AUTOINHIBITION AND ULTRASENSITIVITY IN THE Gαi-PINS-MUD SPINDLE ORIENTATION PATHWAY

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Protein-protein interaction networks translate environmental inputs into specific physiological outputs. The signaling proteins in these networks require regulatory mechanisms to ensure proper molecular function. Two common regulatory features of signaling proteins are autoinhibition and ultrasensitivity. Autoinhibition locks the protein in an inactive state through *cis* interactions with a regulatory module until it is activated by a specific input signal. Ultrasensitivity, defined as steep activation after a threshold, allows cells to convert graded inputs into more switch-like outputs and can lead to complex decision making behaviors such as bistability. Although these mechanisms are common features of signaling proteins, their molecular origins are poorly understood. I used the *Drosophila* Pins protein, a regulator of spindle positioning in neuroblast cells, as a model to study the molecular origin and function of autoinhibition and ultrasensitivity.

Pins and its binding partners, Gαi and Mud, form a signaling pathway required for coordinating spindle positioning with cellular polarity in *Drosophila* neuroblasts. I found Pins switches from an autoinhibited to an activate state by modular allostery. Gαi binding to the third of three GoLoco (GL) domains allows Pins to interact with the microtubule binding protein Mud. The GL3 region is required for autoinhibition, as amino acids upstream and within GL3 constitute this regulatory behavior. This autoinhibitory module is conserved in LGN, the mammalian Pins orthologue.

I also demonstrated that Gαi activation of Pins is ultrasensitive. A Pins protein containing inactivating point mutations to GLs 1 and 2 exhibits non-ultrasensitive (graded) activation. Ultrasensitivity is required for Pins function in vivo as the graded Pins mutant fails to robustly orient the mitotic spindle. I considered two models for the source of ultrasensitivity in this pathway: cooperative or "decoy" Gαi binding. I found ultrasensitivity arises from a decoy mechanism in which GLs 1 and 2 compete with the activating GL3 for the input, Gαi. These findings suggest that molecular ultrasensitivity can be generated without cooperativity. This decoy mechanism is relatively simple, suggesting ultrasensitive responses can be evolved by the inclusion of domain repeats, a common feature observed in signaling proteins.

This dissertation includes previously published and unpublished co-authored material.

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CHAPTER I

INTRODUCTION

NETWORKS OF SIGNALING PROTEINS IMPLEMENT CELLULAR DECISIONS

Living organisms are composed of different cell types that are continuously making important cellular decisions such as whether to proliferate, differentiate, migrate or self-destruct. A fundamental question in cell biology is how do these cells know what to do? Cells are fed a multitude of informative signals (inputs) from their environments and translate them into specific biological responses (outputs). How do cells process all of these signals and translate them into the correct physiological outcome?

To accomplish the arduous task of signal processing, signal transduction pathways have evolved to direct the flow of cellular information. These pathways are largely composed of modular signaling proteins; proteins containing multiple catalytic or protein-protein interaction domains linked in the same polypeptide in a cassette-like fashion (Pawson and Nash, 2003). These proteins change their cellular localization, enzymatic activities or protein binding partners in response to specific signals (Kholodenko, 2006). Changes in protein activities trigger downstream signaling events and eventually lead to a specific response. As the precise integration of cellular information is critical for organismal growth, development and homeostasis, cell biologists have strived to understand how these networks of signaling proteins process

information with extreme precision in the complex environment of the cell (Pawson and Nash, 2003).

Recent studies have highlighted the importance of the modular architecture of signaling proteins in mediating biological functions (Lim, 2002). Cells use a limited number of interaction modules to give signaling proteins specific functions required for translating environmental inputs (Pawson and Nash, 2003). Modularity allows cells to create new signaling behaviors by swapping protein interaction or catalytic domains through recombination events to couple new inputs with different outputs (Dueber et al., 2004). This provides an evolutionary platform to evolve diverse signaling pathways for more efficient signal integration and complex decision-making behaviors. Instead of evolving new genes to mediate different cellular functions, it appears that cells have used modular recombination to create new signaling proteins with novel functions (Pawson and Nash, 2003; Peisajovich et al., 2010). This idea was tested by re-wiring a protein switch to respond to non-native inputs (Dueber et al., 2003) and has been used to reprogram behavior in living cells (Yeh et al., 2007; Peisajovich et al., 2010).

REGULATING SIGNALING OUTPUT THROUGH AUTOINHIBITION

In order to faithfully integrate environmental signals, cells need to ensure that signaling pathways are kept in an "off" state until the correct input has been sensed (Lim, 2002). Misregulation of signaling pathways is often associated with abnormal cellular behavior and disease (Pawson and Nash, 2003). The observations that signaling networks process cellular inputs with high fidelity and that these cellular decisions are

robust implies that signaling proteins have evolved mechanisms to regulate their activities. Through the platform of protein modularity has come a solution to the problem of maintaining an "off" state and transitioning to an "on" state. A common feature of modular signaling proteins is their ability to self-regulate their activities (Dueber et al., 2004). As these modules rarely behave like "beads on a string," the functions of the individual domains are often linked through allosteric mechanisms in which intramolecular interactions between modules can limit signaling output, a regulatory feature known as autoinhibition (Pufall and Graves, 2002). Autoinhibition is defined as the presence of a domain or region within a protein that represses its output activity through an intramolecular interaction. Autoinhibition can be identified if an output domain displays activity when in isolation, but is repressed when present in a folded protein (Pufall and Graves, 2002). This regulatory feature has been described in biological processes such as cell cycle progression, cell polarity, dynamic cytoskeletal rearrangements and kinase cascades regulating growth and differentiation (Lim, 2002). Not only does this solve the problem of ensuring a protein's signaling activity is regulated, but it provides a molecular framework for activating its activity by a specific input signal. The modular architecture of signaling proteins allows for coupling specific inputs into activation if the autoinhibitory region is displaced by an input (Lim, 2002). For instance, autoinhibitory element is often a protein interaction domain. Thus, if ligand binding to this domain disrupts the autoinhibitory interaction, the output is triggered by that specific input. For example, the tumor suppressor Discs large (Dlg) has an autoinhibitory interaction between its SH3 and GK domains that blocks association of the

GK ligand GukH (Qian and Prehoda, 2006). A PDZ domain N-terminal to this region can relieve autoinhibition of SH3-GK depending on its ligand bound state (Marcette et al., 2009). This regulatory feature of Dlg is necessary for proper organismal development (Newman and Prehoda, 2009). Because autoinhibitory mechanisms are critical regulatory features in cell signaling pathways, elucidating the molecular mechanisms at their core is of high importance in the cell signaling field and could lead to potential disease therapies or for reprogramming cellular behavior (Lim, 2002).

AUTOINHIBITION CAN LEAD TO COMPLEX SIGNALING BEHAVIORS

Not only is it important for cells to be able to regulate the "on" and "off" status of signaling pathways, it is also critical that the transition between states occurs correctly; i.e. that the cell senses the signal properly (Tyson et al., 2003). The pathway response profile, defined as the amount of pathway output as a function of input, must correlate with the cellular process the pathway facilitates (Kholodenko, 2006). While some pathways exhibit simple dose-dependent responses, resembling a hyperbolic curve (graded response), others show more complex behaviors such as thresholding and steep activation (i.e. sigmoidal activation curves, (Kim and Ferrell, 2007)). Thresholding can be important for preventing spurious activation in the presence of biological noise while steepness allows cells to respond robustly in the presence of limiting input signal (Illustration 1, (Ferrell, 1999)). Studies of the evolutionarily conserved mitogen activated protein kinase (MAPK) cascade highlight the importance of these input/output relationships. The MAPK cascade has been shown to display either graded or sigmoidal

responses depending on the cellular process that it governs. In the yeast mating pathway, the amount of mating output as a function of mating pheromone exhibits graded signaling behavior (Takahashi and Pryciak, 2008). This is hypothesized to allow yeast to mate efficiently with distant partners because there is no threshold to filter out weak signals and allows them to elongate in the direction of the pheromone, increasing the signal concentration until contacting its partner to form a shmoo (Hao et al., 2008). In the irreversible cell fate decision of *Xenopus* oocyte maturation, the amount of pathway output as a function of progesterone is steeply sigmoidal, such that it is essentially an all or none response (Huang and Ferrell, 1996). This makes the decision to differentiate into a mature oocyte decisive and irreversible after a threshold, to ensure the oocyte does not differentiate prematurely or only partially mature (Ferrell and Machleder, 1998). Thus, it is critical for cells to be able to modulate pathway input/output relationships depending on the cellular context.

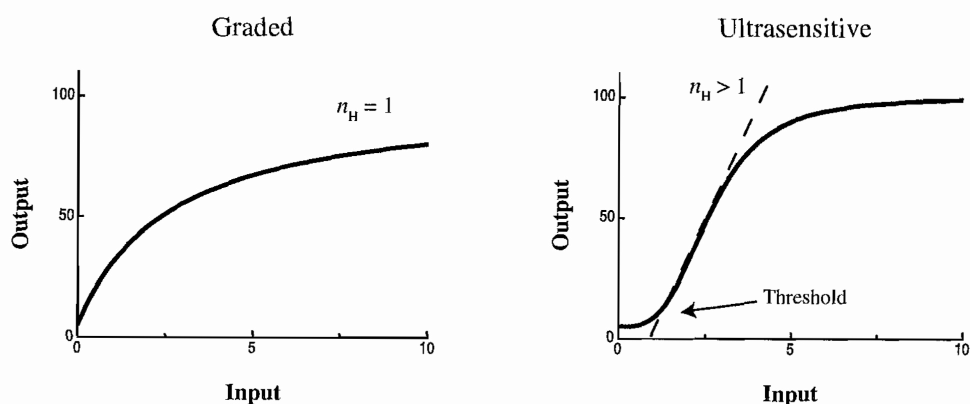

Illustration 1: Examples of graded (left, $n_H = 1$) and ultrasensitive (right, $n_H > 1$) pathway response curves.

ULTRASENSITIVITY IN CELL SIGNALING PATHWAYS

Sigmoidal responses are indicative of signaling behavior known as ultrasensitivity. Ultrasensitivity allows signaling proteins to behave like molecular switches to toggle between off and on more decisively than a protein with a hyperbolic (graded) response curve. The term ultrasensitivity was coined by Goldbeter and Koshland in 1981 after they described how the kinetics of phosphorylation/ dephosphorylation cycles could generate dramatic activation of kinase cascades under certain physiological conditions (Goldbeter and Koshland, 1981). This sharp transition between the inactive and active kinase was sigmoidal, resembling the behavior of an allosteric enzyme with a large Hill coefficient (n_H). Thus, the measure of ultrasensitivity is the apparent Hill coefficient obtained when the response profile is modeled with the Hill equation. Any input/output curve with $n_H > 1$ is deemed ultrasensitive, while a hyperbolic curve will always have $n_H = 1$ (Goldbeter and Koshland, 1984). Goldbetter and Koshland reasoned that this behavior could allow for signal amplification in kinase cascades such that their response to stimuli would be more sensitive after a threshold than a graded response. This idea has withstood the test of time as an increasing body of evidence has demonstrated the importance of ultrasensitive behavior in cell signaling pathways as it allows cells to translate analog information into a more digital response (Kholodenko, 2006). Ultrasensitive behavior of signaling proteins seems to underlie many cellular decisions as it allows for generating more complex decision making behaviors such as bistability (an "all or none" response) and hysteresis, the basis for cellular memories (Burrill and Silver, 2010; Tyson et al., 2003). Although it is well

established that ultrasensitivity is a common feature of signaling pathways, the molecular mechanisms responsible for this behavior are poorly understood. A fundamental question in the cell signaling field is how is ultrasensitivity built into cell signaling pathways?

THE MAPK CASCADE AS A MODEL SYSTEM FOR STUDYING ULTRASENSITIVITY

Two well-characterized ways to incorporate ultrasensitive regulation into signaling pathways is through feedback loops or cooperativity (Kholodenko, 2006). Feedback loops (either positive or double negative) can lead to the observed threshold as the pathway response ramps up with increasing signal concentration, and drives the pathway toward increased activity, leading to a steep transition from off to on (Bashor et al., 2008). Cooperativity, either by input binding having a positive effect on subsequent binding events (i.e.: hemoglobin, Hill, 1910) or by synergistic activation through binding different inputs (additive cooperativity (Goldbeter and Koshland, 1981), or heterotropic cooperativity (Prehoda et al., 2000)) can also yield ultrasensitive responses. This mechanism would make the pathway response more sigmoidal as thermodynamic parameters dictate thresholding and steepness. However, these are not the only documented sources of ultrasensitivity.

The most well studied case of ultrasensitivity is the MAPK cascade. Using *Xenopus* oocyte maturation as a model system, J. E. Ferrell and colleagues demonstrated the molecular basis for the bistable signaling nature of this developmental transition. Oocytes sense the hormone progesterone and rapidly activate the MAPK cascade to

differentiate once a threshold amount of hormone is sensed (Justman et al., 2009). They showed through biochemical and theoretical studies that this switch occurs decisively partially due to a positive feedback loop in the system. However, they noted that the feedback loop was not sufficient for the degree of steepness observed in the transition. Also important was that each step of kinase cascade exhibited sigmoidal activation, i.e. the kinases behaved as ultrasensitive molecular switches ((Huang and Ferrell, 1996), Illustration 2). This behavior arises from a multisite phosphorylation mechanism in which phosphorylation sites (inputs) not coupled to output compete with a key activating site for the upstream activating kinase (Ferrell and Machleder, 1998). Assembling these sigmoidal curves in a cascade has an additive effect on the overall steepness of the transition, leading to dramatic pathway activation (Ferrell, 1997). While multisite phosphorylation does not lead to ultrasensitivity in all systems (Gunawardena, 2005; Pufall et al., 2005), this mechanism has also been described in other biological processes such as cell cycle progression (Nash et al., 2001; Kim and Ferrell, 2007; Salazar and Hofer, 2006). These studies highlight the importance of signaling switches with ultrasensitive behavior at the molecular level. Given that signal transduction networks are largely composed of binary protein-protein interactions, which yield graded responses, how can molecular ultrasensitivity be achieved? Are mechanisms other than cooperativity, which would required thermodynamic coupling of input domains, a complex property to evolve in modular signaling proteins, possible for generating sigmoidal responses? To address these questions as well as sources of autoinhibition, I

used the *Drosophila* Partner of Inscuteable (Pins) protein as a model to study the potential molecular mechanisms of these two regulatory features.

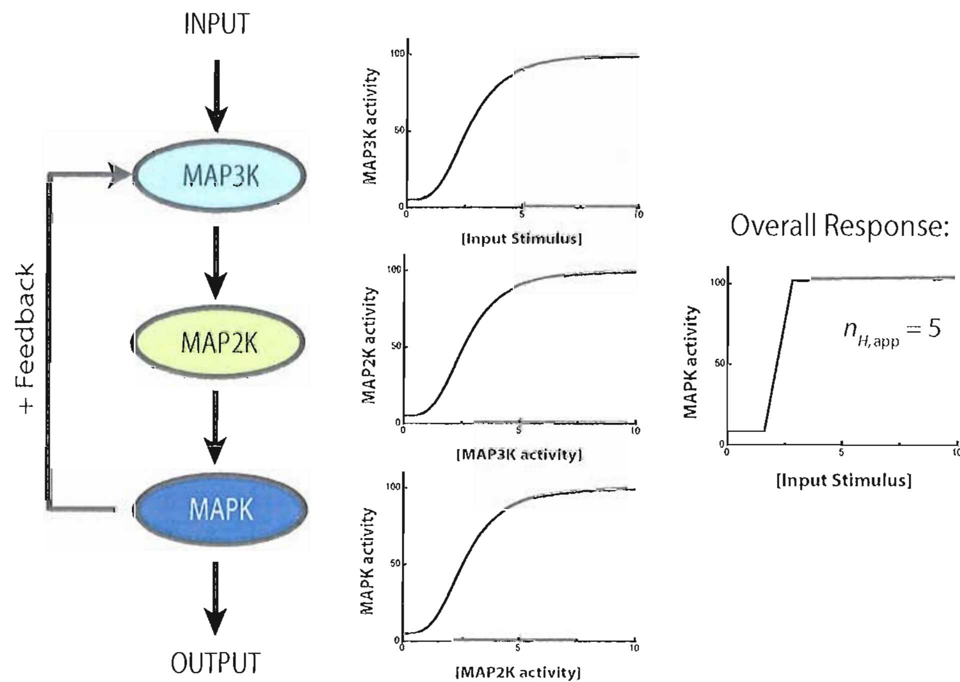

Illustration 2: Molecular ultrasensitivity in the MAPK cascade

The overall input/output relationship of the MAPK cascade for *Xenopus* oocyte maturation is all-or-none from a positive feedback loop and because each kinase displays sigmoidal activation at each step of the pathway. Adapted from Ferrell, 1999.

DROSOPHILA NEUROBLASTS AS A MODEL FOR MECHANISMS OF ASYMMETRIC CELL DIVISION

The Pins protein is a regulator of spindle alignment in *Drosophila* neuroblasts, a type of stem cell required for development of the fly central nervous system (Yu et al., 2006). Neuroblasts are an ideal model system for studying asymmetric cell division, an

evolutionarily conserved mechanism used to generate cellular diversity (Gonczy, 2008). Neuroblasts divide asymmetrically in cell size and fate such that the larger apical cell remains a stem cell and continues to divide asymmetrically, while the smaller basal ganglion mother cell differentiates into neurons or glia (Doe, 2008). Asymmetric divisions require a cell polarization step in which cell fate determinants are segregated to opposite cortical domains. The mitotic spindle is aligned along the polarity axis ensure these fate determinants are properly inherited by each daughter cell (Siller and Doe, 2009). Because spindle misalignment leads to expansion of the stem cell pool, an overgrowth phenotype similar to those observed in cancer, understanding how spindle orientation is regulated is of fundamental importance to cancer cell biology (Cabernard and Doe, 2009).

PINS IS A KEY REGULATOR OF SPINDLE POSITIONING

The protein Pins was first identified in a screen for genes that disrupted spindle alignment in neuroblast cells (Yu et al., 2000). Pins was shown to associate with Inscuteable (Insc), which was originally thought to be the master regulator of spindle positioning, because overexpression of Insc in epithelial cells caused the spindle to be rotated 90° relative to the normal division axis (Kraut et al., 1996). However, proteins acting downstream of Insc were unknown. Further genetic studies revealed roles for Gαi, the alpha subunit of heterotrimeric G-proteins (Yu et al., 2003), and the mushroom body defect (Mud) protein in spindle alignment (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006). Neuroblasts with loss of function mutations to these genes show spindle

alignment and subsequent cellular fate defects. Gαi, Pins and Mud co-localize at the apical cell cortex and can interact in vivo suggesting that Gαi, Pins and Mud form a signaling complex required for proper alignment of the mitotic spindle with the cell polarity axis ((Siller et al., 2006), Illustration 3). The orthologous proteins in the nematode *C. elegans* (GOA1, GPR1/2 and Lin-5 (Colombo et al., 2003; Gotta et al., 2003; Srinivasan et al., 2003)) and mammalian neural progenitors (Gαi, LGN and NuMA (Du et al., 2001; Morin et al., 2007)), other model systems used to study asymmetric cell division, have analogous roles in spindle orientation, suggesting evolutionary conservation of this pathway. Although the gene products involved were identified, it was unclear how the function of this pathway was regulated.

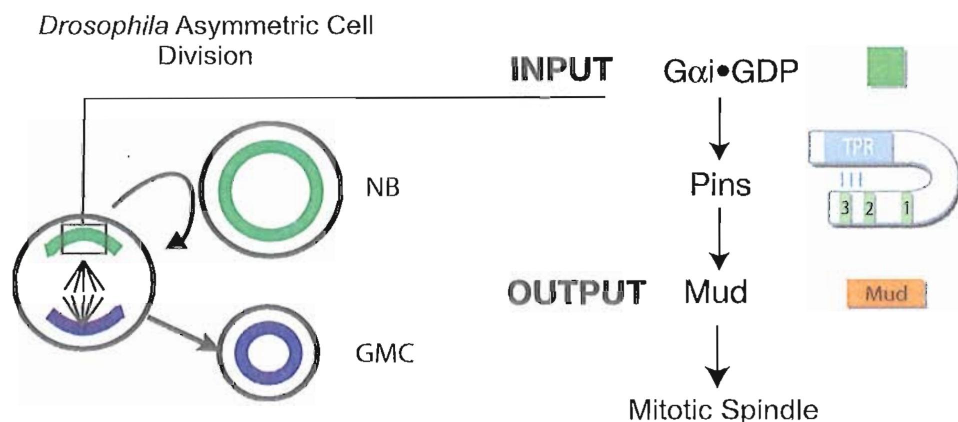

Illustration 3: Proper neuroblast asymmetric division requires spindle-cortex coupling through apical Gαi-Pins-Mud

(Left) Neuroblasts divide asymmetrically in both cell size and fate to self-renew and give rise to the smaller GMC fated to differentiate. (Right) Apical Gαi-Pins-Mud align the spindle along the apical/basal cell polarity axis.

PINS IS A MODULAR SIGNALING PROTEIN

As with many signaling proteins, Pins has a modular domain architecture that hints at its molecular function. Pins contains seven tetratricopeptide repeats (TPRs) in its N-terminal half and three GoLoco domains in the C-terminal region. TPRs are alpha-helical domains that form a super-helical surface for mediating protein-protein interactions (Blatch and Lassle, 1999). GoLoco domains (GL) bind specifically to GDP-bound Gαi had previously been described as modulators of canonical G-protein signaling (Kimple et al., 2002b). This domain architecture is conserved in the mammalian Pins orthologue, LGN, consisting of seven TPRs and four GLs (Du and Macara, 2004). The C-terminal GLs interact with membrane associated Gαi, recruiting Pins to the apical cortex (Yu et al., 2002). Mud is transiently recruited to the apical membrane during mitosis through direct interaction with the N-terminal TPRs (Nipper et al., 2007). Formation of this complex at the apical cortex is required for spindle alignment (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006). This prompted the question: do these modular protein interaction domains behave like beads on a string or is complex assembly regulated?

PINS IS AN AUTOINHIBITED MOLECULAR SWITCH

Nipper and colleagues sought to determine how spindle orientation was achieved through Gαi, Pins and Mud. The authors showed that the N-terminal TPR domains of Pins could interact with Mud in isolation, but not in the context of full-length Pins, indicating Pins is autoinhibited. They demonstrated autoinhibition is relieved through a

modular allosteric mechanism when Gαi associated with the Pins GLs. It remained unclear which regions of Pins were required for repressing the TPRs. The authors also noted that the Pins output (Mud binding) as a function of input (Gαi concentration) appeared to occur rapidly after a threshold suggesting ultrasensitivity in the pathway. In this dissertation, I describe how I used biochemical and cell biological methods to probe the molecular mechanisms responsible for both autoinhibitory and ultrasensitive regulation of Pins. Chapter II describes the Pins autoinhibition and contains previously published and unpublished co-authored material. Chapter III details ultrasensitive Pins regulation and contains previously unpublished co-authored material.

BRIDGE TO CHAPTER II

In the preceding chapter, I described the principle that networks of modular signaling proteins translating cellular information into biological responses. The modular architecture of signaling proteins can allow for evolving new signaling behaviors through recombination of signaling domains. This principle also seems to be important in evolving regulatory mechanisms such as autoinhibition and ultrasensitivity. In the next chapter, I describe how I identified the autoinhibitory region of Pins, show that this region is conserved in mammalian systems and speculate on why autoinhibition is an important feature of the Gαi-Pins-Mud spindle orientation pathway.

CHAPTER II

IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF A CONSERVED PINS AUTOINHIBITORY MECHANISM

*This chapter contains previously published co-authored material taken with permission from:

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INTRODUCTION

Cellular inputs are coupled to specific physiological outputs through networks of dynamically interacting signaling proteins. These proteins allow cellular information to flow either by changing enzymatic activity through post-translational modification,

cellular localization or by associating with different binding partners (Pawson and Nash, 2003). The high degree of fidelity with which these signaling proteins perform their respective functions is largely attributed to the regulatory mechanisms that are built into them. Their activities must be tightly regulated to ensure a certain cellular response does not occur without the corresponding input (Lim, 2002). An emerging theme among signaling proteins is that they often self-regulate their activities through autoinhibition (Pufall and Graves, 2002). Autoinhibition maintains these proteins in an inactive state to keep their output at a basal level. Once a specific input or set of inputs is detected, the protein then adopts an active state and triggers downstream signaling events (Dueber et al., 2004). Understanding the molecular mechanisms responsible for autoinhibition is of increasing importance to cell biology to determine how these behaviors have evolved. In this chapter, I describe using the *Drosophila* Pins protein, a regulator of spindle orientation in neuroblast cells, as a model for determining potential molecular mechanisms leading to autoinhibition.

Drosophila neuroblasts are stem cells required for development of the fly central nervous system. Neuroblasts are an ideal model system for studying asymmetric cell division, an evolutionarily conserved mechanism for generating cellular diversity from a single cell type (Doe, 2008). Neuroblasts divide asymmetrically in both cell size and fate to self renew and give rise to a cell that will differentiate into neurons or glia. In order to specify different daughter cell fates, neuroblasts segregate cellular fate determinants to opposite sides of the cell and align their mitotic spindles along this polarity axis to ensure proper partitioning of these protein complexes into each daughter cell (Illustration 3, (Yu

et al., 2006)). Because spindle misalignment can lead to cellular fate defects, understanding how the spindle is positioned relative to the cell polarity axis is important (Cabernard and Doe, 2009). Genetic data have determined a role for the proteins Pins (for Partner of Inscuteable), the alpha subunit of heterotrimeric G-proteins (Gαi), and the microtubule associated mushroom body defect (Mud) in spindle orientation (Siller et al., 2006; Yu et al., 2003; Yu et al., 2000). These three proteins are co-localized at the apical cortex of mitotic neuroblasts and interact in vivo. It was previously unknown how formation of this complex was regulated (Nipper et al., 2007). Nipper and colleagues showed that Pins undergoes an autoinhibitory intramolecular interaction between its N-terminal tetratricopeptide repeats (TPRs) and C-terminal GoLoco (GL) region that repressed Mud binding to the TPRs. The authors went on to show that Gαi binding to the GLs causes Pins to be activated and allows for Mud association. However it was unclear what elements of Pins were required to mediate this autoinhibition and the importance of this regulatory feature on Pins function in vivo. In this chapter, we describe how we identified the region of Pins required for regulating Mud association to the TPRs and show that this autoinhibitory module is conserved in the mammalian Pins orthologue, LGN. We identify a Pins mutant in which autoinhibition is lost and speculate on its behavior in vivo on spindle orienting function.

METHODS

Protein expression and purification

cDNAs encoding *Drosophila* Pins, mouse Gαi3 (25-354), Mud/NuMA fragments and LGN were subcloned into pBH or pGEX-4T-1 based expression vectors for 6x-histidine or GST-tagged proteins, respectively. Proteins were expressed in *E. coli* and isolated by Ni-NTA agarose affinity chromatography (for his-tagged proteins), anion exchange chromatography and/or size exclusion chromatography (for FRET proteins). Purified protein stocks were stored in binding buffer (20mM HEPES pH 7.5, 100mM NaCl, 1mM DTT and 1mM $MgCl_2$). Protein concentrations were determined by Bradford assay.

Construction and analysis of Pins FRET proteins

A pBH vector with EYFP and ECFP cDNAs was constructed with restriction enzyme sites between the fluorophore open reading frames to create an inframe-fusion of YFP-Pins-CFP. FRET proteins were expressed and purified as described above. 200nM FRET protein was incubated in binding buffer in the presence of Gαi and/or Mud. The FRET ratio (F_{CFP}/F_{YFP}) was determined by exciting the donor CFP at 433nm and observing the emission of CFP at 475nm and the acceptor YFP at 525nm. As a control, FRET proteins were incubated at room temperature for 15min in the presence of 1nM trypsin to cleave Pins and caused a reduction in FRET signal similar to CFP alone (Figure 1B, C). Experiments were conducted on an ISS PC1 spectrofluorometer.

GST pull-down assays

pGEX 4T-1 based expression vectors containing cDNA encoding for GST-fusions of Mud-B isoform residues 1931-1967 and NuMA residues 1889-1913 were generated as described in Newman et al., 2010). Proteins were expressed as described earlier.

GST pull-down assays were performed as described in Nipper et al., 2007. Briefly, glutathione agarose beads were coated with GST-fusions of Mud or NuMA. Pins was incubated at 5μM input in GST pull-down buffer (20mM HEPES pH 7.5, 100mM NaCl, 1mM DTT, 5mM $MgCl_2$ and 0.5% Tween 80) to a total reaction volume of 100μL. Gαi was titrated in at the specified concentrations and proteins were incubated for 15min at room temperature before washing, eluting and analysis by SDS-PAGE.

RESULTS

Pins adopts an inactive "closed" state and requires both Gαi and Mud for "opening"

The observations that the Pins TPRs and GLs can interact in *trans,* and this interaction competes with ligand binding suggested that the GL region folds back on the TPRs to inhibit association with Gαi and Mud (Nipper et al., 2007). To test this model, I created a Pins FRET biosensor consisting of a YFP-Pins-CFP fusion protein to monitor the conformational state of Pins in the absence or presence of ligands (Figure 1A). Pins

exists in a high FRET state as the FRET ratio ($F_{donor}/F_{acceptor}$) is less than the trypsin digest control (Figure 1B). Addition of either Gαi or Mud has little effect on the overall FRET ratio, while addition of both ligands causes Pins to undergo a large conformational change to a low FRET state, consistent with "opening" (Figure 1B). Transitioning to the open state requires Gαi binding at GLs 2 and/or 3 because this FRET change was not observed when these two domains were inactivated by point mutation such that they could no longer bind Gαi (Figure 1C, R570F, R631F respectively, (Adhikari and Sprang, 2003)). We conclude that Pins is locked in an autoinhibited "closed" conformation and transitions into an active "open" state when bound to both Gαi and Mud. Opening requires Gαi binding at GLs 2/3 suggesting that these sites are coupled to Pins activation (Figure 1D). We hypothesize that the "open" Pins, bound to Gαi and Mud, is active in spindle orientation (Nipper et al., 2007, Du and Macara, 2004).

Pins output is repressed by an autoinhibitory mechanism

We next sought to identify elements of Pins required for maintenance of the autoinhibited state with the goal of creating mutant Pins protein that has lost this regulatory feature. In order to achieve this goal and to confirm the results from Nipper et al., 2007, we used a similar GST pull-down assay to test for Mud repression and Gαi activation using a GST-Mud fusion protein containing the minimal TPR interacting sequence of Mud (aa 1931-1961, Newman, et al., 2010, Figure 2A). While the Pins TPR domains alone are unregulated and robustly interact with GST-Mud (Nipper et al., 2007), full-length Pins is unable to interact with the GST-Mud as evidenced by a lack of Pins

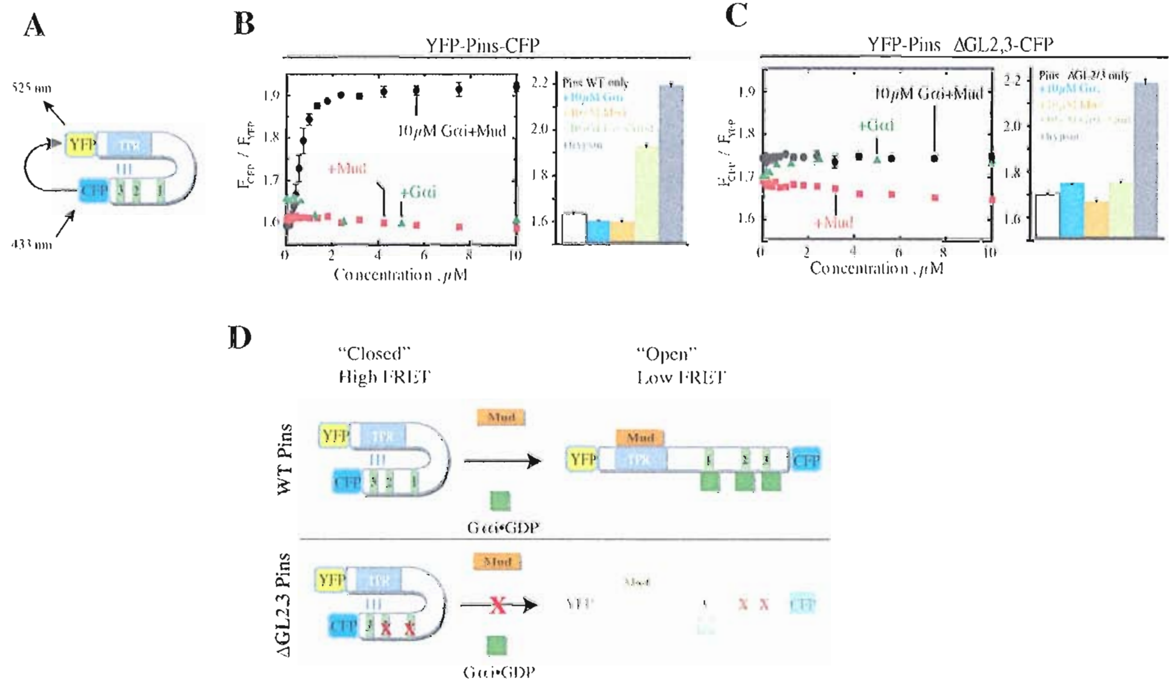
Figure 1: Pins exists in a repressed, "closed" conformational state and requires both Gαi and Mud to adopt an active, "open" state

(A) Molecular diagram of a YFP-Pins-CFP FRET fusion protein used to monitor the conformational state of Pins. FRET is measured by exciting the donor CFP at 433nm and observing the ratio of emission of the donor to the acceptor YFP at 525nm.

(B) Left: The Pins FRET ratio (F_{CFP}/F_{YFP}) was monitored in the presence of increasing amounts of Gαi, Mud or Gαi + Mud. Initially Pins exists in a closed, high FRET state. Addition of either Gαi or Mud to the system causes little change in the Pins conformation. Addition of both ligands causes Pins to adopt and open, low FRET state. Right: Bar graphs denote initial FRET ratio for Pins alone (white), Pins + 10μM Gαi (cyan), Pins + 10μM Mud (orange), Pins + 10μM Gαi and Mud (green) and a trypsin digest control (gray). Error bars represent the standard deviation from the mean of three independent experiments.

(C) The ΔGL2,3 Pins double mutant FRET protein does not undergo the opening observed in WT Pins. ((B) and (C) taken from Nipper et al., 2007 with permission).

(D) Molecular model showing Gαi and Mud disrupt the intramolecular interaction to "open" Pins. Opening requires Gαi binding to GLs 2 and/or 3 as the ΔGL2,3 Pins does not adopt the open, low FRET state.



coming down into the solid phase. At higher concentrations of Gαi input, Pins associates with GST-Mud and is pulled down (Figure 2B, a Pins specific band appears). We conclude that Pins is autoinhibited and requires Gαi for relieving repression of the Pins TPRs to trigger Mud binding output (Nipper et al., 2007).

The GL3 domain couples Gαi input to Pins output

Because autoinhibitory elements are often coupled to input domains, we wanted to test which GL(s) of Pins are required for activation before searching for the inhibitory region. Pins contains three GL domains equally capable of interacting with the input signal Gαi. Which of these GLs are required for the observed Gαi activation of Pins?

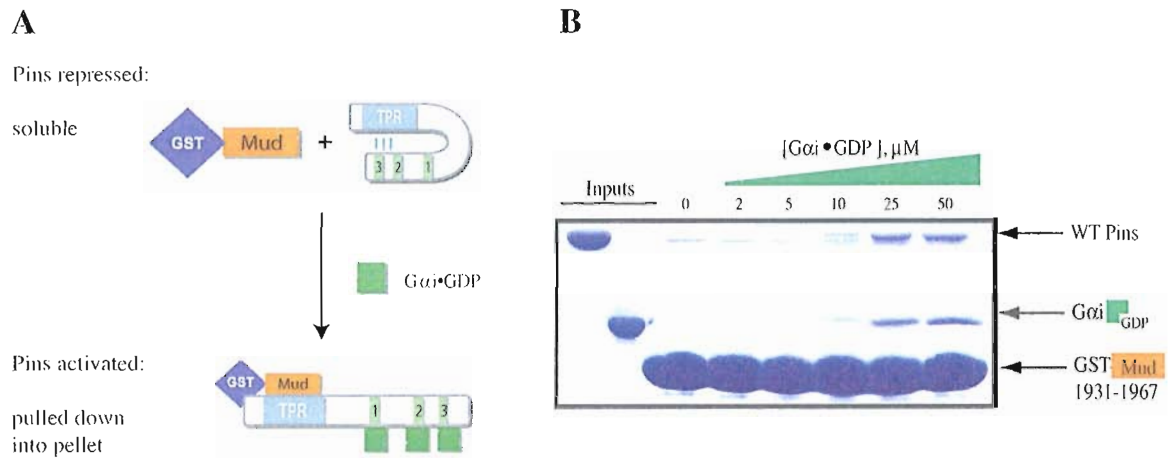


Figure 2: Pins is autoinhibited and requires Gαi for activation

(A) Schematic of the allosteric activation GST pull-down assay used to demonstrate Pins autoinhibition and Gαi activation, originally described in Nipper et al., 2007. Pins undergoes an autoinhibitory intramolecular interaction between the N-terminal TPR domains and the C-terminal GoLoco region. Because the Pins TPRs are repressed, Pins does not interact with a GST-Mud fusion protein and remains in the soluble phase. Addition of Gαi activates Pins by binding the GoLoco domains, relieving the inhibition and allows Pins to bind GST-Mud and be pulled down into the solid phase.

(B) 5μM of WT Pins was incubated with GST-Mud coated glutathione agarose beads in the presence of increasing concentrations of Gαi. At higher Gαi concentrations, Pins begins to be pulled down by the GST-Mud as evidenced by the appearance of the high molecular weight band in the gel, specific to Pins.

The Pins FRET data suggested a role for GLs 2/3 in coupling Gαi binding to Pins output activation. To test this possibility, we made identical point mutations to each GL domain that block association with Gαi and assayed their ability to repress Mud association and be activated by Gαi. A single point mutation to GL3 (ΔGL3 Pins, R631F) renders Pins

unable to be activated by Gαi (Figure 3A), suggesting Gαi binding to GL3 activates Pins. A ΔGL3 FRET construct also failed to adopt the "open" low FRET state (data not shown). Consistent with this, the ΔGL1,2 Pins double mutant (R486F and R570F respectively) is able to be activated for subsequent Mud binding (Figure 3B). We conclude that Pins is activated by a modular allosteric mechanism in which Gαi binding at GL3 triggers a conformational change in the Pins TPRs that makes the Mud binding site available.

Identification of the Pins autoinhibitory module

We next sought to identify the region within the Pins GLs that is required to repress the TPRs with the goal of obtaining a Pins mutant that bound Mud constitutively (i.e., in the absence of Gαi) to assay the role of Pins autoinhibition on spindle orientation in vivo. Because the GLs couple Gαi input into Mud binding output and these domains interact in *trans*, we focused on the GL region. We used the allosteric activation assay depicted in Figure 2A to test if various deletions of the GL region caused Pins to bind Mud constitutively, similar to a Pins TPRs control. Figure 4A shows a summary of the deletions tested and their results. An internal deletion of the linker between the TPRs and GLs or deletion of the GL1 region did not affect Pins autoinhibition, as these constructs were able to repress Mud binding and be activated by Gαi (Pins del linker, Pins del GL1R respectively, Figure 4A, top). These results pointed to the GL2/3 region as being important for repressing the TPRs. A C-terminal truncation of the tail immediately after GL3 also had no effect on autoinhibition (Pins del tail, aa 1-639). However, a deletion of

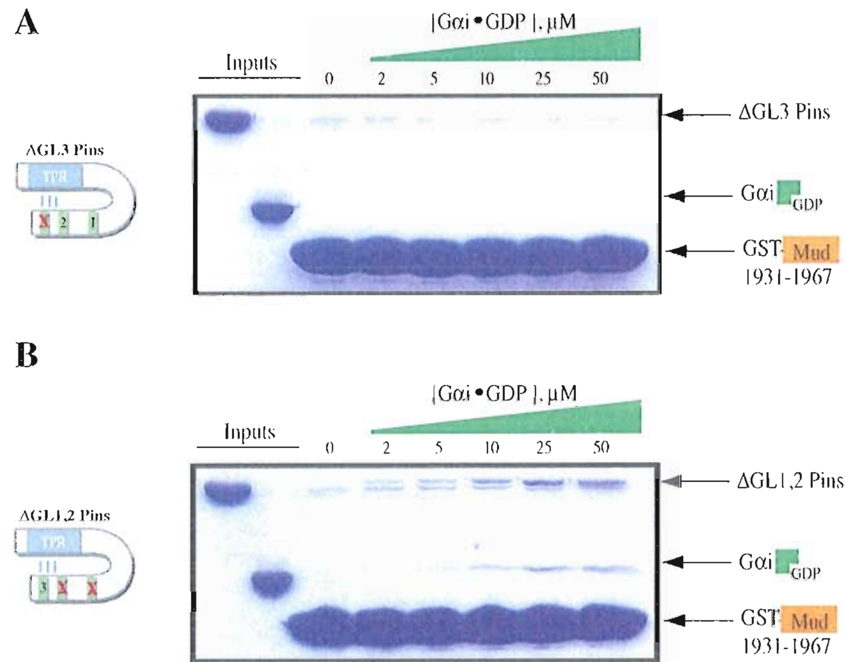


Figure 3: Gαi activation of Pins requires binding to GoLoco 3

(A) Allosteric activation assay of ΔGL3 Pins, which contains an inactivating point mutation to theGL3 domain (R631F) that blocks Gαi binding. This point mutation blocks Pins activation as no Pins band comes down with GST-Mud at high Gαi concentrations.

(B) Binding of Gαi to GL3 is sufficient for Pins activation because a ΔGL1,2 Pins containing inactivating point mutations to GLs 1 and 2 (R486F and R570F respectively) is activated by Gαi as Pins comes down with GST-Mud in a Gαi dose dependent manner.

GL3 caused Pins to bind Mud constitutively, similar to Pins TPRs alone (Pins 1-610, Figure 4A, bottom). This construct, when asymmetrically localized in S2 cells is able to orient the mitotic spindle similar to full length Pins co-transfected with Gαi (Christopher

A. Johnston, unpublished results). Therefore, the autoinhibitory module centers on the GL3 region, the same region coupled to Pins activation.

To further hone in on the residues required to inhibit Mud binding, we made more precise deletions near or within GL3. Deletion of the Pins tail to the C-terminus of GL3 (Pins 1-631) or an internal deletion of GL3 that leaves a predicted alpha helix (Pins 1-623, (Kimple et al., 2002a)) retains the ability to repress Mud association (Figure 4B). These constructs are unable to be activated because Gαi binding requires residues C-terminal to the GL domain proper (Adhikari and Sprang, 2003; Kimple et al., 2002a). This suggests that the first 12 amino acids of GL3 are required for autoinhibition. Deletion of the ten amino acids N-terminal to GL3 (Pins del GL3 NT, Figure 4B) had an intermediate effect on Mud repression, suggesting the autoinhibitory module extends N-terminal to GL3 and that residues within GL3 are not sufficient to lock the TPRs in a closed state. We conclude Pins amino acids 600-623 are required for the observed autoinhibitory regulation mechanism.

The GL3 region is sufficient to repress the Pins TPRs

We then asked if the GL3 region identified earlier was sufficient to repress the TPRs in *cis*. An internal deletion was made removing the nearly 150 amino acid residues comprising the GL1/2 region. This construct, named "mini-Pins," contains the Pins TPRs fused to the GL3 region (amino acids 42-396:590-639, Figure 5A). We tested if mini-Pins could repress Mud binding and be activated by Gαi using the allosteric activation assay described earlier. As seen in Figure 5B, mini-Pins initially does not

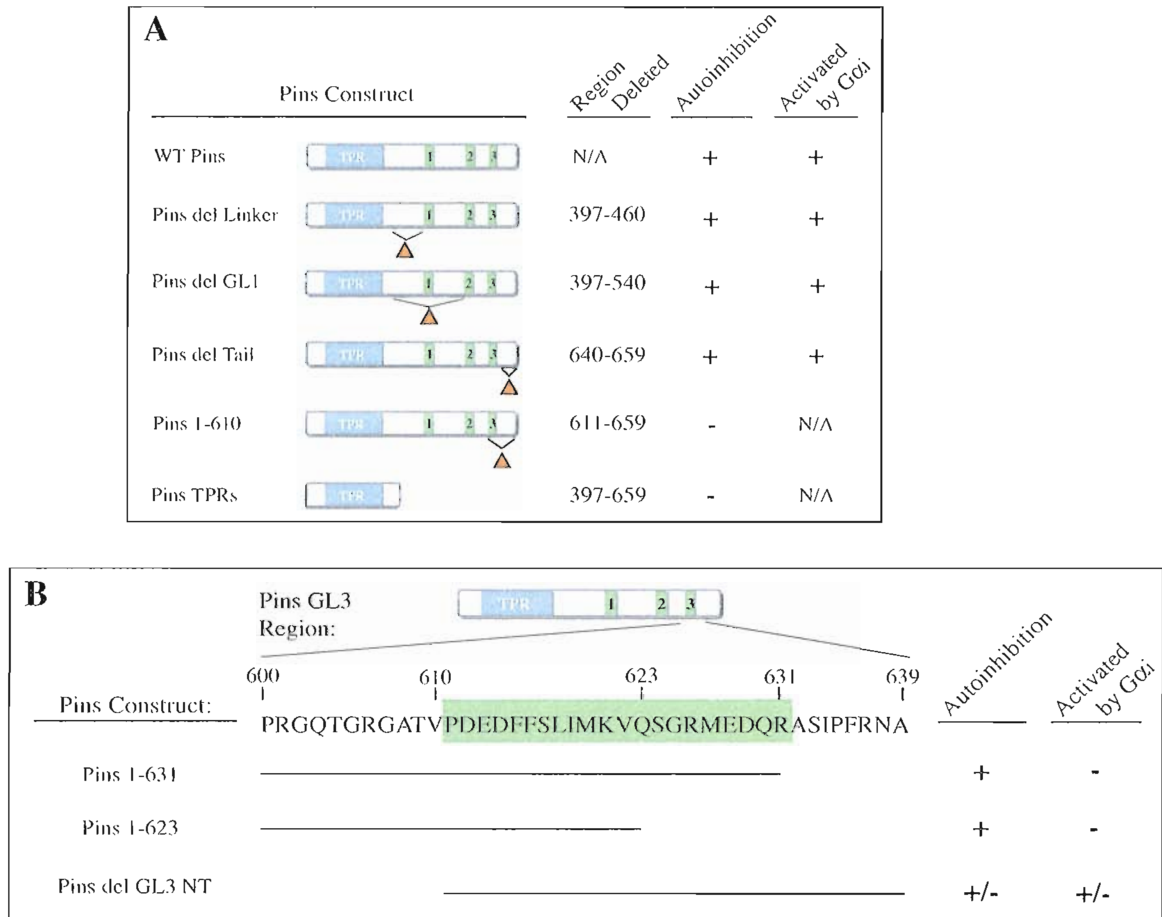


Figure 4: The GoLoco 3 region is required for Pins autoinhibition

(A) Various Pins deletion constructs were assayed for their ability to repress Mud binding and to be activated by Gαi. The deleted regions are noted by an orange triangle. Autoinhibition was scored "+" if little to no Pins is pulled down in the absence of Gαi, similar to WT control. Deletion of the GL3 region (Pins 1-610) shows constitutive Mud binding similar to the Pins TPRs alone.

(B) Deletions within the GL3 region show a role for the N-terminal half of the GL3 domain as well as amino acids immediately N-terminal to GL3. The autoinhibited constructs cannot be activated by Gαi because residues C-terminal to the GL domains are required for Gαi binding.

associate with GST-Mud, but begins to be pulled down in a Gαi dose dependent manner, similar to ΔGL1,2 Pins (Figure 3B). Single point mutations within this region were not sufficient to break autoinhibition (data not shown), suggesting this autoinhibitory region likely contacts the TPRs at multiple sites. We conclude that mini-Pins is autoinhibited, activated by Gαi and the GL3 region identified is sufficient for limiting Pins output when fused to the TPRs in *cis*.

The Pins autoinhibitory module is conserved in the mammalian orthologue, LGN

In mammalian neural stem cells LGN, the Pins orthologue, together with Gαi and NuMA (the Mud homologue) have a conserved role in spindle orientation (Konno et al., 2008; Morin et al., 2007). LGN is also autoinhibited by an intramolecular interaction such that its TPRs cannot associate with NuMA in the absence of Gαi (Du and Macara, 2004). Is the LGN autoinhibitory module conserved with Pins? To test this possibility, we created an analogous LGN deletion construct (despite the high degree of sequence homology between Pins and LGN, we were unable to express recombinant full length LGN protein in *E. coli*). The LGN TPRs were fused to the GL3/4 region in *cis* to create "mini-LGN" (amino acids 1-370:610-672). We tested for autoinhibition by the ability of mini-LGN to associate with a GST-NuMA fusion containing the minimal region for binding to the LGN TPRs (amino acids 1889-1915, Newman et al., 2010). As seen in Figure 5C, mini-LGN requires Gαi for robust interaction with GST-NuMA. We conclude that the autoinhibitory modules of Pins and LGN are conserved in that each is centered on the C-terminal GL region.

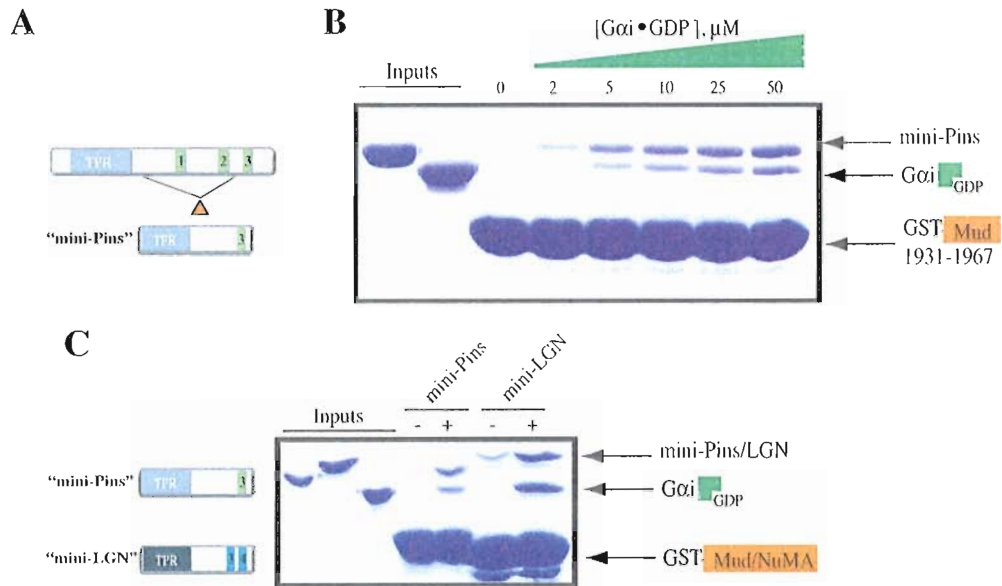


Figure 5: The Pins GoLoco 3 region fused to the TPRs reconstitutes Mud repression

(A) A molecular diagram of the minimal Pins deletion construct that retains autoinhibition ("mini-Pins"). The GL3 region was fused directly to the Pins TPRs (amino acids 42-396:590-639).

(B) Mini-Pins is autoinhibited, and displays Gαi dependent activation of Mud binding.

(C) The autoinhibitory module of Pins is conserved in the mammalian Pins orthologue, LGN.. A mini-LGN construct was created by fusing the LGN TPRs to the GL3/4 region (amino acids 1-370:610-672). This construct is autoinhibited as it weakly associates with GST-NuMA and is activated by Gαi.

DISCUSSION

Autoinhibition in the Gαi-Pins-Mud spindle orientation pathway

Signaling proteins must have limited output in the absence of the appropriate physiological input. Autoinhibition provides a solution to this problem as it maintains

signaling proteins in an "off" state until a specific input or inputs are sensed and allows for more complex regulation such as AND / OR signaling behaviors (Lim, 2002). *Drosophila* Pins is a central component of a spindle orientation pathway in neural progenitor cells. A ternary complex of Gαi-Pins-Mud forms at the apical cortex of mitotic NBs to position the spindle along the cell polarity axis (Izumi et al., 2006, Siller et al., 2006, Bowman et al., 2007). Assembly of this complex is regulated by an autoinhibitory mechanism where the GL region inhibits Mud binding to the Pins TPRs in the absence of Gαi (Nipper et al., 2007). This type of regulation allows for tight temporal and spatial restriction of complex assembly to the apical cortex, where the Gαi input signal is present only during M-phase. We set out to identify the molecular origins of Pins autoinhibition to ultimately determine if this regulatory behavior was required for proper molecular function.

The GL3 region is required for repression and activation of Pins

We developed in vitro binding assays to demonstrate that Pins exists in an autoinhibited "closed" state and undergoes a dramatic conformational change to an active "open" state upon association with Gαi and Mud (Figure 1A, B). Pins opening requires Gαi binding specifically to GL3, suggesting that GL3 is structurally coupled to the Pins TPRs. Consistent with this, a deletion of the GL3 region breaks the autoinhibitory regulation as the Pins 1-610 construct binds Mud even in the absence of Gαi, behaving similar to the TPRs alone. This deletion is sufficient for robust spindle alignment when

asymmetrically localized in S2 cells (Johnston, C.A., unpublished results), suggesting that the unregulated Pins no longer requires Gαi input for triggering its molecular output.

Modular allosteric regulation of Gαi-Pins-Mud complex assembly

Modular allostery is a common feature of autoinhibited signaling proteins as it allows for coupling different inputs to output activity and provides and evolutionary platform for developing new signaling behaviors (Deuber et al., 2004). We have shown that Pins transitions from an autoinhibited state to an active state through modular allostery, in which a physiological input (Gαi) is sensed at a region distinct from the TPR output domain (GL3) and somehow is translated into Mud binding. The precise way in which this input/output coupling occurs is unclear. Nipper and colleagues argue this may occur through a simple competition mechanism in which Gαi and Mud compete with the Pins autoinhibitory intramolecular interaction for binding to the GLs and TPRs, respectively. However, recent data suggest that the mechanism may be more complex. Newman and authors show that Gαi saturated Pins has a higher affinity for Mud than the TPRs alone, suggesting Gαi binding to GL3 causes the TPRs to adopt a different conformation than the free TPRs that makes Mud binding more thermodynamically favorable. They also identify mutations in the TPRs that differentially affect Mud and GL binding, a result inconsistent with a simple competition mechanism. A crystal structure of the repressed mini-Pins/LGN proteins would help determine how Gαi binding is coupled to output by allowing visualization of both the Mud and GL TPR binding interfaces.

Conservation of autoinhibition from flies to mammals

We showed the GL3 region is required for repressing the Pins TPR domains. We demonstrated that this module is evolutionarily conserved as the C-terminal GL3/4 region is required for autoinhibiting the TPRs of LGN, the mammalian Pins orthologue. Conservation of this regulatory behavior suggests it is important for Pins function in spindle orientation. In *C. elegans*, the Pins orthologues GPR1/2 dictate spindle positioning by association with GOA1, the Gαi homologue and bind the coiled-coiled protein Lin-5 (Mud homologue, (Srinivasan et al., 2003), Gonczy, 2008). An open question is whether the analogous GL region mediates autoinhibition in this system as well.

Why is Pins autoinhibition important in the spindle orientation pathway? Nipper et al. speculate that this feature allows for tight temporal and spatial regulation of Pins activity because it restricts Pins-Mud association to the apical cell cortex during M-phase when Gαi is present. We plan to test the functional role of Pins autoinhibition by assaying spindle orientation in neuroblast cells and hypothesize that the constitutively "open" Pins 1-610 will no longer restrict Mud activity to the apical cortex and cause the spindle to be misaligned relative to the cell polarity axis (Figure 6).

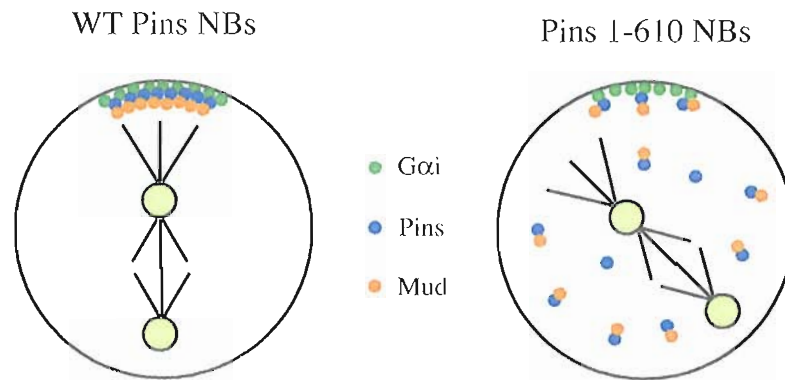


Figure 6: Model for the role of autoinhibition in the Gαi-Pins-Mud spindle orientation pathway

In WT NBs, formation of the Gαi-Pins-Mud complex is restricted to the apical cortex because Pins (blue) requires the membrane tethered Gαi (green) signaling molecule for subsequent binding of Mud (orange). If autoinhibition were lost, as in Pins 1-610, Pins-Mud association would not be restricted to the apical cortex because Gαi is no longer required for Pins activation.

BRIDGE TO CHAPTER III

Autoinhibition is a common mechanism to repress signaling output in the absence of a specific input. However, not only is it important for a signaling protein to transition from inactive to active states, but the amount of output generated as a function of input (i.e., the input/output relationship) is also important. These input/output relationships can exhibit simple graded or more complex sigmoidal responses to cellular inputs. Sigmoidal responses are a behavior known as ultrasensitivity; another common feature of cell signaling pathways, yet the molecular mechanisms responsible for this behavior are poorly understood. Ultrasensitivity allows for complex cellular decision making

behaviors such as bistability, an important feature of irreversible cellular processes. The Pins response to Mud as a function of Gαi appeared to occur in a sigmoidal fashion suggesting ultrasensitivity in the Gαi-Pins-Mud pathway (Figure 2B). In the next chapter, I describe how I used Pins as a model to identify the molecular mechanisms responsible for the sigmoidal response to Mud and test the importance of this regulatory feature in vivo.

CHAPTER III

ULTRASENSITIVE REGULATION OF THE SPINDLE ORIENTING PROTEIN PINS: MECHANISM AND FUNCTION

*This chapter contains unpublished co-authored material prepared for submission to the journal *Cell*

Author contributions: N.R.S. and K.E.P. designed research; N.R.S. performed research; N.R.S. and K.E.P. analyzed data; and N.R.S. and K.E.P. wrote the paper.

INTRODUCTION

Cells exhibit complex decision-making behaviors that are implemented by networks of dynamically interacting proteins (Kholodenko, 2006). Two properties that are commonly found in such pathways are thresholding and ultrasensitivity (Tyson et al, 2003). Thresholding limits output activity until a specific input level is reached, a property which is likely useful for preventing spurious activity in the presence of biological noise (Ferrell, 1996). Ultrasensitivity, in which small variation in input levels leads to a large change in output, can convert graded inputs into more switch-like outputs

and can also be used to generate more complex behaviors such as bistability and hysteresis, the basis of all or none decisions and cellular memory (Goldbetter and Koshland, 1981, Tyson et at, 2003, Burrill and Silver, 2010). Hemoglobin is a classic example of ultrasensitivity in which O_2 binding is enhanced through cooperative interactions (Koshland et al., 1966). Although thresholding and ultrasensitivity are fundamental features of cellular signaling, binary protein interactions exhibit a hyperbolic response profile with large output variation at low input, yet requiring large changes in input levels for maximal output (Figure 1A). A fundamental question in cellular signaling is how complex input-output relationships are built from individual protein-protein interactions. In particular, are alternative mechanisms besides cooperativity, which requires the evolution of thermodynamic coupling between binding sites, used in protein interaction based regulation?

We have investigated ultrasensitivity and thresholding in the regulatory pathway that controls mitotic spindle orientation. As the site of cleavage furrowing, and subsequently the position of the two daughter cells, are determined by mitotic spindle orientation, this fundamental cellular process is important for development and adult physiology (Doe, 2008). For example, epithelial cells divide in a planar fashion with their spindle aligned along the sheet plane such that the two daughter cells remain in the plane (Morrison and Kimble, 2006). During the formation of the epidermal stratified layers, cells in the basement layer switch between proliferative and differentiating divisions by either dividing with their spindle parallel or orthogonal with the plane of the epithelium (Lechler and Fuchs, 2005). Such asymmetric divisions are one mechanism used to

generate cellular diversity (Doe, 2008). *Drosophila* neuroblasts (NBs) divide asymmetrically to generate a self-renewed NB and a ganglion mother cell that divides once more to generate two neurons (Gonczy, 2008). This process requires polarization of cortical factors that specify the two cell fates and rapid alignment of the spindle with the polarity axis such that the cleavage plane precisely bisects the determinants into the two daughter cells (Siller and Doe, 2009). Understanding spindle orientation regulation has implications for cancer biology as failure to align the spindle in NBs can result in an increase in the stem cell pool (Cabernard and Doe, 2009).

In each of these contexts the spindle is positioned by conserved cortically localized factors that are thought to anchor astral microtubules (Johnston et al., 2009). These factors include the heterotrimeric G-protein α subunit (Gαi), partner of Inscuteable protein (Pins), and microtubule associated mushroom body defect protein (Mud) (Yu et al. 2000; Siller and Doe, 2009). Gαi is an upstream component to the pathway that localizes to the apical neuroblast cortex where it binds Pins. Pins is an adapter protein that links signals from GDP loaded Gαi (hereafter "Gαi") to mitotic spindle orientation through the microtubule associated mushroom body defect protein (Mud) (Lin5 in *C. elegans*, NuMA in mammals, (Srinivasan et al., 2003; Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006; Nipper et al., 2007; Du and Macara, 2004), Figure 1B). The ability of Pins to recruit Mud is regulated by an evolutionarily conserved autoinhibitory interaction between the N-terminal tetratricopeptide repeats (TPRs) and C-terminal GoLoco domains (GLs) of Pins that makes its intrinsic affinity for Mud low (Du and Macara, 2004; Nipper et al., 2007). Gαi weakens the Pins intramolecular interaction,

thereby increasing the affinity of Pins for Mud (Nipper et al., 2007; Newman et al., 2010). In a previous study from our lab, we observed Pins activation by Gαi appeared to occur over a narrow range of input after a threshold, suggesting ultrasensitivity in the spindle orientation pathway (Nipper et al., 2007, see Chapter II Figure 2A,B).

In neuroblasts, spindle orientation is remarkably dynamic, as Gαi, Pins, and Mud are transiently polarized at the cortex during mitosis (Izumi et al., 2006; Siller et al., 2006). Although the overall structure of the regulatory pathway is fairly well understood, the quantitative aspects that inevitably support the dynamic spindle orienting behavior in systems such as neuroblasts have not been investigated. To address this gap in our understanding, we reconstituted the Gαi-Pins-Mud regulatory pathway in vitro and, as described below, found that it is highly ultrasensitive. This allowed us to determine the molecular origins of ultrasensitivity and furthermore, by examining a system that had been perturbed to be non-ultrasensitive, to examine the role of this property in vivo.

METHODS

Protein expression and purification

Drosophila Pins cDNA was amplified from an embryonic cDNA library created using the Superscript III kit (Invitrogen). Gαi used in all experiments is mouse Gαi3 amino acids 25-354 (Nipper et al). Gαi cDNA was obtained from a mouse cDNA library. We generated Pins mutants by Quick-change PCR (point mutations), introduction of an

early stop codon (C-terminal deletion) or two-step PCR (internal deletions).

Proteins were expressed in *E. coli* strain BL21(DE3) using pBH4 based vectors encoding a 6x histidine tag. His-tagged proteins were affinity purified on Ni-NTA agarose (Qiagen) and further purified by AKTA FPLC (GE Healthcare) by Anion exchange and/or size exchange chromatography. Protein stocks were stored in binding buffer (20mM HEPES pH 7.5, 100mM NaCl, 1mM $MgCl_2$ and 1mM DTT). Total protein concentrations were determined by Bradford assay (Bio-Rad).

In vitro reconstitution of Pins activation

A synthetic peptide containing the minimal Pins TPR binding domain of Mud isoform B residues 1934-1951 (SNLAMEDEEGEVFNNTYL, Newman et al., 2010) was obtained from EZ-Biolabs. Tetramethylrhodamine (TMR) was conjugated to a cysteine residue added to the C-terminus using TMR-maleimide (Invitrogen Molecular Probes). The conjugation reaction was carried out according to the manufacturers protocol and the TMR-Mud peptide was subsequently purified by RP-HPLC, characterized by MALDI-TOF mass spectrometry and resuspended in binding buffer.

Quantification of the Pins response to Mud as a function of Gαi concentration was conducted under the following conditions: 1μM Pins was incubated with 0.5μM TMR-Mud peptide in binding buffer. Increasing concentrations of Gαi were introduced to the system. Anisotropy was determined by exciting TMR at 555nm and observed emission at 580nm using an ISS-PC1 spectrofluorometer equipped with polarizers and a water bath at 20° C.

The percent of Pins activation was measured relative to the anisotropy of the free TMR-Mud peptide (0%) and to the maximal value of WT Pins observed at 20μM Gαi (100%). For Pins mutants, the maximal percent activation was determined relative to the constitutively active Pins 1-610 value bound to either one or two molecules of Gαi. For "mini-Pins," the maximal percent is relative to the maximum anisotropy value in the presence of 20μM Gαi. The apparent Hill coefficient, $n_{H,\text{app}}$, was obtained by fitting the data to the Hill equation ($y = ([x]^{n_H}/(Kd + [x]^{n_H}))$) (Kim and Ferrell, 2007).

Construction and analysis of the Pins ΔGL1,2 FRET biosensor

The Pins FRET protein was generated as previously described in Nipper et al., 2007. Briefly, Pins ΔGL1,2 cDNA was subcloned into a pBH vector encoding an N-terminal YFP (EYFP 1-239) and C-terminal CFP (ECFP 1-239) to create a YFP-ΔGL1,2 Pins-CFP fusion. The protein was expressed and purified as described above using SEC as a final purification step. 100nM FRET protein was incubated in binding buffer in the presence of increasing concentrations of Gαi. FRET was measured by exciting CFP at 433nm and the emissions of CFP at 475nm and YFP at 525nm respectively were measured. The FRET ratio was determined as the ratio of acceptor (YFP) to donor (CFP) emissions. The dissociation constant (Kd) was determined by fitting the data to a standard binding function.

Fly strains and genetics

The yw strain was used as a control for the analysis of spindle orientation and

Mud recruitment. The transgenic WT Pins strain was obtained from the C.Q. Doe lab. The ΔGL1,2 Pins transgenic fly was created by subcloning into the pUAST vector encoding an N-terminal hemagglutinin (HA) epitope. Transgenic flies carrying UAS-Pins constructs on the second chromosome were balanced over Cyo, Actin-GFP and were crossed with a stock containing the *pins*^{P62} allele (Yu et al., 2000) balanced over TM3 Ser, Actin-GFP on chromosome three. Flies were crossed with the *worniu*-Gal4; *pins*^{P62}/TM3 Ser Actin-GFP driver line (Nipper et al., 2007) for neuroblast specific expression. Homozygous mutant *pins* larvae expressing transgenic Pins protein were identified from lack of expression of GFP in the gut.

The gene trap line G147-GFP, which expresses the GFP-tagged microtubule associated protein Zeus (Siller et al., 2005) was used for live imaging experiments. A stock with this construct recombined with the *pins*^{P62} allele on chromosome three, was obtained from the Doe lab. This stock was crossed with the *worniu*-Gal4; *pins*^{P62}/TM3 Ser, Actin-GFP driver line to obtain *worniu*-Gal4; *pins*^{P62}, GFP-zeus/TM3 Ser, Actin-GFP. The Pins stocks mentioned above were crossed to this new driver line and *pins*^{P62} homozygous larvae were identified from the absence of GFP expression in the gut.

Immunofluorescence

Second to early third instar larval brains were dissected in Schneider's insect medium (Sigma) and fixed in PBS + 4% paraformaldehyde at room temperature for 20 minutes. Brains were washed in 1x PBS-BT (PBS + 2% BSA, 0.3% Triton-X 100, 0.02% sodium azide) three times and incubated with primary antibodies at 4° C

overnight. Brains were washed six times in PBS-BT over 1 hour and incubated with secondary antibodies for 3 hours at room temp. After washing six times for 1 hour, brains were mounted in Vectashield mounting medium (Vector Labs).

The following primary antibodies and dilutions were used: rat anti-Pins (1:500), mouse anti-tubulin DM1A (1:1500), guinea pig anti-Miranda (1:500), mouse anti-HA (Covance, 1:1000), rat anti-tubulin (abCam, 1:500), rabbit anti-Mud (1:1000), rat anti-Par6 (1:250). Secondary antibodies from Invitrogen/Molecular Probes or Jackson Immunoresearch were used according to manufacturer's specifications.

Acquisition and analysis of images for determining spindle orientation

Fixed neuroblast images were acquired on a Leica SP2 confocal microscope equipped with a 63x 1.4 NA oil immersion objective. The reported spindle angle value is the angle between the spindle vector to the cell center and to the center of the apical Pins or Par-6 crescent, as previously described (Siegrist and Doe, 2005). Spindle angles were measured using ImageJ (NIH). Only cells in which the apical Pins signal was 1.5x greater than the cytoplasm were scored in our analysis. Figure panels were arranged using ImageJ, Photoshop and Illustrator (Adobe).

Analysis of apical Mud crescent formation

Images of fixed and stained larval brain neuroblasts were acquired as described above. A mud crescent was scored if the pixel intensity at the apical cell cortex was ≥ 2x the signal intensity at the cell center. Cells expressing WT or ΔGL1,2 Pins were scored if

the apical Pins intensities were ≥ 1.5x that in the cytoplasm.

Live imaging of and analysis of neuroblast spindle dynamics

Second to early third instar larval brains from animals expressing either WT or ΔGL1,2 Pins and GFP-Zeus in the *pins*^{P62} genetic null background were dissected in Schneider's insect medium supplemented with 5% FBS + 0.5μM ascorbic acid (Cabernard and Doe, 2009). Movies were made on a McBain spinning disc confocal microscope equipped with a hammamatsu CCD camera. Images were acquired at four-second intervals with 2μm z-sections. Neuroblasts were identified as large cells in the central brain lobes. Prophase neuroblasts were identified the presence of two centrosomes that did not radiate microtubules into the cell center. Time zero is the start of prometaphase, when the centrosomes begin to nucleate microtubules that penetrate the cell center and form the mitotic spindle. Anaphase onset was determined as the moment when kinetochore microtubules at the center of the spindle began to shorten towards the spindle poles (Siller and Doe, 2008). High velocity spindle movements were scored during the two-minute period immediately prior to anaphase onset. A high velocity spindle movement was scored for either the apical or basal spindle pole if the center of the spindle pole moved ≥ 2 pixels between frames (Siller and Doe, 2008). Movie frames were acquired using Volocity4 software, processed and analyzed in ImageJ, and movies were compiled in Quicktime.

RESULTS

Pins activation by Gαi is ultrasensitive

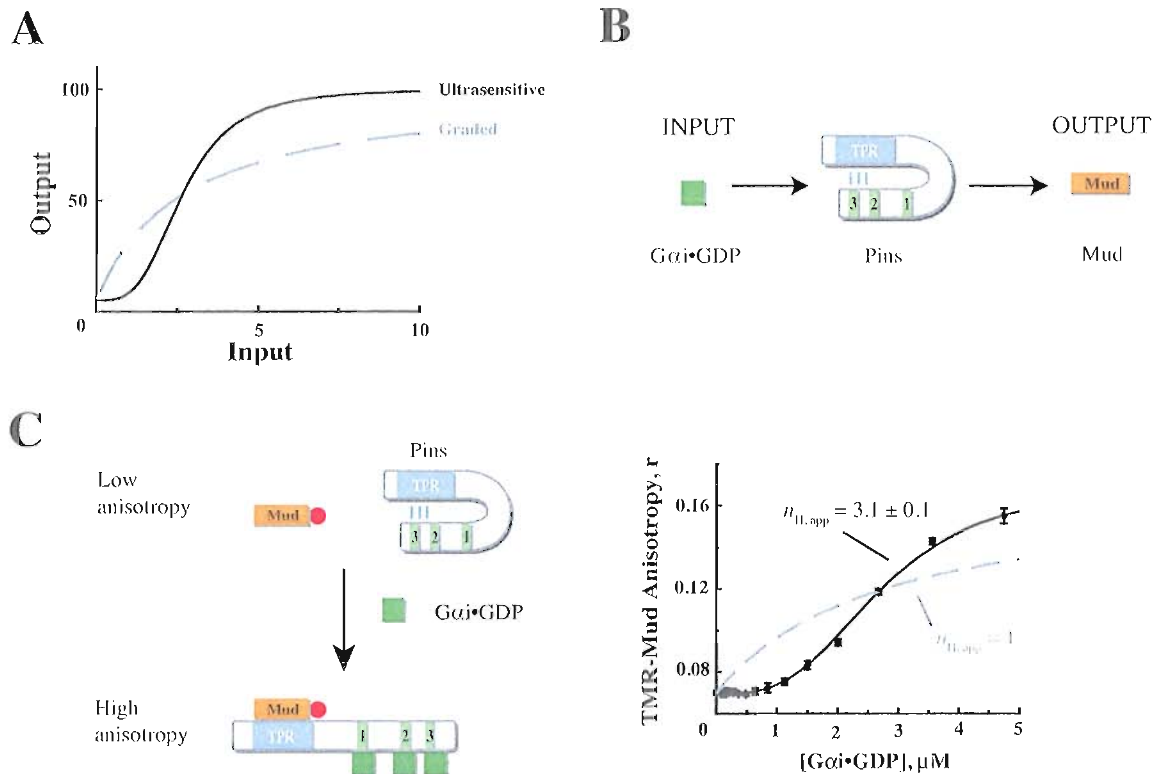
In order to quantitate the relationship of Pins output (Mud binding) and Pins input (Gαi concentration), we reconstituted the Gαi-Pins-Mud pathway in vitro from purified components. A tetramethylrhodamine labeled Mud peptide (TMR-Mud aa1936-1951, isoform B) containing the region necessary and sufficient for association with the Pins TPRs allows detection of Pins-Mud association via the fluorescence anisotropy of the conjugated TMR (Newman et al., 2010, Figure 1C). Because Pins is autoinhibited, its intrinsic affinity for Mud is low such that very little complex forms in the absence of Gαi. Consistent with this, we observed little change in anisotropy upon addition of Pins to TMR-Mud (Fig 1D, 2A). When Gαi is titrated into the system, Pins becomes activated and binds Mud, resulting in a large increase in TMR-Mud anisotropy (Figure 1D, 2A). Activation is highly ultrasensitive as the entire transition occurs between 1 and 5 μM Gαi. We analyzed the Pins activation profile using the Hill equation as the Hill coefficient (n_H) is commonly used to measure ultrasensitivity (Ferrell, JE). We denote the resulting Hill coefficient as "apparent" ($n_{\mathrm{H,\,app}}$) because the Hill equation is a model for cooperative systems (cooperativity can lead to ultrasensitivity, but ultrasensitivity is a general term used to describe sigmoidal responses (Goldbetter, A and Koshland, DE, Jr., 1981, 1983)). The $n_{H,\mathrm{app}}$ for this binding isotherm is 3.1 ± 0.1, which would be very large for a cooperative system (i.e. perfectly cooperative) considering that Pins contains three Gαi binding sites (Figure 1D, 1C).

Figure 1: Gαi activation of Pins is ultrasensitive

(A) Examples of ultrasensitive (black, solid) and graded (gray, dashed) pathway response profiles.

(B) The Gαi-Pins-Mud spindle orientation pathway. Pins is activated by the upstream signal (input) Gαi•GDP and subsequently binds the microtubule associated protein Mud (output).

(C) Left: Schematic diagram of in vitro Pins activation reconstitution. Initially, the system is in a low anisotropy state because Pins is repressed and unable to interact with tetramethylrhodamine (TMR) labeled Mud peptide. Pins is activated upon Gαi binding to the GoLoco domains, leading to increased anisotropy as Pins binds TMR-Mud through the TPRs. Right: Quantification of pathway response shows activation of Pins by Gαi is ultrasensitive. 1μM WT Pins was incubated with 0.5μM TMR-Mud in the presence of increasing concentrations of Gαi. The data was fit using the Hill equation $(y = ([x]^{n_H}/(Kd + [x]^{n_H})))$. The activation profile is well fit with an apparent Hill coefficient $n_{H,\,app} = 3.1 \pm 0.1$, but poorly fit assuming a hyperbolic curve with $n_{H,\,app} = 1$. Error bars and ± values represent the standard deviation from the mean of three independent experiments.



Ultrasensitivity is thought to be a common property of signaling pathways, yet its importance for biological function and the molecular mechanisms by which it arise are not fully understood. In the following sections we use the reconstituted in vitro system to identify the components of the system required for ultrasensitivity and the molecular mechanisms by which ultrasensitivity is achieved.

Pins GoLoco 3 is linked to activation, GoLocos 1 and 2 shape the activation profile

What are the elements of Pins required for ultrasensitivity? We first examined which of the three Gαi-binding GoLoco motifs (GLs) are required for activation by Gαi.

Although we previously used gel filtration to examine Gαi binding (Nipper et al., 2007), the fluorescence anisotropy assay allowed us to more precisely quantify the role of each GL. Of the three GLs, we found that only inactivation of GL3 (by a single point mutation to a critical arginine residue, R631F (Kimple et al., 2002; Adhikari and Sprang, 2003); henceforth ΔGL3) results in a Pins protein that is not activated, as little Pins-Mud complex is formed at saturating Gαi concentrations (25%, Figure 2B). This suggests that Gαi binding at GL3 is required for Pins activation. Consistent with this, Pins with inactivating mutations to GLs 1 and 2 (R486F and R570F for ΔGL1, 2), such that Gαi can only bind to GL3, is nearly fully activated (85%) at the same Gαi concentration. Furthermore, deletion of GL3 (Pins amino acids 1-610) caused Pins to bind Mud in the absence of Gαi (Figure 2B), suggesting that GL3 is structurally coupled to the TPRs and required for Pins autoinhibition. The triple Pins mutant with no functional GL domains shows very little activation (Figure 2A, 10%). These results indicate that Gαi binding to GL3 activates Pins for subsequent Mud binding. We conclude Gαi binding to GL3 is necessary and sufficient for Pins activation by Gαi.

The sequences of the three Pins GLs are highly similar (Nipper et al., 2007), leading us to examine why GL3 is unique in its ability to couple Gαi and Mud binding. A fusion of the Pins TPRs to the GL3 domain (including a 20 residue linker outside of the GL proper), which we term "mini-Pins" (amino acids 42-396 followed by 590-639) recapitulates autoinhibition and activation (Figure 2C). An analogous construct of LGN, the mammalian Pins homologue, also recapitulates autoinhibition (Du and Macara, 2004) and can be activated by Gαi to bind the Mud homologue, NuMA (Chapter II Figure 5B,

C) suggesting conservation of this regulatory module. To determine whether positional information or small sequence differences between each of the GLs are important for coupling to the TPRs, we examined whether each could substitute for GL3 in the context of the mini-Pins. While the GL3 region fused to the TPRs is autoinhibited and able to be activated, GL1 is unable to replace GL3. However, GL2 is nearly able to functionally replace GL3 in this context (Figure 2C). This result suggests that both sequences within and adjacent to the GL domain contribute to the specificity of GL3 interactions with the TPRs. Consistent with this hypothesis, deletion of the linker sequence N-terminal to GL3 in the context of full-length Pins compromises autoinhibition (data not shown).

What is the function of GLs 1 and 2 if not to couple Gαi binding to Pins activation? Although Gαi binding to GL1 or 2 does not activate Pins, we hypothesized that they may be important for shaping the input/output relationship, making it ultrasensitive. To test this possibility, we measured activation of ΔGL1,2 Pins and found that, although this protein can be nearly fully activated, the activation profile has lost all of its sigmoidal character ($n_{H,\text{app}} = 1.0 \pm 0.1$; Figure 2D) with a non-ultrasensitive (graded) response. Individual loss of GL 1 or 2 activity leads to intermediate effects: ΔGL1 reduces $n_{H,\text{app}}$ and thresholding whereas ΔGL2 shifts the response to higher Gαi concentrations relative to ΔGL1,2 Pins (Figure 2E), suggesting a complex interplay between the two binding sites (see below). We conclude that while GLs 1 and 2 are not coupled to Pins activation directly, they are required for the observed ultrasensitivity of the system.

Figure 2: Pins GoLoco 3 is coupled to output while GoLocos 1 and 2 shape the pathway response curve

(A) Schematic diagram the Pins mutants used in this study. Inactivating point mutations to various GL domains (R to F of conserved E/DQR triad) are represented by a red "X" over the mutated domain.

(B) GL3 is the key regulatory element for pathway activation and repression. Bar graphs represent Pins activation (%) in the absence (-, white) or presence (+, gray) of 10μM Gαi for Pins constructs shown in (A). ΔGL3 (R631F) is only weakly activated, while the ΔGL1,2 (R486F and R570F, respectively) is nearly fully activated. Deletion of the GL3 domain (Pins amino acids 1-610) breaks autoinhibition, while the ΔGL1,2,3 Pins is unable to be activated by Gαi.

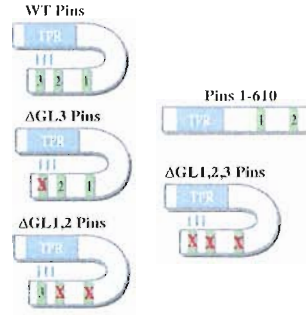
(C) Bar graph representation of Pins activation (%) of the minimally repressed Pins ("mini-Pins") consisting of a fusion of the GL3 region to the Pins TPRs (aa 42-396:590-639). Mini-Pins is able to reconstitute autoinhibition of the TPRs and can be activated by Gαi. Substitution of GL1 for GL3 in this context (mP + GL1) is unable to restore autoinhibition, while GL2 (mP + GL2) has an intermediate effect.

(D) GLs 1 and 2 are required for ultrasensitivity. Inactivating mutations to GLs 1 and 2 in ΔGL1,2 Pins (R486F and R570F respectively) abolishes ultrasensitivity in the system as the profile is graded $n_{H,\text{app}} = 1$ (red curve).

(E) GLs 1 and 2 contribute to the overall ultrasensitivity in different ways. Single mutation of either GL1 or GL2 has differing effects on ultrasensitivity. ΔGL1 (R486F, blue curve, $n_{H,\text{app}} = 1.8 \pm 0.1$) decreases threshold and steepness while ΔGL2 (R570F, green curve, $n_{H,\text{app}} = 2.4 \pm 0.1$) partially decreases thresholding and steepness.

A

Pins Constructs



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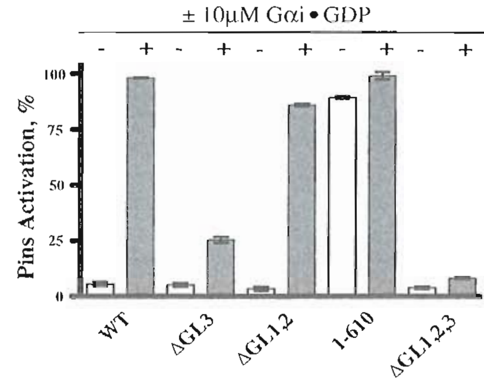

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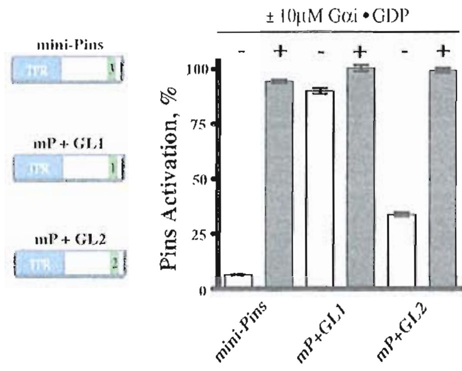

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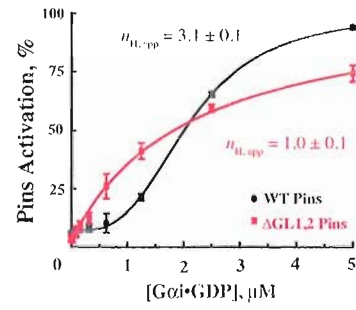

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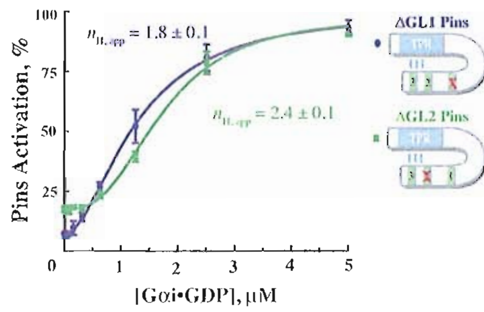


Pins ultrasensitivity is required for robust alignment of the mitotic spindle in *Drosophila* neuroblasts

Ultrasensitivity is a common feature of cell signaling pathways, yet its importance in signal transduction in vivo has rarely been examined. The ΔGL1,2 Pins mutant is activated by Gαi but in a graded fashion. We tested if ultrasensitivity is important for Pins biological function by attempting to rescue the spindle orientation defects of the *pins*^{p62} null allele with ΔGL1,2 Pins (Yu et al., 2000). Larval brain neuroblasts were stained for Pins, tubulin and Miranda (a basal marker) and we scored the spindle angle relative to the apical crescent (Figure 3A-C). WT neuroblasts robustly couple the mitotic spindle position with the cell polarity axis (Siegrist and Doe, 2005). Neuroblasts from *pins*^{p62} mutants often failed to properly align their spindles along the cortical polarity axis (Figure 3C, quantified in D), similar to previous reports (Yu, F. et al., 2000, Siegrist and Doe, 2005). We used the UAS-GAL4 system (Brand and Perrimon, 1993) to express Pins in this genetic background via the neuroblast specific *worniu*-Gal4 driver line (Albertson and Doe, 2003). Although the expression of transgenic Pins proteins are less than the amount of Pins protein in yw control brains (Supplemental Figure S1A), the WT Pins transgene is able to rescue the mitotic spindle orientation defect as previously reported (Figure 3A, D, Nipper et al., 2007). In contrast, neuroblasts expressing ΔGL1,2 Pins exhibited a spindle positioning defect. ΔGL1,2 Pins often failed to localize to the apical cortex of mitotic neuroblasts (~70%), resulting in cytoplasmic staining (Supplemental Figure S1B) consistent with a role for Gαi in cortical Pins recruitment (Yu, F et al., 2003). However, in the 30% of mitotic cells with apically enriched Pins, the spindle is

less coupled to the cell polarity axis compared to neuroblasts expressing WT Pins (Figure 3B, 3D). A similar spindle positioning defect was observed in the 70% of neuroblasts with cytoplasmic Pins when scored relative to Miranda (Supplemental Figure S1C). This phenotype is similar to the null mutant suggesting that ultrasensitivity is an important feature in the Gαi-Pins-Mud spindle orientation pathway.

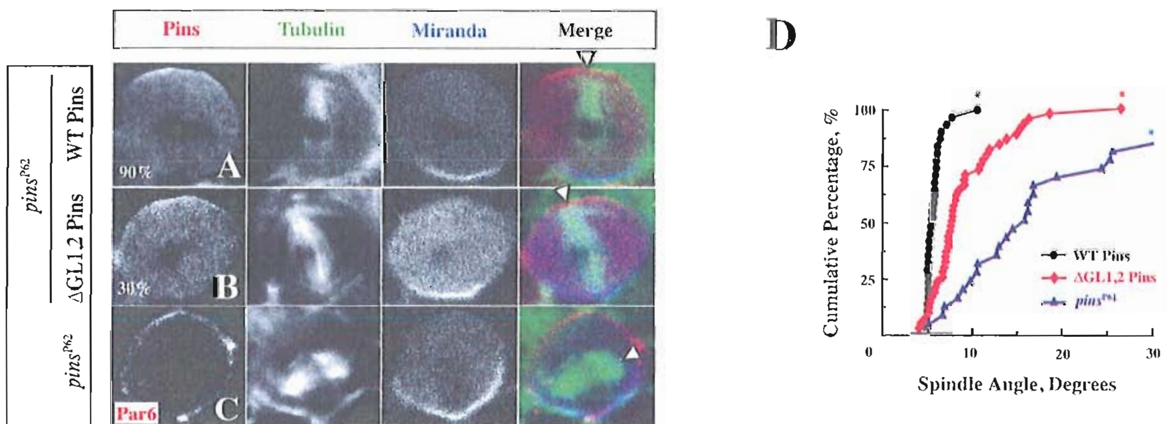


Figure 3: Pins ultrasensitivity is required for robust spindle alignment in vivo

(A-C) Metaphase larval brain neuroblasts (NBs) were fixed and stained for Pins (red, apical), tubulin (green) and Miranda (blue, basal). White arrowheads denote spindle vector. (A,B) NBs expressing WT Pins or ΔGL1,2 Pins in the genetic background of the *pins*^{P62} null allele. (C) *pins*^{P62} NB negative control, Par6 (red) was used to mark the apical cortex.

(D) Cumulative percentage of spindle angle measurements for each experimental condition relative to the center of the apical Pins crescent. *pins*^{P62} NB spindle angles were determined relative to the apical Par6 signal. Spindle angle was measured using ImageJ software. Asterisks: Differences are statistically significant by 1-way ANOVA.

Ultrasensitivity is required to generate maximal pathway output

Why might ultrasensitivity be required in this regulatory pathway? Ultrasensitive responses differ from their hyperbolic counterparts in two respects. At low activator concentration hyperbolic responses actually generate more output which could lead to ectopic activity. At higher concentrations ultrasensitive responses become fully activated over a small concentration range whereas hyperbolic ones require significantly more activator to achieve the same level of activity (Figure 2D, Goldbeter and Koshland, 1981, 1983). Thus, Pins ultrasensitivity may be required for one of two reasons: to repress spurious activity or to achieve a sufficient level of activity at lower Gαi concentrations. We distinguished between these two possibilities by assaying apical Mud recruitment. Mud has been shown to localize to centrosomes by a Pins independent mechanism, but recruitment to the apical cortex requires Pins and Gαi (Izumi et al., 2006, Siller et al., 2006, Bowman et al., 2006, Nipper et al., 2007). Neuroblasts were scored as positive for Mud crescents if the ratio of Mud signal intensity at the cortex exceeded that of the cytoplasm two fold (see methods, Figure 4A-C). In ΔGL1,2 Pins neuroblasts, Mud crescents were observed less often compared to their wild-type counterparts, but more than in null cells (Figure 4D) corroborating our observations in live cells. Because centrosomes are stained in these cells (Izumi et al., 2006; Siller et al., 2006), we were also able to analyze spindle alignment through Mud staining and observed similar results as when tubulin was used to assess spindle position (Johnston, CA et al. 2009, Supplemental Figure S2A). We conclude ΔGL1,2 Pins neuroblasts have decreased spindle orienting activity due to reduced Pins output.

The Gαi-Pins-Mud complex is an evolutionarily conserved protein complex required for generating pulling forces on the mitotic spindle (GOA1-GPR1/2- Lin-5 in *C. elegans* (Colombo et al., 2003), Gαi-LGN-NuMA in mammals (Du and Macara, 2004)) by coupling cell polarity to the dynein/dynactin complex through the adapter protein Lis1 (Siller and Doe, 2008; 2009). Because we observed reduced Mud recruitment in neuroblasts expressing the graded ΔGL1,2 Pins mutant, we hypothesized these cells would have reduced spindle dynamics relative to neuroblasts expressing WT Pins. We measured spindle dynamics using live imaging and visualized spindles of mitotic neuroblasts by expressing a GFP fusion to the spindle associated protein Zeus (Siller et al., 2005). WT and ΔGL1,2 Pins larval brain neuroblasts were imaged from late prophase through telophase (Supplemental movies 1 and 2, respectively). High velocity spindle movements were scored in the two-minute period prior to anaphase onset (Figures 4E-G, see methods). While dynamics at the basal pole of each cell were indistinguishable during this period (Supplemental Figure S2B), the neuroblasts expressing WT Pins had a greater frequency of high velocity spindle movements at the apical pole than ΔGL1,2 Pins cells (Figure 4G). We conclude the observed spindle orienting defect in the graded ΔGL1,2 Pins mutant cells is due to reduced Mud activity at the apical cortex from less Pins output.

Pins ultrasensitivity originates from "decoy" binding at GLs 1 and 2

What is the molecular mechanism by which GLs 1 and 2 cause Pins to be activated in an ultrasensitive manner? Cooperativity can be a source of ultrasensitivity so

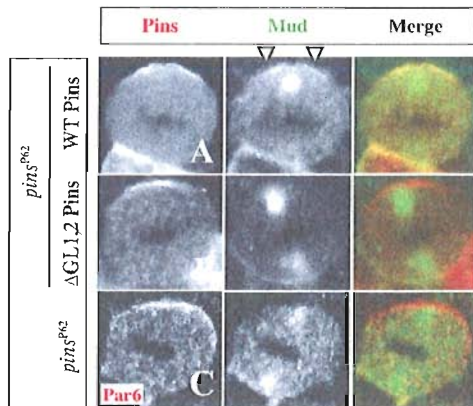
Figure 4: ΔGL1,2 Pins neuroblasts have reduced Gαi-Pins-Mud pathway output

(A-C) Metaphase larval brain neuroblasts (NBs) were fixed and stained for Pins (red, apical), Mud (green) and Miranda (blue, basal). (A,B) NBs expressing WT Pins or ΔGL1,2 Pins in the genetic background of the *pins*^{P62} null allele. (C) *pins*^{P62} NB negative control, Par6 (red) was used to mark the apical cortex. White arrowheads denote the presence of an apical Mud crescent.

(D) Quantification of percent of metaphase NBs imaged that showed a detectable Mud crescent. Mud crescents were scored as a ratio of Mud intensity at the apical cortex greater than or equal to two fold that of the cytoplasm (see supplemental methods). Pixel intensities were measured in ImageJ.

(E,F) Top: Image time course from representative movies capturing dividing NBs expressing WT or ΔGL1,2 Pins in *pins*^{P62} background. GFP-Zeus marks the mitotic spindle. Time is given in minutes relative to prometaphase, the moment when the spindle poles nucleated microtubules that penetrated the cell center. Bottom: Spindle angle relative to position at anaphase onset starting from prometaphase is plotted for each representative movie. Red or blue tick marks denote a rapid spindle movement for the apical or basal spindle pole respectively. The two-minute period prior to anaphase onset analyzed in each movie is marked by horizontal green lines.

(G) NBs expressing ΔGL1,2 Pins have reduced apical spindle pole dynamics. High velocity spindle movements were scored during a two-minute period prior to anaphase onset (see methods). Each point on plot represents an independent measurement. The data from representative movies are represented as blue or red for WT and ΔGL1,2 Pins, respectively. Error bars represent the standard error of the mean. Asterisks denote differences are statistically significant.


Pins
Mud
Merge
WT Pins
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pins P62
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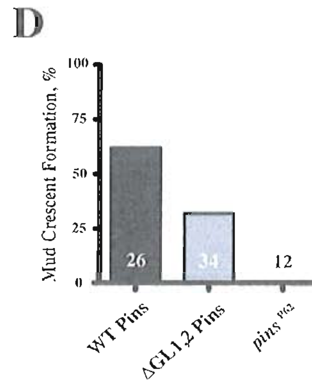
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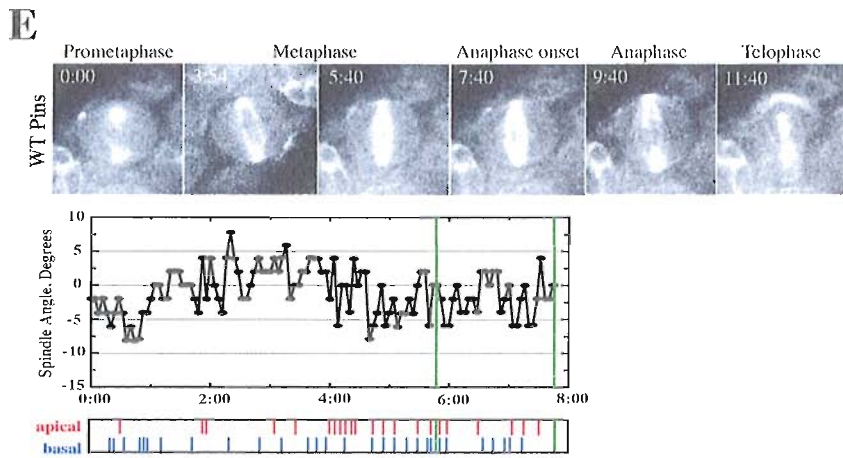
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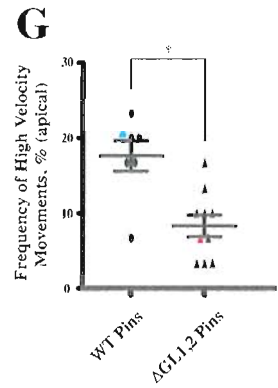
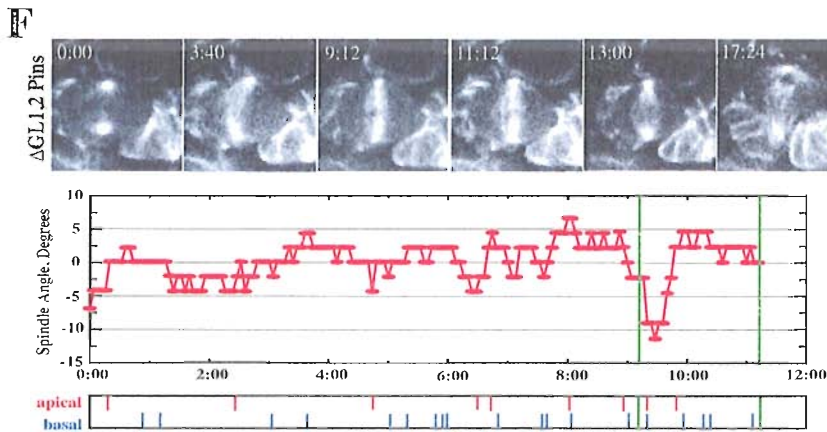

G
Frequency of High Velocity Movements, % (apical)
WT Pins
ΔGL1,2 Pins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ΔGL1,2 Pins
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Spindle Angle, Degrees
apical
basal

we examined this possibility first. In a cooperative mechanism Gαi binding between the Pins GL domains is thermodynamically coupled and would yield the sigmoidal activation profile observed for WT Pins. In fact, the Pins activation profile strongly resembles the behavior of cooperative systems such as hemoglobin, the classic example of cooperativity (n_H = 2.8, Adair, 1925). In the case of Pins, a cooperative mechanism implies that GL3 exists in a low affinity (T) state but Gαi binding to GLs 1 and/or 2 causes a conversion to a high affinity (R) state.

We tested if cooperativity is responsible for Pins ultrasensitivity in two ways. First, we measured the affinity of Gαi for GL3 when GL1 and 2 are not bound to Gαi (i.e. ΔGL1,2 Pins), which should prevent transition into the high affinity state. If cooperativity is responsible for ultrasensitive Pins activation, the GL3 affinity should be significantly lower than the midpoint of the activation threshold observed for the wild type protein (otherwise activation would occur at lower Gαi concentration). By analogy, initial binding events in hemoglobin are of lower affinity than the observed Kd as the system transitions into a higher affinity state. Previously, our lab has used a Pins FRET sensor to observe conformational changes of Pins upon ligand binding (Nipper et al., 2007). We engineered a YFP-ΔGL1,2 Pins-CFP fusion protein to monitor the conformational changes of Pins as Gαi binds to GL3. Using this method, we measured the affinity of GL3 for Gαi to be 3.4 ± 0.3 μM (Figure 5A). This affinity is near the activation transition midpoint, inconsistent with cooperativity being responsible for Pins ultrasensitivity.

We further tested the cooperative model by determining if GLs 1 and 2 must be in *cis* with GL3 for ultrasensitivity, a requirement of cooperativity. We measured ΔGL1, 2

Pins activation in the presence of a ΔGL3 Pins that also contains a mutation in the Pins TPRs rendering it unable to bind Mud (R259A, Newman et al., 2010). The ΔGL3 Pins protein can bind Gαi at GLs 1 and 2, but not enhance the affinity at the ΔGL1, 2 Pins GL3 domain, and thus not influence activation through cooperativity. As shown in Figure 5B, the WT activation profile is largely recapitulated in this experiment ($n_{H,\mathrm{app}} = 2.3 \pm 0.1$), indicating that GL1 and 2 do not need to act in *cis* to generate Pins ultrasensitivity (we believe the difference between the two results from the small amount of activation that occurs from GL2 binding, see ΔGL3 Pins activation data in Figure 2B). Thus, we conclude that Pins ultrasensitivity does not result from cooperative interactions between Gαi binding sites.

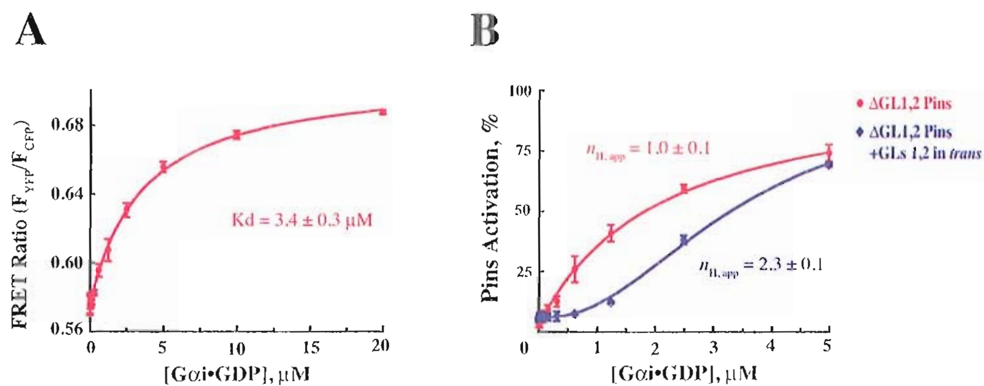


Figure 5: Pins ultrasensitivity is inconsistent with a cooperative mechanism

(A) Forster resonance energy transfer (FRET) was used to approximate the Kd of GL3 for Gαi. 100nM of the YFP-ΔGL1,2Pins-CFP FRET fusion protein was incubated with increasing amounts of Gαi. The binding curve was fit to a Kd = 3.4 ± 0.3µM.

(B) Gαi does not need to bind Pins in *cis* to generate ultrasensitivity. Addition of GLs 1 and 2 in *trans* (Pins R259A, ΔGL3) restores ultrasensitivity to the graded ΔGL1,2 Pins (blue curve) $n_{H,\mathrm{app}} = 2.3 \pm 0.1$.

Because cooperativity is not the source of Pins ultrasensitivity, we considered another possibility. In the MAPK and Wee1 kinase signaling cascades, ultrasensitivity has been demonstrated to result from "decoy" phosphorylation sites that compete with the activating site for the upstream activating kinase (Machleder and Ferrell, 1998, Kim and Ferrell, 2007). These are sites recognized by the enzyme, but not coupled to a functional output. Although such a mechanism has not been reported for protein-protein interaction pathways such as Pins, we tested if decoys might be responsible for Pins ultrasensitivity. In this model, Gαi binding to GLs 1 and 2 would not have any effect beyond competition with GL3. Gαi would bind preferentially to GLs 1 and 2, resulting in the observed threshold. Once nearly saturated, Gαi would begin to populate GL3, resulting in activation and the observed transition steepness (Figure 6A). The observation that the GLs need not act in *cis,* and the relatively high affinity of GL3, as determined above, are consistent with this model. If GLs 1 and 2 are decoys they would not have to be in *cis* with GL3 and the affinity of GL3 could be higher than the activation midpoint, as it will be shifted to lower apparent affinity by competition.

In addition, a key prediction of the decoy mechanism is that the observed activation threshold should be heavily dependent on Pins concentration, as this will determine the amount of decoy present. We observed a dose dependent change in the threshold when the Pins concentration was varied consistent with GLs 1 and 2 acting as decoys (Figure 6B). Decreasing the concentration of Pins by one half or doubling the concentration in our anisotropy assay caused a proportional shift in the threshold (Figure 6B, red and blue curves, respectively). It has been argued that competition mechanisms

similar to multisite phosphorylation should only allow for thresholding, but not the apparent steep activation we observe with Pins (Gunawardena, 2005). We sought to determine the reason why decoys can generate ultrasensitivity in our system and not simply build a threshold. We reasoned the relative affinities of each Pins GL domain would determine the order of binding and that high affinity decoy domains should make a thresholded response as shown in a report describing engineering of a synthetic protein switch (Lu et al., 2010). We tested this idea by adding a high affinity GL peptide in *trans* to the non-ultrasensitive ΔGL1,2 Pins protein. Under these conditions, we generate a strong threshold in the response with no ultrasensitivity as the hyperbolic curve is simply shifted to the right and is well approximated assuming $n_{H,\text{app}} = 1$ (Figure 6C). These results suggest that the affinities of the Pins GL domains are tuned in order to shape the response profile from pure thresholding to a sigmoidal shape. To test this idea in the context of full length Pins, we lowered the affinity of the activating site, GL3 using a point mutation (R631A) that will decrease the affinity for Gαi, but not completely abolish binding. In this case, the affinities of the decoy domains are much higher than GL3 and we would expect the see loss of ultrasensitivity. As seen in Figure 6D, ultrasensitivity is nearly completely eliminated ($n_{H,\text{app}} = 1.2 \pm 0.1$), supporting our model that the affinities of the Pins GLs are "tuned" to achieve the observed sigmoidal activation instead of a hyperbolic response with a threshold (Figure 6A).

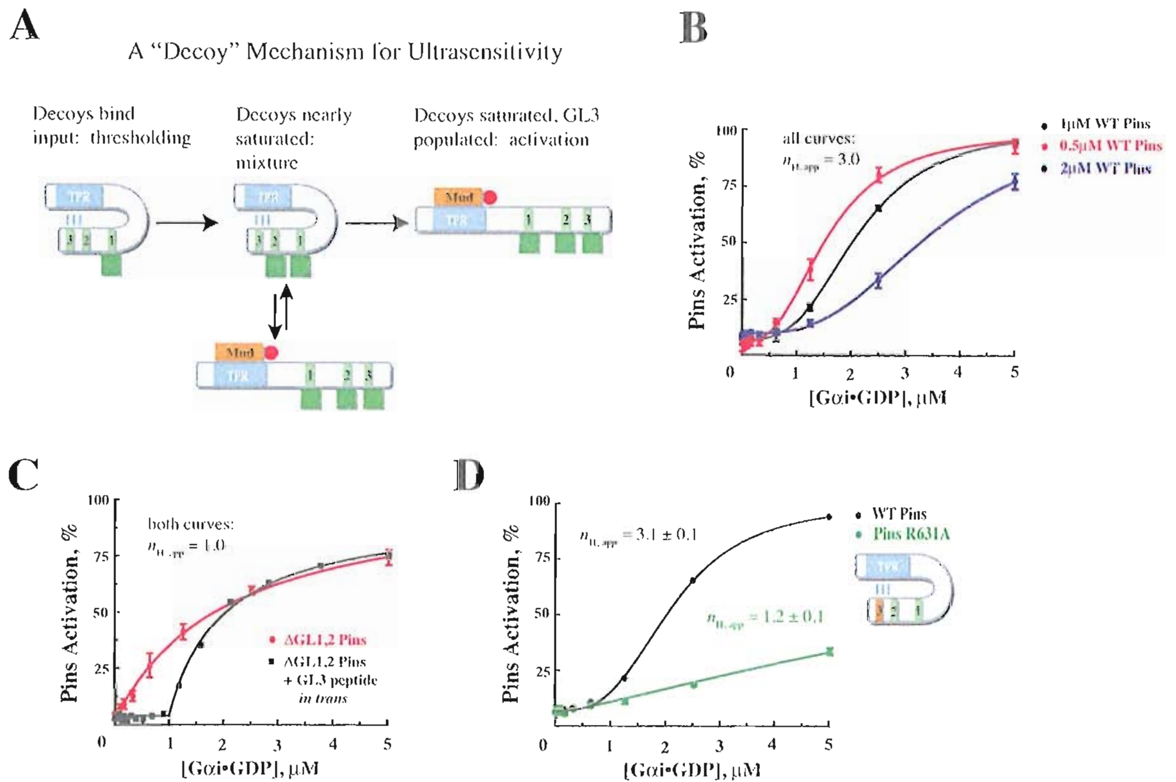
Figure 6: A "decoy" mechanism generates ultrasensitivity in the Gαi-Pins-Mud spindle orientation pathway

(A) Schematic diagram of the decoy mechanism: Decoy GLs 1 and 2 generate ultrasensitivity by competing for the Gαi input with the regulatory domain, GL3. At low Gαi inputs, the decoy GLs 1 and 2 are preferentially bound, leading to an observed threshold. At intermediate Gαi concentration, the decoys are nearly saturated, and GL3 begins to be populated, yielding a slight amount of Pins activation. At higher Gαi concentration, the decoys are fully saturated and Gαi binds to GL3, leading to Pins activation and observed steepness.

(B) The Pins activation threshold is directly proportional to Pins input concentration. Addition of half (0.5μM, red curve) or double (2μM, blue curve) the amount of Pins leads to a proportional change to the activation threshold, defined as a 5% increase in Pins activation above the initial value. Each curve is well approximated by $n_{H,\text{app}}$ = 3.0.

(C) High affinity decoy domains can lead to thresholding without steepness. Addition of the high affinity Gαi GL3 peptide in *trans* to ΔGL1,2 Pins builds a strong threshold without adding ultrasensitivity to the system (black curve, $n_{H,\text{app}} = 1$).

(D) The Kds of each GL domain of Pins are tuned to generate ultrasensitivity. A point mutation to GL3 (R631A) that lowers the affinity of GL3 for Gαi abolishes Pins ultrasensitivity with $n_{H,\text{app}} = 1.2 \pm 0.1$ (green curve).



DISCUSSION

Pins ultrasensitivity arises from a GoLoco "decoy" mechanism

The complex input/output relationships generated by cell signaling networks allow for a multitude of cellular decision making behaviors necessary to implement the diverse physiological phenomena necessary for life such as bistability or hysteresis (Kholodenko, 2006). Ultrasensitivity is a building block for these types of behaviors, yet its molecular origins are poorly understood (Kim and Ferrell, 2007). We have used the *Drosophila* Pins protein as a model to investigate potential mechanisms of generating

ultrasensitivity at the molecular level. We were able to reconstitute Pins activation by Gαi and the subsequent response to Mud in vitro from purified components to elucidate the molecular mechanisms responsible for ultrasensitivity. We have found that Pins activation is highly ultrasensitive (Figure 1D, $n_{H,\text{app}} = 3.1 \pm 0.1$) and that this ultrasensitivity arises from a decoy mechanism as GLs 1 and 2 compete with the activating GL3 for the input, Gαi. It is generally assumed that cooperativity is required for ultrasensitivity in protein-protein interaction networks and in protein-DNA interactions (Dueber et al., 2007; Giorgetti et al.). However, our findings do not agree with a cooperative mechanism for three reasons. First, activation of ΔGL1,2 occurs at a lower Gαi concentration than WT (Figure 2D). Second, the sigmoidal response can be largely recapitulated by adding GLs 1 and 2 back in *trans* (Figure 5C). Lastly, the thresholding behavior is entirely dependent on the concentration of Pins present, rather than the thermodynamics of the system (Figure 6B). These findings suggest that a sigmoidal response curve can be generated without cooperativity from binary protein-protein interactions through a simple competition mechanism, similar to the competition that occurs in kinase signaling cascades (reviewed by (Salazar and Hofer, 2006)).

Although competition and cooperativity are both mechanisms that can generate ultrasensitive responses, there are inherent differences between them. Ultrasensitivity generated by cooperativity should dramatically reduce the amount of input necessary to reach maximal output. This is because, without cooperativity, the amount of output generated by a limited input would be minimal, as the activating site is held in a repressed state, similar to the O_2 binding site of hemoglobin. If hemoglobin could not

transition from the repressed, low affinity T state, the amount of O_2 required to fully populate the binding site would be much higher than the amount of O_2 in the atmosphere. On the other hand, the competition mechanism described here and in kinase cascades generates ultrasensitive responses from a threshold, as activation would occur in a graded fashion without competition. Therefore, this type of mechanism, while yielding sigmoidal responses with high apparent Hill coefficients, may be more important for thresholding than the observed apparent steepness. While multisite phosphorylation is required for the bistable signaling nature of *Xenopus* oocyte maturation (Huang and Ferrell, 1996) and cell cycle progression (Kim and Ferrell, 2007), it is unclear if the decoy mechanism described here can lead to bistability in protein-protein interaction networks.

Pins ultrasensitivity is required for robust alignment of the mitotic spindle in vivo

We examined the requirements of ultrasensitivity on spindle alignment in *Drosophila* neuroblasts. Expressing the graded ΔGL1,2 Pins in the $pins^{P62}$ null background led to a defect in spindle positioning, relative to WT Pins (Figure 3A-D), suggesting that ultrasensitive regulation of Pins is important for proper molecular function. The reduced spindle orienting activity of the graded Pins mutant is likely from reduced pathway output as we observed decreased apical Mud recruitment and spindle pole dynamics relative to WT neuroblasts (Figure 4A-G). The ΔGL1,2 Pins spindle phenotype is similar to loss of Lis1 function, an adaptor protein that physically links the Gαi-Pins-Mud complex at the apical cortex to the dynein/dynactin complex, which

generates the pulling forces on the spindle (Siller and Doe, 2008). It is likely that the ΔGL1,2 Pins phenotype is not fully penetrant because the mitotic spindle is not completely random in *pins*^{P62} null neuroblasts (Siegrist and Doe, 2005, Fig 3C), the presence of a secondary Pins-dependant spindle orientation pathway, (Pins-Dlg-Khc73, Siegrist and Doe, 2005, Johnston et al., 2009) and the observation that Lis1 dependent pulling forces on the basal pole are still functional (Siller and Doe, 2008, Supplemental Figure 4B).

How can the decoy mechanism allow for generating maximal pathway outputs? Given that the observed Pins ultrasensitivity arises largely from competition, we were surprised the ΔGL1,2 Pins mutant had reduced spindle orienting activity because it showed more output relative to WT Pins at low Gαi concentration from loss of thresholding. This result supports a model in which the apparent steepness in Pins activation is important (Figure 7, bottom right). Steep activation profiles allow for increased output in the presence of limited inputs, and perhaps this is critical in Pins spindle orienting function. Alternatively, the threshold may still be important as it allows for spatial restriction of Pins activity to the apical cortex at metaphase. We observed a recruitment defect in the graded Pins mutant that lacked functional GLs 1 and 2, highlighting a role for these decoy domains in targeting Pins to the apical cortex (Supplemental Figure S1B). Loss of the threshold could decrease Pins output by reducing the total amount of Pins at the cortex and subsequently leading to less Mud recruitment (Figure 7, bottom center). Thus, ultrasensitivity may be an important feature of the Gαi-Pins-Mud spindle orientation pathway as it allows for generating maximal

pathway output through temporal and spatial restriction of Pins activity.

Protein modularity can shape cell signaling behavior

The decoy mechanism described here is similar to what has been demonstrated in kinase cascades where multiple phosphorylation sites either in *cis* or *trans* can compete with an activating site for the upstream activating kinase (Machleder and Ferrell, 1998; Kim and Ferrell, 2007; Salazar and Hofer, 2006). However, it has been argued that multisite phosphorylation should only allow for thresholding and not the apparent steepness we observed with Pins (Gunawardena, 2005). We have shown in our system that decoys can lead to ultrasensitivity or pure thresholding depending on the affinities relative to the activating site for the input. A high affinity decoy sets a strong threshold, but a lower affinity decoy domain can change thresholding into a more sigmoidal shaped curve, simply by blending the inflection point between thresholding and activation. A separate study from our lab using a synthetic biology approach has corroborated these results (Lu et al., 2010). This type of ultrasensitivity may be a fairly common component of cell signaling pathways because autoinhibition and domain repeats are common features of cell signaling proteins (Pufall et al., 2002; Dueber et al., 2004). Thus, incorporating more domain repeats through genetic recombination events can modulate the response profile. The relative affinities of these sites could then be "tuned" through point mutations to build either thresholding behavior or steepness into the signaling pathway. The mammalian Pins homologue, LGN, contains four GL domains (Adhikari and Sprang, 2003). As LGN has an analogous role as Pins in spindle positioning in

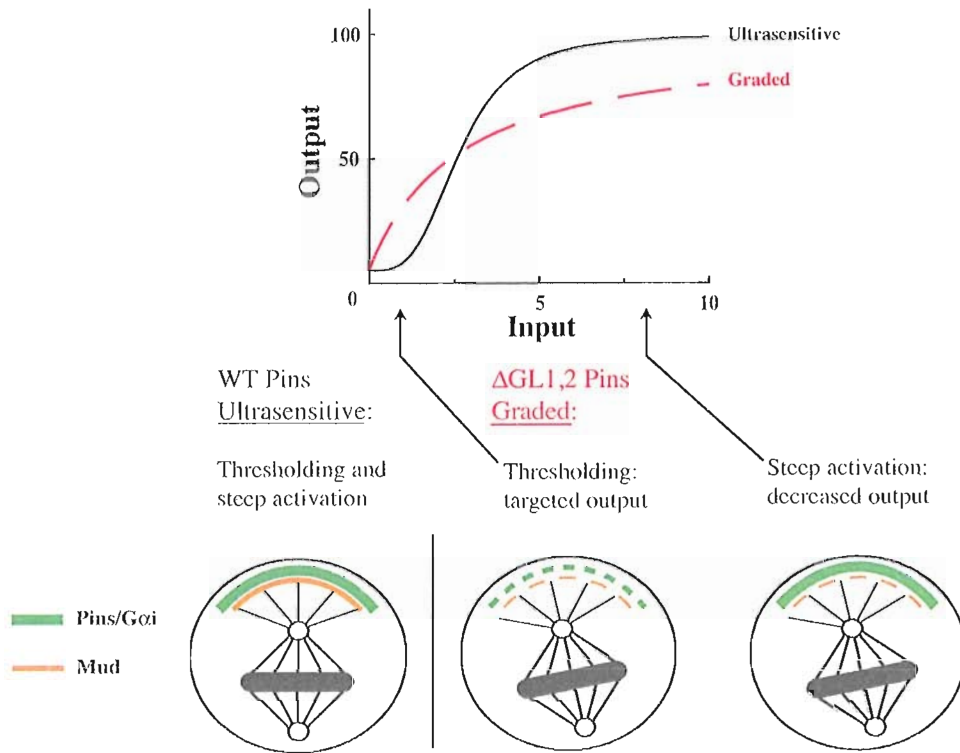


Figure 7: Models for in vivo requirement of ultrasensitivity in the Gαi-Pins-Mud spindle orientation pathway

(Left, bottom) WT Pins robustly orients the spindle along the cell polarity axis through Mud recruitment while the ΔGL1,2 Pins has reduced spindle orienting activity from decreased Mud recruitment. WT Pins displays both thresholding and steep activation of ultrasensitive curve (black) while the graded ΔGL1,2 Pins curve has neither thresholding nor steep activation (red). Either feature could be required for generating maximal pathway output. Thresholding may ensure targeting of Pins activity to the apical cortex (center, bottom). Loss of this feature could lead to reduced Mud recruitment if there is less Pins present on the apical cortex. Steep activation could lead to reduced Mud recruitment from less total activation of the apically restricted Pins (right, bottom).

mammalian systems (Du and Macara, 2004; Morin, et al., 2007), we speculate that evolution has tuned the response of LGN to be more ultrasensitive to improve spindle orienting function in neural progenitor cells. This mechanism for incorporating ultrasensitive regulation into cell signaling pathways is much simpler than cooperativity which would require that domain repeats be thermodynamically coupled to one another.

BRIDGE TO CHAPTER IV

In the preceding chapter, I described how used the Gαi-Pins-Mud pathway as a model to study the molecular mechanisms leading to sigmoidal activation and the importance of this feature on spindle orientation in neural stem cells. In the next chapter, I will summarize the findings contained in this dissertation and discuss how these results increase our knowledge of regulatory mechanisms used by cell signaling proteins to efficiently translate cellular information. I also discuss potential future studies regarding the questions raised by these results.

CHAPTER IV

SUMMARY AND FUTURE CONSIDERATIONS

Summary

My thesis work focused on identifying regulatory mechanisms used by modular signaling proteins to facilitate the flow of cellular information. The two regulatory features I investigated were autoinhibition (output regulation through *cis* inhibition) (Pufall and Graves, 2002) and ultrasensitivity (sigmoidal pathway responses) (Tyson et al., 2003). I used the protein Pins as a model system to identify the molecular mechanisms responsible for these two behaviors and to assay their importance on Pins function in spindle orientation in *Drosophila* neuroblast cells. I found that Pins output (Mud association) is regulated by an autoinhibitory intramolecular interaction between the Pins TPRs and GL3 domain. This repression is relieved upon input (Gαi) binding to the regulatory GL3, suggesting Pins is activated through modular allostery. This regulatory feature is conserved in the mammalian Pins orthologue, LGN. As GL3 is coupled to Pins activation, GLs1 and 2 shape the pathway response, making it ultrasensitive with apparent Hill coefficient, $n_{H,\text{ app}} = 3.1 \pm 0.1$. Inactivating point mutations to GLs 1 and 2 abolish ultrasensitivity and make the Pins response profile graded ($n_{H,\text{ app}} = 1.0 \pm 0.1$). Ultrasensitive Pins regulation is required for in vivo function because the graded Pins mutant fails to robustly orient the mitotic spindle and has

reduced spindle dynamics from decreased pathway output (apical Mud recruitment). The in vitro Pins activation data did not support a cooperative mechanism because GL3 is a high affinity Gαi binding site and ultrasensitivity could be restored to the graded Pins mutant by adding GL domains in *trans*. We propose a simpler mechanism as the source of ultrasensitivity in the system where GLs 1 and 2 act as "decoys" to compete with GL3 for Gαi. This competition mechanism leads to the observed thresholding and steepness and is analogous to what has been described in kinase cascades through multisite phosphorylation (Salazar and Hofer, 2006). The data suggests that ultrasensitive responses can be incorporated into signaling proteins through modular recombination of repeat interaction domains, a common feature of signaling proteins (Lim, 2002; Pawson and Nash, 2003).

Future considerations

The data presented in Chapter II identifying the autoinhibitory module responsible for limiting Pins output supports two models for how repression could occur. The GL3 region could directly compete with Mud for the same binding site on the TPRs. Alternatively, GL3 may bind the TPRs at a site distal to the Mud binding interface, but hold the TPRs in a conformation that occludes the Mud interaction surface. Studies by Newman et al., 2010 suggest the latter mechanism may be working, in which case it would be interesting to learn more about how this autoinhibitory interaction can function and how it was evolved. A crystal structure of Pins would help illuminate which model is correct. As no crystals of full-length Pins have been generated (unpublished results),

the "mini-Pins" constructs described in Chapter II would be an alternative strategy since this is the minimal construct that displays autoinhibition and can be activated by Gαi. Newman and colleagues have identified residues in the TPRs required for interaction with Mud, as well as the minimal amino acid sequence of Mud required to interact with the TPRs. A crystal structure would allow for visualization of these residues in the TPRs and their local conformation in the inhibited Pins.

I demonstrated that the GL3 regulatory module was conserved in mammalian systems in that LGN repression of NuMA required the GL3/4 region. This suggests this strategy for limiting association of the downstream microtubule associated protein may be of general importance. As such, the Pins orthologues in *C. elegans*, GPR1/2, contains an N-terminal helical-rich region (potentially TPR-like) and a C-terminal GL domain. It was shown that GPR1/2, along with the Gαi and Mud orthologues (GOA-1 and Lin-5, respectively) comprise an analogous signaling pathway required for spindle positioning in the asymmetric division of the one-cell zygote (Couwenbergs et al., 2004). It has never been shown if formation of this complex is regulated by an analogous autoinhibitory mechanism. I plan to test if the Lin-5 association is regulated by the GL domain and if it can be activated by Gαi, similar to what we observe in Pins and LGN. This could be addressed by asking if the three proteins form a ternary complex in vivo by co-immunoprecipitation experiments in the absence and presence of GOA-1 RNA*i*. I hypothesize that this autoinhibitory module is conserved in GPR1/2 and that the three proteins would only form a ternary complex in the absence of GOA-1 knockdown.

Another question raised by my studies is what is the functional importance of autoinhibition on spindle orienting activity? I identified a Pins deletion construct (Pins 1-610) that lacks the autoinhibitory GL3 region and binds Mud constitutively similar to the free TPR domains. As misregulation of signaling pathways is often associated with disease (Pawson and Nash, 2003), I will test the ability of this mutant to rescue spindle orientation defects of the *pins-* neuroblasts. My hypothesis is that this mutant will fail to align the spindle along the cell polarity axis because Pins-Mud association will no longer be restricted to the apical cell cortex. Loss of spindle cortex coupling may lead to defects in cell fate specification.

The ultrasensitive behavior of Pins appears to be important for generating maximal pathway activation in the response to a limited amount of Gαi input. This feature arises from the three GL repeats in the C-terminal half of Pins. As stated earlier, the *C. elegans* Pins orthologues, GPR1/2, only contain one GL domain. Therefore, the Pins proteins in this system should display graded signaling pathway dynamics. How does the spindle orientation pathway generate the necessary amount of output in this system? There may be more input signal present at the cortex in these cells or there could be a feedback mechanism at work. Conversely, LGN has evolved a fourth GL domain. This suggests that this feature was incorporated through a recombination event to make the pathway more switch-like in nature. This could be addressed by a similar method used in our in vitro analysis of Pins. Newman et al. have identified the minimal NuMA sequence of interacting with the LGN TPRs. I plan to test if LGN displays ultrasensitive regulation through a decoy mechanism similar to Pins utilizing the "mini-

LGN" construct introduced in Chapter II. I hypothesize that the additional GL domain in LGN will make it more ultrasensitive than Pins with $n_{H,\mathrm{app}} > 3.1$.

Concluding remarks

My thesis research has identified molecular mechanisms responsible for autoinhibition and ultrasensitivity in the Gαi-Pins-Mud spindle orientation pathway. These findings support the hypothesis that modular recombination provides an evolutionary platform to incorporate new regulatory features into cell signaling proteins. These results are the first to demonstrate that sigmoidal pathway responses can be incorporated into binary protein-protein interaction networks without cooperativity. This may be a more common strategy of cell signaling proteins to build sigmoidal activation into regulatory pathways as it is much simpler to incorporate repeat protein interaction domains than it is to evolve thermodynamic coupling between these repeats.

APPENDIX

SUPPLEMENTAL MATERIALS FOR CHAPTER III

SUPPLEMENTAL METHODS

Western blot of larval brain lysate

Twelve second to early third instar larval brains were dissected in SIM and lysed in 1x PBS + 0.1% NP-40 by homogenization. Samples were separated by SDS-PAGE, transferred to nitrocellulose and probed for Pins or tubulin (loading control). Antibodies used were rat anti-Pins (1:1000), mouse anti-tubulin (DMIA, 1:1000). HRP-conjugated secondary antibodies (from Santa Cruz Biotechnology), followed by enhanced chemilluminescence (Thermo Fisher) were used for visualization.

Analysis of spindle orientation by Mud staining

Images of fixed and stained larval brain neuroblasts were acquired as described earlier. Because Mud associates strongly to centrosomes in a Pins independent manner, we were able to analyze spindle position by measuring the angle between a line drawn through the centrosomes to the cell center and back to the center of the apical Pins crescent. As described earlier, cells expressing WT or ΔGL1,2 Pins transgenes were only scored if the apical Pins intensities were ≥ 1.5x that in the cytoplasm.

SUPPLEMENTAL FIGURES

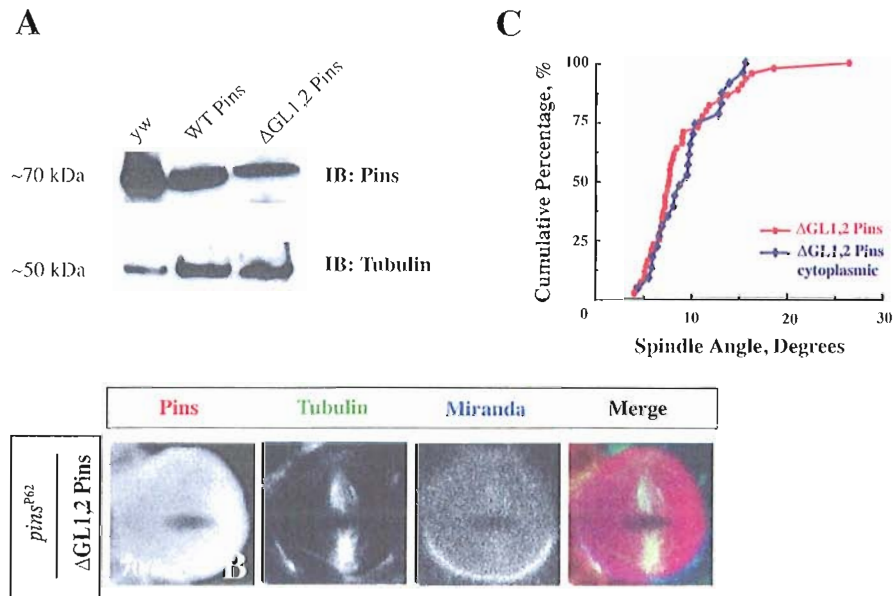


Figure S1: ΔGL1,2 Pins expression and localization in neuroblasts

(A) A western blot of larval brain lysate shows that the transgenic Pins proteins are expressed at the predicted molecular weights. These samples were probed for tubulin as a loading control.

(B) Representative image of the ~70% of fixed larval brain neuroblasts with cytoplasmic ΔGL1,2 Pins staining.

(C) Cumulative percentage plot comparing spindle angles determined for mitotic neuroblasts with apically enriched (red) or cytoplasmic ΔGL1,2 Pins (blue).

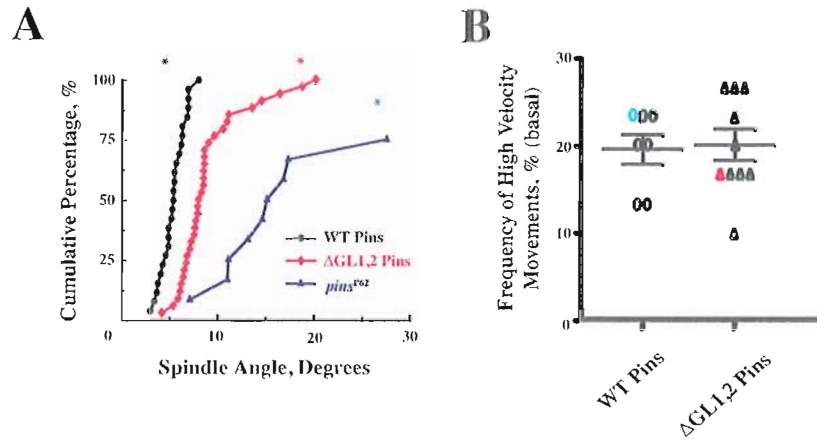


Figure S2: Analysis of spindle angle in ΔGL1,2 Pins neuroblasts by Mud staining and quantification of basal spindle pole dynamics

(A) Cumulative percentage of spindle angle measurements for each experimental condition from Mud stains relative to the center of the apical Pins crescent. *pins*^{P62} NB spindle angles were determined relative to the apical Par6 signal. Spindle angle was measured using ImageJ software. Asterisks: Differences are statistically significant.

(B) Plot of frequency of high velocity spindle movements for the basal spindle pole in WT or ΔGL1,2 Pins movies (see methods). The data from representative movies are represented as blue or red for WT and ΔGL1,2 Pins, respectively.

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