MOLECULAR FUNCTION OF THE CELL POLARITY PROTEIN PARTNER OF INSCUTEABLE IN *DROSOPHILA* NEUROBLASTS

by

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A DISSERTATION

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Asymmetric cell division (ACD) is a unique mechanism employed during development to achieve cellular diversity from a small number of progenitor cells. Cells undergoing ACD distribute factors for self-renewal at the apical cortex and factors for differentiation at the basal cortex. It is critical for proper development that the mitotic spindle be tightly coupled to this axis of polarization such that both sets of proteins are exclusively segregated into the daughter cells.

We use ACD in *Drosophila* neuroblasts as a model system for understanding the molecular mechanisms that govern spindle-cortical coupling. Neuroblasts polarize Partner of Inscuteable (Pins), Gαi and Mushroom Body Defect (Mud) at the apical cell cortex during mitosis. Gαi and Pins are required for establishing cortical polarity while Mud is essential for spindle-cortical alignment. Gαi and Mud interact through Pins GoLoco domains and tetratricopeptide repeats (TPR) respectively, however it is unclear

how Mud activity is integrated with Pins and Gαi to link neuroblast cortical polarity to the mitotic spindle.

This dissertation describes how Pins interactions with Gαi and Mud regulate two fundamental aspects of neuroblast ACD: cortical polarity and alignment of the spindle with the resulting polarity axis. I demonstrate that Pins is a dynamic scaffolding protein that undergoes a GoLoco-TPR intramolecular interaction, resulting in a conformation of Pins with low Mud and reduced Gαi binding affinity. However, Pins TPR domains fail to completely repress Gαi binding, as a single GoLoco is unaffected by the intramolecular isomerization. Gαi present at the apical cortex specifies Pins localization through binding this "unregulated" GoLoco. Liberation of Pins intramolecularly coupled state occurs through cooperative binding of Gαi and Mud to the other GoLoco and TPR domains, creating a high-affinity Gαi-Pins-Mud complex. This autoregulatory mechanism spatially confines the Pins-Mud interaction to the apical cortex and facilitates proper apical-spindle orientation. In conclusion, these results suggest Gαi induces multiple Pins states to both properly localize Pins and ensure tight coupling between apical polarity and mitotic spindle alignment.

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CHAPTER I

INTRODUCTION TO CELL POLARIZATION AND ASYMMETRIC DIVISION

History and Background

The existence of cellular polarization was initially postulated in the mid to late 17th century, with E. Ray Lankester proclaiming: "All differentiation of cells, the development of one kind of cell from another, is dependent on internal movements of physiological molecules of the protoplasm of such cells." Although this statement was prescient, biologists had no available molecular tools to discern how cells separate factors promoting cellular differentiation. Biologist Edwin Grant Conklin achieved a major coup in 1905 when he traced the retention of pigmented materials in the ascidian *Styela*. In particular, Conklin identified a "yellow material" in *Styela* tail muscles that was inherited from specific cells during early embryogenesis (1). His results provided the first circumstantial evidence that cells could spatially position, or polarize determinants which promote cellular differentiation, organ growth and development. For the intervening century, cell biologists have wondered; what are the fundamental processes that enable a cell to segregate these "fate determinants" in such a precise manner? Research on the mechanisms underlying asymmetric polarization of the cell

has accelerated in the past decade and remains an area of intense biological research in both basic science and cancer biology. This dissertation will explore the topic of cellular polarization through study of asymmetric cell division, a developmental mechanism in which cells unequally partition proteins as a mechanism for creating cellular diversity.

Biological Features of Asymmetric Cell Division

Asymmetric cell division (ACD) is a developmental process that results in cell polarization and asymmetric distribution of proteins and cytoplasm. At specific points in development, a stem cell like progenitor first creates an axis of intrinsic cellular polarity by segregating fate determinants, such as transcription factors or mRNA, to opposite poles of the cell, then aligning the mitotic spindle with this axis of polarity and concluding with an asymmetric cleavage. The result of this fissioning is the production of two daughter cells that adopt different developmental fates (Illustration 1). A hallmark of ACD is that the larger cell will self-renew, maintaining its stem cell like identity, while the other cell contains determinants which lead to cellular differentiation. Because the self-renewing cell is able to undergo multiple rounds of ACD, this division allows for multiplicative expansion of specific cell types in a directed manner. Thus, asymmetric divisions create cellular diversity from a small number of progenitor cells.

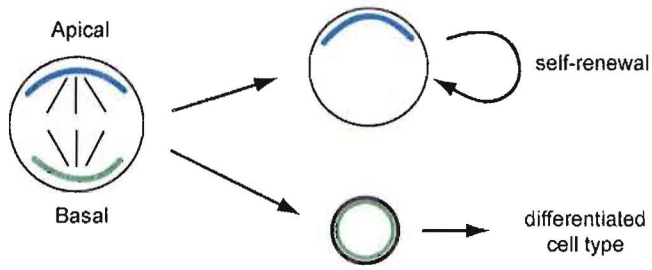

Illustration 1. Overview of asymmetric cell division Cells undergoing asymmetric division deposit determinants for self-renewal (blue) or differentiation (green) on the apical and basal poles of the cell. At metaphase, the mitotic spindle aligns with this axis of polarity, fostering segregation of fate determinants into the resulting daughter cells.

For proper development of the organism, it is imperative that both sets of polarized fate determinants be exclusively segregated into the resultant daughter cells. Improper distribution of fate determinants can lead to proliferative defects and developmental abnormalities (2, 3). To demarcate the positions of fate determinants, we define the localization of these complexes in the cells as either apical or basal. The apical pole can be defined through extrinsic (proximal to the ventral neuroepithelium) or intrinsic cues (based on the distribution of self-renewal markers); while the basal pole is, by definition, considered diametrically opposed to the apical. In order to achieve this precise spatial positioning of determinants, cells localize an amalgam of proteins that are distributed to the apical or basal pole of the progenitor cell. Therefore, cells employing ACD need robust mechanisms to localize determinants to each cell pole and maintain tight spatial coupling of the mitotic spindle to these complexes, such that during mitosis both sets of fate determinants are properly directed into each daughter cell.

Use of *Drosophila melanogaster* as a Tool for Studying Asymmetric Division

Neuroblasts in the fruit fly, *Drosophila melanogaster,* are stem cell-like progenitors that undergo asymmetric division to produce a larger self-renewing neuroblast (NB) and a smaller ganglion mother cell (GMC) that terminally differentiates into two neurons or glia (4). The neurons and glial cells that germinate from the GMC ultimately form the central nervous system of the fruit fly. As in other cells undergoing ACD, neuroblasts deposit factors promoting self-renewal or differentiation at the apical and basal poles of the cell, respectively. Requisite alignment of the mitotic spindle with this neuroblast apical/basal polarity is essential such that during mitosis, apical and basal fate determinants are properly partitioned into the daughter cells. A number of genetic mutants have been identified in *Drosophila* exhibiting defects in neuroblast polarity and ACD. Cells lacking these components of the apical complex show aberrant ACD phenotypes, such as symmetric division, defective distribution of fate determinants and misaligned spindle orientation. These findings suggest protein complexes at the apical or basal cortex function to polarize the cell, align the mitotic spindle with this cortical polarity and partition the NB unequally.

Molecular Components Linking Cortical Polarity to the Mitotic Spindle

A fundamental question in neuroblast biology is how factors at the apical or basal cortex function to align the mitotic spindle with intrinsic cortical polarity. *Drosophila* neuroblast division is a prime model system for examining the molecular

machinery required for asymmetric cell division, as genetic studies have uncovered revealed several pathways that are responsible for aligning cortical polarity with mitotic spindle alignment (5). The principal pathway linking neuroblast cortical polarity with mitotic spindle alignment is the Pins, Gαi and Mud complex (5-7). Interestingly, there is biological homology between the molecular components that specify ACD in metazoan development with, GPR-1/2, GOA-1, Lin-5 in C. elegans (8-10), and LGN, Gαi, NuMA (11) in mammals, believed to work in the same fashion as Pins, Gαi and Mud. The conservation of this domain architecture across a variety of organisms suggests an essential function for this family of molecules in cell polarity and development.

Although we have an understanding of the basic network of gene products involved in ACD, we have little understanding of how the signal outputs from Pins, Mud and Gαi are integrated to promote neuroblast mitotic alignment. The next section of the introduction will explore the protein domains that allow Pins, Gαi and Mud to interact, how these interactions are specified through discrete protein domains and how these domains influence protein function.

The Pins, LGN, GPR-1/2 Family of Cell Polarity Proteins

A major regulator of neuroblast cell polarity and spindle orientation is Partner-of-Inscuteable (Pins; LGN or mPins in mammals, GPR-1/2 in C. elegans). Pins is localized to the apical cortex of metaphase neuroblasts where it functions to promote cell polarity and cortical-microtubule coupling (6) Bowman, 2006, (7) Siller, 2006, (5) Izaki

2006). Pins function is regulated by two sets of protein domains, which have specific molecular functions. Pins seven tetratricopeptide repeats (TPRs) have been shown to be the essential scaffolding element for interacting with several other neuroblast polarity components; the spindle-associated coiled-coil Mushroom body defect protein (Mud; NuMA in mammals, Lin-5 in C. elegans) and Inscuteable (Insc), which is posited to act as a molecular linker between the Par polarity complex and Pins (12). Furthermore, the TPR domains of the Pins homologue LGN has been reported to interact with the mammalian Lethal giant larvae (Lgl) tumor suppressor protein, which assists in segregation of fate determinants (13-15). The other domain present within Pins, the carboxyl-located GoLoco motifs, serves to sequester the Gαi class of heterotrimeric G protein subunits. Heterotrimeric G protein components have been shown to be essential players in neuroblast asymmetric cell division and play a major role in mitotic spindle dynamics.

Gαi Subclass of Signal Transduction Proteins

Heterotrimeric G proteins (HGP) are an essential component in *Drosophila* neuroblasts and other cell lineages exhibiting ACD. HGP complexes consist of an alpha subunit (Gα) in complex with a beta-gamma (Gβγ) heterodimer anchored at the cell membrane. G protein complexes are coupled to a transmembrane receptor that responds to external stimuli by dissociating the HGP complex. Pins localizes to the cell cortex through interactions with Gαi (adenyl-cyclase inhibitory) subunit (16). The Pins-Gαi

interaction occurs through the GoLoco protein motif, a 19 amino acid domain whose function is sequestering GDP-bound Gαi subunits (17). Pins GoLoco domains act as a guanidine dissociation inhibitor (GDI) to prevent ADP-ATP nucleotide exchange, and been shown to dissociate Gαi-GDP subunits from their cognate Gβγ partner in a nucleotide independent state (18). This mechanism of G-protein activation differs from activation by canonical G protein coupled receptors (GPCR) where ligand binding at the GPCR induces Gαi nucleotide exchange and dissociation from Gβγ. This "receptor-independent" G-protein signaling mechanism constitutes an intrinsic polarity cue for the neuroblast. Downstream effectors of Gαi and Gβγ affect microtubule dynamics in mammalian cells, indicating that regulation of G proteins may modulate spindle formation/orientation in NBs (19). Consistent with this hypothesis, ectopic expression of Gβ and Gγ has been shown to reduce spindle size in NBs, whereas null mutations of a Gγ isoform cause a large spindle phenotype (20). These studies indicate that G protein signaling contributes to the formation and regulation of the mitotic spindle, suggesting that regulation of Gα or Gβγ activity through Pins may affect spindle dynamics.

Mud, NuMA, Lin-5 Microtubule Binding Proteins

The Mud family of proteins are essential molecular components for linking cortical polarity to mitotic spindle alignment in asymmetric divisions in several model organisms. (Mud in *Drosophila*, NuMA in mammals and Lin-5 in *C. elegans*) The Mud

orthologue NuMA is normally retained in the nucleus during interphase of both symmetrically and asymmetrically dividing cells, but is released to the cytoplasm at nuclear envelope breakdown (21). Soon thereafter, Mud, like NuMA becomes associated with the mitotic centrosomes. Mud is thought to function near the centrosome, where it cross links astral microtubules and promotes aster focusing (22). Recombinant Mud has also been shown to enhance the stability or frequency of microtubule (MT) polymerization (5, 6). Both embryonic and larval brain neuroblasts exhibit a transient apical enrichment of Mud protein at metaphase (6, 7). Neuroblasts lacking Mud display randomized spindle alignment; cortical-spindle coupling is defective (6, 7). As a result of this spindle defect, fate determinants are not partitioned into the daughter cells and cells do not adopt their proper fate. As a result, *mud* neuroblasts show a neuroblast hyperproliferation phenotype resulting in large brain size (3). Mud has been shown to interact and align the mitotic spindle with Pins apical crescents, however little is understood as to how this interaction is regulated within neuroblasts.

Pins molecular architecture lends itself to scaffolding various components of the apical cortex; Pins engages in a number of functions; it serves to polarize the neuroblast through interactions with Gαi and Inscuteable, and link cortical polarity to the mitotic spindle through interactions with Mud. Pins ability to associate with multiple members of the apical complex through an intrinsic protein-protein interaction module, coupled with Pins array of Gαi signaling domains in the same molecule implies Pins may serve

as a scaffold to sequester cell polarity components and signaling molecules in close proximity.

Bridge to Chapter II

In the preceding chapter, we defined the role of cell polarization and asymmetric cell division in contributing to organ and tissue development and introduced the concept of *Drosophila* neuroblasts as a model system for studying the molecular components inducing asymmetric division. In Chapter II, using data from a previously published manuscript, I will describe how Pins is localized to the cell cortex though interactions with Gαi and how regulated Pins-Gαi complex formation induces cortical-spindle capture through interactions with the microtubule-associating protein Mud.

CHAPTER II

GαI GENERATES MULTIPLE PINS ACTIVATION STATES TO LINK CORTICAL POLARITY AND SPINDLE ORIENTATION IN *DROSOPHILA* NEUROBLASTS

Introduction

Asymmetric cell division occurs when the mitotic spindle is aligned with the cell polarity axis, resulting in molecularly distinct sibling cells. Asymmetric divisions generate cell diversity, including during mammalian neurogenesis and epidermal lineages (4, 23-25). More recently, asymmetric cell division has been proposed to regulate stem cell pool size during normal development, adult tissue homeostasis, and in cancer (26). Thus, understanding how the mitotic spindle is coupled to the cell polarity axis is relevant to stem cell and cancer biology. Here we investigate this question in *Drosophila* neuroblasts, a model system for studying asymmetric cell division.

Drosophila neuroblasts are stem cell-like progenitors that divide asymmetrically to produce a larger self-renewing neuroblast and a smaller ganglion mother cell (GMC) that differentiates into neurons or glia (24). Mitotic neuroblasts segregate factors that

promote neuroblast self-renewal to their apical cortex and differentiation factors to their basal cortex. Precise alignment of the mitotic spindle with the neuroblast apical/basal polarity is required for asymmetric cell division and proper brain development: spindle misalignment leads to symmetric cell divisions that expand the neuroblast population and brain size (2, 4, 27).

A key regulator of neuroblast cell polarity and spindle orientation is Partner-of-Inscuteable (Pins; LGN or mPins in mammals, GPR-1/2 in *C. elegans*). In metaphase neuroblasts, Pins is co-localized at the apical cortex with the heterotrimeric G-protein subunit Gαi and the spindle-associated coiled-coil Mushroom body defect protein (5-7) (Mud; NuMA in mammals, Lin-5 in *C. elegans*). Pins and Gαi are interdependent for localization and for establishing cortical polarity (16, 18). Pins also binds to and recruits Mud to the apical cortex; Mud is specifically required to align the mitotic spindle with Gαi/Pins, but has no apparent role in establishing cortical polarity (5-7).

The mechanism underlying Pins regulation of cortical polarity and spindle-cortex coupling is unclear. It is unknown how (or if) the formation of the Gαi-Pins-Mud complex is regulated. Pins has the potential to bind multiple Gαi•GDP molecules via three short GoLoco motifs (Fig. 1*A*), as do mammalian Pins homologs (17), but the role of these multiple binding sites is unknown. Moreover, via its tetratricopeptide repeats (TPRs), Pins can bind Mud (6-7), but the stoichiometry and regulation of this interaction has not been explored. Furthermore, we show below that, just as in it's mammalian homolog LGN (11), the regions of Pins containing the TPRs and GoLocos interact, raising the possibility of cooperative "opening" of Pins by Gαi and Mud ligands. Here

we test the role of Pins intra- and intermolecular interactions in coupling cortical polarity with spindle orientation. We use biochemistry, genetics, and *in vivo* live imaging to test the role of Pins intramolecular interactions, and whether Gαi and Mud bind Pins independently, cooperatively, or antagonistically. We conclude that Pins has multiple functional states – a form recruited by a single Gαi to the apical cortex that is unable to bind Mud, but sufficient to induce cortical polarity; and a form saturated with Gαi that recruits Mud and links cortical polarity to the mitotic spindle. The multiple Pins states are due to cooperative binding of Mud and Gαi to Pins, and result in a tight link between apical cortical polarity and mitotic spindle orientation.

Methodology

Protein Expression and Purification

DNA encoding full length *Drosophila* Pins was amplified from an embryonic cDNA library. *Drosophila* Gαi was completely insoluble and therefore mouse Gαi 3 25-354 (which is 76% identical to the *Drosophila* protein) cloned from a macrophage cDNA library was used for these studies. A plasmid containing Mud residues 1825-1997 was generated as previously described (7). Pins GoLoco mutants were generated by utilizing a previously described arginine to phenylalanine substitution that renders GoLoco motifs incapable of binding Gαi (17). These substitutions were introduced into Pins at residue numbers: 486 for GoLoco1, 570 for GoLoco2 and 631 for GoLoco3.

All proteins were expressed using the *E. coli* strain BL21(DE3) as a host strain with pGEX 4T-1 based vectors for GST fusions and pBH based vectors for hexahistidine fusions, which were isolated and purified as previously described (28).

Gαi was either used directly after purification, or loaded with GDP or GMPPNP subsequent to purification (GDP-loaded and unloaded behaved identically). Nucleotide was added at a 5-fold molar excess in 10 mM HEPES, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, pH 7.5 and incubated at room temperature for 30 minutes. The final buffer conditions contained 10 mM $MgCl_2$.

In Vitro Binding Assays

GST pull-down assays, were performed as previously described (28). Briefly, ligands were added to a GST/Glutathione agarose mixture at the indicated concentrations to a final reaction volume of 50μL and incubated at room temperature for 15 minutes before washing and elution.

Fluorescence anisotropy binding assays were performed on an ISS PC1 fluorimeter. For labeling of the Pins GoLocos, a cysteine was added at the COOH terminus of residues 372-658 and the two naturally occurring cysteines in this fragment, 487 and 561, were mutated to serines. This purified fragment was incubated with tetramethyl-rhodamine maleimide (Molecular Probes T-6027) at a 5-fold molar excess in the presence of the reducing agent TCEP. The reaction was quenched with 1 mM BME and unreacted dye was removed by gel filtration. For binding experiments, solutions were prepared with increasing amount of ligand and constant dye-labeled component

(100 nM) in a buffer of 10 mM HEPES, 100 mM NaCl, 1 mM DTT. The anisotropy at each ligand concentration was measured with excitation and emission wavelengths of 550 nm and 578 nm respectively. The temperature was maintained at 20°C using a circulating water bath. Data series were fit to an equation describing binding to three independent, equivalent sites using non-linear regression.

Gel filtration studies were carried out on a Superdex 200 molecular sizing column (GE Healthcare) equilibrated in 10 mM HEPES, 100 mM NaCl, and 1 mM DTT. 100 µL protein reactions were mixed on ice and incubated at 4°C for 15 min before being loaded on the column. The column was run at a flow rate of 0.5 mL/min with 300µL fractions collected for analysis. Protein elution was detected by absorbance at 280 nm. The column was calibrated with a series of molecular weight standards (GE Healthcare).

Construction and Analysis of the Pins Fluorescence Resonance Energy Transfer Sensor

Full length Pins, with Yellow Fluorescent Protein (EYFP 1-239) and Cyan Fluorescent Protein (ECFP 1-239) coding sequences at the NH_2- and COOH-terminuses, was expressed and purified as described above except that the protein was purified by gel filtration chromatography. Fluorescence Resonance Energy Transfer (FRET) measurements were performed as described above but with an excitation wavelength of 433 nm (to minimize direct YFP excitation). FRET controls using trypsin (Sigma) were obtained by incubating 200nM of the appropriate FRET sensor with 0.9 nM of trypsin at 18°C for 15 min. The amount of energy transfer was measured by taking the ratio of CFP (475 nm) and YFP (525 nm) fluorescence.

Fly Strains

The *Oregon R* strain was used as wild type control for the analysis of cell polarity and spindle orientation in larval neuroblasts of fixed specimens. The full length Pins cDNA containing either the wt or the Pins GoLoco Δ GL2/3 open reading frame were subcloned into pUAST containing an amino-terminal hemagluttinin (HA) epitope. Transgenic flies carrying *P{UAS-HA:Pins} or P{UAS-HA:PinsΔ GL2/3}* insertions on the 2nd chromosome were balanced and crossed to the $pins^{P62}$ allele using standard genetic methods to form *P{UAS-HA:Pins};*$pins^{P62}$*/TM3-Sb* or *P{UAS-HA:PinsΔ GL2/3};*$pins^{P62}$*/TM3-Sb lines.* These flies were crossed at 18°C to the *worniu-GAL4;* $pins^{P62}$*/TM3-actin-GFP Ser* driver line and mutant larvae in the progeny were analyzed. Newly hatched mutant larvae were identified based on the absence of GFP expression and the presence of smaller central brain neuroblasts.

Immunocytochemistry

Freshly hatched wild type, $pins^{P62}$ and Gai^{P8} zygotic mutant larvae were aged for 96-120h at 25°C and prepared for immunofluorescent antibody labeling as described previously, with the modification that 5% normal goat serum was added to the larval blocking and primary antibody solutions (29). Primary antibodies were: rat anti-Pins (#2, 1:500; W. Chia); rabbit anti-Gαi (raised against peptide, amino acids 327-355; 1:500); rabbit anti-Insc (1:1000, W. Chia); rabbit anti-Mud (raised against amino acids 375-549, 1:2000, H. Nash); mouse anti-α-tubulin (DM1A, Sigma, 1:2000) and mouse-anti-HA

(Covance, 1:1000). Fluorescently-conjugated secondary antibodies were obtained from Jackson ImmunoResearch (Charlottesville, VA) and Molecular Probes (Eugene, OR). For DNA labeling, fixed specimens were incubated in PBS 0.1% Triton-X100 containing 1 mg/ml RNAse A for 1hr at room temperature and counterstained with 4 ⌈g/ml propidium iodide. Confocal images were acquired on a Leica TCS SP2 microscope equipped with a 63x 1.4 NA oil-immersion objective. Panels were arranged using ImageJ, Adobe Photoshop, and Adobe Illustrator.

Analysis of Spindle Orientation in Fixed and Live Larval Neuroblasts.

In fixed specimens, spindle orientation was measured at metaphase as the angle between the spindle axis (defined by position of the two spindle poles) and the cell polarity axis (defined as a line through the cell center and the center of the Miranda /Pins crescent).

Results

Gαi and Mud Bind Cooperatively to Pins

The NH_2-terminal half of Pins contains seven tetratricopeptide repeats (TPRs) and the COOH-terminal half contains three GoLoco motifs which we term the GoLoco Region, or GLR (Figure 1*A*). Each of the three GoLocos has the potential to bind GDP-bound Gαi (17) whereas the TPRs bind the Mud protein (5-7). Prior to testing whether the Pins intramolecular interaction regulates Pins-Gαi-Mud complex assembly, we first characterized each of the relevant individual domain interactions: TPR-GLR, TPR-Mud,

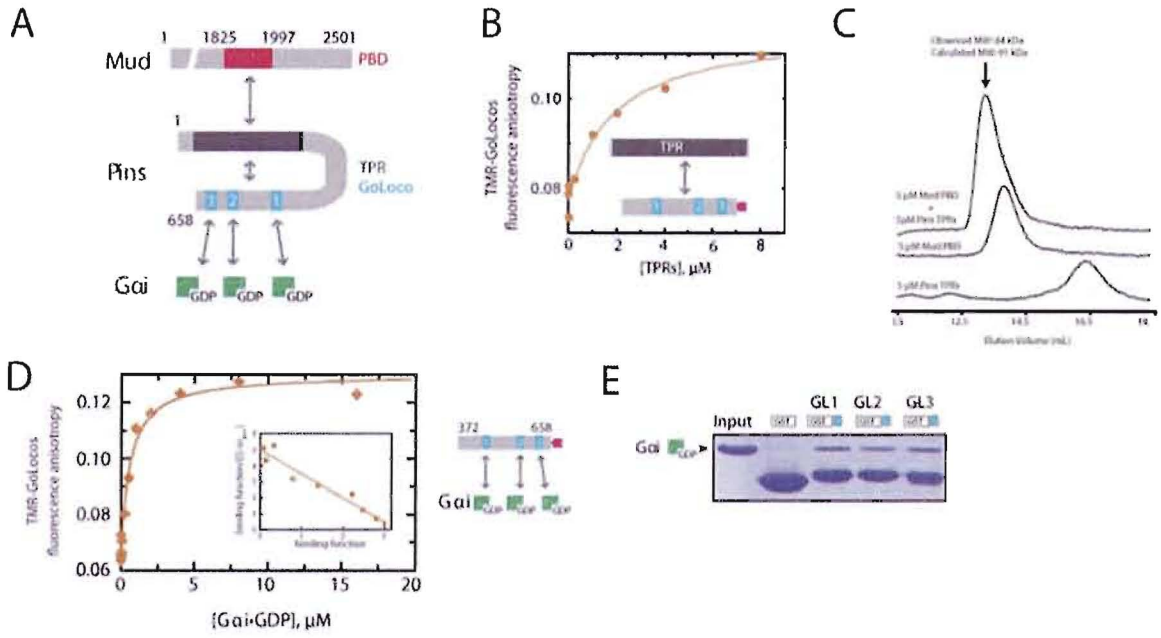

Figure 1. Gαi and Mud exhibit simple binding to Pins GoLoco and TPR domains
(***A***) Domain structure of Pins and intra- and intermolecular interactions (TPR: tetratricopeptide repeat, PBD: region of Mud that binds Pins). (**B**) The Pins TPRs and GoLocos bind to form a *trans* complex. The extent of TPR binding to the GoLocos was followed by fluorescence anisotropy using a tetramethylrhodamine attached to the GoLoco COOH-terminus. The best-fit curve is for a bimolecular association reaction with a K_d of 1.8 μM. (**C**) Mud binds the Pins TPRs with a 1:1 stoichiometry. Gel filtration chromatography is shown for the Mud PBD domain, the Pins TPRs, and a combination of both. The resulting complex has an elution volume consistent with a 1:1 complex. (***D***) The Pins GoLocos are intrinsically independent, equivalent Gαi binding sites. The extent of Gαi•GDP binding to the Pins GoLocos was monitored by the fluorescence anisotropy of a tetramethylrhodamine attached to a cysteine at its C-terminus. The curve represents a model with three equivalent binding sites of affinity $K_d = 530 \pm 80$ nM. A Scatchard analysis is shown in the inset where the binding function is equal to the concentration of Pins-bound Gαi•GDP divided by the total concentration of Pins. (***E***) Each of the Pins GoLoco motifs can bind Gαi. Individual GST-fusions of the three GoLocos bind Gαi•GDP at qualitatively similar levels.

and GLR-Gαi. First, using the fluorescence anisotropy of tetramethylrhodamine (TMR) attached to the COOH-terminus of the Pins GLR, the Pins TPR-GLR intramolecular interaction has an affinity of K_d = ~2 μM *in trans* (Fig. 1*B*), which may be significantly stronger *in cis* due to effective concentration. Second, the Pins TPRs bind Mud with a 1:1 stoichiometry as judged by the elution profile of the TPR-Mud complex on a calibrated gel filtration column is shown in Figure 1*B*, indicating that these seven repeats form a single Mud binding site. Next, to assess if Gαi binding to the GLR is cooperative, noncooperative or antagonistic, we titrated TMR-labled GLRs with GαI (Fig 1*D*). The binding isotherm is fit well with by a model describing three equivalent, independent sites with sub-micromolar Gαi affinities (K_d = 530 ± 80 nM) and yields a linear Scatchard relationship (Fig. 1*D*, inset). Finally, we show each of the three Pins GoLoco domains binds Gαi•GDP (hereafter Gαi) equally well in a qualitative pull-down assay (Fig. 1*E*). We conclude that the three GoLocos in the Pins GLR bind Gαi with similar affinities and without cooperativity in the absence of the TPRs, similar to a three GoLoco region of the protein AGS3 (30).

To test whether the Pins intramolecular interaction regulates Pins-Gαi-Mud complex assembly, we first determined if it is affected by Gαi or Mud binding. Using a qualitative assay in which the TPRs and GLR are expressed as separate fragments, we find that increasing concentrations of Gαi completely disrupt the *trans* TPR-GLR complex (Fig. 2*A*). This is consistent with a model where Gαi populates the GLR, displacing it from the TPRs. The region of Mud that binds to Pins (Pins Binding Domain

or PBD; contained within Mud residues 1825-1997) also disrupts the TPR-GLR complex, but not as efficiently as Gαi (Fig. 2*B*). Thus, Pins contains an intramolecular interaction that competes against both Gαi and Mud binding.

Because Gαi and Mud are both coupled to the Pins intramolecular interaction, we tested whether the two proteins bind cooperatively to Pins by determining if Gαi could enhance the affinity of Pins for Mud. As shown in Figure 2C, 1 μM Pins binds weakly to a GST fusion of the Mud PBD domain. However, addition of Gαi induces a large increase in Pins binding and formation of a Mud-Pins-Gαi ternary complex. We conclude that Gαi increases the affinity of Pins for Mud (i.e. Gαi and Mud bind cooperatively to Pins).

Differential GoLoco regulation by the Pins TPRs

As Pins contains three Gαi binding sites and the Pins intramolecular interaction competes against Gαi binding, we next tested if the TPRs repress the Gαi binding sites equally. Biochemical *in trans* studies detailed in Figure 1 indicate the TPR-containing region of Pins is the molecular domain which moderates the Pins intramolecular isomerization and henceforth we refer to the TPR repeats (TPRs) as the functional unit of this interaction.

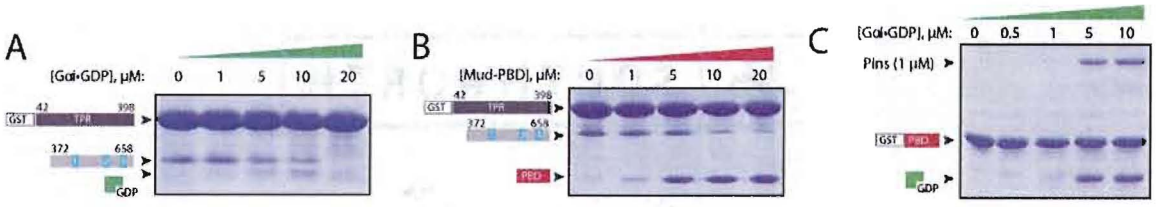

Figure 2. Pins intramolecular interaction regulates Gαi and Mud binding (*A*) Gαi disrupts the Pins intramolecular interaction. In a qualitative "pull-down" assay, Gαi•GDP competes with the Pins GoLocos for binding to the Pins TPRs. The presence of Gαi•GDP indicates that a Gαi-GLR-TPR complex can be formed at certain Gαi concentrations (5-10μM), suggesting that the GoLoco domains are unequally regulated by the TPRs. At a higher concentration (20μM), occupation of all three GoLoco motifs by Gai interferes with the interaction of the GLR-TPR region. Proteins are stained with coomassie brilliant blue. (*B*) The Mud PBD domain disrupts the Pins intramolecular interaction. Binding of Mud to the Pins TPRs (as in panel b) competes with the Pins GoLocos. (*C*) Gαi increases the affinity of Pins for Mud. Full-length Pins binds weakly to the Mud PBD domain, but binding is enhanced by the presence of Gαi•GDP indicating that Gαi and Mud bind cooperatively to Pins.

Unequal regulation of the GoLocos by the TPRs would alter how Gαi regulates Mud-Pins interactions. To determine how the TPRs modulate Gαi-GoLoco binding, we used gel filtration chromatography of full-length Pins and Gαi. We find that Pins elutes as a single peak with an elution volume consistent with the molecular weight for a monomer (Fig. 3*A*). (The protein composition of the peaks, as determined by SDS-PAGE, is shown beneath each figure panel). Addition of low Gαi concentrations leads to formation of a 1:1 Gαi:Pins complex peak (we assigned peaks to 1:1 or 2:1 complexes using Pins mutants with one or two GoLocos inactivated). Higher Gαi concentrations lead to formation of a 3:1 Gαi:Pins complex with a very broad peak, suggestive of a lower affinity interaction. We conclude that full-length Pins contains a single high-affinity Gαi-binding GoLoco and two low affinity GoLocos.

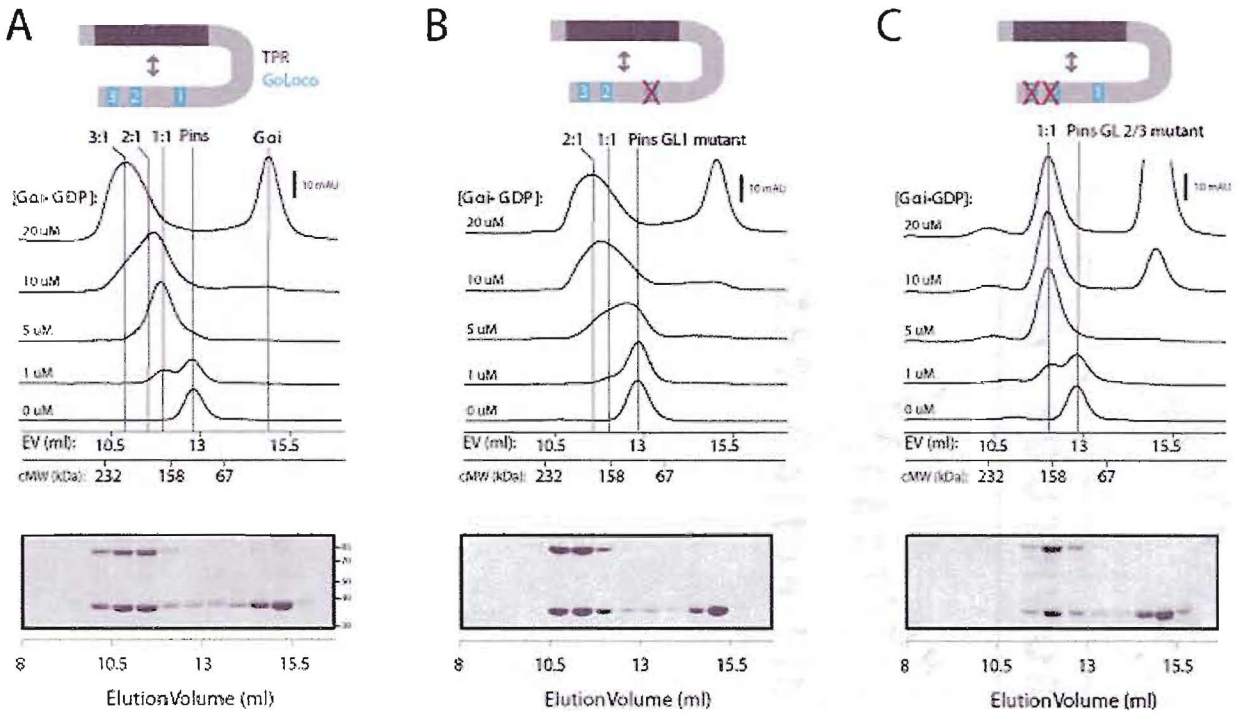

Figure 3. Differential repression of the three GoLocos by the Pins intramolecular interaction (*A*) Analysis of Pins binding to Gαi•GDP by gel filtration chromatography. Pins and mixtures of Pins and Gαi•GDP were separated by gel filtration. Marks indicating the elution volumes of 2:1 and 1:1 Gαi:Pins complexes were determined using single and double Pins GoLoco mutants, respectively. The column elution volumes (EV) of standard proteins (cMW) are shown on the x-axis. The protein composition of the 20 μM Gαi eluate for this and b,c are shown in below each figure panel. (*B*) Analysis of Pins with an inactive GoLoco 1 (Pins Δ GL1) binding to Gαi•GDP by gel filtration chromatography. Loss of GoLoco 1 causes loss of the high affinity peak that occurs at low Gαi concentrations. (*C*) Analysis of Pins with an inactive GoLoco 2 and 3 (Pins Δ GL 2/3) binding to Gαi•GDP by gel filtration chromatography. Only the high affinity interaction remains after loss of GoLocos 2 and 3.

As the three GoLocos are intrinsically equivalent, independent Gαi binding sites (Figure 1*D*), the distinct Gαi binding behavior in full-length Pins suggests that Pins contains one GoLoco domain that is unregulated or only partially regulated by the TPRs and two GoLoco domains that are cooperatively repressed by the TPRs. In order to further explore this model, we inactivated one or more GoLocos by mutating a single critical arginine residue (17) to phenylalanine in the context of full-length Pins. These mutations do not inhibit the ability of the TPRs and GoLocos to interact. Inactivation of GoLoco 1 (Pins Δ GL 1; note use of "Δ" refers to a nonfunctional domain, not deletion) binding to Gαi specifically abolishes the high affinity 1:1 complex (Fig. 3*B*); whereas inactivation of either GoLoco 2 or 3 has no effect on the high affinity complex (unpublished observations). We therefore classify GoLoco 1 as a high affinity GoLoco in the context of full-length Pins. Pins that lacks a functional GoLoco 1 forms a broad 2:1 complex at higher Gαi concentrations, indicating that GoLocos 2 and 3 bind more weakly, but cooperatively to Gαi in the presence of the TPRs. Disruption of GoLocos 2 and 3 (Pins Δ GL 2/3) leads to formation of a 1:1 complex at low concentrations of Gαi, further confirming that GoLoco 1 not repressed by the TPRs (Fig. 3C). We conclude that the TPRs differentially regulate the three GoLoco domains: Gαi shows unregulated high-affinity binding to GoLoco1 and low affinity, cooperative binding to GoLocos 2 and 3.

We next asked how Gαi binding to the different Pins GoLoco domains affects the ability of Pins to bind Mud. When GoLoco1 is specifically inactivated, Gαi can still enhance Mud binding (Fig. 4*A*), in a manner similar to the wild-type Pins (Fig. 2C). The activation is more efficient however, presumably due to the lack of Gαi "buffering" by

GoLoco 1. In contrast, in the Pins Δ GL2/3 mutant, Gαi does not enhance Mud binding (Fig. 4B) even though it binds GoLoco1 with high affinity (Fig. 3*B*). Thus, the Pins TPRs differentially regulate the ability of Gαi to promote Pins-Mud binding: Gαi binding to GoLoco1 has no effect on Pins-Mud binding, whereas Gαi binding to GoLocos 2 and 3 strongly enhances Pins-Mud association.

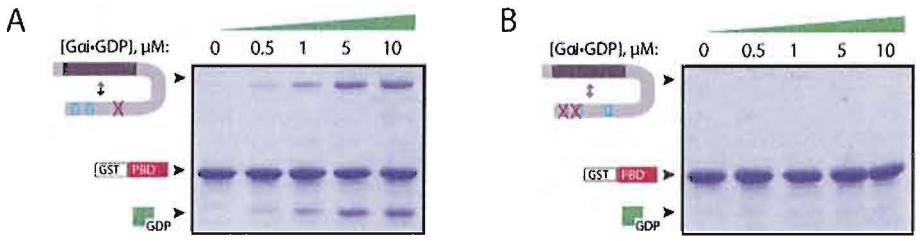

Figure 4. Pins-Mud coupling requires cooperative Gαi binding to GoLocos 2 and 3
(***A***) Cooperative binding of Gαi and Mud to Pins does not require GoLoco 1. In the absence of GoLoco 1, Gαi•GDP enhances the affinity of Pins for Mud. (***B***) GoLocos 2 and 3 are required for cooperative binding of Gαi and Mud to Pins. Although Gαi•GDP can bind to Pins in which GoLocos 2 and 3 are inactivated (panel c), binding does not lead to cooperative Mud binding.

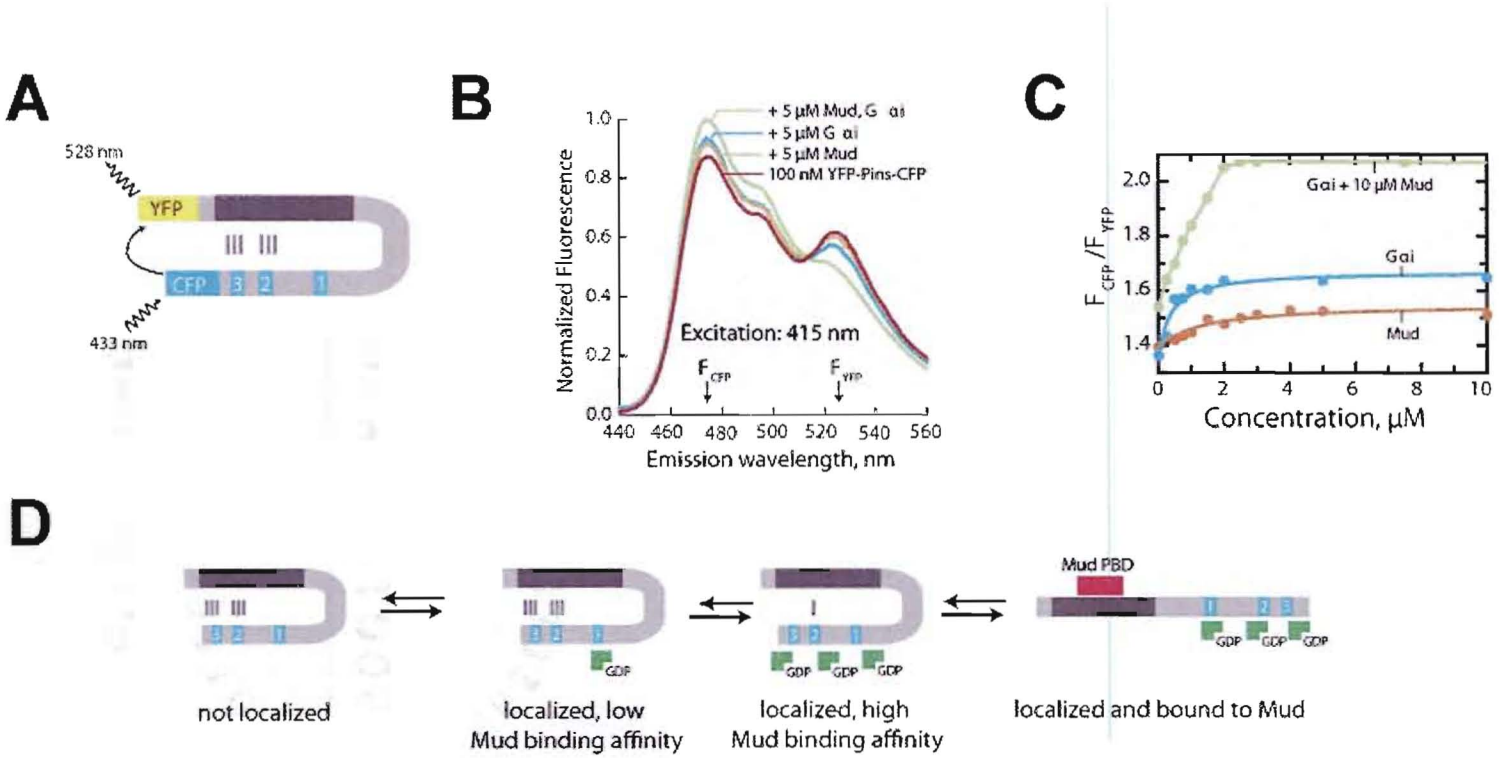

Figure 5. Pins undergoes a conformational change into a high Mud binding affinity state in response to Gαi binding to GoLocos 2 and 3 (*A*) Architecture of a Pins FRET sensor. (*B*) Spectral profile of the Pins FRET sensor in response to Gai and Mud ligand titration. (*C*) Plot of Pins FRET change in response to Gai or Mud ligands added alone or in tandem. (*D*) Model for coupled Gαi•GDP and Mud binding and relationship to Pins conformational states.

The Conformational Transition to "Open" Pins Requires both Mud and Gαi

Our results suggest that Gαi binding to GoLocos 2 and 3 "opens" Pins to allow Mud binding to the TPRs. In order to directly monitor the Pins conformational transition between "closed" and "open" states, we constructed a Pins fluorescence resonance energy transfer (FRET) sensor with YFP and CFP at the NH_2 and COOH termini, respectively (Fig. 5*A*). This type of sensor has been successfully to monitor the conformational transition of a mammalian Pins homolog, LGN (11). Surprisingly, addition of Gαi or Mud alone did not cause a significant change in the YFP-Pins-CFP FRET signal, even at high concentrations (Fig. 5*B*), suggesting that Gαi or Mud alone is insufficient to "open" Pins. The addition of both ligands together, however, leads to a large change in the FRET signal (nearly complete loss of energy transfer) indicating that Mud and Gαi are both required to induce the "open" Pins conformation (Fig. 5*C*).

Because Mud or Gαi alone are not able to "open" Pins, we can exclude a simple model in which Mud and Gαi directly compete in a mutually exclusive fashion (e.g. sterically) with the intramolecular interaction. Although we observe disruption of the Pins TPR-GLR interaction *in trans* (Fig. 2A,B), this is likely to result from effective concentration effects in which the interaction is weaker when the two domains are not in the same polypeptide. We conclude that Mud and Gαi allosterically modulate the TPRs and GoLocos, respectively, in a manner that leaves the intramolecular interaction intact but in a weakened state, poised to open upon binding of the second ligand. Thus, Pins can exist in a "closed" state (no Gαi or Mud bound), a "potentiated" closed state (with Gαi or Mud bound), and an "open" state (with both Gαi and Mud bound) (Fig. 5*D*).

Neuroblasts Expressing Pins with Inactive GoLocos 2 and 3 Fail to Induce Pins-Mud Apical Coupling

To address if Pins with inactivated GoLocos 2 and 3 is able to recruit Mud to the apical cortex of mitotic neuroblasts, we generated UAS-controlled transgenic lines containing either HA:Pins wt or HA:Pins Δ GL 2/3. The localization of ectopic Pins protein was assessed in a *pins* background in 3rd instar larval central brain neuroblasts. Both HA:Pins wt and HA:Pins Δ GL 2/3 showed cortical crescents Fig. 6*A,B*. Metaphase neuroblasts with HA:Pins wt or HA:Pins Δ GL 2/3 apical crescents were then scored for Mud localization. In wild type mitotic neuroblasts, Mud forms an apical crescent (100%, n > 20, reference 22). Similarly, in *pins* mutants expressing HA:Pins wt protein, Mud forms an apical crescent (Fig. 6A, 86%; n = 15). In contrast, in *pins* mutant neuroblasts expressing HA:Pins Δ GL 2/3, Mud does not form an apical crescent (Fig. 6B, 69%; n = 13).

In order to understand how cortically localized and Mud-recruiting Pins states are populated as Gαi accumulates at the apical cortex, we simulated the Pins Gαi response profile based on the parameters described earlier (Fig. 6D). At low Gαi concentration, Pins with Gαi bound to GoLoco 1 predominates because of its higher affinity relative to the other two GoLocos (which are repressed by the TPRs). Although this Pins form does not to bind Mud with high affinity, we hypothesize that it is sufficient to induce aspects of cortical polarity (e.g. Insc polarization). At higher Gαi concentrations GoLoco 1 becomes saturated and binding can occur at GoLocos 2 and 3 allowing for Mud recruitment to the apical cortex (Fig. 3*D*). Thus, we predict that as Gαi

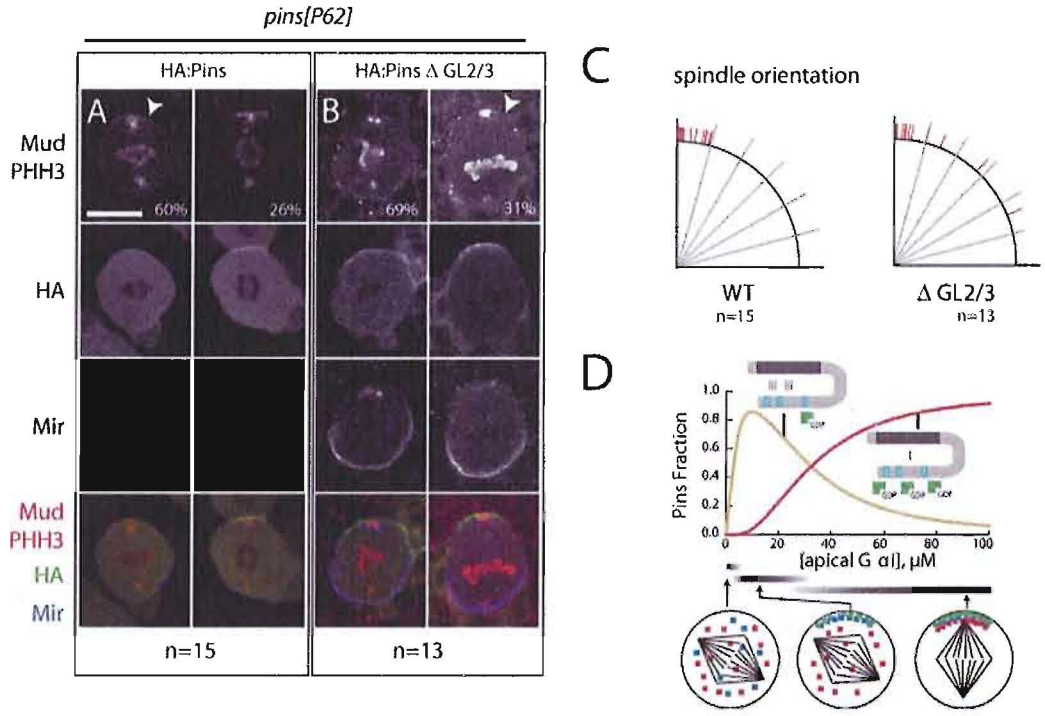

Figure 6. *pins* neuroblasts expressing Pins Δ GL2/3 fail to recruit apical Mud and show defects in metaphase spindle orientation (***A***) *Left and right columns:* Ectopic Pins can rescue Mud localization in *pins* larval neuroblasts *First row:* anti-Mud and anti-phospho H3 antibody (α-PHH3). Moderate (60%) or strong (26%) Mud apical crescents were observed in metaphase neuroblasts. Apical Mud signal was not detectable in 14% of the neuroblasts. Mud spindle association during mitosis is normal and independent of Pins activity. *Second row:* anti-HA antibody was used to detect ectopic protein expression. *Third row:* Miranda was used as a basal marker. Scale Bar, 5μM. (***B***) *Left and right columns:* Transgenic Pins with inactivated GoLocos 2 and 3 fails to rescue Mud apical enrichment in *pins* larval neuroblasts. *First row:* anti-Mud and anti-phospho H3 antibody (α-PHH3). No Mud crescents were observed in 69% of metaphase neuroblasts expressing Pins Δ GL2/3. Weak or moderate Mud crescents were detected in 31% of the population. (***C***) *pins* zygotic mutant neuroblasts expressing ectopic Pins Δ GL2/3 show spindle alignment defects. Quantification of apical spindle pole alignment (red ticks) relative to the center of the Miranda cortical crescent (vertical line). *pins* neuroblasts expressing ectopic wild type Pins have spindles that are tightly aligned, but Pins Δ GL2/3 mutant spindles are frequently misaligned. (***D***) Pins Gαi response profile. The concentration of Pins with Gαi bound at GoLoco 1 and Gαi-saturated Pins is shown as a function of Gαi concentration. This model uses the intrinsic affinity of Gαi for the GoLocos (K_d = 530 nM) and assumes that the inactive form of Pins is favored 1000:1 over active Pins in the absence of Gαi or Mud. Simulations were performed with Berkeley Madonna. Predicted neuroblast phenotypes with the state of cortical polarity (Mud = red, Pins = blue, Gαi = green) and spindle positioning are shown at low, medium, and high, Gαi concentration ranges.

accumulates at the apical cortex, it first recruits Pins in a form that is competent for cortical polarization, but not spindle positioning. As Gαi levels further increase, however, GoLocos 2 and 3 become populated, weakening the intramolecular interaction and freeing the TPRs to recruit Mud to the apical cortex.

Discussion

Through interactions with Gαi and Mud, Pins regulates two fundamental aspects of asymmetric cell division: cortical polarity and alignment of the spindle with the resulting polarity axis. In this study, we have investigated the mechanism by which Gαi regulates Pins interactions with the spindle orientation protein Mud. We have found that, although the three Pins GoLocos are intrinsically equivalent, independent Gαi binding sites, an intramolecular interaction with the Pins TPRs leads to differential Gαi binding. Gαi binding to GoLoco 1 is not coupled to the Pins intramolecular interaction and therefore does not influence Mud binding but is sufficient to localize Pins to the cortex for Mud-independent functions (e.g. recruitment of Insc to the apical cortex). Gαi binding to GoLocos 2 and 3 destabilizes the Pins intramolecular interaction leading to cooperative Mud binding, and together the ligands induce an "open" Pins conformational state. This leads to a model in which Gαi induces multiple Pins activation states: one that localizes cortically but is not competent for Mud binding, and one that binds Mud linking localized Gαi to the mitotic spindle (Fig. 3*D*).

The Pins Intramolecular Interaction as a Mechanism for Localizing Mud Activity to the Cortex

Intramolecular interactions are common features of signaling proteins that typically act through "autoinhibition" of an enzymatic or ligand-binding activity (31). Such interactions allow for coupling of regulatory molecule binding to an increase or decrease in downstream function, a critical aspect of information flow in signaling pathways (32-34). Pins is involved in the regulation of multiple downstream functions and our results support the notion that the multiple Gαi binding sites present in Pins allow for the signal to branch into two pathways, one controlling cortical polarity and the other one spindle positioning. A notable exception to the multiple GoLocos present in Pins-like proteins is the *C. elegans* Pins homologue GPR-1/2 which contains a single GoLoco domain. The lack of multiple GoLocos in GPR-1/2 may be consistent with their more limited role in *C. elegans* asymmetric cell division, where they regulate spindle positioning but not cortical polarity (8-10).

In the model presented here, the Pins intramolecular interaction serves to regulate Mud binding. This may occur for several reasons. First, localization of Mud activity to the apical cortex appears to be important for aligning the spindle with the axis of cortical polarity (5-7). In this context, the Pins intramolecular interaction may be important for restricting Mud activity to the apical cortex. This observation is consistent with previous observations that too little Mud (in *mud* mutant neuroblasts) results in spindle position defects without any rotation (7). Second, Mud activity may be affected by its interaction with Pins. For example, LGN binds to a region of NuMA near its

microtubule binding site such that LGN binding to NuMA competes with microtubule binding (35).

Unequal Regulation of the GoLocos by the TPRs Leads to a Complex Gαi Response Profile

A unique feature of the Pins intramolecular interaction is that autoinhibition is incomplete. Binding of GoLocos 2 and 3 to Gαi is repressed by the TPRs, but binding to GoLoco 1 is not. This has two important consequences. First, whereas the three GoLocos are intrinsically equivalent and independent Gαi binding sites, TPR repression of GoLocos 2 and 3 significantly lowers the affinity of these GoLocos relative to GoLoco 1. This leads to preferential population of GoLoco 1 which may be important for temporal regulation of asymmetric cell division by ensuring that cortical polarity is established before the spindle is positioned. Second, the TPRs appear to repress GoLocos 2 and 3 cooperatively (Gαi binding to 2 or 3 increases the affinity at the other site). Cooperativity is a common property of signaling pathways that is used generate complex input-output profiles (36). Pins exhibits both homotropic (Gαi) and heterotropic (Gαi and Mud) binding cooperativity. In both cases, cooperativity is not an inherent property of the binding sites but is generated through the competition that results from the intramolecular interaction between the TPRs and GoLocos. Such "cooperative repression" of inherently equivalent binding sites through intramolecular interactions may be a general mechanism for generating cooperativity in signaling proteins. The multiple Pins states are due to cooperative binding of Mud and Gαi to Pins, and result in a tight link between apical cortical polarity and mitotic spindle orientation.

Bridge to Chapter III

In the preceding chapter we have shown that the regions of Pins containing the TPRs and GoLocos interact, leading to cooperative "opening" of Pins by Gαi and Mud ligands. We conclude that Pins has multiple functional states – a form recruited by a single Gαi to the apical cortex that is unable to bind Mud, but sufficient to induce cortical polarity; and a form saturated with Gαi that recruits Mud and links cortical polarity to the mitotic spindle. In Chapter III we will detail how modulation of Pins functional states by Gαi and Mud affects the larger network of Pins interaction partners.

CHAPTER III

PINS TETRATRICOPEPTIDE REPEATS EXHIBIT DYNAMIC PROTEIN BINDING SPECIFICITY

Introduction

Pins occupies a central role in neuroblast asymmetric cell division, establishing cortical polarity though interactions with Gαi and orienting the mitotic spindle though binding of Mud (37). Loss of Pins function results in a variety of neuroblast phenotypes, including spindle misalignment and symmetric divisions (38). Both Pins and the mammalian Pins homologue, LGN, engage in a host of apical protein interactions through their tetratricopeptide repeat (TPR) domains (Illustration 2). Pins interaction with Mud, as shown in the previous section, is a simple binding event where one Mud

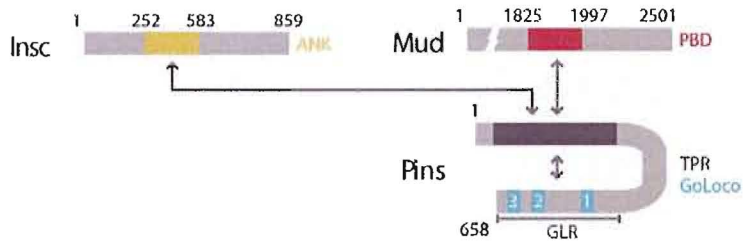

Illustration 2. Pins TPR domains serve as a scaffold for interactions with multiple polarity members. Pins TPR domains (black box) interact with Mud and Inscuteable. The Pins Binding Domain of Mud (red) and the ankyrin repeats of Insc (yellow) are the minimal determinants that confer Pins interaction.

molecule interacts with the TPR array within Pins. We have previously shown that Mud binding to Pins TPRs is negatively regulated by an intramolecular interaction (Chapter II). Relief of this interaction enhances TPR affinity for Mud. One fundamental question stemming from our prior research is if Pins network of interaction partners exhibit regulated Pins binding using the same autorepressive mechanism.

The neuroblast apical protein Inscuteable (Insc) links the cortical polarity established by Bazooka/Par6/aPKC with Mud-Pins-Gαi activity (12). Illustration 2 displays how the Insc-Pins complex forms through Pins TPR domains and a series of five ankyrin-like repeats (subsequently referred to as Insc-ANK) within the Insc molecule (39). The ankyrin region has been shown to be functionally equivalent to the full length Insc molecule in neuroblasts, and is able to interact with Bazooka and apically focus Pins (39). Does Pins intramolecular interaction regulate Inscuteable binding, as in the case of Mud or Gαi, or do Pins and Insc interact in a static, or unregulated manner.

Gαi binding proteins have recently been shown to behave as dynamic scaffolding molecules in photoreceptor complexes (40). I have shown Pins regulates binding to different binding partners during neuroblast division, leading to the question that Pins might act as a dynamic scaffold, switching between apical binding partners in a regulated manner. Underscoring this potential Pins-mediated plasticity, is that asymmetric cell division itself is a complex, dynamic event. Apical proteins are shunted to the neuroblast cortex in early prophase, maintain their localization through metaphase and dissociate in early anaphase. This period represents an eleven-minute

window in which apical proteins must concentrate, perform their prescribed function and then disperse (7). This dynamic process indicates proper spatiotemporal regulation of apical components is critical to inducing cortical polarity and spindle positioning. For instance, Pins TPRs have been shown to bind the Pins GoLoco region, Inscuteable and Mud. A fundamental question is how Pins is able to "select" different ligands out of this sea of binding partners.

TPR Domains Facilitate Interaction Plasticity

Pins TPRs are a short repeating sequence motif, similar in secondary structure to Armadillo (ARM), HEAT, Leucine-Rich-Repeats (LRRs) and Ankyrin domains. This class of domains are characterized by a helix-turn-helix motif which forms a modular unit (Figure 7A). These modules can stack upon one another, forming a superhelical structure with a central binding cleft (Figure 7B) (41). The number of repeats therefore determines the length of the binding cleft. The Drosophila genome contains 47 TPR containing proteins, varying in array length of between 3 to 24 TPR domains. Preliminary NMR studies have concluded that the Pins Binding Domain (PBD) of the Mud protein is likely disordered (Newman, R.; personal communication, 2006). This result is consistent with predicted and observed TPR binding properties; as the central binding core of the TPR array is likely to recognize disordered regions within proteins (41).

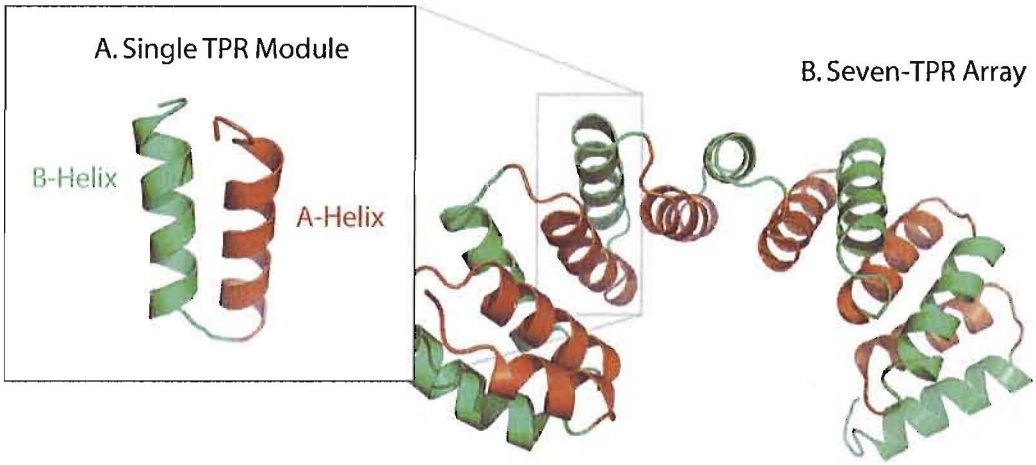

Figure 7. Representation of Pins TPR Structure (***A***) Single TPR domain structure from the 12 TPR domain protein, O-linked GlcNAc transferase (PDB ID: 1W3B) A-helix is colored orange, B-helix in green. (***B***) Molecular structure of an array of seven TPR repeats. The A-helices are positioned toward the interior of a large central binding cleft that can accommodate peptide ligands.

The residues in the TPR A-helix are capable of coordinating interactions with a diverse set of ligands (42). Although there is no published Pins TPR structure, we can gain mechanistic insight into how Pins functions by comparing the structural features of two different TPR containing proteins, O-linked GlcNAc transferase (OGT; PDB ID: 1W3B) and protein phosphatase 5 (Ppp5; PDB ID: 2BUG) (41, 43). Although these two proteins share little overall homology, the overarching structures are remarkably similar. Moreover, both these TPR containing molecules interact with ligands using different sets of residues in their superhelical ampiphathic groove. OGT coordinates ligands using an "asparagine ladder" at positions 3 and 6 in the A-Helix which lines the TPR binding cleft (41). The asparagines are thought to form hydrogen bonds with the peptide main chain in the TPR acceptor in a manner similar to how asparagines in Importin-α recognize

nuclear localization signals (41). Alternatively, acidic residues on Hsp90 interact with basic residues on the A-Helix at positions 2 and 12 of Ppp5 (43). These results suggest solvent-exposed residues in TPRs use electrostatic complementation and hydrogen bonding to interact with different classes of target ligands. Interestingly, Pins contains both an asparagine ladder similar to OGT and an array of basic residues like that of Ppp5 in it's TPRs (Illustration 3). Interestingly, both the Mud PBD and GLR contain both basic and acidic stretches of residues that might serve to complement the asparagines or acidic residues in Pins TPRs. Our research will attempt to dissect the contribution of each of these sets of residues in regulating Pins interactions with Mud and the GLR.

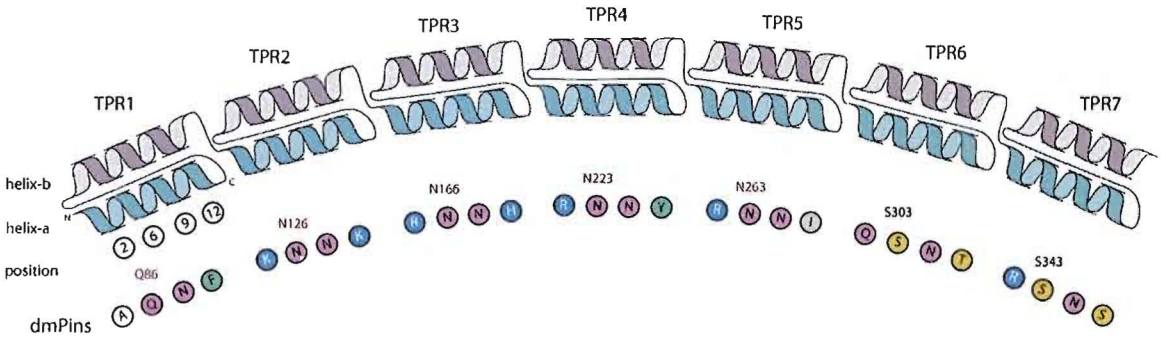

Illustration 3. Pins TPR A-Helix contains two sets of solvated residues Asparagine residues at positions 6 and 9 in Helix-A are denoted in purple. Basic residues which flank the core asparagines at helix positions 2 and 12 are labeled in blue.

Methodology

Protein Expression and Purification

DNA encoding full length *Drosophila* Pins was amplified from an embryonic cDNA library. *Drosophila* Gαi was completely insoluble and therefore mouse Gαi 3 25-354 (which is 76% identical to the *Drosophila* protein) cloned from a macrophage cDNA library was used for these studies. A plasmid containing Mud residues 1825-1997 was generated as previously described (7). *Drosophila* Inscuteable was cloned from an embryonic fly cDNA library. Asparaine to alanine mutations in Pins TPRs were generated by site directed mutagenesis. Pins ORF asparagines mutated to alanine were residues N86, N89, N126, N129, N166, N169, N223, N226, N263, N266 and N306.

All proteins were expressed using the *E. coli* strain BL21(DE3) as a host strain with pGEX 4T-1 based vectors for GST fusions and pBH based vectors for hexahistidine fusions, which were isolated and purified as previously described (28).

Gαi was either used directly after purification, or loaded with GDP or GMPPNP subsequent to purification (GDP-loaded and unloaded behaved identically). Nucleotide was added at a 5-fold molar excess in 10 mM HEPES, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, pH 7.5 and incubated at room temperature for 30 minutes. The final buffer conditions contained 10 mM $MgCl_2$.

In Vitro Binding Assays

GST pull-down assays, were performed as previously described (28). Briefly, ligands were added to a GST/Glutathione agarose mixture at the indicated concentrations to a final reaction volume of 50μL and incubated at room temperature for 15 minutes before washing and elution.

Results

Inscuteable Binding to Pins is Unregulated by Pins Intramolecular Interaction.

In Chapter II we showed that Pins undergoes an allosteric activation in response to Gαi binding which potentiates Mud binding. Here we attempt to assess if Insc-Pins formation is regulated by a similar mechanism. Using a pull down assay we show in Figure 8*A,B* that Pins and Gαi interact specifically with the minimal Mud PBD and Insc-ANK domains and that Mud-Pins binding is responsive to Gαi input. By comparison, Insc-ANK interacts with Pins equivalently in the absence or presence of Gαi (Fig 8C).

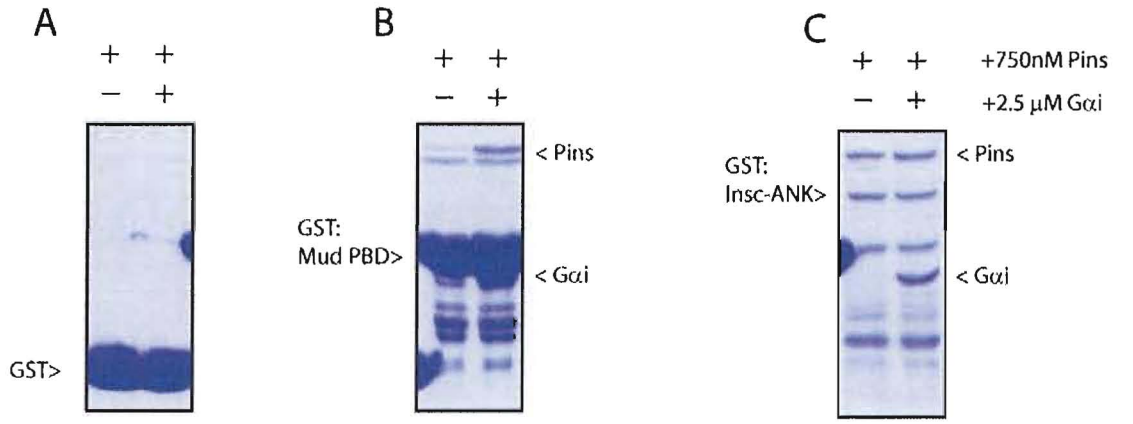

Figure 8. Inscuteable exhibits static binding to Pins (A) 750 nM Pins and 2.5 υM Gαi do not interact with GST alone. (B) As shown in Chapter II, the Pins-Mud interaction is responsive to Gαi input. The band immediately beneath the Pins band is a GST contaminant. (C) Pins exhibits static binding to GST:Insc-ANK in the absence and Presence of Gαi.

Pins Asparagine Ladder Serves to Coordinate Mud binding but not GoLoco Binding

Pins engages in a number of protein interactions though its TPR repeats. Principal among these are an intramolecular TPR-GLR interaction and TPR-Mud interaction. We have shown in Chapter II that the Mud and GLR binding with Pins TPRs are mutually exclusive events *in trans*, and therefore likely share a common binding site. Furthermore, both the Pins GLR and Mud PBD are known to be disordered, flexible proteins, as they are easily degraded by proteolysis, or NMR studies (Nipper, personal observations). This flexible trait and the previously described TPR interaction makes them excellent candidates for being a TPR binding cleft ligand (41).

To assess the role of Pins solvated A-Helix residues in contributing to ligand specificity, we performed a series of alanine scanning experiments to determine what residues were critical for TPR ligand recognition (44). Although these residues have

been posited as "essential" for coordinating TPR-ligand interactions in other proteins, an exhaustive search of the literature failed to demonstrate the requirement of an aspargine ladder for ligand binding. Initially, we mutated each TPR asparagines (Asn) pair separately at residues 6 and 9 to assay the contribution of each individual Asn tandem to foster either GoLoco or Mud binding. These mutations failed to yield any appreciable difference in GoLoco or Mud binding; no individual TPR appeared to specify ligand binding. To assess if different combinations of TPR asparagines contribute to GoLoco or Mud binding, I tested a mutant with both TPRs 3 and 4 having asparagines substituted with alanine. This mutant also behaved identically to wild type TPRs. Finally, we mutated the entire asparagine array within TPRs one though six (GST:TPR, N->A), to generate an asparagine-dead mutant. When we tested the binding of this mutant in a pull down assay, I discovered that Mud-TPR binding was completely abrogated (Fig. 9). Interestingly, the TPR-GLR interaction was maintained at wild type levels in this mutant suggesting that the TPR domains remain properly folded, as GoLoco recognition remains intact.

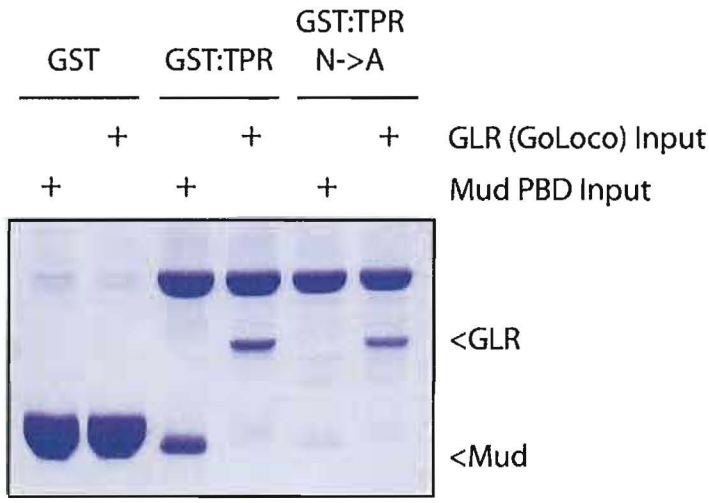

Figure 9. Pins TPR asparagine ladder is essential for Mud but not GoLoco binding. WT Pins TPR bind Mud and the GoLoco region (GLR), forming a TPR-ligand complex. Mutation of the central binding cleft asparagines in six Pins TPRs results in loss of Mud binding while GLR binding remains at WT levels.

Discussion

The finding that the Inscuteable-Pins interaction is independent of Gαi levels is consistent with established data and known Pins behavior. Pins-Insc apical polarity is known to be maintained in the presence of low levels of Gαi in larval neuroblasts (37). Future experiments will be aimed at understanding how other protein complexes formed with Pins, such as Dlg and Lgl are regulated.

I have also demonstrated Pins TPR domains exhibit binding plasticity to cell polarity ligands. We have shown that TPR-Mud interactions require a conserved set of asparagines in Pins. These asparagines residues may serve to coordinate interactions with the Mud PBD. Moreover, these asparagines are not critical for GLR-TPR binding. Future experiments will seek to discern if electrostatic complementation between acidic

residues in the GLR and basic residues in TPRs act to sequester the GoLoco region in the TPR binding cleft. I will also attempt to isolate the binding determinants in the Mud PBD, with an emphasis on attempting to crystallize a TPR-Mud duplex to derive an atomic resolution structure.

Bridge to Chapter IV

In Chapter III I noted the structural determinants in Pins TPRs that influence Mud coupling and noted that Inscuteable binds to Pins regardless of the binding occupancy of the TPRs. In Chapter IV I will summarize our findings and finalize my conclusions on how Pins molecular conformations regulate Mud, Gαi and Inscuteable coupling.

CHAPTER IV

FINDINGS AND FUTURE CONSIDERATIONS

Summary

The research presented within this dissertation explores the molecular function of Pins in aligning the neuroblast mitotic spindle to cortical polarity through interactions with Mud and Gαi. Far from being a monolithic scaffold, which simply aggregates and concentrates proteins adjacent to the cell cortex, I show that Pins displays dynamic binding properties to other apical polarity members. I describe in detail how Pins initially assumes a conformational state with low Mud and Gαi binding capacity, but that Gαi relaxes this conformation, resulting in Pins with robust Mud binding affinity. I also show that the cell polarity protein Inscuteable is a constitutive Pins binding protein, which supports its purported role in genetic studies as a linker between Bazooka-induced polarity and Pins localization. This research also contributes to understanding how the structural elements of the Pins TPR domains are able to recognize and specify binding of either the GoLoco array or Mud proteins.

Future Research Considerations

My immediate plans for expanding the research presented herein are to confirm Inscuteable's role as a linker between Bazooka polarity and Pins polarity. We will

examine Inscuteable distribution in *pins* larval neuroblasts expressing our Pins Δ GL2/3 mutant transgene, which cannot be allosterically activated by Gαi. We expect that Pins and Inscuteable will be colocalized at the apical cortex of mitotically active neuroblasts, but that this axis of polarity will be misaligned with metaphase mitotic spindle alignment. There may also be hyperproliferation defects due to improper fate determinant segregation as seen in *mud* mutant larval brains. These results would buttress our current biochemical data and further strengthen our biochemical model for Pins function at the apical cortex. Subsequent studies will seek to confirm the role of basic residues in Pins TPRs as essential elements for binding Pins GoLoco array. We will also seek to identify resides in Pins TPR Helix-B which confer the Insc-TPR interaction.

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