S2 cells and Ajuba with LATS1/2 in mammalian cell lines. In a more recent study Rauskolb et al. found that dJub is important for junctional localization of Warts [313]. Moreover, when cytoskeletal tension was elevated, Warts demonstrated increased junctional localization. This finding is in line with the recent work of Yin et al. who found that LATS localized to the cell membrane and that this localization was Merlin/NF2 dependent [295]. These studies are in contrast to other articles that suggest Hippo and Warts are normally predominantly cytoplasmic [282, 316].

Tight junctions (TJs), also referred to as occluding junctions or zonulae occludentes, act as a semipermeable barrier to the paracellular transport of ions, solutes, and water and are thought to function as a divider that separates apical and basolateral domains of plasma membranes [317]. In *Drosophila* septate junctions are equivalent to TJs. The Hippo pathway is connected to TJs via Angiomotin (AMOT) which associates with TJ components, and is required for TJ maturation [318]. Angiomotin and its family members AMOTL1 and AMOTL2 are prominent negative regulators of YAP1/TAZ activity. AMOT proteins directly interact with YAP1 and TAZ via their PPXY motifs, which directly bind to the WW domains in YAP1 and

TAZ. This interaction results in the sequestration of YAP1 and TAZ outside of the nucleus [319-321]. In addition, AMOT proteins can positively regulate Hippo pathway-mediated phosphorylation of YAP1 and TAZ, decreasing their nuclear localization and transcriptional activity [319].

Apical-basal cell polarity and the Hippo signaling pathway

Studies in *Drosophila* have documented a clear connection between apical-basal cell polarity and the Hippo signaling pathway. *dlg*, *lgl*, and *scrib* mutant flies all develop neoplastic tumors [5, 46, 47, 157, 322]. Imaginal discs mutant for *scrib* demonstrate hyperactivation of the Hippo pathway effector Yorkie, which drives the formation of neoplastic masses [323]. *dlg*, *lgl*, and *scrib* exhibit a strong genetic interaction with *warts* and the loss of *dlg*, *lgl*, and *scrib* in ovarian follicular epithelial cells results in overexpression of Yorkie target genes *CycE* and *DIAP1* [324]. *scrib* or *lgl* mutant clones in the developing eye exhibit increased expression of the key cell-cycle regulator *CycE* and ectopic cell proliferation [325, 326]. Still, the precise mechanisms by which *dlg*, *lgl*, and *scrib* regulate the Hippo pathway are not yet known. In *lgl* mutants, increased cell proliferation occurs without loss of apical-basal cell polarity, revealing separable roles for Lgl in cell polarity and regulation of cell proliferation [326]. Conversely, tissue overgrowth results when aPKC is overexpressed [6]; in *Drosophila* larva epithelial activation of *aPKC* increases cell proliferation [327, 328], and in brain neuroblasts *aPKC* mutants show reduced proliferation and suppress most *lgl*$^{-}$ cell overproliferation and polarity phenotypes [193].

The apical-basal cell polarity proteins regulate the Hippo pathway through at least two distinct mechanisms. First, in Drosophila aPKC and Lgl have been shown to act antagonistically

on the Hippo pathway: aPKC overexpression or Lgl depletion does not affect Ft or Ex localization, but causes Hippo and dRASSF to be mislocalized [278, 329]. When active aPKC is overexpressed in imaginal disc cells, Yorkie localization becomes more nuclear [330, 331]. The polarity protein Crb is also able to regulate the Hippo pathway. However, the directionality and mechanism of its action appears to be rather complex because both loss-of-function and gain-of-function of *crb* can lead to elevated Yorkie activity [329, 332]. Overexpression of *crb* results in mislocalization of Crb and reduced levels of Ex, which is likely due in part to Crb mediated turnover of Ex [329, 332]. Hippo and dRASSF localization is not disrupted when Crb is overexpressed, suggesting that Crb acts on the Hippo pathway via a distinct mechanism from aPKC and Lgl [329]. Interestingly, loss of *crb* also results in Yorkie overexpression [332]. Ex protein levels were increased as expected in *crb* mutants but also mislocalized to the basolateral domain. Hence, it appears that Crb may be playing dual roles in Hippo pathway regulation: localizing Ex to the membrane where it can sequester Yorkie and promote Hippo function as well as mediating degradation of Ex.

In general, it appears that apical polarity proteins tend to promote Yorkie activity, while basal polarity proteins attenuate it; however, this is a bit of an oversimplification as the loss of basolateral polarity protein Par-1 results in a phenotype that is opposite to the phenotype that results from loss of basolateral polarity genes *dlg*, *lgl*, and *scrib* and both loss-of-function and gain-of-function of apical polarity protein Crumbs result in the same phenotype. In *Drosophila* overexpression of Par-1 results in elevated Yorkie activity, increased expression of Hippo target genes, and tissue overgrowth, while loss of Par-1 results in reduced expression of Hippo target genes and reduced organ size [333]. In addition, Huang et al. also found that Par-1 physically

interacts with Hippo and Salvador and restrict the activity of Hippo by phosphorylating Hippo at Ser30 [333].

The connection between apical-basal cell polarity and Hippo signaling may be quite direct, because some of the core polarity proteins are physically interacting with several components of the Hippo pathway. For instance, human Kibra was recently identified as a substrate of aPKC and new interactions could come to light in the future [334]. In addition, in mouse cells, Scribble can bind to MST1/2 and LATS1/2 and it is thought that it might be able to promote LATS1/2 dependent inhibition of TAZ and YAP [335].

Interestingly, 14-3-3 and phosphatase PP2A, whose roles we have already discussed in Hippo pathway signaling, also help play in regulating apical-basal cell polarity. Par1 inhibits Par3 by phosphorylating it and generating two 14-3-3 binding sites [30]. Par1 phosphorylation of Par3, restricts it to the apical membrane of *Drosophila* photoreceptors, and this action is antagonized by the phosphatase PP2A, which removes Par-1-mediated phosphorylation of Par-3 targets it and its binding partners to adherens junctions [336]. Thus there appear to be a number of connections between apical-basal polarity and Hippo pathway signaling.

Interestingly, in addition to responding to the regulation by the apical basal polarity proteins, it appears that Hippo pathway components may also regulate apical domain size in *Drosophila*. Cells that lack *mer*, *ex*, *wts*, or *hpo* have enlarged apical domains [205, 207, 316, 337, 338]. This enrichment of apical receptors depends on Yki, which indicates that this is a downstream effect of the Hippo pathway. In addition, the apical polarity proteins aPKC, Crb, and Patj are overexpressed in cells in which Yki levels are increased or Hippo pathway components are mutated [316, 338]. Similarly, in mammalian epithelial cells knockdown of

KIBRA or activation of YAP1 both cause apical domain expansion [339]. Thus, apical domain size is regulated by the Hippo pathway in both flies and mammalian cells.

Aside from *dlg* not much is known about the about the connection between Discs Large gene family and the Hippo pathway and one of the questions I will be addressing in this work is whether Dlg5 is connected to Hippo pathway signaling.

Planar cell polarity and the Hippo pathway

In addition to apical basal polarity, epithelial tissues also possess planar cell polarity (PCP), which allows cells to sense and orient themselves relative to antero-postero, dorso-ventral, and proximo-distal axis [297]. One example of PCP is the directional orientation of hairs in the wings of Drosophila and in the mouse epidermis (Figure 8C). There still appears to be substantial debate about the precise relationship between some of the pathways that contribute to planar cell polarity [340-342]. It has been proposed that the core PCP pathway is thought to amplify asymmetry and coordinate polarization between neighboring cells while the global PCP pathway is responsible for converting tissue-level gradients to subcellular gradients (Figure 8A, 8B). Frizzled (FZ), Dishevelled (DSH), Diego (DGO), Van Gogh (VANG; also known as strabismus), the LIM domain protein Prickle (PK), and Flamingo (FMI; also known as Starry night STAN) function in the core PCP pathway while Dachsous (DS), Fat (FT), and Four-jointed (FJ) are thought to function in the global PCP pathway. Within the core PCP pathway Flamingo, Prickle, and Van Gough localize to the proximal regions of cell-cell junctions while Frizzled, Dishevelled, and Diego localize to the distal regions [340]. This polarization is amplified and reinforced by both intracellular and intercellular interactions, the latter of which is thought be

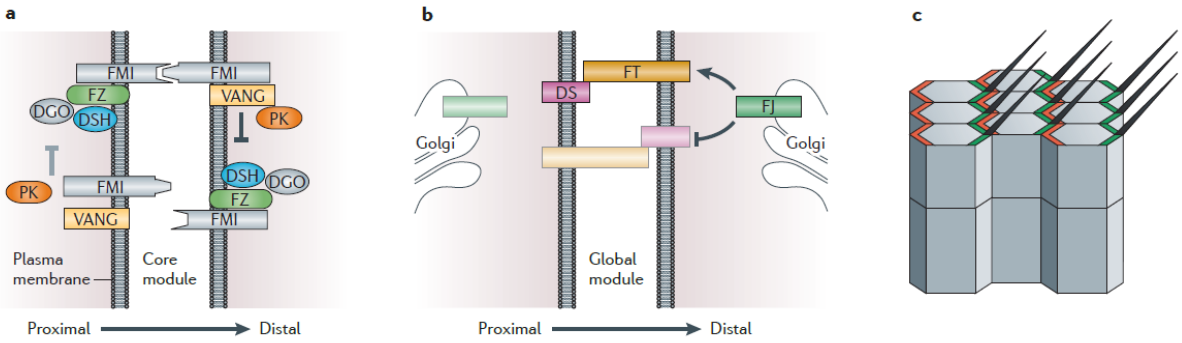


Figure 8: **A model of the PCP signaling mechanism based on work in *Drosophila.*** The mechanism of PCP signaling is proposed to consist of three functional modules: a core module, a global directional cue and one of many tissue-specific effector modules that respond to the upstream modules to produce morphological asymmetry in individual tissues. A) The core module acts to amplify asymmetry and to coordinate polarization between neighboring cells, producing a local alignment of polarity. Proteins in the core signaling module adopt asymmetric subcellular localizations and include: Frizzled (FZ), Dishevelled (DSH), Diego (DGO), Van Gogh (VANG; also known as strabismus), the LIM domain protein Prickle (PK), Flamingo (FMI; also known as Starry night STAN). It is thought these asymmetric localizations determine eventual morphology by orienting downstream effectors. B) The global module functions to translate tissue-level expression gradients to subcellular gradients of Dachsous (DS) –Fat (FT; also known as cadherin-related tumor suppressor) heterodimer expression. DS and atypical cadherin FT form heterodimers that may orient in either of two directions at cell–cell boundaries. The golgi protein Four-jointed (FJ) phosphorylates the cadherin domains of Fat and Dachsous as they transit through the Golgi. The graded expression of FJ and DS results in a larger fraction of FT–DS heterodimers in one orientation relative to the other. C) Asymmetric core protein localization, with proximal proteins (shown in red) and distal proteins (shown in green) on opposite sides of cells. Localization of proteins corresponds to morphological polarity; in this example, polarized hair growth is shown. Source: [340]

mediated by Flamingo, Van Gough, and Frizzled [341]. Within the global PCP pathway, Fat is a large atypical cadherin that heterodimerizes with Dachsous (Ds), another atypical cadherin [343]. The golgi kinase Four-jointed modulates the interaction between Fat and Dachsous by phosphorylating both of their extracellular domains [344-347]. Four-jointed and Dachsous are expressed in opposite gradients along the proximo-distal axis of *Drosophila* limbs and eye, such that cells have more Dachsous-bound Fat on their proximal side [348-350]. This difference in Fat activity is thought to be important for proper planar polarization of the atypical myosin Dachs (D). The membrane localization of Dachs is antagonized by Fat, which causes Dachs to localize to the distal sides of cells [351].

In addition to its role in the global PCP pathway, atypical cadherin Fat also functions upstream of the core Hippo Pathway [352-356]. It is thought that Fat modulates PCP independently of the Hippo pathway [282, 284, 288, 357, 358]. Fat is able to regulate the Hippo pathway in two different ways: Fat signaling prevents Dachs-dependent degradation of Warts and promotes apical localization of Ex [282, 284, 357-359]. Taken together, these studies reveal an interesting connection between PCP and the Hippo pathway.

Hippo pathway and Cancer

Massive tissue overgrowth in Hippo mutant *Drosophila* flies has suggested that this signaling pathway may play an important role in human cancer [205, 215, 337]. In recent years multiple studies in mammalian model systems confirmed this hypothesis. Mutations in core Hippo pathway components result in tumorigenesis in mice and mutations, deletions, and amplifications of a subset of Hippo pathway genes are observed in human cancers [360].

Studies that have sought to identify germline mutations that contribute to human cancer have identified TP53, RB1, BRCA1, BRCA2, and PTEN as tumor suppresser genes [360]. However, to date, there have been few germline or somatic mutations in the Hippo pathway genes that have been frequently observed in human cancers. One notable exception is neurofibromin 2 (NF2), which is frequently inactivated in human cancer and hence fits the classical definition of a bona fide tumor suppressor gene [360]. Mutations in *NF2* occur in approximately 1/25,000 individuals, with half of the mutations occurring as a result of germline inheritance and half due to de novo mutations [242]. By age 60 nearly 100% of individuals with mutations in NF2 develop tumors in the nervous system, eyes, and skin [361]. Interestingly, the PCP genes FAT and DCHS (Dachsous) also appear to play a role in cancer. The FAT family of genes is commonly mutated in colorectal cancer, and the DCHS (Dachsous) family has been associated with ovarian and colorectal cancer [360]. However, the FAT and DCHS genes are extremely large and hence would be expected to experience a higher number of mutations, particularly in colorectal cancers which have a high rate of mismatch repair defects. It is also noteworthy that YAP1 and TAZ (WWTR1) are frequently amplified in human cancer: TAZ is amplified in 30% of lung squamous cell carcinomas, 21% of breast cancers, 17% of ovarian cancers, 15% of head and neck squamous cell carcinomas while YAP1 is amplified in 11% of cervical squamous cell carcinomas and endocervical adenocarcinomas, 9% of head and neck squamous cell carcinomas, and 8% of ovarian serous cystadenocarcinomas (source: http://www.cbioportal.org/).

A large number of Hippo pathway genes have been identified as tumor suppressors in mice. Sav1 conditional knockout mice develop tumors of the liver and the bile ducts [266] as well as gastrointestinal hyperplasia [265]. Mob1a/1b null/heterozygous mice develop tumors in skin, liver, lungs, and salivary glands [362]. YAP1 is frequently amplified in human cancers and

YAP1 overexpression in mice results in gastrointestinal dysplasia, pancreatic ductal metaplasia, hepatocellular carcinoma, and squamous cell carcinoma [220, 262, 267, 363]. Knockout of *Lats1* causes mice to develop soft tissue sarcomas and ovarian tumors [364]. *Mst1*$^{-/-}$ *Mst2*$^{-/-}$ mice develop liver and bile duct tumors [263, 264, 266]. Mst1/2 also restrain intestinal stem cell proliferation and tumorigenesis of the colon by inhibiting YAP1 [365]. In addition, Mst1 has been shown to be required for faithful chromosome segregation and loss of Mst1 results in lymphoma [366]. It is important to note, that while *Mst1/2* and *Lats1/2* are functioning as tumor suppressor genes in mice, these genes are not frequently mutated in human cancers. LATS1 is homozygously deleted in 16% of adenoid cystic carcinomas and LATS2 is homozygously deleted in 8% of metastatic prostate tumors (source: http://www.cbioportal.org/). Interestingly, several studies have indicated that *Mst1/2* and *Lats1/2* may be epigenetically silenced in human cancers [367-369].

The biological functions of the Hippo pathway are intimately tied to tumor development and progression. In the previous sections, we examined the connection between apical basal polarity and cancer as well as the role of stem cells and progenitor cells in tumor formation. It makes sense that since the Hippo pathway interacts with core polarity proteins and is important for maintenance of pluripotency that it might also mediate cancer development through either or both of these mechanisms. In addition to regulation of pluripotency and polarity, the Hippo pathway also connects to other pathways of tumor development via several other mechanisms. First, a fundamental characteristic of neoplasia is unrestricted cell proliferation. In flies and in mice, mutations in the Hippo pathway result in excess proliferation in a number of organs [220, 262, 267, 370]. Second, the Hippo pathway is required for contact inhibition and loss of contact inhibition is frequently observed in transformed cells. Normal adherent cells cease to proliferate

when they come in contact with their neighbors, and this is referred to as contact inhibition. Transformed cells frequently lose contact inhibition, and loss of NF2 or overexpression of YAP can provoke cells to overcome contact inhibition [221, 371, 372]. Third, the Hippo pathway participates in regulating cell cycle exit, which is one way organisms can regulate growth. Irreversible cell cycle exit, or senescence, is one way in which organisms restrict inappropriate proliferation. LATS2 is able to activate p53. In turn, p53 increases transcription of LATS2, which is necessary for arresting cells that have become inappropriately tetraploid [373]. LATS2 depletion is also associated with suppression of Retinoblastoma protein (RB)-induced senescence markers [374]. Together these studies suggest that the Hippo pathway may be playing a role in the maintenance of tumor suppressor checkpoints. A fourth way in which the Hippo pathway is tied to the cancer development is through its regulation of survival. In the mouse liver, induction of apoptosis by CD95 and tumor necrosis factor is blocked by overexpression of YAP [220]. Similarly, YAP overexpression in cancer cell lines causes resistance to apoptosis induced by chemotherapeutic agents [375]. Finally, the Hippo pathway is connected to the development of cancer through its association with metastasis. Metastasis is a feature of advanced tumors and is a multistep process that often includes an epithelial-mesenchymal transition (EMT) of cells before they leave the primary tumor site. YAP overexpression can promote EMT of cultured cells [375]. Likewise, YAP and TAZ levels are elevated in high grade metastatic breast cancer as compared to low grade non-metastatic breast cancer [335]. Additionally, LATS1 and LATS2 are both lower in metastatic prostate cancers than in non-metastatic ones [306]. In summary, there is a plethora of potential mechanisms by which the Hippo pathway could be influencing tumor development or progression.

CHAPTER 2: DLG5 NEGATIVELY REGULATES THE HIPPO SIGNALING PATHWAY

Introduction

The establishment and maintenance of apical-basal cell polarity is critical for proper morphogenesis. Studies in Drosophila and C. elegans have identified several key complexes of proteins that localize to and help define the apical and basolateral membrane domains. However, many of the mechanisms of apical-basal cell polarity are still poorly understood [376]. The aPKC-Par3-Par6 and Crumbs-Pals1-Patj complexes localize apically, while Dlg-Lgl-Scrib and Par1 proteins localize basolaterally [1-4]. Par1 and the Dlg-Lgl-Scrib proteins are known to antagonize the function of apical aPKC-Par3-Par6 and Crumbs-Pals1-Patj complexes [7, 8]. Dlg (discs large) is a member of the basolateral polarity proteins which interacts with Lgl and Scrib and is crucial for the establishment and maintenance of the basolateral membrane domain [46, 47].

Dlg5 is a member of the discs large family of proteins. Dlg5's PDZ, SH3, and GUK domains identify it as a MAGUK (membrane associated guanylate kinase) family protein; however, Dlg5's N-terminal CARD, DUF, and coiled-coil domains also distinguish it from other discs large proteins. Dlg5 knockout mice develop hydrocephalus, renal cysts, and an emphysema-like lung phenotype [120]. Dlg5 is functionally important for the maintenance of apical basal cell polarity and epithelial tubes and *Dlg5*$^{-/-}$ mice exhibit brain and kidney abnormalities due to the loss of polarity in the neural progenitor cells and epithelial cells lining the kidney collecting ducts [120]. *Dlg5*$^{-/-}$ lung epithelial cells show defects in apical basal polarity and *Dlg5*$^{-/-}$ mice go on to develop enlarged air sacs and an emphysema-like phenotype

[121]. Dlg5 disrupts polarity, in part, by causing mislocalization of aPKC; immunofluorescence staining of newborn kidneys revealed that aPKC demonstrates normal apical localization in wild type collecting ducts but was no longer localized apically in *Dlg5*$^{-/-}$ kidneys [120]. Similarly, in *Dlg5*$^{-/-}$ lungs aPKC is no longer localized apically in epithelial cells [121]. The mislocalization of apical polarity protein aPKC and disruption of epithelial tube structures indicates that Dlg5 is critical for the establishment and maintenance of apical basal polarity.

So far, little is known about how the apical-basal polarity regulates downstream cell signaling. Recently an interesting connection between apical basal polarity and Hippo pathway has emerged. The Hippo pathway plays a significant role in regulating apoptosis, proliferation, and cell differentiation [201, 245]. At the core of the Hippo pathway, mammalian homologues of the *Drosophila* Hippo kinase, MST1/2 auto-phosphorylate at T183/T180, which increases their kinase activity [232]. MST1/2 then phosphorylate kinases LATS1/2 at T1079, priming them to phosphorylate YAP1 and TAZ at S127 and S89, respectively [234]. When mammalian YAP1 and TAZ are phosphorylated, they are either cytoplasmically sequestered or ubiquitinated and degraded [240, 241]. When YAP1 and TAZ are not phosphorylated, they are able to enter the nucleus and trigger activation of downstream targets such as *CTGF* and *CYR61,* by binding to transcription factors such as TEAD [201, 242, 244].

Recent studies have demonstrated that apical-basal polarity proteins are able to regulate the Hippo pathway through at least two distinct mechanisms. aPKC and Lgl are both able to act on the Hippo pathway in opposing fashions [278, 329]. Overexpression of aPKC or loss of its antagonist, Lgl, induces cytoplasmic mislocalization of an apical pool of Hippo (the *Drosophila* homologue of MST1/2) and increases colocalization of Hippo and its negative regulator RASSF. These results suggest that aPKC can reduce Hippo pathway activation, a function that is

antagonized by Lgl, but the precise mechanism of aPKC-mediated regulation of the Hippo pathway remains to be elucidated. The polarity protein Crumbs is also able to regulate the Hippo pathway via a distinct mechanism [329, 332]. Studies in *Drosophila* have identified that Crumbs is able to bind to Expanded (Ex), an upstream agonist of the Hippo pathway, and that this association is required for the apical localization of Ex [329, 377, 378]. In cells mutant for Crumbs, Ex is basally mislocalized, resulting in a down regulation of the canonical Hippo pathway and subsequent tissue overgrowth [329, 377, 378]. Interestingly, in *Drosophila,* Crumbs overexpression also results in a mislocalization of Ex and overproliferation due to Hippo pathway inactivation [329, 377, 378]. The mechanism of Crumb's dual role in the Hippo pathway is not entirely understood, although current studies make it apparent that many of the mechanistic connections between the apical-basal polarity and Hippo pathways remain to be discovered.

In order to identify new potential binding partners and functions for Dlg5, we collaborated with Dr. Andrew Emili's research group in at the University of Toronto. Dr. Emili's group overexpressed a tagged Dlg5 construct in HEK 293 T cells and used Affinity Purification-Mass Spectrometry (APMS) to reveal a novel interaction between Dlg5 and the Mst1/2 kinases of the Hippo pathway. Inactivation of *Dlg5* in mice *in vivo* and in primary cells *in vitro* (loss-of-function experiments) results in hyperactivation of Hippo pathway and decreased total levels and activity of YAP1 and TAZ. Overexpression of DLG5 (gain-of-function experiments), inhibits Hippo signaling and results in overexpression of Hippo pathway target genes. In genetic epistasis experiments in mice, *Dlg5* shows strong a genetic interaction with both *Taz (Wwtr1)* and *Yap1*. Mechanistically, I found that DLG5 negatively regulates the Hippo pathway by inhibiting the binding of MST to LATS. This study increases our

understanding of the connection between apical-basal polarity and the Hippo pathway and identifies apical-basal polarity family protein DLG5 as a novel interactor and regulator of the Hippo signaling transduction pathway.

Results

Mass Spectrometry analysis of Dlg5 protein complexes

Dlg5 is required for proper mammalian morphogenesis; however, the molecular mechanisms of Dlg5 are not well understood. We hypothesized that Dlg5 likely functions by interacting with other proteins and the identification of Dlg5 interacting partners may help reveal its molecular mechanisms. To identify Dlg5 binding partners in mammalian cells we decided to use unbiased Mass Spectrometry analysis of Dlg5 protein complexes. For this purpose, we collaborated with Dr. Andrew Emili at the University of Toronto, who is a specialist in Mass Spectrometry based protein analysis and identification. A graduate student in Dr. Emili's laboratory, Julian Kwan, generated a line of HEK293 T cells stably expressing full-length Flag-tagged Dlg5 protein. Julian performed several Flag-pull down experiments followed by Mass Spectrometry based protein identification using this cell line, along with Flag-GFP and multiple other HEK293 lines expressing irrelevant Flag-tagged proteins. Bioinformatics analysis of the Mass Spectrometry data helped to eliminate the background proteins, which can be seen in pull downs from control cell lines. Overall, 25 proteins were identified in Dlg5 protein complexes (Table 2). While multiple novel, putative Dlg5 interacting partners were identified through the Mass Spectrometry experiments, we were especially intrigued by the presence of multiple

Table 2: Novel interactors of Dlg5 as identified by AP-MS

Each column indicates a separate affinity purification (AP), for a total of 6 for Dlg5. V1 represents a pool of stable 293T cells transduced with Dlg5-expressing lentivirus. V2 represents a separate pool generated at a different time (a fully independent replicate). TX represents 293T cells transiently transfected with Dlg5-expressing plasmid using Fugene6. The proteins listed were scored as specific using compPASS. The values are spectral counts for each protein. "sp" indicates that a Swiss-Prot (uniprot) ID follows. Source: Julian Kwan

Gene Name	v1 AP1	v1 AP2	v2 AP1	v2 AP2	tx AP1	tx AP2	Locus	MW
DLG5	421	355	123	128	124	126	sp|Q8TDM6|DLG5_HUMAN	213868.27
STK3	23	30	19	24	36	26	sp|Q13188|STK3_HUMAN	56301.13
MARK3	26	33	17	15	17	21	sp|P27448|MARK3_HUMAN	87004.66
YME1L1	11	14	19	20	14	10	sp|Q96TA2|YMEL1_HUMAN	86455.29
CAMK2D	21	19	7	13	9	7	sp|Q13557|KCC2D_HUMAN	56369.43
MARK2	17	14	11	8	11	9	sp|Q7KZI7|MARK2_HUMAN	87910.87
CAMK2A	18	20	7	9	7	7	sp|Q9UQM7|KCC2A_HUMAN	54029.73
SAV1	20	16	5	5	14	8	sp|Q9H4B6|SAV1_HUMAN	44634.09
PRPSAP2	0	3	11	4	31	16	sp|O60256|KPRB_HUMAN	40925.78
CAMK2G	10	15	8	11	7	6	sp|Q13555|KCC2G_HUMAN	62609.21
CEP97	19	13	9	7	5	3	sp|Q8IW35|CEP97_HUMAN	96981.17
STK4	0	4	13	6	12	7	sp|Q13043|STK4_HUMAN	55630.19
FAM135A	11	5	3	3	7	11	sp|Q9P2D6|F135A_HUMAN	169839.91
SEC16A	19	10	0	0	5	5	sp|O15027|SC16A_HUMAN	233518.47
TRIM27	5	13	3	4	7	6	sp|P14373|TRI27_HUMAN	58489.88
USP15	5	4	7	7	6	5	sp|Q9Y4E8|UBP15_HUMAN	112419.16
CDC37	5	7	8	7	3	0	sp|Q16543|CDC37_HUMAN	44468.42
NT5C2	2	1	9	10	0	0	sp|P49902|5NTC_HUMAN	64969.97
HOOK3	5	6	0	2	3	5	sp|Q86VS8|HOOK3_HUMAN	83126.02
DNAJC13	0	2	5	5	3	3	sp|O75165|DJC13_HUMAN	254431.31
C20orf117	3	3	5	0	4	0	sp|O94964|CT117_HUMAN	159760.57
CAMK2B	4	5	3	3	0	0	sp|Q13554|KCC2B_HUMAN	72726.97
CCDC18	5	7	0	2	0	0	sp|Q5T9S5|CCD18_HUMAN	168962.64
KIAA0802	0	2	0	0	3	5	sp|Q9Y4B5|K0802_HUMAN	208778.26
PROSC	0	4	2	0	0	4	sp|O94903|PROSC_HUMAN	30343.9
MARK1	3	3	0	2	0	0	sp|Q9P0L2|MARK1_HUMAN	89002.7

peptides from STK3 (MST2), STK4 (MST1) and Salvador proteins, which represent one of the critical nodes of the core Hippo signal transduction pathway (Figure 9). We noted that $Dlg5^{-/-}$ mice have several phenotypes that are similar to phenotypes of mice with mutations in the Hippo pathway components. For example, $Taz^{-/-}$ mice are smaller than their wild type littermates and develop cysts in their kidneys and an emphysema-like lung phenotype [248, 249, 379]. Therefore, we hypothesize that Dlg5 may function as a regulator of the Hippo signaling pathway and decided to investigate the potential connection between Dlg5 and Hippo kinases in more detail.

Immunoprecipitation experiments confirm the interaction between DLG5 and MST kinases in mammalian cells.

Mass Spectrometry experiments represent a very powerful approach in the identification of individual proteins in complex protein mixtures; however, they are notoriously prone to false positive findings. Therefore, to verify the protein-protein interaction that was identified by mass spectrometry, we examined the ability of DLG5 to bind to MST kinases using co-immunoprecipitation experiments. For this purpose we utilized HEK293 FT cells expressing exogenous tagged proteins and mouse primary neural progenitor cells expressing endogenous Dlg5 and MST1/2. As expected, the transfection of constructs encoding V5-tagged DLG5 and nVA (3xFlag-2xTEV-6xHIS-StepII-Beacon)-tagged MST2 into HEK293FT cells resulted in expression of the corresponding proteins (Figure 10A and10B). Importantly, when nVA-Mst2 was pulled down with Strep-Tactin sepharose, V5-DLG5 was also pulled-down, but only in samples that expressed nVA-Mst2 (Figure 10A). Similarly, when V5-DLG5 was pulled down with anti-V5-sepharose, nVA-Mst2 was also pulled-down, but only in samples that expressed V5-DLG5 (Figure 10B). Thus, I detected a specific physical interaction between exogenously

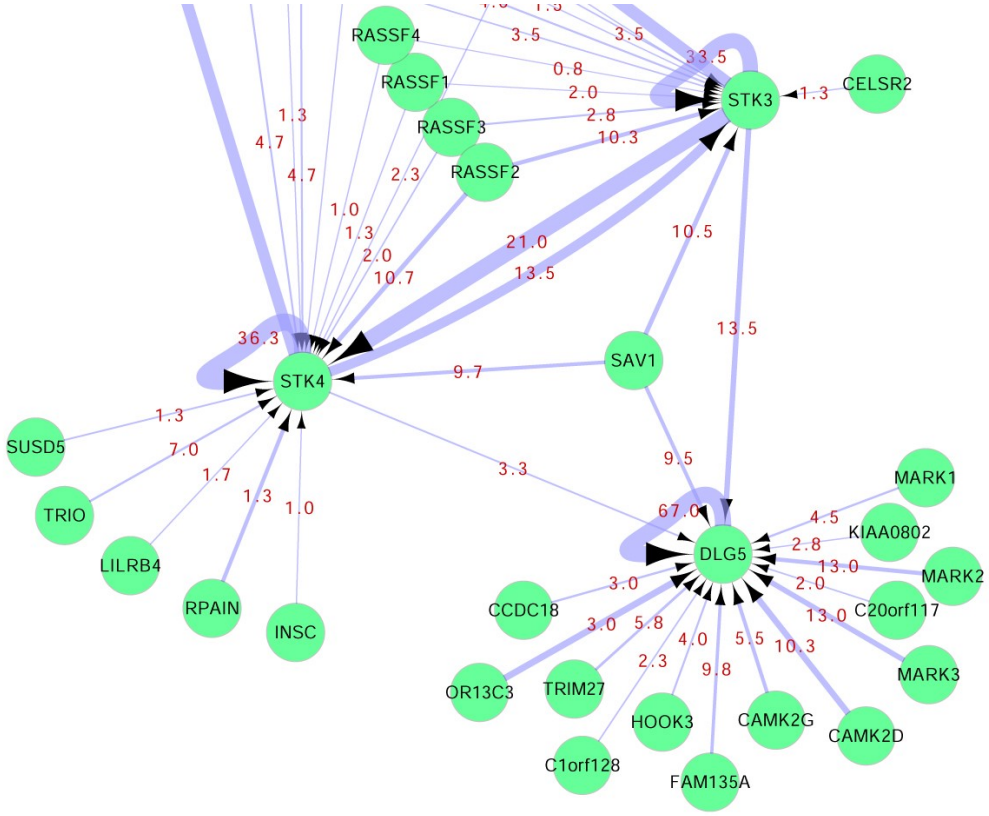


Figure 9: The Dlg5 interactome as defined by AP-MS.
The edges are labeled with the average number of unique peptides identified and edge width corresponds to the average number of times a peptide corresponding to that protein was identified (spectral counts). Source: Julian Kwan

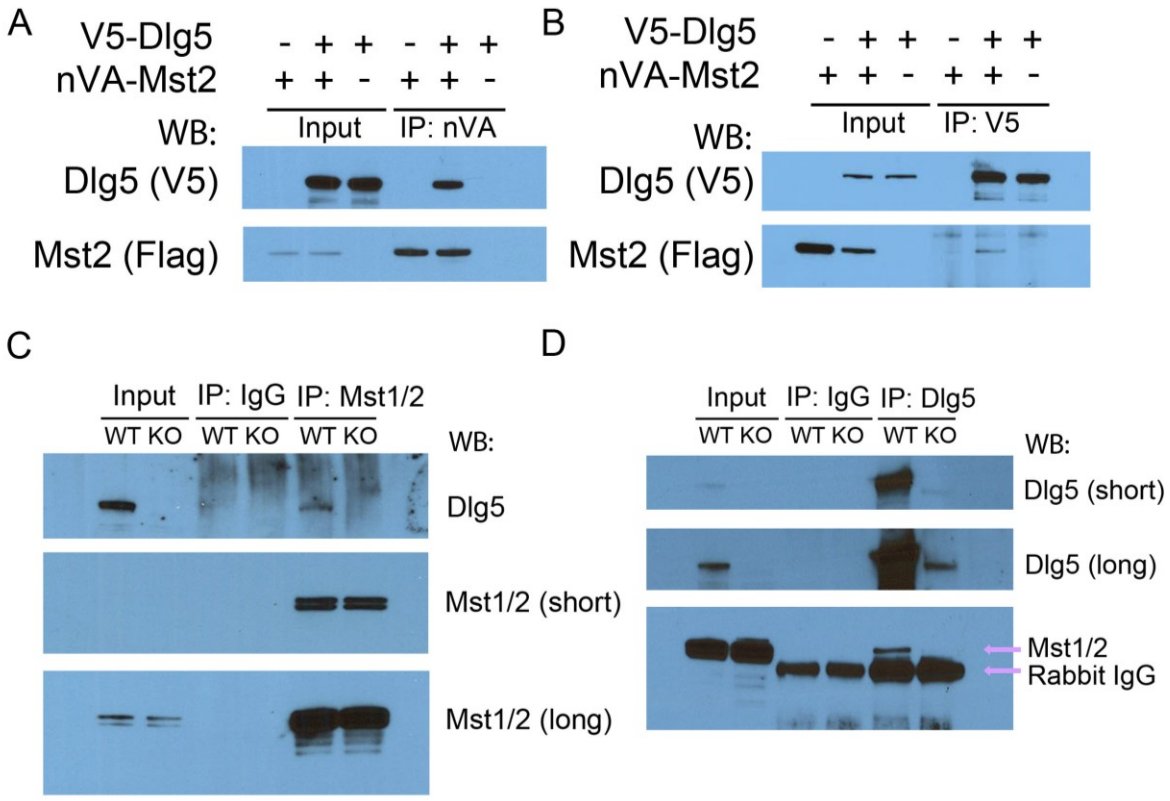


Figure 10: Dlg5 binds to Mst1/2, a Hippo pathway kinase.
A) Western blot (WB) analysis of total (input) and immunoprecipitated (IP) with Strep Tactin sepharose (pulls down the nVA tag) proteins from HEK 293 FT cells transfected with V5-Dlg5 and nVa-Mst2 expression constructs, as indicated. Note, DLG5 was pulled down only, when nVA-MST2 was present in cell lysates. B) Western blot (WB) analysis of total (input) and immunoprecipitated (IP) with V5- sepharose proteins from HEK 293 FT cells transfected with V5-Dlg5 and nVa-Mst2 expression constructs, as indicated. Note, MST2 was pulled down only, when V5-Dlg5 was present in cell lysates. C) Western blot (WB) analysis of total (input) and immunoprecipitated (IP) with control IgG or anti-MST1/2 antibodies proteins from wild-type (WT) or *Dlg5*-knockout (KO) neural progenitors, isolated from E11.5 embryos. Note, endogenous DLG5 was pulled down only with anti-MST1/2, but not with control IgG antibodies. D) Western blot (WB) analysis of total (input) and immunoprecipitated (IP) with control IgG or anti-DLG5 antibodies proteins from wild-type (WT) or *Dlg5*-knockout (KO) neural progenitors, isolated from E11.5 embryos. Note, endogenous MST1/2 was pulled down only with anti-DLG5, but not with control IgG antibodies.

expressed DLG5 and MST2 proteins, which confirmed the Mass Spectrometry data. Exogenous proteins expressed in transfected HEK293 cells are usually overexpressed and an interaction between overexpressed proteins in HEK293 cells does not necessarily prove an interaction between endogenous proteins. Therefore, I wanted to determine if the interaction between DLG5 and MST1/2 is also present in an endogenous setting. For this purpose, I utilized primary mouse neutral progenitor cells isolated from brains of wild-type and *Dlg5*$^{-/-}$ embryos at day 11.5 of development (embryonic day 11.5, E11.5). This model system was highly relevant to my studies, because *Dlg5*$^{-/-}$ embryonic neural progenitor cells display cell polarity defects resulting in a prominent brain phenotype in *Dlg5*$^{-/-}$ newborns. This model also provided me with cells lacking endogenous Dlg5 that can be used as a negative control in my co-immunoprecipitation experiments. Thus, I isolated neural progenitor cells (NPCs) from wild type (WT) and *Dlg5*$^{-/-}$ (KO) E11.5 brains and established them in culture. As expected, DLG5 protein was prominently expressed in wild-type but not in *Dlg5*$^{-/-}$ cells (Figure 10C and 10D). When MST1/2 was immunoprecipitated with anti-MST1/2 antibodies on Protein A-sepharose beads, DLG5 was also pulled-down, but only in samples from wild-type cells (Figure 10C). Importantly, DLG5 was not pulled down by non-specific IgGs on Protein A-sepharose beads (negative control), indicating specificity of the pull down (Figure 10C). Similarly, when DLG5 was pulled down with anti-Dlg5-, but not with control IgG antibodies, MST was co-immunoprecipitated with Dlg5 (Figure 10D). Taken together with the results from co-immunoprecipitation experiments with tagged proteins above, these data confirm a physical interaction between DLG5 and MST.

Western blot analyses of endogenous Hippo pathway proteins reveal activation of the Hippo signaling pathway in *Dlg5*$^{-/-}$ cells.

Since DLG5 interacts with MST1/2, two of the core protein kinases of the Hippo signaling pathway, I decided to analyze whether Hippo pathway is impacted in *Dlg5*$^{-/-}$ cells. To begin this work, I performed western blot analysis of total and phosphorylation-specific levels of Hippo pathway proteins YAP1 and TAZ in total protein lysates from E14.5 control and Dlg5$^{-/-}$ brains, livers, lungs, and skins. The primary output of the Hippo signaling pathway is the phosphorylation of YAP1 by LATS1/2 (on S127 and S397) and subsequent degradation of YAP1 and TAZ proteins. Analysis of total brain protein extracts revealed a prominent decrease in YAP1 and, to a lesser extent, TAZ proteins in *Dlg5*$^{-/-}$ brains (Figure 11). A similar analysis of total protein extracts revealed a decrease in total TAZ in livers, skin and, to a lesser extent, lungs of *Dlg5*$^{-/-}$ embryos (Figure 11). While the differences in total levels of YAP1 and TAZ between wild type and *Dlg5*$^{-/-}$ samples showed organ-specific variability, protein lysates from *Dlg5*$^{-/-}$ organs consistently showed higher PhosphoS127-YAP1/total-YAP1 and PhosphoS89-TAZ/total-TAZ protein ratios than those from wild-type controls (Figure 11). These differences indicate potential activation of the Hippo signaling pathway, which would result in increased phosphorylation at S127 and degradation of YAP1 and TAZ proteins. Interestingly, the phenotype of smaller overall body size, polycystic kidneys, and premature differentiation of neural progenitors in *Dlg5*$^{-/-}$ mice is consistent with decreased activity of YAP1/TAZ.

To continue my investigation of the Hippo pathway in *Dlg5*$^{-/-}$ mice, I isolated total protein extracts from additional E14.5 wild-type (n=5) and *Dlg5*$^{-/-}$ (n=5) brains and performed western blot analyses of DLG5, NF2, phospho (T1079 and S909) and total LATS1/2, phospho

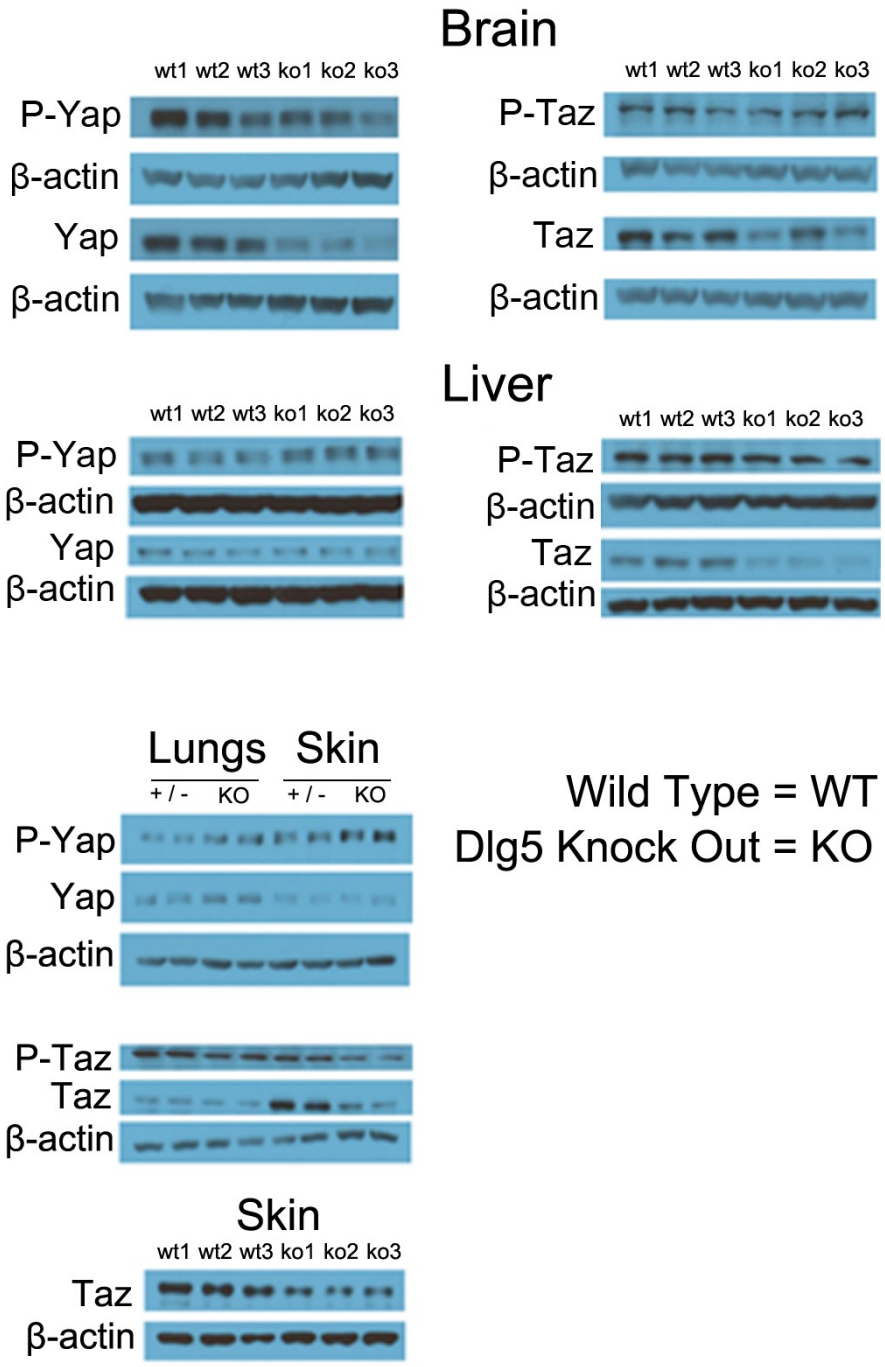


Figure 11: Changes in total levels and S127 phosphorylation of YAP1 and TAZ in Dlg5 $^{-/-}$ mice.
Western blot analyses of total protein extracts from E14.5 wild type (WT) and *Dlg5*$^{-/-}$ (KO) brain, liver, lung, and skin with anti-phospho-YAP1, total YAP1, anti-phospho-TAZ, total TAZ, and beta-actin antibodies.

(T183) and total MST1/2, phospho-(S127) and total YAP1, and TAZ. β-actin was used as a protein loading control. Consistent with earlier data (Figure 11), protein lysates from *Dlg5*$^{-/-}$ brains showed higher ratios of phospho (S127)-YAP1/total-YAP1 (P=0.015), as well as lower total levels of YAP1 (P=0.0003) and TAZ (P=0.009) proteins (Figure 12-15). Moreover, the analysis of LATS1/2 proteins revealed an increase in ratios of phospho-(T1079) LATS/total LATS1 (P=0.03) and phospho (T1079) LATS/total LATS2 (P=0.002) proteins in *Dlg5*$^{-/-}$ brains (Figures 12-15). In the canonical Hippo signaling pathway, the T1079 on LATS1/2 is the site for activating phosphorylation by MST1/2 [234] and absolute levels of phospho-(T1079) Lats1/2 appear to be slightly higher in *Dlg5*$^{-/-}$ brains (Figure 12). I also detected an increase in the ratios of phospho-(T183) MST/total MST1 (P=0.002) and phospho-(T183) MST/total MST2 (P=0.01) proteins in *Dlg5*$^{-/-}$ brains. The T183 is the auto-phosphorylation site on MST1/2 kinases, which becomes hyperphosphorylated upon activation of MST kinases [232]. Overall, these data indicate activation of the MST1/2-LATS1/2 kinases of the canonical Hippo signal transduction pathway in *Dlg5*$^{-/-}$ brains.

Expression of endogenous gene targets of the Hippo pathway is decreased in *Dlg5*$^{-/-}$ mice.

While the exact endogenous gene targets of the Hippo signaling pathway are tissue-specific, the *Ctgf* and *Cyr61* genes are direct gene targets that are almost always affected by changes in the transcriptional activity of YAP1/TAZ [380, 381]. To analyze whether the expression of *Ctgf* and *Cyr61* genes is affected in in *Dlg5*$^{-/-}$ brains, I performed qRT-PCR analysis of total RNAs isolated from E14.5 wild-type (n=3) and in *Dlg5*$^{-/-}$ (n=3) brains (Figure 16). The quantitation of the data demonstrates significantly lower levels of Cyr61 (P=0.02) and

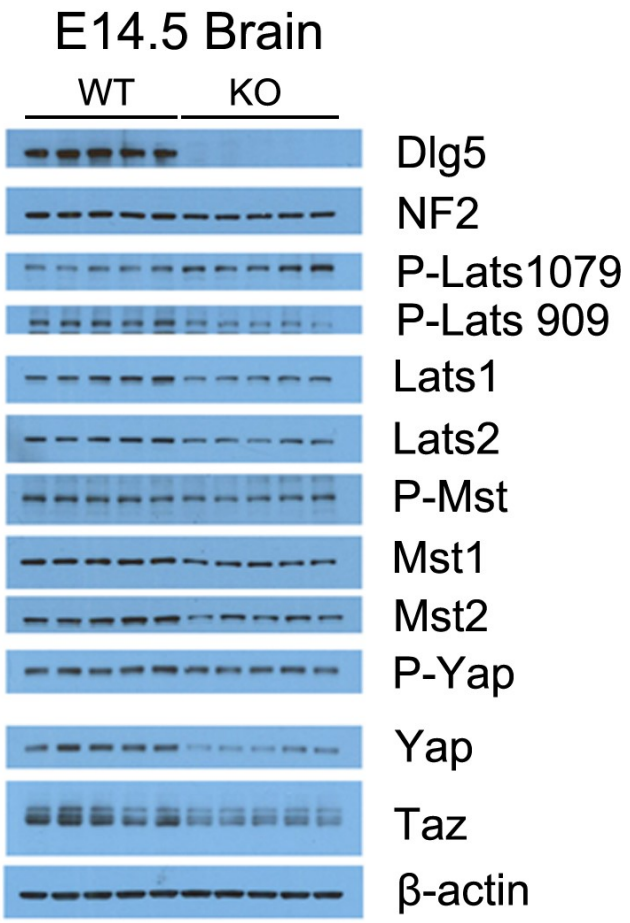


Figure 12: Changes in total levels and phosphorylation of Hippo pathway proteins in E14.5 brains from wild-type (WT, n=5) and Dlg5-knockout (KO, n=5) embryos.
Western blot analyses of total protein extracts with anti-DLG5, anti-NF2, anti-phospho-LATS(1079), anti-phospho-LATS(909), anti-total-LATS1, anti-total-LATS2, anti-phospho-MST, anti-total-MST1, anti-total-MST2, anti-phospho-YAP1(127), total YAP1, total TAZ, and beta-actin antibodies.

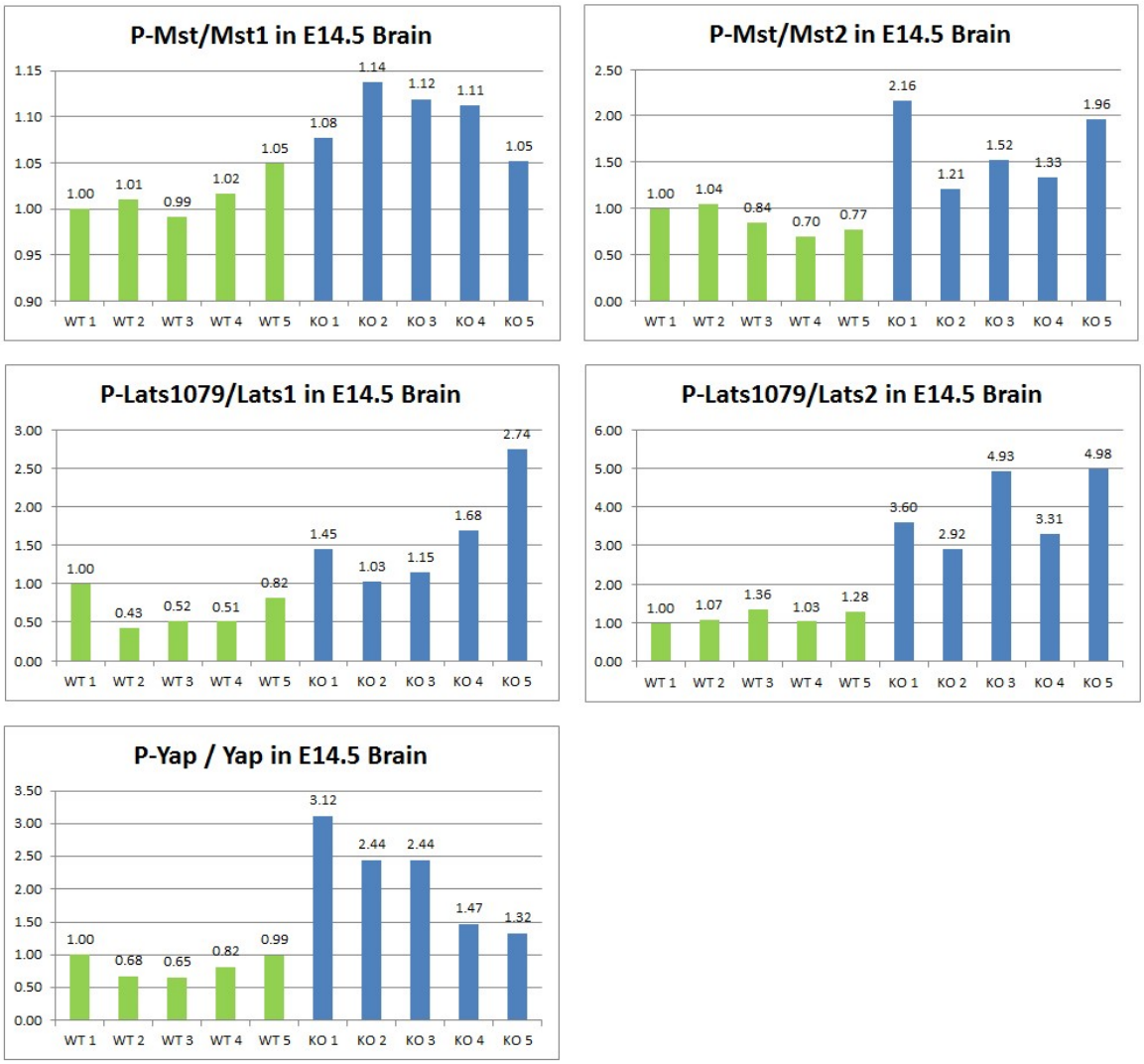


Figure 13: Quantitation of specific phosphorylation of Hippo pathway proteins in E14.5 brains from wild-type (WT) and Dlg5-knockout (KO) embryos.
The Western blot data for total and phospho-specific antibodies shown in Fig. 12 were scanned and analyzed by Image J. The graphs show phospho to total ratios for MST, LATS and YAP1 in individual protein samples from 5 wild type (WT) and 5 Dlg5-knockout (KO) brains, with ratio in the first wild-type sample (WT1) arbitrary adjusted to 1.

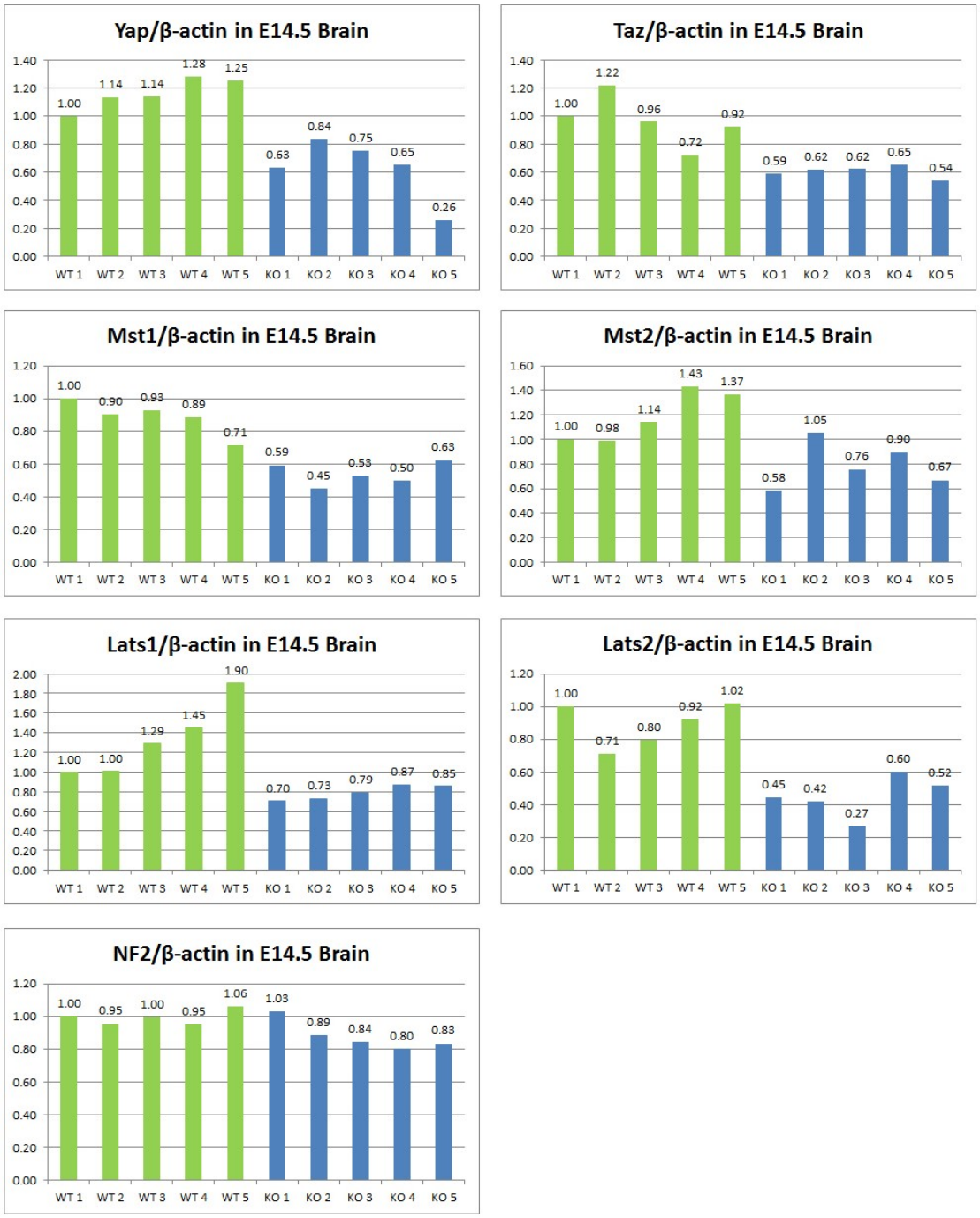


Figure 14: Quantitation of total levels of Hippo pathway proteins in E14.5 brains from wild-type (WT) and Dlg5-knockout (KO) embryos.
The Western blot data shown in Fig. 12 were scanned and analyzed by Image J. The graphs show total indicated protein to β-actin ratios for YAP1, TAZ, MST1, MST2, LATS1, LATS2, and NF2 in individual protein samples from 5 wild type (WT) and 5 Dlg5-knockout (KO) brains, with ratio in the first wild-type sample (WT1) arbitrarily adjusted to 1.

E14.5 Brain	
	T test P Value:
NF2/β-actin	0.047
P-Lats 1079/Lats1	0.031
P-Lats 1079/Lats2	0.0024
P-Lats 909/Lats1	0.079
P-Lats 909/Lats2	0.98
Lats1/β-actin	0.031
Lats2/β-actin	0.00067
P-Mst/Mst1	0.0024
P-Mst/Mst2	0.011
Mst1/β-actin	0.00049
Mst2/β-actin	0.013
P-Yap/Yap	0.015
Yap/β-actin	0.00034
Taz/β-actin	0.0091
	Decreased
	Increased

Figure 15: Activation of the canonical Hippo signaling pathway and decrease in total levels of YAP1 and TAZ in E14.5 brains from Dlg5$^{-/-}$ mice.
The statistical analysis (t-test) of differences in total levels and phospho-versus-total protein ratios between wild-type and Dlg5$^{-/-}$ groups shown in Figs. 13-14. Note the statistically significant increase in phospho-versus-total protein ratios for MST, LATS and YAP proteins in Dlg5$^{-/-}$ brains. Note the statistically significant decrease in total levels of YAP1 and TAZ in Dlg5$^{-/-}$ brains.

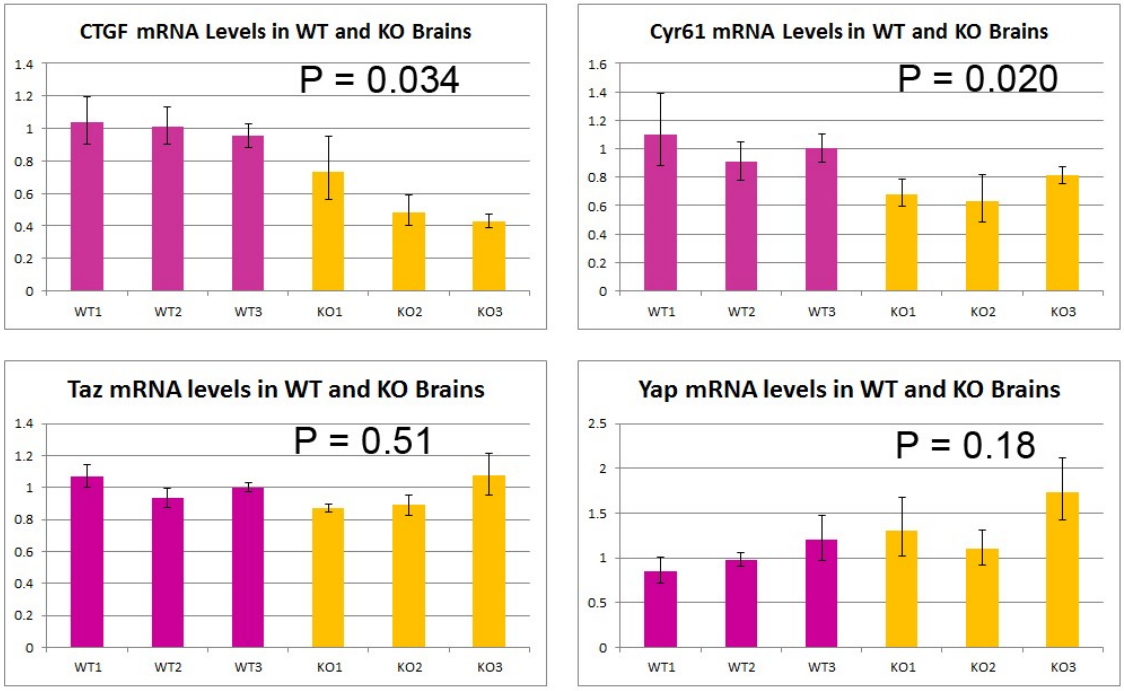


Figure 16: Decreased expression of direct gene targets of the Hippo pathway in Dlg5$^{-/-}$ brains.
mRNA levels of *Ctgf* and *Cyr61* (direct downstream targets of the Hippo pathway), as well as *Yap1* and *Taz* were determined by RT qPCR in wild-type (WT) and Dlg5-knockout (KO) E14.5 brains. Note the statistically significant decrease in the levels of *Cyr61* and *Ctgf* mRNAs, but static levels of *Yap1* and *Taz* in Dlg5-knockout brains. These data confirm activation of the Hippo pathway signaling in Dlg5-knockout brains. The data also demonstrate that the decrease in total YAP1 and TAZ protein levels in Dlg5$^{-/-}$ brains (Fig. 15) occurs at the post transcriptional level, which is consistent with the activation of Hippo signaling in Dlg5-knockout brains.

CTGF (P=0.03) mRNAs in in *Dlg5*$^{-/-}$ brains. Importantly, the total mRNA levels of YAP1 and TAZ were unchanged. These data are consistent with the activation of the canonical Hippo signaling and decreased transcriptional activity of YAP1/TAZ in *Dlg5*$^{-/-}$ mice.

Gain-of-function experiments reveal negative role of Dlg5 in regulation of the canonical Hippo signaling pathway.

Analysis of *Dlg5*$^{-/-}$ mice revealed activation of the canonical Hippo pathway signaling in cells lacking Dlg5. These loss-of-function experiments suggest a negative role for Dlg5 in the regulation of the Hippo pathway. However, these data do not necessarily prove that Dlg5 is a member of the Hippo signaling pathway, because the connection between Dlg5 and Hippo signaling can be rather indirect. For example, we already know that Dlg5 is necessary for proper maintenance of cell polarity and it is possible that changes in apical-basal cell polarity or in some other general cellular mechanisms are responsible for the increase in Hippo kinase activity in *Dlg5*$^{-/-}$ mice. Therefore, I decided to complement the loss-of-function experiments, with gain-of-function studies. For this purpose, I utilized overexpression of Dlg5, LATS, MST, YAP1 and TAZ proteins in non-polarized HEK293 FT cells.

I transfected HEK293 FT cells with Flag-LATS1, nVA-MST2, and Flag-YAP1 expression constructs with and without V5-DLG5 and analyzed resulting levels of YAP1 (Figure 17A). To determine the consistency of the resulting changes, each transfection experiment was performed in duplicates. In each combination of YAP1 with MST2 and LATS1 constructs, the overexpression of Dlg5 did not affect the total levels of MST2 and LATS1, but prominently increased the levels of YAP1 (Figure 17A). These Dlg5-mediated changes were especially prominent when both MST2 and LATS1 were overexpressed in the same cells (Figure 17A, red

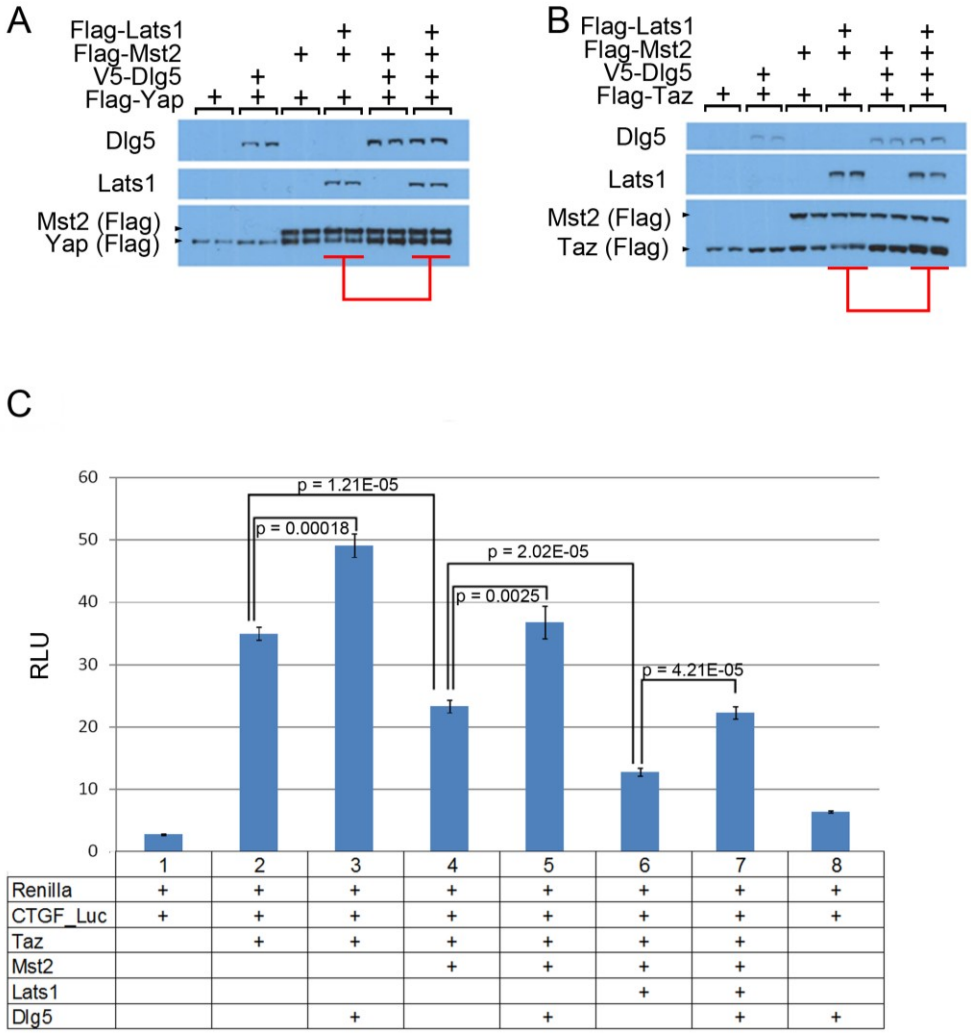


Figure 17: Dlg5 negatively regulates the Hippo signaling pathway in gain-of-function experiments.

A-B) Western blot analyses of total protein extracts from HEK 293 FT cells transfected (in duplicates) with indicated expression constructs. Note that presence of Dlg5 antagonizes Lats1 function and increases YAP1 (A) and TAZ (B) protein levels. C) Luciferase transcriptional activity assay in HEK 293 FT cells. The CTGF promoter was fused to luciferase and fluorescence was used to assess CTGF promoter activity when Flag-Lats1, Flag-Mst2, and Flag-Taz were expressed in the presence or absence of V5-Dlg5. Note the consistent and statistically significant increase in CTGF promoter activity in the presence of DLG5.

brackets). Similar changes were observed in experiments with TAZ (Figure 17B, red brackets). Therefore, overexpression of Dlg5 increased the levels of YAP1 and TAZ, indicating that Dlg5 is a negative regulator of the Hippo pathway, which normally promotes degradation of YAP1 and TAZ proteins.

In addition to the analysis of YAP1 /TAZ protein levels, I also performed Luciferase transcriptional assays to measure the impact of DLG5 on transcriptional activity of Hippo pathway effectors in gain-of-function experiments. In these assay I decided to utilize a CTGF promoter, which is a known direct target of YAP1/TAZ transcriptional activity. The CTGF-firefly luciferase, TAZ, MST2, LATS1 were transfected with and without DLG5 into HEK 293 FT cells. The levels of transfection were normalized using Renilla-Luciferase luminescence and the activity of CTGF promoter was determined by quantitation of firefly luciferase luminescence (Figure 17C). As expected, expression of MST2 and LATS1 negatively impacted TAZ-mediated activation of CTGF promoter. Overexpression of DLG5 consistently attenuated the activity of MST and LATS and increased the activity of CTGF promoter, demonstrating a negative role for DLG5 in regulating the Hippo signaling pathway.

Overall, my gain-function-experiments revealed a negative role of Dlg5 in regulation of the canonical Hippo signal transduction pathway. These findings are consistent with activation of Hippo signaling in *Dlg5*$^{-/-}$ mice and suggest a direct involvement of Dlg5 in regulation of this signaling pathway.

Dlg5-mediated cleavage of MST2

While it was not our intent to focus on caspase cleavage of MST2, gain-of-function experiments in HEK 293 FT cells consistently yielded a prominent lower molecular weight

MST2 fragment when Dlg5 was coexpressed with MST2 (Figure 18), leading us to pursue this phenomenon in more detail. Based on the size of the Mst2 fragment and the surrounding protein marker it was estimated the N-terminally tagged nVA-MST2 fragment was approximately 45kDa in size. Endogenous mouse MST2 protein is 55kDa and if the protein is being cleaved, the surrounding protein markers can be used to estimate the site of cleavage. The approximate location of the cleavage site was estimated to be at amino acid 306, which sits between the N-terminal kinase domain and C-terminal SARAH domain. MST2's caspase cleavage site, which recognizes a DXXD motif, is located 16 amino acids away from this estimated cleavage site (Figure 19). In an attempt to identify the protease that could be generating this MST fragment, I performed a pilot inhibitor assay. I utilized the following inhibitors: Calpeptin, PD15606, Epoxomycin, MG-132, and Z-vad-fmk. I hypothesized that if one of these inhibitors targeted a protease responsible for MST2 cleavage, then I would observe a decrease in the levels of cleaved MST2 fragment. However, none of the inhibitors changed the proportion of higher and the lower molecular weight MST2 fragments. However, increased MST2 cleavage in the presence of Dlg5 is a reproducible phenomenon that warrants further investigation.

The connection between aPKC, Dlg5, and the Hippo pathway

Previous studies in the Vasioukhin lab found that Dlg5 is able to affect the localization, but not activation, of apical polarity protein aPKC [120, 121]. It has been published that aPKC is able to regulate the Hippo pathway, but the precise mechanism of this action is still unknown [278, 329-331]. In order to determine if I can detect aPKC-mediated regulation of the Hippo pathway, I performed gain-of-function experiments in HEK 293 FT cells transfected with constructs expressing Hippo pathway proteins in the presence or absence of aPKC. I found that

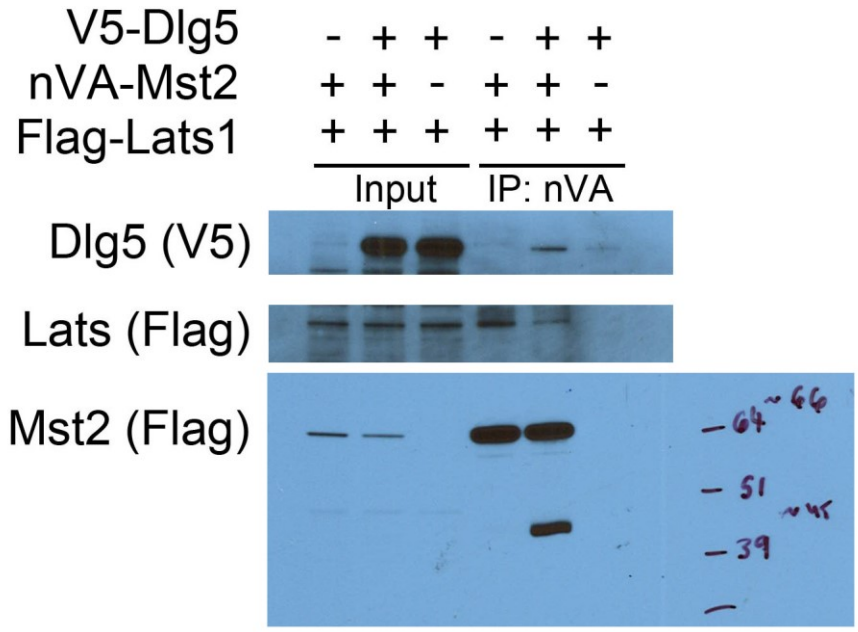


Figure 18: Overexpression of Dlg5 induces partial cleavage of Mst2 protein.
Western blot analyses of total (input) and immunoprecipitated (IP) with Strep Tactin sepharose (pulls down the nVA tag) proteins from HEK 293 FT cells transfected with V5-Dlg5, nVa-Mst2, and Flag-LATS1 expression constructs, as indicated. Note the appearance of an additional low molecular weight fragment of MST2 in samples containing DLG5.

Mouse Mst2/Stk3
Accession:
NP_062609.2

```
  1 meqppasksk lkklsedslt kqpeevfdvl eklgegsygs vfkaihkesg qvvaikqvpv
 61 esdlqeiike isimqqcdsp yvvkyygsyf kntdlwivme ycgagsvsdi irlrnktlte
121 deiatilkst lkgleylhfm rkihrdikag nillntegha kladfgvagq ltdtmakrnt
181 vigtpfwmap eviqeigync vadiwslgit siemaegkpp yadihpmrai fmiptnpppt
241 frkpelwsdd ftdfvkkclv kspeqratat qllqhpfikn akpvsilrdl iaeameikak
301 rheeqq|rele eeeensdede ldshtmvkts sesvgtmrat stmsegaqtm iehnstmles
361 dlgtmvinse eeeeeeeeee edgtmkrnat spqvqrpsfm dyfdkqdfkn kshencdqsm
421 repgpmsnsv fpdnwrvpqd gdfdflknls leelqmrlka ldpmmereie elhqrysakr
481 qpildamdak krrqqnf
```

| - Estimated cleavage site
Protein Kinase ATP binding region
Protein Kinase
NES – Nuclear Export Sequence
SARAH - Sav/RASSF/Hpo Domain
NLS – Nuclear Localization Sequence
Mst2 - Caspase cleavage site DXXD

Figure 19: Estimated position of Mst2 cleavage based on resulting protein fragment size. The size of the Mst2 fragment observed in GOF experiments was used to estimate the position of cleavage. It appears that Mst2 is being cleaved between the N-terminal protein kinase and C-terminal SARAH domains.

aPKC, just like Dlg5, stabilizes TAZ protein levels and antagonizes LATS-mediated depletion of TAZ (Figure 20). Preliminary data by another member of the Vasioukhin lab found that aPKC is capable of phosphorylating Dlg5 at three sites, S1689, S1700, and S1702. Since aPKC and Dlg5 both have the same effect on total TAZ levels in gain-of-function experiments, I hypothesized that aPKC-mediated phosphorylation of Dlg5 could be playing an important role in Dlg5-mediated attenuation of the Hippo pathway. Full length Dlg5 constructs with S1689, S1700, and S1702 residues mutated to either 3A or 3D were generated to serve as non-phosphorylatable and phosphor-mimicking isoforms of Dlg5. In order to determine if aPKC's phosphorylation of Dlg5 could be regulating its interaction with the Hippo pathway, I analyzed wild-type, 3A mutant, and 3D mutant Dlg5 in gain-of-function experiments in HEK 293 FT cells. Activation of the TEAD by TAZ was then assessed by luciferase assays. I found that overexpression of both the Dlg5SA and Dlg5SD constructs resulted in increased activation of TAZ, similar to wild type Dlg5 (Figure 21). These findings suggest that aPKC-mediated phosphorylation of Dlg5 is dispensable for Dlg5-mediated negative regulation of the Hippo signaling pathway.

Negative genetic interaction between mammalian *Dlg5* and *Yap1* or *Taz* genes

To confirm the involvement of Dlg5 in regulation of the Hippo signaling pathway, I performed genetic epistasis experiments in mice. In mice, the *Dlg5* gene is located on chromosome 14 position A3, *TAZ (WWTR1)* on chromosome 3 position D, and *YAP1* on chromosome 9 position A1. $Dlg5^{-/-}$ mice are smaller than their wild type littermates and develop hydrocephalus, polycystic kidneys, and an emphysema-like lung phenotype [120, 121]. $TAZ^{-/-}$ mice are also smaller than their littermates and develop polycystic kidneys and an emphysema-

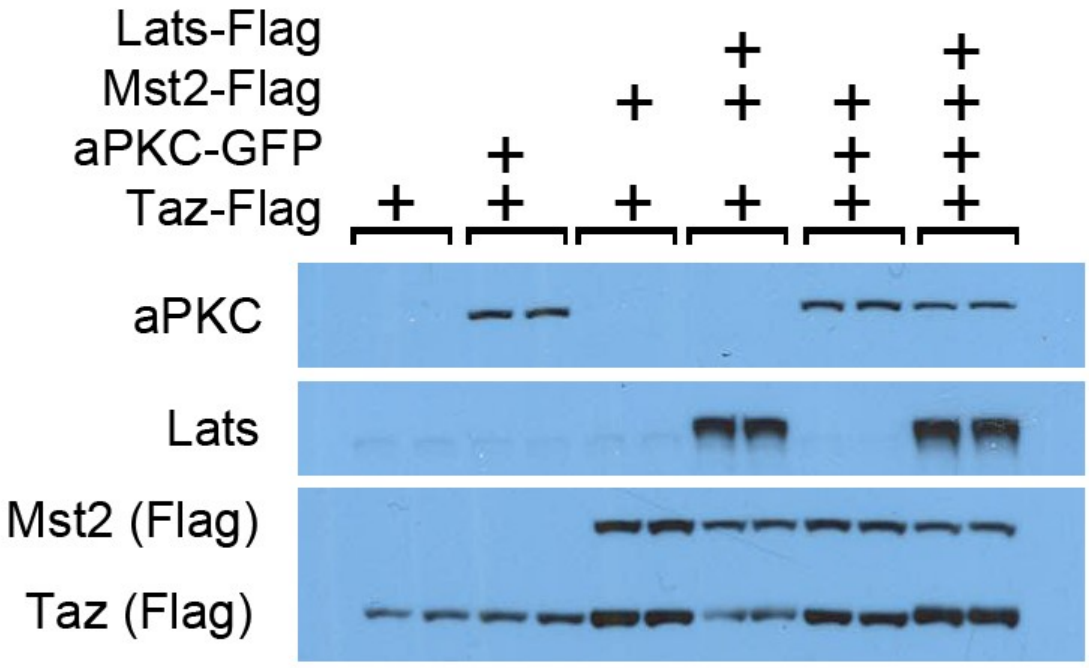


Figure 20: **aPKC negatively regulates the Hippo signaling pathway in gain-of-function experiments.**
Western blot analyses of total protein extracts from HEK 293 FT cells transfected (in duplicates) with the indicated expression constructs. Note that presence of aPKC antagonizes LATS1 function and increases TAZ protein levels.

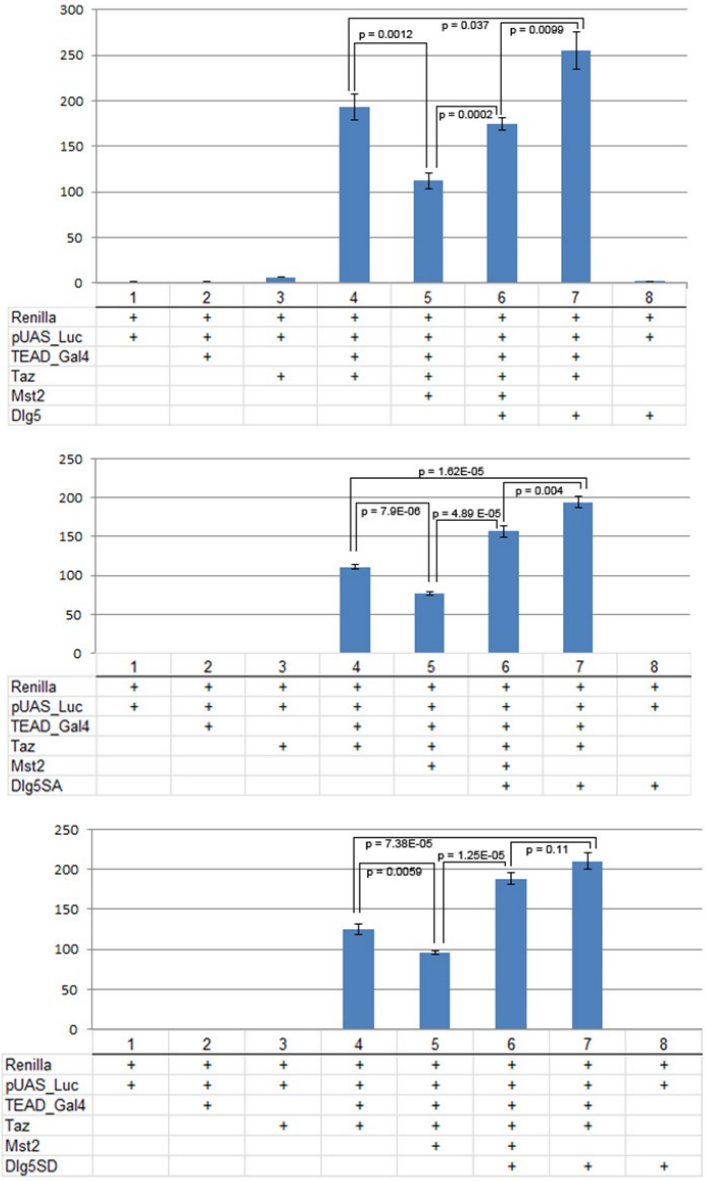


Figure 21: **aPKC-mediated phosphorylation of Dlg5 has no impact on its function in regulation of TAZ transcriptional activity.**
Luciferase transcriptional activity assay in HEK 293 FT cells. The pUAS promoter was driving luciferase and fluorescence was used to assess TEAD-GAL4 fusion transcriptional activity in the presence of indicated expression constructs. Wild-type (DLG5), phosphorylation defective (DLG5SA) and phosphor-mimetic (DLG5SD) forms of DLG5 were equally potent in promotion of TEAD-GAL4 transcriptional activity, indicating that phosphorylation at this Serine has no impact on DLG5 function in regulation of the Hippo signaling pathway.

like lung phenotype [248, 249, 379]. *Yap1*$^{-/-}$ mice, by contrast, die by embryonic day E8.5 due to yolk sac defects [247]. I decided to generate double mutant *Dlg5/Yap1* and *Dlg5/Taz* (*Wwtr1*) mice and determine whether Dlg5 suppresses or enhances the effect of reduced Yap or Taz. I reasoned that if *Dlg5* functions as a negative regulator of the Hippo pathway, then I should expect an aggravation of the *Dlg5*$^{-/-}$ phenotypes when the loss of *Dlg5* is accompanied by decreased expression levels of either the *Yap1* or *Taz* genes.

Our laboratory already had *Dlg5*$^{-/-}$ and *Taz*$^{-/-}$ mice; however, we did not have *Yap1*$^{-/-}$ animals. To generate the *Yap1*$^{+/-}$ allele, I crossed *Yap1*$^{floxed/floxed}$ mice from Dr. Duojia Pan [273] with *Meox2tm1(cre)Sor*/J mice that were obtained from Dr. Philippe Soriano. *Meox2tm1(cre)Sor*/J mice display Cre recombinase activity in early embryogenesis [382]. These mice are utilized as a deleter strain for floxed genes. As expected, double mutant *Yap1*$^{flox/+}$/Meox2tm1*(cre)* animals displayed recombination of the *Yap1* gene effectively generating the germline *Yap1*$^{+/-}$ allele, which was utilized in my subsequent epistasis experiments.

I initially crossed *Dlg5*$^{+/-}$ mice with *Yap1*$^{+/-}$ mice and obtained *Dlg5*$^{+/-}$*Yap1*$^{+/-}$ animals, which themselves did not display any overt phenotypes. To analyze the genetic interaction between *Dlg5* and *Yap1*, I crossed *Dlg5*$^{+/-}$ females to *Dlg5*$^{+/-}$ *Yap1*$^{+/-}$ males and observed the relative frequency of offspring genotypes at postnatal day 7-10 (P7-10). I reasoned that, if a given combination of genes were completely or partially lethal, than that genotype would not

appear or would appear with lower than expected frequency. Likewise, an innocuous combination of genes is predicted to appear at the expected or higher frequency. According to calculated probability, in the case of a lack of genetic interaction between *Dlg5* and *Yap1*, I would have expected 12.5% of the offspring to be $Dlg5^{-/-}$ $Yap^{+/-}$. However, after collecting and genotyping 64 pups, I found no $Dlg5^{-/-}$ $Yap^{+/-}$ pups, which was highly statistically significant (P = 0.0025) (Figure 22). This finding suggests a prominent genetic interaction between *Dlg5* and *Yap1*. To determine whether $Dlg5^{-/-}$ $Yap^{+/-}$ pups are born alive, but die within few days after birth, I analyzed the genotypes of newborn pups (P0). Indeed, I found that $Dlg5^{-/-}$ $Yap1^{+/-}$ pups were born alive; however, they die within a few days of birth (Figure 23). Interestingly, $Dlg5^{-/-}$ $Yap^{+/-}$ animals also have curly, piglet-like tails (Figure 23).

To determine if *Dlg5* genetically interacts with *Taz*, I crossed $Dlg5^{+/-}$ $Taz^{+/-}$ females to $Dlg5^{+/-}$ $Taz^{+/-}$males. According to calculated probability, in the case of a lack of genetic interaction between *Dlg5* and *Taz*, I would have expected 6.25% of the offspring to be $Dlg5^{-/-}$ $Taz^{-/-}$ and 12.5% to be $Dlg5^{-/-}Taz^{+/-}$. However, out of 125 P7-10 pups, 0% were $Dlg5^{-/-}Taz^{-/-}$ (P = 0.0039) (Figure 24). $Dlg5^{-/-}Taz^{+/-}$ animals were also present at lower than expected frequency of 1.6% (P=0.0002). To determine whether some of the missing genotypes were born alive but failed to survive to P7, I again analyzed newborn pups. Out of the 48 genotyped newborn pups, there was one pup with the $Dlg5^{-/-}Taz^{-/-}$ genotype (significantly lower than expected, P=0.029) (Figure 25). This $Dlg5^{-/-}Taz^{-/-}$ pup was found dead at P0. Thus, it is unclear whether $Dlg5^{-/-}Taz^{-/-}$ mice are stillborn or are born alive and die shortly thereafter. In contrast to $Dlg5^{-/-}Taz^{-/-}$ pups, at P0 $Dlg5^{-/-}Taz^{+/-}$ animals appeared with higher than expected frequency (16.67% as opposed to

♀ *Dlg5*$^{+/-}$ x ♂ *Dlg5*$^{+/-}$ *Yap*$^{+/-}$

		Expected:		Observed:		
Dlg5	Yap	Number	Percent	Number	Percent	
Dlg5 +/+	Yap +/+	8	12.50%	11	17.19%	
Dlg5 +/+	Yap +/-	8	12.50%	11	17.19%	
Dlg5 +/-	Yap +/+	16	25.00%	21	32.81%	
Dlg5 +/-	Yap +/-	16	25.00%	12	18.75%	
Dlg5 -/-	Yap +/+	8	12.50%	9	14.06%	
Dlg5 -/-	Yap +/-	8	12.50%	0	0.00%	P = 0.0025
P7-P10 total:		64	100.00%	64	100%	

Figure 22: A prominent negative genetic interaction between *Dlg5* and *Yap1*.
Dlg5$^{+/-}$ females were crossed to *Dlg5*$^{+/-}$/*Yap1*$^{+/-}$ males and the resulting progeny were genotyped at postnatal days 7-10. This figure shows the expected and observed genotypes. Note the statistically significant absence of *Dlg5*$^{-/-}$/*Yap1*$^{+/-}$ pups (as assessed by chi-squared analysis). The *Yap1*$^{-/-}$genotype is embryonic lethal and was not included in this analysis.

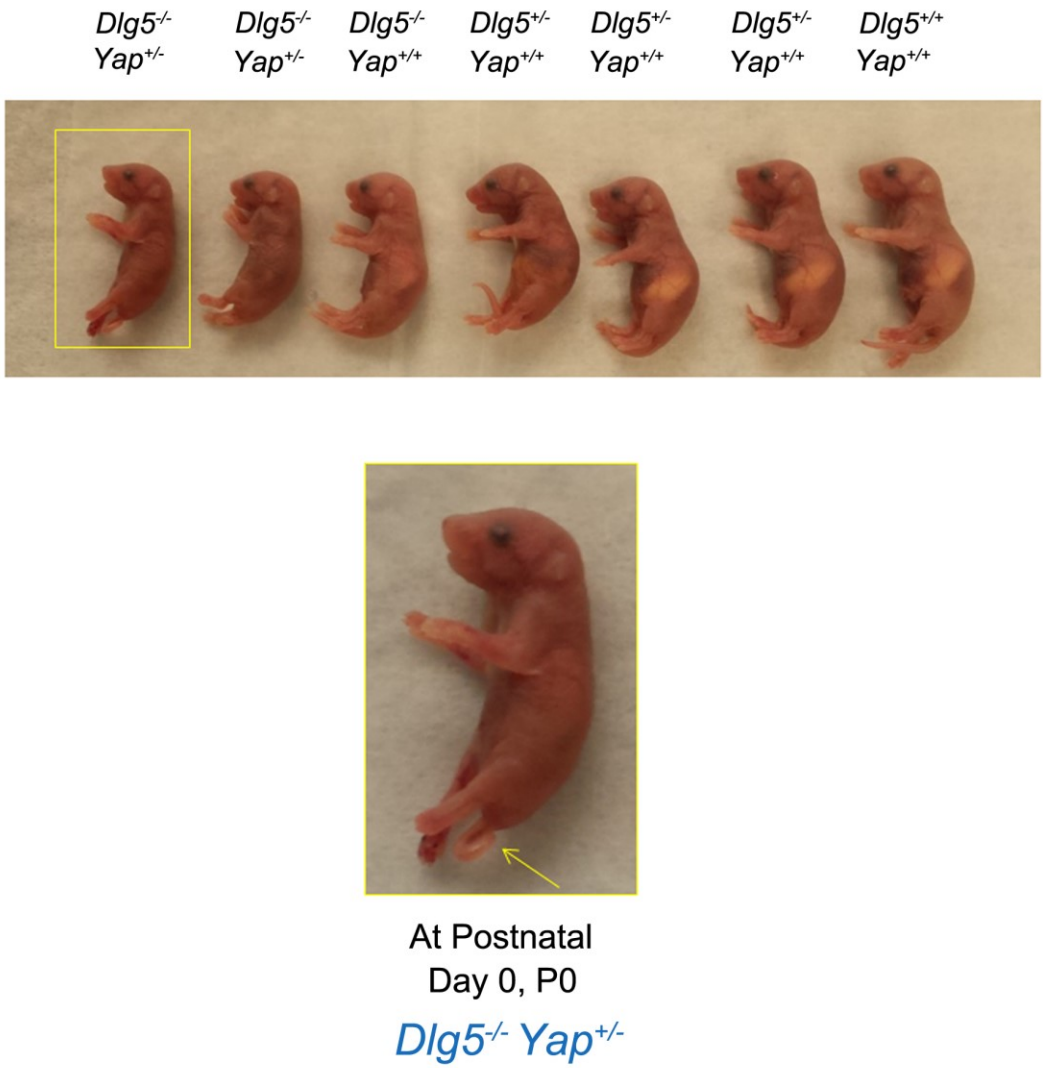


Figure 23: Representative images of Postnatal Day 0 (P0) pups with indicated genotypes. *Note, Dlg5*$^{-/-}$ *Yap1*$^{+/-}$ pups are born alive and have curly tails; however, they do not survive and die within few days after birth.

♀ *Dlg5*$^{+/-}$ *Taz*$^{+/-}$ x ♂ *Dlg5*$^{+/-}$ *Taz*$^{+/-}$

Expected Ratios:				Observed Ratios:		
Dlg5	Taz	Number	Percent	Number	Percent	
Dlg5 +/+	Taz +/+	7.8125	6.25%	16	12.80%	
Dlg5 +/+	Taz +/-	15.625	12.50%	20	16.00%	
Dlg5 +/+	Taz -/-	7.8125	6.25%	8	6.40%	
Dlg5 +/-	Taz +/+	15.625	12.50%	17	13.60%	
Dlg5 +/-	Taz +/-	31.25	25.00%	43	34.40%	
Dlg5 +/-	Taz -/-	15.625	12.50%	15	12.00%	
Dlg5 -/-	Taz +/+	7.8125	6.25%	4	3.20%	
Dlg5 -/-	Taz +/-	15.625	12.50%	2	1.60%	P = 0.0002
Dlg5 -/-	Taz -/-	7.8125	6.25%	0	0.00%	P = 0.0039
P7-P10	Total:	125	100.00%	125	100.00%	

Figure 24: A prominent negative genetic interaction between *Dlg5* and *Taz (Wwtr1)*. *Dlg5*$^{+/-}$*/Taz*$^{+/-}$ females were crossed to *Dlg5*$^{+/-}$*/Taz*$^{+/-}$ males and the resulting progeny were genotyped at postnatal days 7-10. Table shows expected and observed genotypes. Note the statistically significant absence of *Dlg5*$^{-/-}$*/Taz*$^{-/-}$ and decreased numbers of *Dlg5*$^{-/-}$*/Taz*$^{+/-}$ pups as assessed by chi-squared analysis. *Taz*$^{-/-}$ mice are viable and fertile, and, therefore, the *Taz*$^{-/-}$ genotype was included in this analysis.

A

B

♀ *Dlg5*$^{+/-}$ *Taz*$^{+/-}$ x ♂ *Dlg5*$^{+/-}$ *Taz*$^{-/-}$

Offspring at P0

Expected Ratios:				Observed Ratios:		
Dlg5	Taz	Number	Percent	Number	Percent	
Dlg5 +/+	Taz +/-	6	12.50%	10	20.83%	
Dlg5 +/+	Taz -/-	6	12.50%	7	14.58%	
Dlg5 +/-	Taz +/-	12	25.00%	12	25.00%	
Dlg5 +/-	Taz -/-	12	25.00%	10	20.83%	
Dlg5 -/-	Taz +/-	6	12.50%	8	16.67%	
Dlg5 -/-	Taz -/-	6	12.50%	1	2.08%	P = 0.0291
P0 Total:		48	100.00%	48	100.00%	

Expect 6/48 offspring to be *Dlg5*$^{-/-}$ *Taz*$^{-/-}$ but only observe 1/48

Figure 25: *Dlg5*$^{-/-}$ *Taz*$^{-/-}$ pups are present among newborns but at a lower than expected frequency.

A) Representative images of Postnatal Day 0 (P0) pups with indicated genotypes.
Note, *Dlg5*$^{-/-}$ *Taz*$^{-/-}$ pups are present among newborns; however, they are found dead and absent from the litters at later time points after the birth. Pups with an * next to their genotype were found dead.

B) A prominent negative genetic interaction between *Dlg5* and *Taz(Wwtr1)*.
Dlg5$^{+/-}$/*Taz*$^{+/-}$ females were crossed to *Dlg5*$^{+/-}$/*Taz*$^{-/-}$ males and the resulting progeny was genotyped at postnatal days 0. This table shows the expected and observed genotypes. Note the statistically significant decrease in *Dlg5*$^{-/-}$/*Taz*$^{-/-}$ pups (as assessed by chi-squared analysis). A *Dlg5*$^{-/-}$/*Taz*$^{-/-}$ pup was found dead. Thus, it is unclear whether *Dlg5*$^{-/-}$ *Taz*$^{-/-}$ pups are stillborn or are born alive and die immediately thereafter.

12.5%) and were alive, indicating that pups with this genotype died after P0 but before P7-10.

Taken together, these data indicate a strong genetic interaction between mammalian *Dlg5* and both *Yap1* and *Taz* genes. This is consistent with my biochemical data, which revealed the negative role of DLG5 in the regulation of Hippo pathway signaling, which negatively regulates YAP1 and TAZ levels, in both gain-of-function and loss-of-function experiments.

Immunohistochemical analysis of Hippo pathway proteins in developing *Dlg5*$^{-/-}$ brains

Profound abnormalities in brain development were previously documented in *Dlg5*$^{-/-}$ embryos. *Dlg5*$^{-/-}$ brain neural progenitor cells lose apical-basal polarity and prematurely differentiate [120, 125]. Loss of polarity causes the fusion of the aqueduct and subsequent accumulation of the cerebrospinal liquid in the brain ventricles, which culminates in prominent hydrocephalus. Hippo signaling plays an important role in brain development, where YAP1 promotes self-renewal and inhibits differentiation of neural progenitor cells [275, 383, 384]. I hypothesized that some of the phenotypes in *Dlg5*$^{-/-}$embryos may be due to hyperactivation of Hippo signaling and depletion of YAP1/TAZ proteins in developing progenitor cells. Since *Dlg5*$^{-/-}$embryos develop prominent phenotypes in the developing brain, I decided to concentrate on brain neural progenitor cells and focus on the area between developing 3rd and 4th brain ventricles (future aqueduct), which is the most affected area in *Dlg5*$^{-/-}$ brains.

As expected, immunohistochemistry (IHC) staining with Sox2 antibodies (a marker of neural progenitor cells) revealed neural progenitors lining the ventricular structures in both E15.5 wild-type and *Dlg5*$^{-/-}$ brains (Figure 26). However, *Dlg5*$^{-/-}$ progenitors appeared disorganized and often lost the intimate connection with the ventricular surface that was evident in the wild-

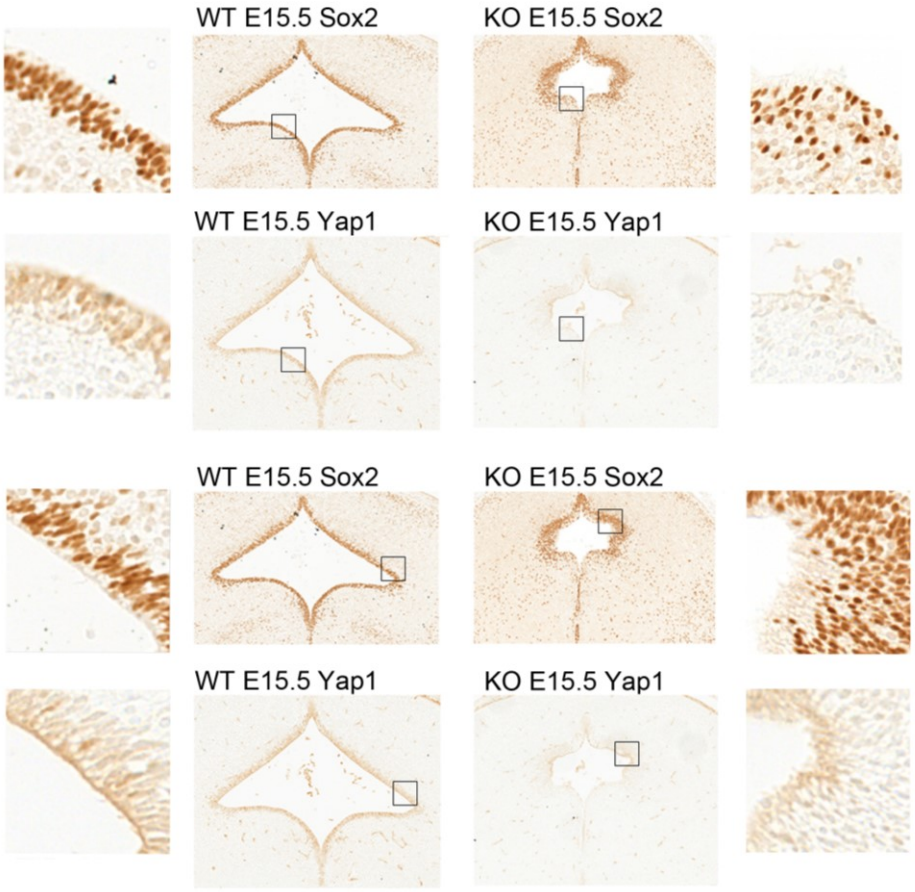


Figure 26: Loss of YAP1 precedes loss of Neural Progenitor Cells (NPCs) in brains from *Dlg5*$^{-/-}$ embryos.
Immunohistochemical staining for Yap1 and Sox2 (a marker of NPCs) on coronal sections from E15.5 wild-type (WT) and Dlg5-knockout (KO) mouse brains at the level of 4th ventricle. Serial sections were used to identify similar brain positions in wild-type and Dlg5-knockout brains. Low power images are shown at the center and indicated by black squares regions are shown at higher magnification on left and right sides. Note, Sox2+ NPCs are still present at the lining the 4th ventricle; however, they display a prominent decrease in the staining for YAP1.

type progenitor cells. This is consistent with the loss of apical-basal cell polarity and disruption of apical-junctional complexes in *Dlg5*$^{-/-}$ progenitors in these areas of developing brain, which was previously documented [120]. IHC staining for YAP1 revealed a prominent decrease in the levels of YAP1 in *Dlg5*$^{-/-}$ progenitors (Figure 26). Therefore, *Dlg5*$^{-/-}$ progenitors display decreased levels of YAP1, which precedes their premature loss in *Dlg5*$^{-/-}$ brain. Since YAP1 is necessary for the maintenance of neuronal progenitor cells, decreased levels of YAP1 may be responsible for the premature differentiation and loss of progenitors in *Dlg5*$^{-/-}$ brains.

In addition to staining for YAP1 in *Dlg5*$^{-/-}$ brains, I also performed IHC staining for LATS and MST proteins. Localization of MST and LATS to the plasma membrane is known to play an important role in regulation of the Hippo pathway signaling. For example, NF2 regulates the Hippo pathway by targeting LATS to the plasma membrane where it can be phosphorylated and activated by MST [295]. In Drosophila, the JUB (AJUBA) protein inhibits WARTS (LATS) by sequestering it at the adherens junctions [313]. IHC staining for LATS revealed prominent localization of LATS at the apical junctional complexes at the surface of the ventricles in wild-type brains (Figure 27). In contrast, the staining was diffuse throughout the cytoplasm in progenitors in *Dlg5*$^{-/-}$brains (Figure 27). MST was prominently localizing to the nucleus in both wild-type and *Dlg5*$^{-/-}$ brains. I did not observe prominent changes in total MST localization in *Dlg5*$^{-/-}$brains; however, it was noticeable that while phospho-MST antibodies displayed localized nuclear staining in wild-type brains, more diffuse cytoplasmic staining was prevalent in *Dlg5*$^{-/-}$ brains (Figure 27).

Overall, from my IHC analysis of the developing wild-type and *Dlg5*$^{-/-}$ brains I conclude that DLG5 is necessary for the proper sequestration of LATS at the apical junctional complexes in neural progenitor cells. In *Dlg5*$^{-/-}$ progenitors LATS was localizing to the cytoplasm where it

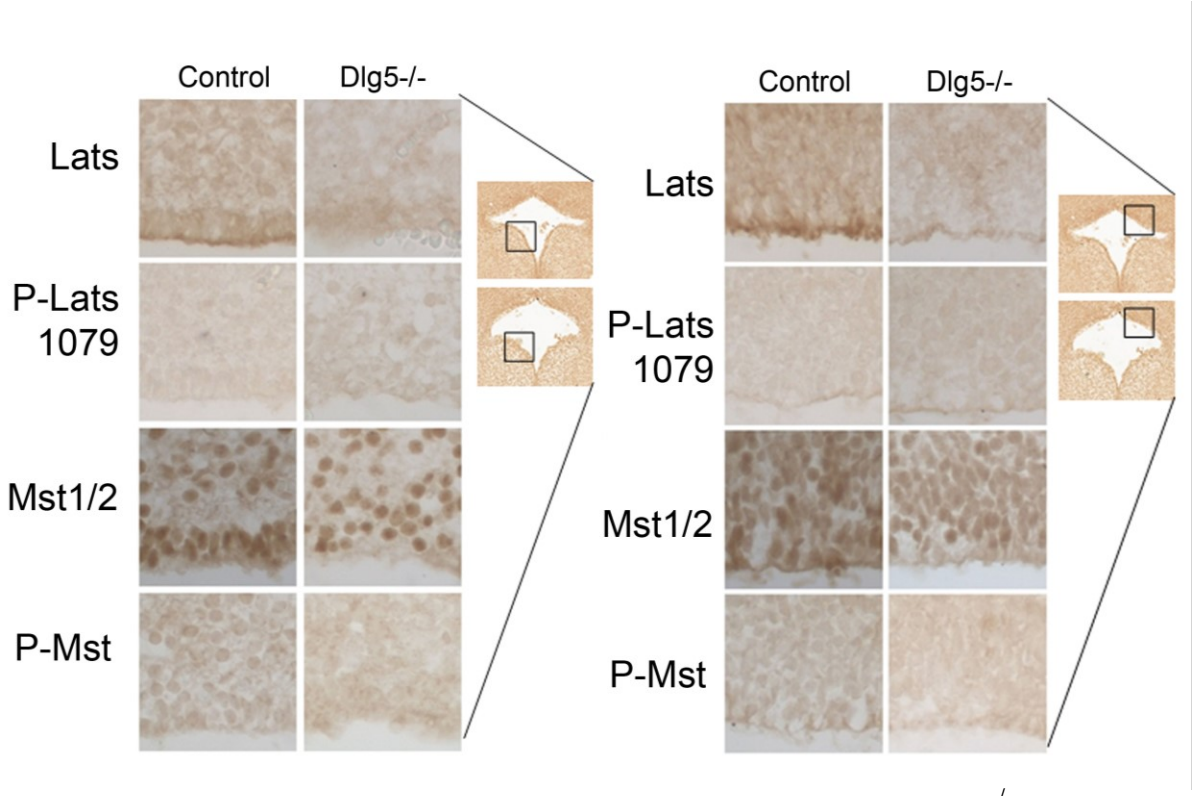


Figure 27: Localization of MST and LATS of the Hippo pathway in Dlg5$^{-/-}$ brains. Immunohistochemical staining for LATS, phospho-LATS, MST, and phospho-MST on coronal sections from E15.5 wild-type (WT) and Dlg5-knockout (KO) mouse brains at the level of 4th ventricle. Serial sections were used to identify similar brain positions in wild-type and Dlg5-knockout brains. Low power images are shown at the right hand side and regions indicated by black squares are shown at higher magnification on left hand side of each panel, respectively. Note the loss of strong apical LATS staining in *Dlg5*$^{-/-}$ brains. In addition, phospho-MST staining appears predominantly nuclear in WT brains, but is more diffuse in KO brains.

was overlapping with its activated (phosphorylated) upstream kinase MST. Consistent with my biochemical analysis of *Dlg5*$^{-/-}$ brains, I found decreased levels of nuclear YAP1 in *Dlg5*$^{-/-}$ progenitors, which may be responsible for premature loss of self-renewal and differentiation of neural progenitors in *Dlg5*$^{-/-}$ brains.

Non-polarized, primary neural progenitors cultured in a 2D system maintain differences in the Hippo signaling between wild-type and *Dlg5*$^{-/-}$ cells.

I have already demonstrated significant differences in Hippo signaling between wild-type and *Dlg5*$^{-/-}$ developing brains. The immunostaining for Hippo pathway proteins revealed prominent expression of YAP1 in neural progenitor cells; however, developing brains contain a large number of other cell types (primarily neurons) which do not express YAP1. Therefore, to concentrate on the relevant cell type, I decided to isolate and analyze primary wild-type and *Dlg5*$^{-/-}$ neural progenitor cells in culture.

Primary neural progenitor cells (NPCs) can be expanded indefinitely in culture without differentiation when grown on laminin-coated plates in serum-free advanced DMEM media containing B27, FGF2 and EGF [385]. I isolated neural progenitor cells from wild-type (n=3) and *Dlg5*$^{-/-}$ (n=3) brains and established them in this culture system. Western blot analysis of cultured neural progenitor cells revealed significantly higher ratios of phospho-LATS/total-LATS (P=0.004) and lower levels of total YAP1 and TAZ (P = 0.024 and P = 0.023, respectively) in *Dlg5*$^{-/-}$ cells (Figure 28-30). I have also analyzed the levels of direct transcriptional targets of YAP1/TAZ *Ctgf* and *Cyr61*. qRT-PCR experiments reveal significant decrease in the expression levels of both *Ctgf* (P=0.006) and *Cyr61* (0.01) in *Dlg5*$^{-/-}$ cells (Figure 31). The mRNA levels of *Yap1* and *Taz* were not affected by the loss of *Dlg5* indicating that the

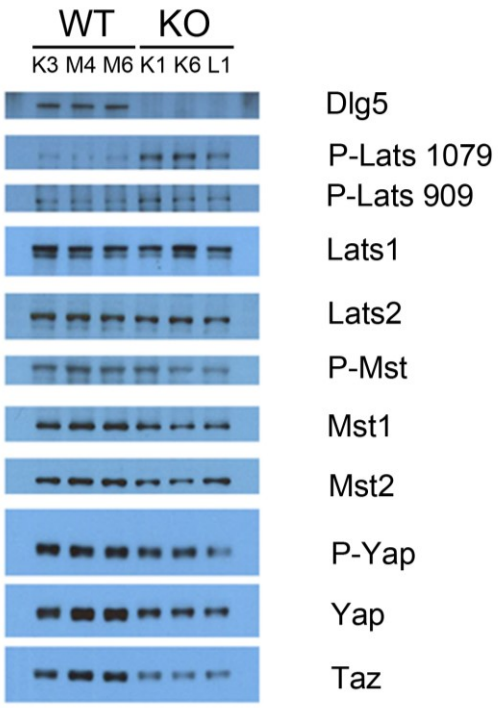


Figure 28: Differences in total and phosphorylation levels of Hippo pathway proteins in wild-type and Dlg5$^{-/-}$ NPCs

Changes in total levels and phosphorylation of Hippo pathway proteins in cultured NPCs isolated from telencephalon from E11.5 wild-type (WT, n=3) and Dlg5-knockout (KO, n=3) embryos. Western blot analyses of total protein extracts with indicated antibodies. Note that the activation of Hippo signaling and decrease in total levels of YAP1 and TAZ proteins previously identified in Dlg5-knockout brains (Figs. 12-15) are preserved in cultured NPCs.

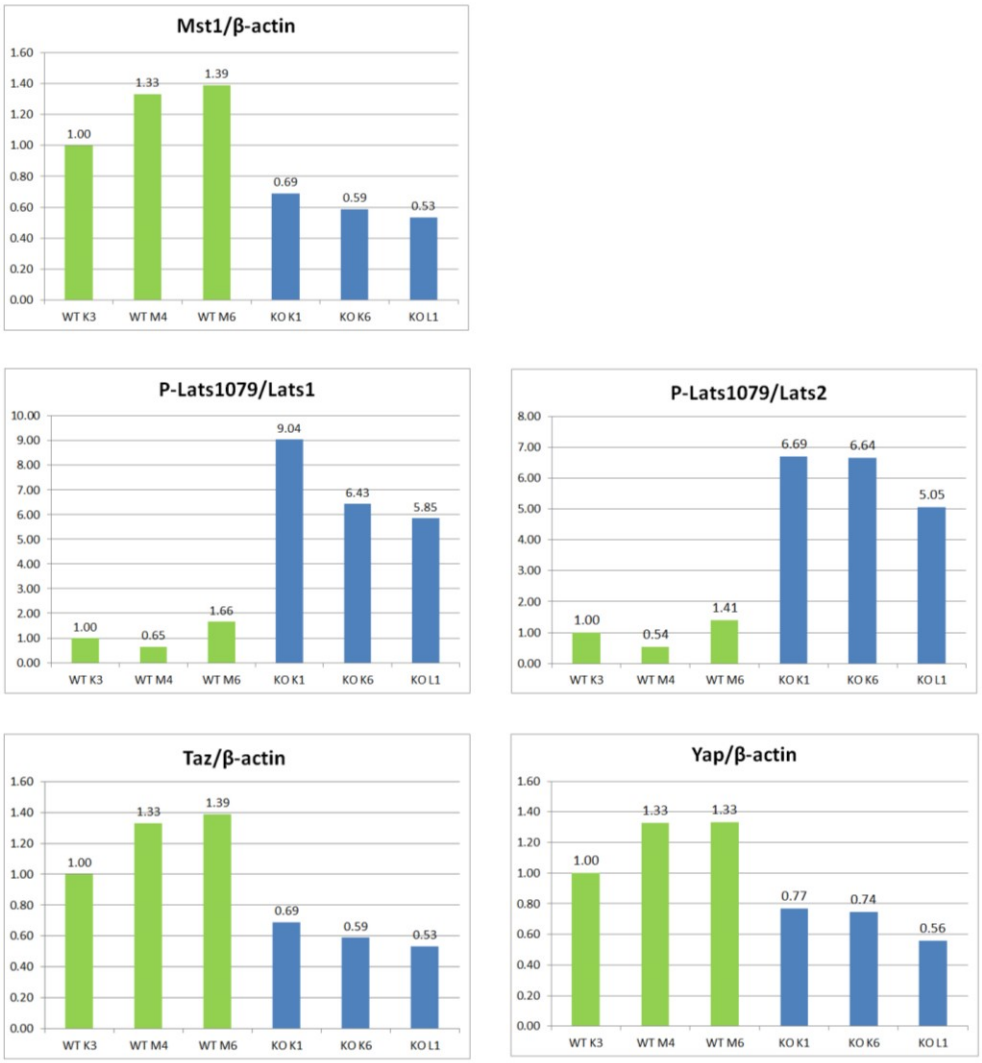


Figure 29: Hippo pathway signaling is activated and total levels of Yap and Taz are lower in Dlg5$^{-/-}$ NPCs.

Relative protein levels of Mst1/β-actin, P-Lats 1079/Lats1, P-Lats 1079/Lats2 Taz/β-actin, and Yap/β-actin in NPCs isolated from WT and Dlg5$^{-/-}$ KO brains were quantified by ImageJ. Protein extracts from NPCs isolated from Dlg5$^{-/-}$ KO brains have lower total levels of Yap, Taz, and Mst1 and higher levels of P-Lats 1079/Lats1 and P-Lats 1079/Lats2 than WT controls.

NPCs	
	T test P Value:
NF2/β-actin	0.014
P-Lats 1079/Lats1	0.015
P-Lats 1079/Lats2	0.0034
P-Lats 909/Lats1	0.26
P-Lats 909/Lats2	0.13
Lats1/β-actin	0.617
Lats2/β-actin	0.179
P-Mst/Mst1	0.82
P-Mst/Mst2	0.91
Mst1/β-actin	0.02
Mst2/β-actin	0.072
P-Yap/Yap	0.75
Yap/β-actin	0.024
Taz/β-actin	0.023
	Decreased
	Increased

Figure 30: Statistical analyses of changes in total levels and phosphorylation of Hippo pathway proteins in cultured NPCs.
Statistical analyses of Western blot data in part A as quantified by Image J (t-test).
Note that the activation of Hippo signaling and decrease in total levels of YAP1 and TAZ proteins previously identified in Dlg5-knockout brains (Figs. 12-15) are preserved in cultured NPCs.

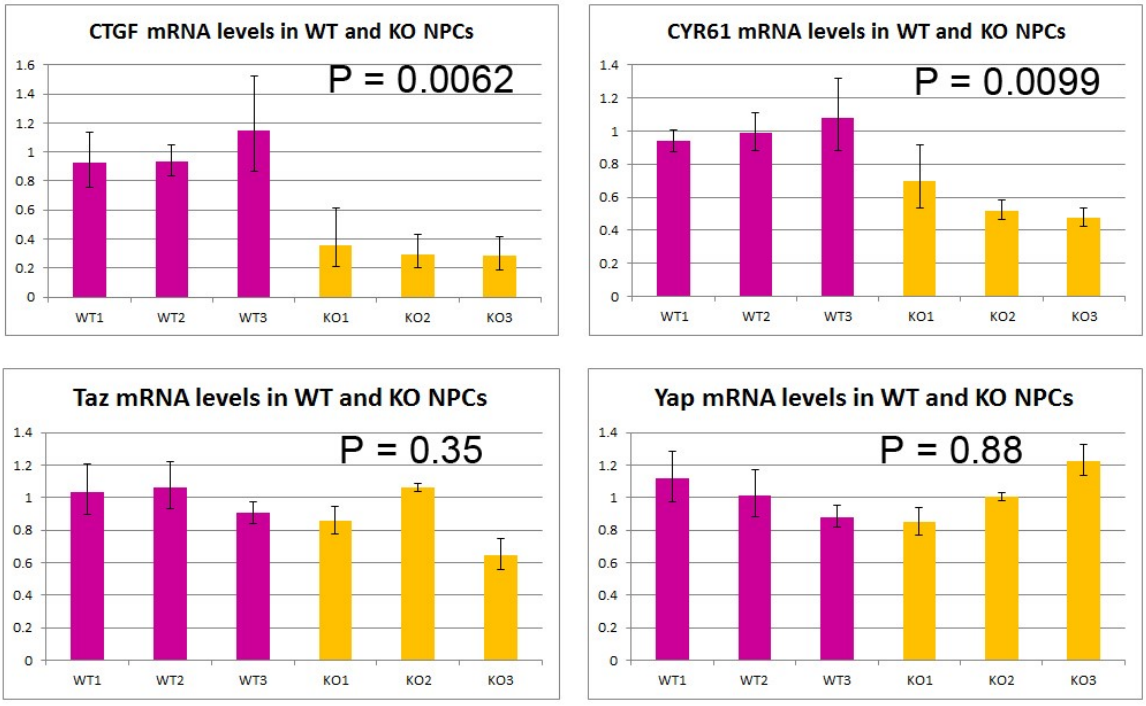


Figure 31: **Decreased expression of direct gene targets of the Hippo pathway in cultured *Dlg5*$^{-/-}$ NPCs.**
mRNA levels of *Ctgf* and *Cyr61* (direct downstream targets of the Hippo pathway), as well as *Yap1* and *Taz* were determined by RT qPCR in wild-type (WT) and Dlg5-knockout (KO) NPCs. Note the statistically significant decrease in the levels of *Cyr61* and *Ctgf* mRNAs, but static levels of *Yap1* and *Taz* in Dlg5-knockout cells.

decreased protein levels of YAP1 and TAZ are not due to decreased transcriptional activity of these genes (Figure 31).

Overall, these data demonstrate that, similar to what is observed in developing brains *in vivo*, Dlg5 is necessary for the negative regulation of the Hippo pathway in cultured progenitor cells. Since apical-basal cell polarity and apical-junctional protein complexes are not maintained in culture, similarly to the data obtained in gain-of-function experiments in 293FT cells, these data indicate that changes in Hippo pathway signaling are unlikely to be the secondary events due to the loss of cell polarity in *Dlg5*$^{-/-}$ progenitor cells.

DLG5 negatively regulates the association between MST and LATS in the canonical Hippo signaling pathway.

Since the negative impact of Dlg5 in the activity of the Hippo signaling pathway is preserved in primary neural progenitor cells in culture, I decided to use these cells as a model to study the mechanisms responsible for Dlg5 function in the Hippo pathway. First I sought to determine whether differences between wild-type and *Dlg5*$^{-/-}$ progenitor cells in YAP1/TAZ protein levels and specific phosphorylation can be rescued by the knockdown of MST1/2 and/or LATS1/2. Neural progenitor cells were transfected with siRNA oligos targeting YAP1, GFP, MST1/2, or LATS1/2 and 2 days later the resulting cells were analyzed by Western blot with DLG5, phospho and total YAP1, TAZ, MST1/2 and LATS1/2 antibodies (Figures 32-33). While I observed some differences between the experiments, the differences in levels of TAZ between wild-type and *Dlg5*$^{-/-}$ progenitor cells were completely or partially rescued by the knockdown of LATS1/2, while they were not significantly affected by the knockdown of MST1/2. These

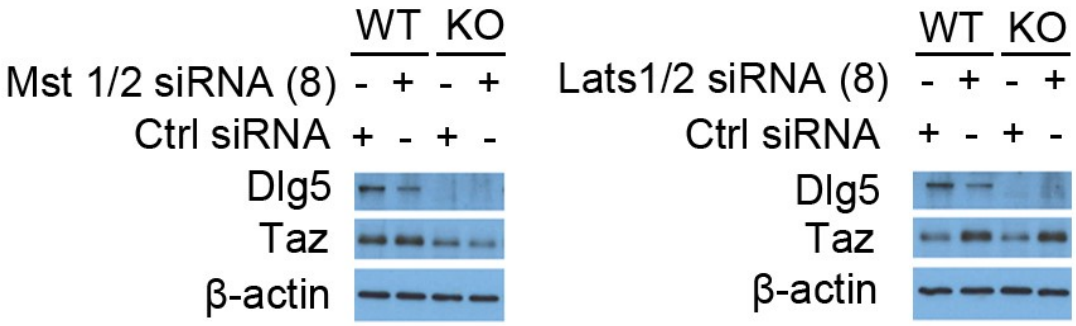


Figure 32: Changes in TAZ protein levels after siRNA knockdown of Mst1/2 and Lat1/2 suggest that in Hippo pathway Dlg5 is acting downstream of Mst1/2 and upstream of Lats1/2 in NPCs.

Wild-type (WT) and Dlg5-knockout (KO) NPCs were transfected with control (Ctrl), MST1/2 targeting (combination of 8 oligos) or LATS1/2 targeting (combination of 8 oligos) siRNA oligos. Total protein extracts were prepared 2 days after transfection and analyzed by Western blot with anti-Dlg5, anti-TAZ and anti-β-actin antibodies. Note rescue of differences in Taz protein levels between wild-type and $Dlg5^{-/-}$ cells after *Lats1/2* knockdown, but preservation of these differences after *Mst1/2* knockdown.

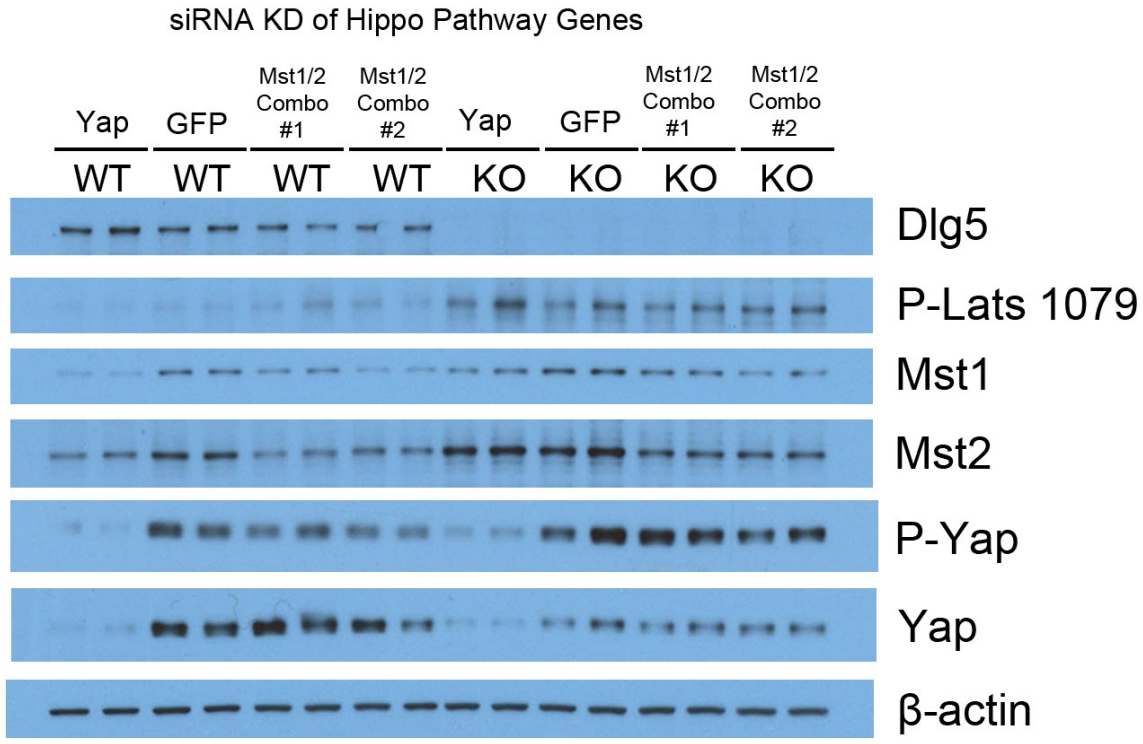


Figure 33: Changes in YAP1 protein levels after siRNA knockdown of Mst1/2 suggest that in Hippo pathway Dlg5 is acting downstream of Mst1/2
Western blot analyses of proteins from wild-type (WT) and Dlg5-knockout (KO) NPCs transfected (in duplicates) with siRNAs knocking down (KD) Yap1 (Yap), GFP, and Mst1/2 (2 sets of oligos, each targeting MST1 and MST2). Note, Mst1/2 knockdowns do not rescue the differences in phosphor/total Yap1 ratios and total YAP1 levels between WT and KO cells.

findings suggested that Dlg5 impacts the Hippo pathway upstream from LATS1/2 but downstream from MST1/2 (Figure 32-33).

Since Hippo signaling can be impacted by localization of MST1/2 or LATS1/2 proteins, I performed cell fractionation studies. Wild-type and *Dlg5*$^{-/-}$ neural progenitor cells were fractionated to separate nuclear, cytoplasmic, and membrane fractions, which were then analyzed by Western blot with YAP1, TAZ, MST1/2, LATS1/2 antibodies as well as markers of cytoplasmic (RabGDI), nuclear (Lamin B1) and membrane (N-cadherin) fractions. While the total levels of TAZ and YAP1 were lower in *Dlg5*$^{-/-}$ cells, as expected, I did not find significant differences in subcellular compartment localization of MST1/2, LATS1/2, YAP1 and TAZ proteins. These data indicate that DLG5 does not regulate Hippo pathway by changing subcellular compartment localization of MST1/2 (Figure 34).

I hypothesized that by binding to MST1/2, DLG5 may compete with MST1/2-LATS1/2 interaction and this may be the primary mechanism responsible for the DLG5-mediated negative regulation of the Hippo pathway. To test this hypothesis, I decided to analyze LATS1/2-MST1/2 protein complex formation in wild-type and *Dlg5*$^{-/-}$ neural progenitor cells. However, all my attempts to co-immunoprecipitate endogenous LATS1/2 and MST1/2 from neural progenitor cells in culture or in vivo were unsuccessful. Consistent with this finding, the overwhelming majority of co-immunoprecipitation experiments in the published literature between LATS1/2 and MST1/2 were performed have used exogenously expressed proteins. Thus, I tested my hypothesis using exogenously expressed DLG5, LATS1 and MST2 proteins. As expected, the specific interaction between LATS1 and MST2 was readily detectable in these conditions as LATS1 was pulled down via MST2 (Figures 35) and MST2 was pulled down via LATS1 (Figure

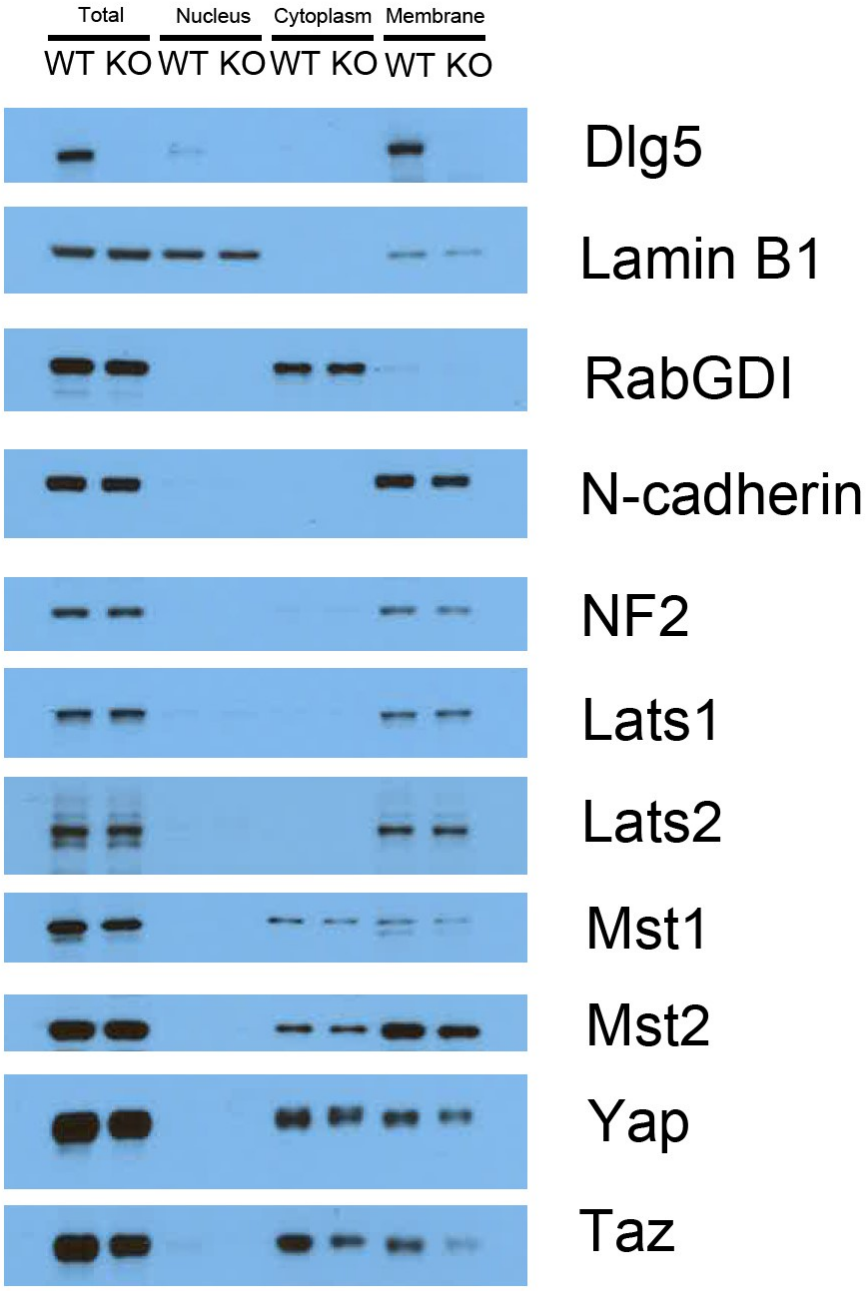


Figure 34: Cell fractionation experiments reveal no significant differences in subcellular localization of Hippo pathway proteins between wild-type and Dlg5$^{-/-}$ NPCs.
Western blot analyses of total, nuclear, cytoplasmic and membrane proteins from wild-type (WT) and Dlg5-knockout (KO) NPCs with indicated antibodies.

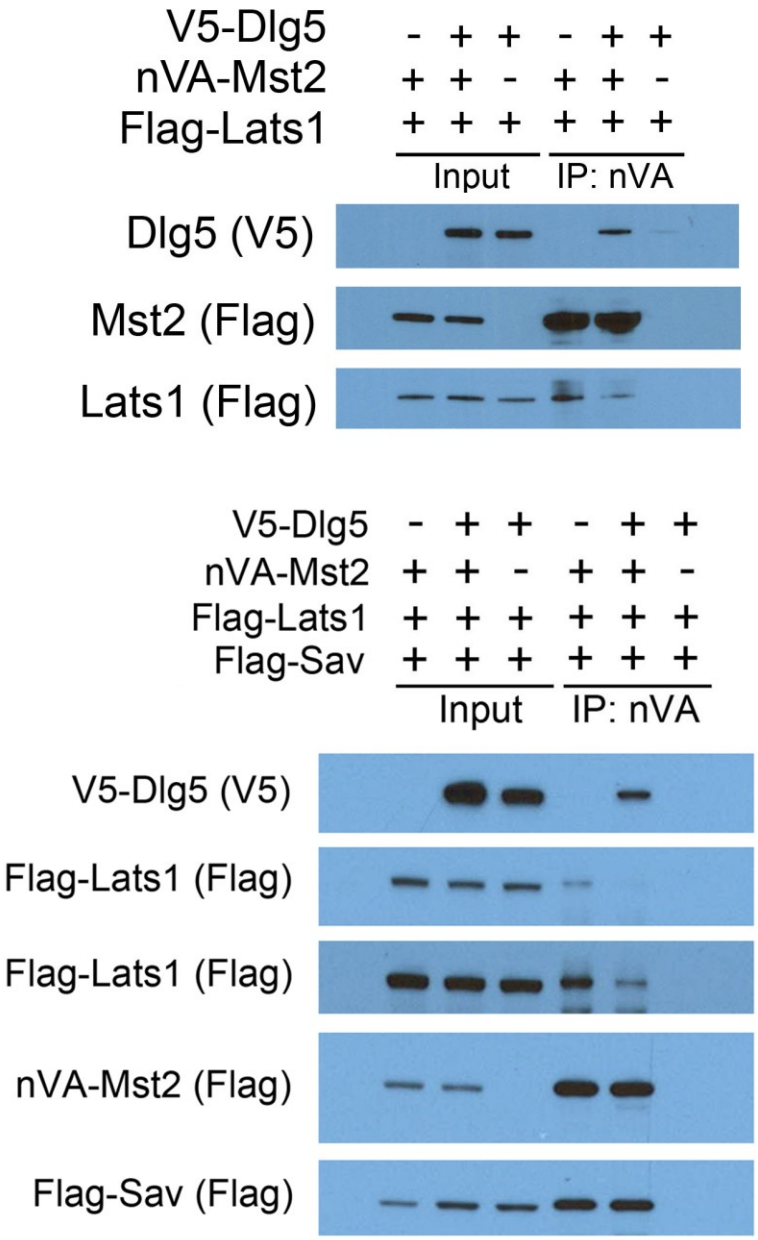


Figure 35: Dlg5 interferes with the binding of MST to LATS.
Western blot (WB) analyses of total (input) and immunoprecipitated (IP) with Strep Tactin sepharose (pulls down nVA tag) proteins from HEK 293 FT cells transfected with V5-Dlg5, nVa-Mst2, Flag-LATS1 and Flag-Salvador (Sav) expression constructs, as indicated. The nVa tag on Mst2 contains also contains Flag. Note, the presence of DLG5 inhibits the binding of MST2 to LATS1. Presence of Salvador had no impact on Dlg5-mediated inhibition of the MST2-LATS1 interaction.

36). Interestingly, in both cases, co-expression of DLG5 decreased binding between MST2 and LATS1. Taken together, these data support the model that Dlg5 negatively regulates the Hippo pathway by preventing the binding of MST to LATS and interfering with the propagation of the activating kinase cascade in the canonical Hippo signaling pathway (Figure 37).

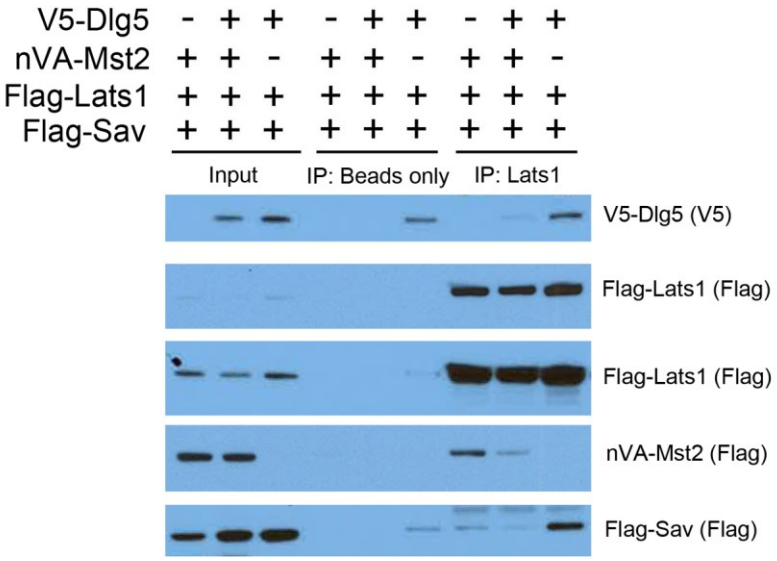


Figure 36: DLG5 interferes with the binding of LATS to MST.
Western blot analyses of total (input) and immunoprecipitated (IP) with either beads alone (negative control) or anti-LATS1 antibodies proteins from HEK 293 FT cells transfected with indicated expression constructs. Note, presence of DLG5 inhibits binding of LATS1 to MST2.

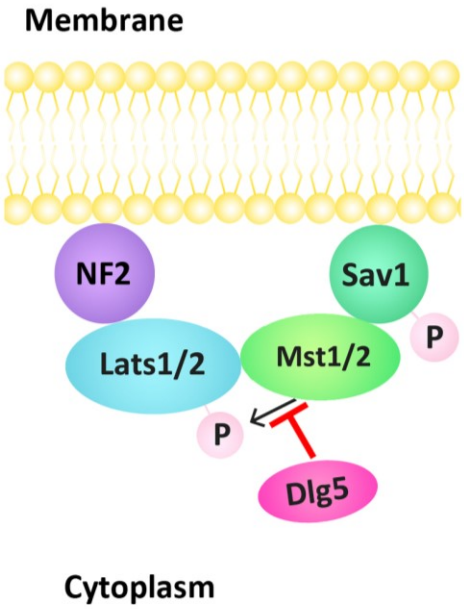


Figure 37: Model of Dlg5's action on the Hippo pathway.
Dlg5 functions at the membrane to inhibit binding of Mst to Lats.

Discussion

The Hippo pathway plays a critical role in regulation of normal tissue development, adult organ homeostasis, and cancer. Increased activity of the primary targets of Hippo signaling, YAP1 and TAZ, stimulates self-renewal of stem and progenitor cells and, in some cases, causes an increase in the organ size. In contrast, decreased activity of YAP1 and TAZ usually takes place in differentiating cells and it promotes cell-cycle withdrawal and cellular differentiation. Because constitutive activation of YAP1 or TAZ results in cancer development and YAP and TAZ are frequently amplified in human cancers, the *YAP1* and *TAZ(WWTR1)* genes are considered to be proto-oncogenes. To properly regulate tissue homeostasis, the Hippo pathway needs to be able to sense the cellular microenvironment, and multiple studies have uncovered connections between Hippo pathway activation and cell density, polarity, and the physical properties of the extracellular matrix. Exactly how the Hippo pathway is connected to cellular density and polarity is only beginning to be understood. This study identifies a novel mechanism directly connecting the Hippo pathway with apical-basal cell polarity. With the help of my collaborators, I found that an important apical-basal cell polarity protein, DLG5, interacts with and negatively regulates the activity of mammalian Hippo kinases MST1/MST2. I show that in cells lacking DLG5 the MST1/2-LATS1/2 canonical Hippo pathway is hyperactive and this results in a prominent decrease in YAP1/TAZ transcriptional activity in *Dlg5*$^{-/-}$ mice. I demonstrate that the negative connection between DLG5 and the Hippo pathway is maintained in gain-of-function experiments and is also evident in cell culture conditions lacking cell polarization. This strongly suggests that Dlg5 impact on Hippo signaling is not indirect, for

example secondary to the loss of cell polarity in *Dlg5*$^{-/-}$ cells, but instead, Dlg5 is a genuine regulator that is hardwired into the system.

Interestingly, the phenotype of *Taz* (*Wwtr1*) knockout mice is remarkably similar to that of *Dlg5*$^{-/-}$ mice. *Dlg5*$^{-/-}$ pups are smaller than their wild type littermates and they develop kidney cysts, hydrocephalus, and an emphysema-like lung phenotype [120, 121]. Strikingly, *Taz*$^{-/-}$ mice are smaller than their wild type counterparts and develop kidney cysts and an emphysema-like lung phenotype very similar to *Dlg5*$^{-/-}$ [248, 249, 379]. Considering the connection that was found here between Dlg5 and Hippo signaling pathway, it is likely that many of the phenotypes in *Dlg5*$^{-/-}$ mice are due to the decreased activity of YAP1/TAZ proteins. Rescue of *Dlg5*$^{-/-}$ phenotypes by increasing the activity of YAP1 or TAZ proteins would strongly support this hypothesis. I have attempted to address this genetically and generated *Dlg5*$^{-/-}$ mice with whole-body DOX-induced genetic activation of YAP1; however, these experiments were unsuccessful. Unfortunately, the whole-body expression of activated YAP1 proved to be highly noxious to the animals. The pups with DOX-induced YAP1 were born at a lower than expected frequency and when activated YAP1 was induced by DOX post weaning, the mice died within 5-6 days of beginning DOX treatment (data not shown).

Hippo signaling can be regulated via multiple mechanisms. My results demonstrate that DLG5 is likely to inhibit the MST1/2-LATS1/2 kinase cascade by binding to MST1/2 and preventing their interaction with downstream LATS1/2 kinases. It is possible, that this is not the only mechanism of DLG5 function in the Hippo pathway. MST1/2 activity can be regulated by its intercellular localization; however, this mechanism is unlikely, because I did not find significant differences in MST1/2 or LATS1/2 localization between wild-type and *Dlg5*$^{-/-}$ cells. Alternatively, DLG5 may connect MST1/2 with important upstream regulators, which negatively

regulate MST1/2. Upstream regulation of MST1/2 activity is an extremely poorly understood area of the Hippo signaling pathway. For example, most of the upstream Hippo pathway regulators affect F-actin cytoskeleton and cellular contractility; however, this connection is believed to be either completely independent from the Hippo pathway [300] or affect LATS1/2 but not MST1/2 kinases. Phosphoinositide 3-kinase/Akt inhibits MST1 via direct phosphorylation [386, 387]. In addition, Raf-1 binds and inhibits MST2 kinase [388], whereas dissociation from Raf-1 and binding to the tumor suppressor protein RASSF1A activates MST2 [389]. Therefore, MST1/2 may function as an important signaling hub integrating both the Ras-Raf-MAPK and PI3K-AKT pathways [387]. If DLG5 functions as a linker protein that brings together MST1/2 and AKT or MST1/2 and Raf-1, then decrease in DLG5 abundance or availability would result in MST1/2 activation. While many proteins were identified in Dlg5-protein complexes by Mass Spectrometry, AKT or RAF-1 peptides were not detected, suggesting that bridging MST1/2 with either AKT or Raf-1 is an unlikely mechanism of DLG5 function.

Interestingly, Mass Spectrometry analysis of DLG5 protein complexes identified multiple peptides from MARK3 (PAR1A), MARK2 (PAR1B) and MARK1 (PAR1C) proteins. PAR1 proteins are serine/threonine-protein kinases involved in cell polarity and regulation of microtubule dynamics. Since PAR1 is so important in the establishment of apical-basal cell polarity and DLG5 is also necessary for the maintenance of apical-basal cell polarity, it will be interesting to explore the functional significance of DLG5-PAR1 interaction. For example, it is possible that DLG5 regulates PAR1 localization or activity and the abnormalities in PAR1 signaling ultimately responsible for apical-basal cell polarity defects in *Dlg5*$^{-/-}$ mice. While the cell polarity connections here are quite interesting, it is also intriguing that PAR1 has been identified as a negative regulator of Hippo (MST1/2) in Drosophila model system [333]. PAR1

was shown to physically interact with Hippo and phosphorylate it at S30 to restrict its activity. This finding is contrasted by finding the in mammalian HEK 293 cells, where the knockdown of MARK1, MARK3 and MARK4 resulted in hyperactivation of YAP1, suggesting that PAR1 kinases were not negative, but positive regulators of Hippo signaling pathway [390]. Nevertheless, it would be very interesting to analyze whether DLG5 can bridge MARK proteins with MST1/2 and whether this may be involved in DLG5 function in the negative regulation of MST1/2.

The connection between Dlg5 and the Hippo pathway components also gives us some insight about the ways in which apical basal polarity is tied to Hippo pathway signaling. The gain-of-function experiments I performed in cell culture experiments suggests that Dlg5 and aPKC could both negatively regulating the Hippo pathway through the same mechanism and this warrants further investigation. While it is known that aPKC is able to regulate the Hippo pathway, the precise mechanism is still unknown [278, 329-331]. Recent studies have shown that aPKC can interact with Kibra to regulate vesicle trafficking [391], which is very interesting considering that Dlg5 is also known to play a role in vesicle trafficking [120]. aPKC can phosphorylate Kibra [334] and Kibra can in turn inhibit the kinase activity of aPKC [339]. Furthermore, protein kinase M (PKM) ζ, a brain-specific constitutively active form of aPKC that is transcribed from an alternative promoter, can also bind to and be inhibited by Kibra [392, 393]. If we discard our former assumption that the Hippo pathway proceeds as a linear cytoplasmic kinase cascade and instead consider newer evidence that suggests that Hippo pathway phosphorylation occurs at the cell membrane [295], then it might be possible that aPKC and Dlg5 are regulating the Hippo pathway via the same mechanism, which may involve Kibra.

However, more work is needed to elucidate the precise mechanism by which Dlg5 and aPKC are regulating the Hippo pathway.

Since I found that DLG5 is a negative regulator of Hippo signaling, overexpression of DLG5 may potentially promote human cancer initiation or progression. Analysis of the human cancer datasets reveals amplification of DLG5 in 10% of breast cancers, 9% of human uterine carcinosarcomas, 7% of prostate adenocarcinomas, and 6% ovarian cancers (www.cbioportal.org). It may be interesting to analyze whether overexpression of DLG5 in these cancers contributes to activation of YAP1/TAZ and tumor initiation. In addition to the evidence of DLG5 overexpression, there is significant literature on DLG5 mutations or loss of expression in human cancer. DLG5 is mutant in 11% of human colorectal carcinomas, 9% of stomach adenocarcinomas, and 5% melanomas (www.cbioportal.org). Moreover, functional studies demonstrated that loss of DLG5 promotes tumor cell migration and invasion, which may be important in tumor progression and metastasis [132]. Interestingly, not down-regulation but overexpression of YAP1 promotes cell migration and invasion. Thus, it is unlikely that increased Hippo signaling in *Dlg5*$^{-/-}$ cells is responsible for the augmentation of cell migration in cells missing Dlg5.

Analysis of Hippo pathway kinases revealed lower total levels of MST1/2, LATS1/2, and NF2 in the brain protein extracts and neutral progenitor cells from Dlg5$^{-/-}$ mice. This can be either due to rapid degradation of constitutively activated kinases or activation of a negative feedback mechanism. Several negative feedback mechanisms within the Hippo pathway have already been identified. For example in Drosophila, proteins upstream of the Hippo pathway, Expanded, Merlin, and Kibra, are also transcriptional targets of Yorkie, revealing a negative feedback mechanism [283, 285, 292]. In mammalian cells, negative regulators of YAP1, AMOT

and AMOT-like proteins are the frequent gene targets of the Hippo signaling pathway [244, 390].

My gain-of function experiments consistently demonstrated presence of both full-length and lower molecular weight MST2 in cells overexpressing DLG5 (Figure 36). The pilot protease inhibitor experiment failed to identify the putative protease that may be involved in cleavage of MST2 in DLG5 expressing cells. The majority of protease inhibitors that I utilized were acquired from Dr. Robert Eisenman's laboratory and were used at the concentrations deemed to be physiological relevant. It is possible that the concentrations employed in this experiment were not sufficient to see a difference in our particular model system. Additional possibilities are that a protease outside of the inhibited pathways may be responsible or that alternative splicing may be responsible. In order to rule out the proteolytic enzymes tested in this experiment, positive controls are needed to verify that each of the inhibitors was used at a physiologically relevant concentration and that the inhibitors were in fact inhibiting their intended proteolytic enzymes.

It is tempting to speculate that caspases might be responsible for Dlg5-mediated cleavage of MST2; Dlg5 has a CARD domain which in other proteins has been shown to be involved in caspase signaling and the estimated cleavage site sits within 16 amino acids of a confirmed caspase cleavage site. However, one important caveat is that previous studies have identified that the cleaved form of MST2 is *more* active than the full-length form of MST2, and Dlg5 negatively regulates the Hippo pathway while facilitating MST2 cleavage. Therefore, it is unlikely that a caspase is responsible for DLG5-mediated cleavage of MST2. One potential candidate is YME1L1, an ATP-dependent mitochondrial metalloprotease, which was identified by AP-MS by our collaborators to be a strong interactor of DLG5. However, additional experiments are needed to test this hypothesis.

To summarize, there were several key findings that came to light as a result of this study. Dlg5 physically interacts with the Mst1/2 kinases of the Hippo signaling pathway and Mst1/2, Lats1/2 and Yap1/Taz are hyperphosphorylated in Dlg5$^{-/-}$ mice, indicating activation of Hippo signaling. Hippo pathway is also hyperactive in non-polarized Dlg5$^{-/-}$ neural progenitor cells *ex vivo* and Dlg5 suppresses Hippo signaling and increases levels and activity of Yap1 and Taz in gain-of-function experiments in cultured non-polarized HEK 293 FT cells *in vitro*. Taken together these findings indicate that activation of Hippo signaling is not a secondary result of the disruption of apical-basal cell polarity in Dlg5$^{-/-}$ mice. Genetic epistasis experiments in mice demonstrate a strong genetic interaction between Dlg5 and Yap1 and between Dlg5 and Taz genes. In addition, siRNA-mediated experiments in neural progenitor cells indicate that Dlg5 is acting upstream of Lats1/2 and downstream of Mst1/2 and gain-of-function experiments demonstrate that Dlg5 inhibits the binding of Mst1/2 to Lats1/2. Taken together, these results reveal that Dlg5 is a novel member of the Hippo signal transduction pathway, which inhibits the signaling by binding to Mst1/2 and preventing its association with Lats1/2.

Materials and Methods

Antibodies

Antibodies used to assess protein levels by western blot: Ms YAP (Santa Cruz sc-101199), Rb P-YAP S127 (Cell Signaling 4911), Ms TAZ (BD Pharmingen 560235), Rb MST1 (abcam ab97399), Rb MST1 (Millipore [Upstate] 07-061), Rb MST2 (Thermo Scientific PA5-28567), Rb P-MST1/2 183/180 (Cell Signaling 3681), Rb LATS1 (Cell Signaling 3477), Rb LATS2 (Bethyl Labs A300-479A), Rb LATS1/2 S909 (Cell Signaling 9157), Rb P-LATS1/2 T1079

(Cell Signaling 9159), Rb DLG5 (generated by Nechiporuk et al. [120]), Rb Lamin B1 (abcam ab16048), Ms N-Cadherin (Invitrogen 33-3900), Ms RabGDI (Synaptic Systems 130 001), Ms FLAG (Sigma F1804), Ms V5 (AbD Serotec MCA1360), and Ms β-actin (sigma A5441).

Antibodies used for co-immunoprecipitation: Rb Dlg5 (generated by Nechiporuk et al. [120]), Rb MST1/2 (Bethyl Laboratories A300-468A), and Rb LATS1 (Cell Signaling 3477).

Antibodies used for immunohistochemistry: Rb Sox2 (Chemicon AB5603), Rb Yap (Cell Signaling 4912), Rb MST1 (abcam ab97399), Rb LATS1 (Cell Signaling 3477).

Cell Culture

HEK 293 FT cells were grown in MEF media (DMEM, 10% FCS, NEAA, Pen/Strep, Sodium Pyruvate). Neural progenitor cells NPCs were isolated from E11.5 mouse brains. The NPCs were grown on laminin-coated plates in NSC growth media: 100 ml NS-A basal medium (Stem Cell Technologies, #05750), 100 ml DMEM-F12 medium (Invitrogen, #12634-010), 2 ml B27, (Invitrogen, #17504-044, kept at -20°C), 2 ml N2 (Invitrogen, #17502-048, kept at -20°C), 2 ml Pen/Strep (Invitrogen), 2 ml L-glutamine (Invitrogen), 10 ng/ml mEGF (Peprotech, #315-09), and 10 ng/ml hFGF2 (Peprotech, #100-18B).

Co-immunoprecipitation

For endogenous co-immunoprecipitation experiments, cells were lysed and immunoprecipitation was performed in the following buffer: 50mM Tris, 100mM NaCl, 0.5% NP-40, 0.1mM EDTA, 0.5mM $MgCl_2$, phosphatase inhibitors (Roche 04906837001), and protease inhibitors (Roche

11836170001). Protein extracts from wild-type and *Dlg5*$^{-/-}$ NPCs were precleared by incubation with Protein A Beads (Millipore 16-156), that were pre-blocked with BSA, for 1 hour prior to overnight incubation with either Dlg5 or Mst1/2 antibody. The following day, the lysates were incubated with Protein A Beads for 2 hours prior to washings.

For gain-of-function co-immunopreipitation experiments, cells were lysed and immunoprecipitation was performed in the following buffer: 50mM Tris, 75mM NaCl, 1% Triton X-100, 0.1mM EDTA, 0.5mM $MgCl_2$, phosphatase inhibitors (Roche 04906837001), and protease inhibitors (Roche 11836170001).
Tagged gain-of-function constructs in HEK 293 FT cells were immunoprecipitated with either V5 Sepharose (Sigma A7345-1ML) or Strep-Tactin Sepharose (IBA 2-1201-002). Lysates were incubated with beads for 1-2 hours prior to washings.

In one GOF experiment Lats1 antibody was used to precipitate Flag-Lats1. In this experiment cells were lysed and immunoprecipitation was performed in the following buffer: 50mM Tris, 75mM NaCl, 0.5% NP-40, 0.1mM EDTA, 0.5mM $MgCl_2$, phosphatase inhibitors (Roche 04906837001), and protease inhibitors (Roche 11836170001). Cell lysates were incubated with Lats1 antibody at +4˚C overnight prior to incubation with Protein A Beads (Millipore 16-156) for 2 hours and subsequent washing.

Cleavage Inhibitor assays

HEK 293 FT cells were plated on a 6-well plate precoated with gelatin. The following day the cells were transfected with nVa-Mst, V5-Dlg5, and Flag-Lats constructs via PEI and OPTI-

MEM. The following evening, the cleavage inhibitors were added to yield the following final concentrations: Calpeptin 20μM, PD15606 100nM, Epoxomycin 200μM, MG-132 20 μM, Z-vad-fmk 200 μM. Vehicle control was treated with the same volume of DMSO as the inhibitor treated conditions. Cells were harvested 18 hours after treatment.

Immunohistochemistry

Sections of E15.5 brains were treated using a Citrate-Based Antigen Retrieval Solution (Vector laboratoriesH-3300) prior to immunohistochemistry staining. Vectastain Elite ABC Kit Rabbit IgG (Vector Laboratories PK-6101) was used to stain the samples and DAB Substrate Kit (Vector Laboratories SK-4100) was used to visualize the immunohistochemistry staining.

Cell Fractionation

Wild-type and $Dlg5^{-/-}$ NPCs were allowed to reach confluence and were harvested the following day. The cells were collected by repeatedly rinsing the plate with RSB buffer using a 1000μL micropipette tip. Cell pellets were resuspended in ice-cold buffer RSB, containing: 10mM Hepes, 10mM NaCl, 1.5mM $MgCl_2$, 1mM DTT, 0.5mM PMSF, and 20μM Mg132 + phosphatase and protease inhibitors. Cells were allowed to swell for 30 minutes on ice and then were homogenized with 20 passes of a dounce homogenizer (Sigma D8938). 200μL of lysed cells were removed a total fractionation control. The remaining solution was spun at 400g at +4°C for 2 minutes to isolate the nuclear fraction. The supernatant was transferred to ultracentrifuge-safe thick-wall tubes (Beckman Coulter 349622 Polycarbonate) and spun for 90 minutes at 52,700 rpm (150,000g) in a TLA100.3 rotor. The pellet was saved as the membrane fraction and the supernatant as the cytoplasmic fraction. The membrane and nuclear pellets were

resuspended in EBC buffer containing: 50mM Tris, 250mM NaCl, 1% Triton X-100, 1mM DTT, 0.5mM PMSF, and 20μM Mg132 + phosphatase and protease inhibitors. 2x LDS loading buffer was added to the protein samples. Samples were boiled for 7 minutes and sonicated for 15 seconds prior to freezing.

Gain-of-Function Cell Culture

HEK 293 FT cells were plated on 6-well plates precoated with gelatin. The following day cells were transfect with nVA-Mst2, Flag-Taz, and Flag-Lats in the presence and absence of GFP-aPKC by using PEI and OPTI-MEM. The cells were collected two later and aPKC and Hippo pathway protein levels were assessed by western blot.

Luciferase Assays

Luciferase experiments were conducted in HEK 293 FT cells using the Promega Dual-Glo® Luciferase Assay System E2920. The day prior to Lipofectamine transfection, 20,000 HEK 293 T cells were plated in each well of a 96 well plate (Falcon 353219). The next day, Renilla (BOXIT #107) and CTGF Promoter fused to Luciferase (BOXIT #1531) constructs were transfected in the presence and absence of different combinations of Taz (BOXIT # 1523), Mst2 (BOXIT #1366), Lats1 (BOXIT #1313), and Dlg5 (BOXIT #556) constructs with Lipofectamine and OPTI-MEM. Two days later fluorescence was assessed using Biotek Synergy 2 using Gen5 software.

For the experiments that examined the role of aPKC-mediated phosphorylation of Dlg5.

Renilla (BOXIT #107), pUAS-Luciferase (BOXIT#1315), and Gal4-TEAD CTGF (BOXIT #1316) constructs were transfected in the presence and absence of different combinations of Yap (BOXIT# 1307) and Mst2 (BOXIT #1366) in the presence or absence of either wild type Dlg5 (BOXIT #556), Dlg5SA (BOXIT #1092), or Dlg5SD (BOXIT #1096). Two days later fluorescence was assessed using Biotek Synergy 2 using Gen5 software.

Mice

The *Dlg5*$^{-/-}$ mice were generated by Tamilla Nechiporuk [120]. *Taz*$^{-/+}$ mice, 129S-*Wwtr1*^{tm1Benj}/J, were purchased from Jackson laboratories (Strain # 011120). *Yap*$^{-/+}$ mice were generated by breeding *Yap*$^{fl/fl}$ mice with mice carrying *More-Cre*. The *Yap*$^{fl/fl}$ mice were a gift from Dr. Duojia Pan's laboratory and the *More-Cre* mice were a gift from Dr. Philippe Soriano's laboratory.

For the genetic interaction experiments Dlg5$^{+/-}$ female mice were bred with *Dlg5*$^{+/-}$ *Yap*$^{+/-}$ male mice. The resulting offspring were examined at Postnatal Day 7-10 (P7-10). *Dlg5*$^{+/-}$ *Taz*$^{+/-}$ female mice were bred with *Dlg5*$^{+/-}$ *Taz* $^{+/-}$ males. The genotype frequency of their offspring was examined at P7-P10. At P0 *Dlg5*$^{+/-}$ *Taz*$^{+/-}$ female mice were bred with *Dlg5*$^{+/-}$ *Taz* $^{-/-}$ male mice and the relative frequencies of their offspring were quantified.

RT-qPCR

mRNA of WT and Dlg5$^{-/-}$ brains was isolated using Trizol, purified using the RNeasy MinElute Cleanup Kit (Qiagen 74204), and cDNA was made using the SuperScript III First strand cDNA Synthesis System (Invitrogen 18080-051). Relative cDNA levels were assessed using Universal

Probes (Roche) and Invitrogen SuperMix-UDG (Invitrogen # 11730-025). RT-qPCR reactions were run on 7900 Real-Time PCR System (Applied Biosystems).

The following oligos and probes were used for qRT-PCR:

CYR61 forward: GGATCTGTGAAGTGCGTCCT, Universal Probe # 66

CYR61 reverse: CTGCATTTCTTGCCCTTTTT, Universal Probe # 66

CTGF forward: TGACCTGGAGGAAAACATTAAGA, Universal Probe # 71

CTGF reverse: AGCCCTGTATGTCTTCACACTG, Universal Probe # 71

DLG5 forward: GAGCAATTCGAAACAGCACA, Universal Probe # 62

DLG5 reverse: ACCCTGGACAACCCCTGT, Universal Probe # 62

RPS16 forward: GATATTCGGGTCCGTGTGA, Universal Probe # 77

RPS16 reverse: TTGAGATGGACTGTCGGATG, Universal Probe # 77

TAZ forward: TGCTACAGTGTCCCCACAAC, Universal Probe # 73

TAZ reverse: TGACCGGAATTTTCACCTGT, Universal Probe # 73

YAP forward: AAATGCTCCAAAATGTCAGGA, Universal Probe # 47

YAP reverse: CATTCGGAGTCCCTCCATC, Universal Probe # 47

siRNA Experiments

A 96-well plate was coated with laminin prior to rapid, reverse siRNA transfection. siRNA oligos were diluted with Dharmafect in OPTIMEM and applied directly to the wells. NPCs were then placed in the wells. 48 hours later, the wells were washed with PBS and the cells were collected with 2x LDS, boiled, and sonicated.

Western blots

Protein gels were run at 200V for 1 hour and 15 minutes. The resulting blots were transferred for 2 hours and 15 minutes (250mA for two midi-sized blots and 120mA for two regular-sized blots). The long transfer time was necessary because of the large molecular weight of Dlg5.

CHAPTER 3: STUDY CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

This study demonstrates that the apical-basal cell polarity protein Dlg5 interacts with MST1/2 and negatively regulates the canonical Hippo signaling pathway. Mass Spectrometry analysis followed up by multiple co-immunoprecipitation experiments identified MST1/2 as a prominent DLG5 interacting partner. Analyses of *Dlg5*$^{-/-}$ mice and primary neural progenitor cells from *Dlg5*$^{-/-}$ embryos revealed increased phosphorylation of LATS1/2 (Thr1079), decreased total levels of YAP1/TAZ and decreased expression of gene targets of the Hippo pathway in DLG5$^{-/-}$ animals. Moreover, genetic epistasis experiments revealed a strong genetic interaction between *Dlg5* and *Yap1* and between *Dlg5* and *Taz* genes. These findings indicate activation of the canonical Hippo pathway in *Dlg5*$^{-/-}$ cells both *in vivo* and in cell culture. Consistent with these loss-of-function experiments, gain-of-function data with exogenously expressed DLG5 revealed increased total levels of TAZ and activation of endogenous TAZ gene targets in cells overexpressing DLG5. Since DLG5 maintained a negative impact on the Hippo pathway in both gain-of-function and loss-of-function experiments and in both polarized tissues and non-polarized cultured cells, I conclude that DLG5 is a direct regulator of Hippo signaling and its impact on the pathway is not a secondary effect due to the loss of polarity and tissue disorganization in *Dlg5*$^{-/-}$ mice.

Mechanistically, DLG5 negatively impacts canonical Hippo pathway signaling by interacting with MST1/2 and inhibiting its interaction with LATS1/2. While cell fractionation experiments did not reveal significant changes in localization of MST or LATS in *Dlg5*$^{-/-}$ mice,

overexpression of Dlg5 consistently decreased interaction between MST and LATS proteins. This provides a simple mechanistic explanation for DLG5-mediated negative regulation of the Hippo signaling pathway.

Future directions

This study identified polarity protein DLG5 as a novel regulator of the Hippo signaling pathway. While the connection between DLG5 and the Hippo signaling is clearly demonstrated in this study, significant future work will have to explore the exact mechanisms responsible for this connection. Is DLG5 directly interacting with MST1/2, Salvador or does it use an additional intermediate protein to physically bind to the Hippo pathway members? In order to investigate this interaction in more detail, *in vitro* co-immunoprecipitation experiments which utilize purified proteins will have to be performed.

Which domains of DLG5 and MST1/2 are required for their interaction? The Vasioukhin lab has already generated numerous truncated Dlg5 constructs which contain different DLG5 domains. By performing gain-of-function co-immunoprecipitation experiments in which he overexpressed the different DLG5 fragments in conjunction with MST2, Julian Kwan was able to identify that the third PDZ domain of DLG5 is required for the interaction between DLG5 and MST2.

Is DLG5 blocking the interaction between MST1/2 and LATS1/2 because it binds to the domain of MST1/2 that directly interacts with LATS1/2? *In vitro* kinase assays with purified MST1/2, LATS1/2, and DLG5 proteins will be necessary to address this question. If DLG5 is in fact preventing the binding of MST to LATS by directly competing with LATS for binding to

MST, one would expect that less LATS would be phosphorylated at T1079 whenever DLG5 is present in these *in vitro* experiments and this can easily be assessed by western blot.

It is possible that DLG5's interaction with MST is not direct, but is instead mediated by another protein. One candidate protein that should be explored in further detail is the Kibra. Kibra has been shown to regulate vesicle trafficking by interacting with aPKC [391], which is noteworthy when one considers that DLG5 is known to play a role in vesicle trafficking [120]. It is also known that aPKC can phosphorylate Kibra [334] and that Kibra can in turn inhibit the kinase activity of aPKC [339]. If we consider newer evidence that suggests that Hippo pathway phosphorylation occurs at the cell membrane, rather than proceeding as a linear kinase cascade in the cytoplasm [295], then it might be possible that aPKC and DLG5 are regulating the Hippo pathway via the same mechanism, which could potentially involve Kibra. A simple gain-of-function co-immunoprecipitation experiment in which exogenous Kibra and DLG5 proteins are overexpression in HEK 293 cells could be performed to determine if this hypothesis warrants further investigation. Alternatively, an endogenous co-immunoprecipitation experiment could also be performed on wild-type and *DLG5*$^{-/-}$ NPCs.

DLG5's connection to tight junctions could also use further investigation. Phylogenetic analysis of DLG5 finds that the terminal PDZ domains of DLG5 and ZO1-3 subfamily are very closely related [112]. In addition, Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2) protein structural analysis of DLG5 suggests a strong similarity to PATJ (INADL) protein (data not shown). Proteins with similar protein structures often interact with one another and amino acids 1480-1590 of Dlg5 are structurally nearly identical to several of INAD-like's PDZ domains. Thus it could be possible that Dlg5 binds to PATJ or ZO1-3 and this interaction could be serving as a link between the tight junctions and the Hippo pathway. PATJ, a member of the Crumbs - Pals -

Patj complex, is also a potentially interesting target because of its connection to TAZ and polycystic kidney disease (PKD). Three independent studies identified that *TAZ*$^{-/-}$ mice develop renal cysts characteristic of PKD [248, 249, 379]. In humans, mutations in polycystin-1 (PKD1/PC-1) or polycystin-2 (PKD2/PC-2) can result in autosomal dominant polycystic kidney disease (ADPKD) [249]. PC-1 and PC-2 are large transmembrane proteins that interact through their C-termini to form a receptor–ion channel complex [394, 395]. The coiled-coil domain of PC-1 is thought to be important for its interaction with PC-2 which has been shown to function as a nonselective Ca^{2+}-permeable cation channel protein. [396]. Duning et al. observed an interaction between TAZ and PATJ and noted that both TAZ and PATJ impair PC-2 channel activity when co-expressed with PC-2 in oocytes of *Xenopus laevis* [397], while Tian et al. found that TAZ promotes PC2 degradation through a $SCF^{\beta\text{-Trcp}}$ E3 ligase complex, suggesting a clear link between the Hippo pathway and APKD [379]. Given that *DLG5*$^{-/-}$ animals develop a polycystic kidney phenotype that is very similar to that of *TAZ*$^{-/-}$ animals and that TAZ has been shown to interact with PATJ, it might be worthwhile to explore the connection between DLG5 and PATJ. For this a simple gain-of-function co-immunoprecipitation in which HEK 293 FT cells are transfected with either V5-tagged DLG5 or V5-tagged LacZ and immunoprecipitated with V5-sepharose would be sufficient. Co-immunoprecipitation of endogenous PATJ could be easily assessed by western blot to determine if the PATJ-DLG5 connection warrants further investigation. If DLG5 does bind to PATJ, this interaction could be relevant for the polycystic phenotype observed in *DLG5*$^{-/-}$ kidneys.

In addition to the exploration of DLG5-MST1/2 interactions, it will be interesting to determine whether DLG5 also binds to MARK1-3 kinases and whether it can bridge MST1/2 with MARK1-3. MARK1-3 (PAR1 kinases) play an important role in apical-basal cell polarity

and regulation of the microtubule cytoskeleton. MARK1-3 are also implicated in regulation of the Hippo signaling pathway. It would be interesting to explore whether DLG5 is necessary for the connection between MARK1-3 and Hippo signaling. In addition, since we know that both DLG5 and PAR1 are playing an important role in apical-basal cell polarity, it would be interesting to determine whether interaction between DLG5 and MARK1-3 is necessary for DLG5 function in cell polarity maintenance. To analyze this possibility, a cell culture model capable of establishing apical-basal cell polarity would be required. I have attempted to generate polarizable models of kidney cell lines, to study the function of DLG5 with respect to apical-basal polarity and Hippo pathway, but unfortunately my efforts were unsuccessful. Numerous attempts to use shRNA constructs to generate Dlg5 loss-of-function lines in MDCK (*Madin-Darby* Canine Kidney) and IMCD3 (Inner Medullary Collecting Duct) cells were ineffective because these lines either failed to show a dramatic enough reduction of DLG5 protein levels or a robust difference in the ability of WT and DLG5 loss-of-function lines to polarize. It is important to note that the generation of these lines was attempted before CRIPSER/Cas-9 technology became mainstream. It is possible that if reattempted with the CRIPSER/Cas-9 system that the generation of these lines would be successful. If so, these cells lines could serve as models to separate out DLG5's roles in apical-basal polarity and the Hippo pathway.

Gain-of-function experiments with DLG5 and MST1/2 proteins revealed DLG5-mediated cleavage of MST1/2. It would be interesting to explore this in more detail to determine the nature of this cleavage, ascertain its role in the Hippo signaling pathway, and identify the protease responsible. One potential protease to follow up on is the mitochondrial YME1L1 metalloprotease, which was identified as a potential DLG5 binding partner by Julian Kwan via AP-MS. It is also an attractive candidate because the stable knockdown of YME1L1 in HEK

293 cells leads to impaired cell proliferation and apoptotic resistance [398], which are two processes that are highly relevant to Hippo pathway function. A co-immunoprecipitation experiment in which V5-Dlg5 is overexpressed in HEK 293 FT and immunoprecipitated could reveal whether DLG5 binds to YME1L1. If DLG5 does in fact bind to YME1L1, siRNA oligos could be used to target YME1L1. If YME1L1 is indeed responsible for cleaving MST2, then one would expect less cleavage of MST in HEK 293 FT cells treated with siRNA oligos against YME1L1. It would also be interesting to explore what conditions facilitate the activity of this protease. For example, does high or low cell density impact MST cleavage? For this, HEK 293 FT cells transfected with nVA-MST in the presence and absence of V5-DLG could be grown in low and high density conditions and the levels of MST cleavage in these two conditions could be assessed by western blot. Is the cleavage of MST serving to activate or inhibit the Hippo pathway? This could be determined by analyzing TAZ and YAP protein levels by western blot or CTGF and CYR61 mRNA by RT-qPCR in cells in which YME1L1 activity has been either repressed or activated.

Another finding that warrants further investigation is that different tissues in *Dlg5*$^{-/-}$ mice reveal a differential impact of the loss of DLG5 on YAP1 and TAZ protein levels. This is potentially very interesting, because it is believed that YAP1 and TAZ should be regulated by Hippo pathway in a similar manner particularly if DLG5 is regulating the pathway at the level of MST1/2 and SAV. If this is the case, then what is responsible for the differences between DLG5-mediated impact on YAP1 and TAZ?

There is substantial evidence in the literature to suggest that YAP1 and TAZ may have distinct functions in different spatial temporal contexts [251-255]. While we did not explicitly seek to differentiate between the functions of YAP1 and TAZ in our study, some differences

became apparent nonetheless. For example, siRNA knockdown of LATS1/2 in WT and DLG5 KO neural progenitor cells results in a dramatic increase in endogenous protein levels of TAZ, but not YAP1 (data not shown). In addition, Dlg5 KO NPCs have decreased levels of both YAP1 and TAZ, but TAZ levels are reduced much more dramatically in DLG5 KO NPCs *ex vivo*. DLG5 also appears to regulate TAZ- but not YAP-mediated activation of TEAD in luciferase experiments (data not shown). Interestingly, *Dlg5*$^{-/-}$ mice also bear a much stronger resemblance to *Taz*$^{-/-}$ mice [248, 249, 379] than to *Yap1*$^{-/-}$ mice [247], suggesting that Dlg5 may be preferentially acting through TAZ. However, given that we've demonstrated that Dlg5 is able to negatively regulate the Hippo by binding to MST1/2, the mechanism by which this occurs is not clear. It is possible that DLG5 is interacting with the Hippo pathway via an additional mechanism. It is also possible that some of the aforementioned potential DLG5 binding partners, such as PATJ, could be responsible because this would provide a mechanism through which DLG5 could be selectively interacting with TAZ but not YAP in addition to regulating the Hippo pathway by binding to MST2. If there are differences in YAP1 and TAZ regulation, then understanding the nature and mechanisms responsible for these differences may significantly extend our knowledge about the Hippo pathway and manner in which it functions.

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CURRICULUM VITAE

ANNA K. SCZANIECKA

EDUCATION AND EXPERIENCE

Ph.D. Molecular and Cellular Biology, U of Washington, Seattle, WA — Expected Mar 2015
M.S. Epidemiology, University of Washington, Seattle, WA — 2010
B.S. Biology, California Institute of Technology (Caltech), Pasadena, CA — 2004
13+ years of laboratory research experience — 2001-present

RESEARCH EXPERIENCE

Graduate (PhD) Research with Dr. Valeri Vasioukhin — June 2009 – Present
Fred Hutchinson Cancer Research Center, Seattle, WA

Demonstrated that Dlg5, a Discs Large-related cell polarity protein, plays a role in linking cell polarity (apico-basal) and cell growth (Hippo) pathways, by using both an *in vivo* mammalian mouse model and *ex vivo* mammalian cell lines.
Employed a siRNA screen in primary neural progenitor cell lines to identify specific Hippo pathway interactions with Dlg5.
Used western blot and co-immunoprecipitation techniques to demonstrate the effects of Dlg5 loss on Hippo pathway protein levels and protein-protein interactions.
Used cell fractionation, immunohistochemistry, and immunofluorescence techniques to identify altered cellular localizations of proteins in Dlg5 knockout mice.

Graduate (MS) Epidemiology Research with Dr. Emily White — Sept 2009 – Sept 2010
Fred Hutchinson Cancer Research Center, Seattle, WA

Examined the association of specific dietary fatty acid intake with breast cancer incidence among postmenpausal women in the VITAL cohort, and compiled and analyzed data from 40,000+ food frequency questionnaires using STATA statistical software.
First-authored a publication demonstrating that different fatty acids were differentially associated with incidence of breast cancer (see Sczaniecka et al., 2012).

Research Technician in the Laboratory of Dr. Marc Van Gilst — Aug 2007 – Aug 2008
Fred Hutchinson Cancer Research Center, Seattle, WA

Studied the relationship between gene regulatory networks and a nuclear hormone receptor responsible for inducing a fasting response in *C. elegans*.
Conducted a genetic screen to identify new pathway components and/or downstream effectors of this nuclear hormone receptor that affect development.
Compared mRNA levels in fed and fasted samples of mutant and wild-type *C. elegans*, using microarray analysis and QPCR.
Cloned, injected, and imaged GFP tagged proteins related to ceramide and sphingolipid metabolism.

Research Assistant in the Laboratory of Dr. Ulrich Mueller July 2004 – July 2007
The Scripps Research Institute, La Jolla, CA

Assisted in a genetic screen to identify and characterize genes related to hearing loss in mice.
Maintained a mouse colony of ~1500 mice and tested mice electronically for hearing defects by measuring ABR (Auditory Brainstem Response) and DPOAE (Distortion Product OtoAcoustic Emissions).
Performed micro-dissections of the embryonic inner ear for morphological analysis by scanning electron microscopy.
Performed RNA extractions and RT-PCR to isolate cDNA from inner ear tissue for further cloning and incorporation into a cDNA microarray.
Designed and cloned GFP, His, and Myc tagged constructs for use in cell expression studies.
Co-authored five publications.

Research Assistant in the Laboratory of Paul W. Sternberg July 2001 – March 2004
Caltech/Howard Hughes Medical Institute in Pasadena, CA

Studied *C. elegans* mating behavior to understand how sensory inputs and motor control coordinate to create a defined series of behavioral steps, such as mating.
Assisted in an RNAi screen and conducted mating assays to characterize the range of mating phenotypes and determine at role of mechanosensory genes in *C. elegans* mating.
Tested the effect of various neurotransmitters on tail curling (important to mating behavior) in males.

TEACHING AND MENTORING EXPERIENCE

Teaching Assistant

Biochemistry 442 "Cellular and Molecular Biology", U of Washington Spring 2011
Wrote quizzes, taught two weekly sections, and held weekly office hours.

Biology 1 "Drugs and the Brain", Caltech Spring 2004
Taught a course section, held office hours, and attended weekly TA meetings.

Research Mentor

BioQuest Academy, Seattle Biomed (Biomedical Research Institute) Summer 2009
Served as a mentor for Bioquest, an intensive summer research program for driven high school students from underrepresented backgrounds.

TRAINING GRANTS

NIH Cell and Molecular Biology Training Grant 2011-2014
NCI Cancer Prevention Training Grant 2009-2010