The Role of Wnt Signaling in C. elegans Neuronal Development
by
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by

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Abstract
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Cellular polarization is critical for many stages of neuronal development, including the asymmetric division of the neuroblast and the axon-dendrite specification of the mature neuron. In C. elegans, Wnt glycoproteins control the polarization of the ALM mechanosensory neurons, but the molecules that mediate Wnt signaling are unclear. I found that the Ror kinase CAM-1, which has been shown to bind to Wnts, is required for ALM polarity. CAM-1 acts in the ALM to promote its polarity and CAM-1 may also have a non-autonomous function in sequestering Wnts. Furthermore, I implicated actin regulators unc-34 and two known components of the Rac pathway, ced-10/Rac and mig-10/lamellipodin, in ALM polarity. Expression of unc-34 and mig-10 from a neuron-specific promoter unc-86 significantly rescued the ALM polarity defect, indicating these genes can function in the neurons. Lastly, I identified that the MIG-15 kinase, a member of the Ste20 kinase superfamily, and potential components of a MIG-15 signaling pathway that might be novel Wnt effectors in neuronal polarity.

Wnts also regulate the asymmetric cell divisions (ACD) of many non-neuronal cell types and the Q neuroblast lineage (Teuliere & Garriga, unpublished observation). The molecules that mediate Wnt signaling in ACD in the Q lineage are unclear. One possible candidate is PIG-1, which is orthologous to vertebrate MELK and belongs to a family of serine/threonine kinases including PAR-1, SAD-1 and AMPK (Cordes et al., 2006). This group of kinases can be phosphorylated and activated by the polarity-regulating kinase LKB1. LKB1 kinase, along with its binding partners STRAD and MO25, have been shown to be master regulators of polarity in many different contexts, and I find that C. elegans orthologs of LKB1, STRAD and MO25 regulate the asymmetric cell division of the Q.p lineage and are potential regulators of PIG-1 in this process.
To My Family
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CHAPTER ONE

AN INTRODUCTION TO NEURONAL POLARIZATION AND WNT SIGNALING
Cellular polarization is critical for the specialized functions of many cell types, including epithelial cells, migrating cells and cells undergoing asymmetric cell division. Neurons are among the most highly polarized cells in our bodies, often exhibiting multiple branched dendrites receiving and integrating synaptic inputs and a single long axon that has presynaptic sites that can release neurotransmitters into the synaptic cleft. In addition to differences in morphology and function, axons and dendrites exhibit distinct molecular markers. For instance, axons express the microtubule-binding tau whereas dendrites express microtubule-associated protein MAP-2. How neurons establish polarity has fascinated neuroscientists since the time of Ramón y Cajal, but only in the last decade have we begun to unravel the molecular mechanisms governing neuronal polarity. An enhanced understanding of this process has already begun to provide exciting clues for promoting regeneration of severed neurons following injury (Hellal et al., 2011).

**Development of neuronal polarization in vitro**

Pioneering studies by Banker and colleagues in the 1980s using cultured rat embryonic hippocampal neurons provided us with significant insights into the process of neuronal polarization. (Dotti et al., 1988). In this system, post-mitotic hippocampal neurons are isolated from embryonic day 18 rats, plated onto coverslips, and observed over a 10-day period. The development from an unpolarized neuron into a neuron with a single axon and multiple dendrites is highly stereotypical and can be divided into five characteristic stages. Shortly after the neurons are plated, they spread filopodia and lamellipodia (stage 1), which develop into immature neurites with similar lengths (stage 2). At this stage, neurites continuously grow and retract until one of them starts to break the morphological symmetry and grows faster than the others to become the final axon (stage 3). A few days later, the remaining neurites develop into mature dendrites (stage 4). Approximately 10 days after plating, neurons form synapses and establish a neuronal network (stage 5) (Arimura and Kaibuchi, 2007). In this cultured system, the neurons can polarize spontaneously in the absence of external cues, suggesting a cell-intrinsic program for specifying axons and dendrites. External cues are also important to direct axon-dendrite specification in vivo (see later sections).

Transition from stage 2 to 3 signals a major symmetry-breaking event in a neuron when one axon is specified. However, such fate specification is not fixed; even neurons integrated into functional circuits can still generate axons from dendrites (Gomis-Ruth et al., 2008). Such neuronal plasticity was first demonstrated with stage 3 neurons (Goslin and Banker, 1989). The researchers found that if they transected the axon so that it was of similar length to the other processes, a scenario similar to stage 2 of normal development, a round of competition ensued until one process (not necessarily the original axon) exhibited accelerated growth and became an axon. If the transected axon was still longer than other processes, it had a good chance of regenerating. Interestingly, when the transected axon was transected 10 µm shorter than the other processes, the shortened axon became a dendrite and the longest process, which previously destined to be a dendrite, grew rapidly and became an axon. These axon transection studies suggest that all neurites during early development (stages 1 and 2) have the potential to become an axon and once one neurite exceeds the others by a critical length through stochastic polarization, it will be specified as an axon (in stage 3).
Models of neuronal symmetry-breaking: stochastic vs. pre-determined

Neuronal polarization of cultured hippocampal neurons is thought to be a stochastic event; a neurite which by chance accumulates the threshold level of cytoplasmic determinants will amplify this initial asymmetry through a local positive feedback loop and becomes an axon. The developing axon also simultaneously sends a long-range feedback inhibitory signal to other processes to prevent them from becoming axons (Andersen and Bi, 2000). This is known as the “activator-inhibitor” model. An alternative “one-takes-all” model postulates that the negative feedback may not be necessary if the developing axon depletes the limiting pool of axon-specifying components so that the neuron cannot support growth of more than one axon (Fivaz et al., 2008; Inagaki et al., 2010; Toriyama et al., 2010). Although many signaling molecules that can specify axons have been identified (see later sections), how positive and negative feedback loops work remain elusive.

One of the earliest molecular markers for neuronal polarization in terms of development is shootin1 (Toriyama et al., 2006). The protein initially shows stochastic accumulation in multiple neurites but preferentially localizes to a single neurite before the neuron breaks its symmetry. Through quantitative live-cell imaging and mathematical modeling, Toriyama et al. found that the selective shootin1 accumulation depends on stochastic anterograde transport and retrograde diffusion (Toriyama et al., 2010). shootin1 concentration rises in the longest neurite because it exhibits the slowest diffusion rate. This shootin1 upregulation induces further neurite outgrowth, eventually leading to the specification of the axon. This nicely illustrates the concept of positive feedback and suggests a neurite-length sensing mechanism for neuronal polarization.

In the abovementioned system, the initial asymmetry is generated by differential retention of the determinant among neurites of different lengths. It is conceivable that the asymmetry can also be generated by enhanced transport into the prospective axon. Fivaz et al. (2008) proposed that the positive feedback between small GTPase HRas and downstream signaling lipid kinase PI3K (detailed in a later section) would enhance anterograde transport of HRas into the developmental axon, although this has not been directly proven. Interestingly, the authors noted that an increase in HRas level in the developing axon is coupled to a decrease in HRas level in other neurites, suggesting the amount of HRas is limiting in a neuron. This finding is consistent with the “one-takes-all” model where one axon is formed by out-competing other neurites for components necessary for axonal outgrowth.

However, other lines of evidence suggest that long-range inhibitory signals have to exist to prevent formation of multiple axons. For instance, that mechanical elongation of a neurite from a stage 3 neuron (with an axon already specified) leads to a neuron with two axons (Lamoureux et al., 2002). Furthermore, treating stage 4 neurons with actin-depolymerizing drug cytochalasin D results in a multiple axon phenotype (Bradke and Dotti, 2000). These studies argue for the existence of inhibitory signals to prevent dendrites from becoming axons.
In support of the so-called “activator-inhibitor” model, Shelly et al. (2010) found that levels of secondary messengers cAMP and cGMP are critical in specifying axons and dendrites in the cultured hippocampal neurons. Developing axons accumulate high levels of cAMP but low levels of cGMP whereas dendrites exhibit the opposite patterns. The observation that cAMP and cGMP are regulated antagonistically suggests that axon formation is accompanied by dendrite inhibition, and vice versa. Notably, targeted elevation of cAMP in one immature neurite caused a decrease in cAMP level (and concomitant increase in cGMP level) in all other neurites of the same neuron. This ensures formation of a single axon through a long-range negative feedback mechanism.

Although “one-takes-all” and “activator-inhibitor” models differ in whether the long-range inhibition exists, they essentially agree that local activation of a single neurite to become an axon is a result of a stochastic event. A competing model argues that hippocampal neurons can use a pre-mitotic mechanism to specify axons (de Anda et al., 2005). The authors from the study cultured the embryonic day 16 (E16) hippocampal neurons, instead of the older conventional E18 neurons, and consistently found that the first developing neurite from the neuron became the future axon. Furthermore, they noticed that the close clustering of centrosomes, Golgi and endosomes opposite to the plane of the last mitotic division and the positions of these organelles were predictive of where the future axon would appear. A similar correlation between centrosome localization and axon formation was observed in cultured cerebellar granule neurons (Zmuda and Rivas, 1998). Manipulation of centrosome numbers in hippocampal neurons led to a corresponding change in the number of axons, arguing the centrosomal polarized activity is both necessary and sufficient for axon formation (de Anda et al., 2005). It is hypothesized that centrosomes as microtubule organizing centers (MTOCs) can support polarized growth more efficiently in the prospective axon (de Anda et al., 2005). These findings suggest that a pre-existing cell polarity can dictate axon specification and changes in cleavage plane orientation during mitosis may disrupt neuronal polarization.

**Neuronal polarization in vivo**

Studies in cultured hippocampal neurons have shed light on the mechanism of neuronal polarization but questions arise as to whether these findings are relevant in vivo. As opposed to living in a homogenous environment, neurons in vivo often have to come in contact with other types of cells and respond to extrinsic polarizing factors. These environmental constraints may cause the neurons in vivo to behave differently than when they are cultured. Indeed, Zolessi et al. found that zebrafish retinal ganglion cells (RGC) in vivo do not display numerous immature neurites prior to polarization like they did in vitro (Zolessi et al., 2006). Furthermore, the first neurite that appears from the grasshopper Ti1 neuron becomes the future axon (Lefcort and Bentley, 1989). In contrast, neurons from mammalian neocortex do exhibit a multi-polar morphology before axon initiation (Noctor et al., 2004), suggesting there is a wide range of cell-type specific patterns of neuronal polarization in vivo.

One common theme emerging from studies of various in vivo models is that post-mitotic neurons often migrate extensively before they polarize, and the leading and trailing processes formed during migration are subsequently specified as the dendrite and the
axon, respectively or vice versa, depending on the cell type (Barnes and Polleux, 2009). In the case of zebrafish RGCs, neuroepithelial progenitor cells first undergo asymmetric cell division near the apical surface. Then the nucleus of the neuron translocates toward the basal surface, followed by retraction of its apical process. Eventually the basal process of the neuron is specified as the axon while dendrites extend from its apical side (Zolessi et al., 2006). Mutations that disrupt the apical/basal polarity of neuroepithelium can cause an axon to emerge from the apical side of the neuron, highlighting the importance of the integrity of neuroepithelium in establishing neuronal polarity.

Another well-studied in vivo model is the mammalian neocortex, thanks to the pioneering work in fetal monkeys by Pasko Rakic in the 1970s. After being generated by the glial progenitors in the ventricular zone, the cortical neurons migrate radially toward the pial surface, each cell exhibiting both a leading and a trailing process. Following a transient multi-polar phase, the trailing process of the neuron adopts the axonal fate and elongates rapidly. The leading process eventually gives rise to apical dendrites (Barnes and Polleux, 2009; Hatanaka and Murakami, 2002; Rakic, 1971). In the rat neocortex, the centrosome has been observed to translocate from the apical side to the basal side of the neuron during the multi-polar phase prior to axonal outgrowth and laser inactivation of centrosome disrupts axon formation (de Anda et al., 2010). This finding echoes the in vitro studies that centrosome positioning helps determine the site of axon formation.

However, there are clearly cell type-specific differences because the positioning of the centrosome doesn’t correlate with the site of axon emergence in zebrafish RGCs (Zolessi et al., 2006). In that case, the centrosome stays on the apical side while the axon projects basally. Furthermore, the observation that Drosophila mutants lacking centrioles form a grossly normal nervous system (Basto et al., 2006) argues against centrosomes being essential for all types of neuronal polarization.

**Intrinsic regulators of neuronal polarity**

How asymmetry is generated from an initially symmetric state is a fascinating question in developmental biology. The leading edge of migrating cells and highly motile structures called growth cones that tip the axons both exhibit polarized behaviors that are conceptually similar to neuronal symmetry breaking (von Philipsborn and Bastmeyer, 2007). Indeed, a number of molecules important for chemotaxis and axon guidance are also involved in neuronal polarization. These regulators of neuronal polarization were identified, in many cases, by analyzing (1) their selective localization in the developing axon and (2) effects of their up-regulation or down-regulation on the formation of axons and dendrites (Barnes and Polleux, 2009).

**The role of cytoskeleton in neuronal polarity**

Actin and microtubules have inherent structural polarity, and their ability to undergo dynamic assembly and disassembly allows the cell to respond rapidly to polarity signals. Along with a large number of cytoskeleton-associated proteins (i.e. motor proteins), actin and microtubules are critical in establishing both morphological as well as functional asymmetry within a cell (Li and Gundersen, 2008). The axon and dendrites are highly
polarized structures within a neuron: whereas axons are characterized by a more dynamic actin structure at the growth cone (Bradke and Dotti, 1999) and stable microtubules in the shaft (Witte et al., 2008), dendrites usually have a more stable actin network and less stable microtubules. It is thought that a less stable actin structure at the growth cone would allow more efficient penetration of microtubules, resulting in elongation of the axon (Bradke and Dotti, 2000). This model is supported by numerous studies which have shown that axon/dendrite specification and outgrowth can be altered through modification of cytoskeletal dynamics. First, treating a single immature neurite in cultured hippocampal neuron with actin-depolymerizing drug cytochalasin D (Bradke and Dotti, 2000) or microtubule-stabilizing drug taxol (Witte et al., 2008) induces axon formation. Strikingly, taxol promotes functional axon regeneration in CNS of rodents (Hellal et al., 2011), raising the exciting possibility that this treatment can be used in humans following spinal cord injury. Second, over-expression of profilin, a positive regulator of actin polymerization, reduces process outgrowth (Da Silva et al., 2003). Conversely, over-expression of coflin, a negative regulator of actin polymerization, promotes process outgrowth (Meberg and Bamburg, 2000). Third, the kinesin-1 motor domain, which preferentially binds to stable microtubules marked by acetylation (Reed et al., 2006), accumulates in the prospective axon before neuronal polarization (Jacobson et al., 2006). With the recent discovery of tubulin acetyltransferases (Akella et al., 2010; Shida et al., 2010), it will be interesting to see whether inactivation of these enzymes disrupts axon formation both in vitro and in vivo. The affinity of kinesin-1 for stable microtubules may also explain an earlier observation that organelles, membrane vesicles and cytoplasmic components are preferentially targeted to the prospective axon (Bradke and Dotti, 1997). In addition to acetylation, microtubule stability can be regulated by the phosphorylation of microtubule-associated proteins such as tau (discussed in later sections).

**Lipid signaling: PI3-kinase and potential effectors**

Studies of Dictyostelium and neutrophil migration have shown that PI3-kinase and its lipid product phosphatidylinositol-3,4,5-triphosphate (PIP3) are concentrated near the leading edge of the cell and this localized PI3K activation is essential for proper chemotaxis (von Philipsborn and Bastmeyer, 2007). Similarly, PI3K is important for neuronal polarization as pharmacological inhibition of this enzyme blocks axon specification (Menager et al., 2004; Shi et al., 2003). By contrast, over-expression of a constitutively active form of PI3K induces formation of multiple axons (Yoshimura et al., 2006). Local activation of PI3K leads to production of PIP3, which can bind to pleckstrin homology (PH) domain-containing proteins such as Akt. The translocation of Akt to the PIP3-enriched membrane allows it to be subsequently activated by kinases PKD1 and PKD2 (Fayard et al., 2010). Using the PH domain of Akt fused to GFP (PH Akt-GFP) as a biosensor for PIP3 formation, Menager et al. (2004) shows that when coming in contact with extracellular matrix protein laminin, a single immature neurite from a stage 2 hippocampal neuron selectively accumulates PIP3 at its tip and then elongates rapidly to become an axon. Akt is enriched at the tip of the axon (Shi et al., 2003) and over-expression of membrane-tethered (constitutively active) Akt causes multiple axons to form (Jiang et al., 2005; Yoshimura et al., 2006). Taken together, these data suggest that there is an axon-promoting pathway comprised of PI3K, PIP3 and Akt. The enzyme that
antagonizes PI3K activity is the lipid phosphatase PTEN, which converts PIP3 to PIP2. Over-expression of PTEN disrupts axon formation (Jiang et al., 2005; Shi et al., 2003) whereas reducing levels of PTEN causes a multiple axon phenotype (Jiang et al., 2005). These results echo findings in chemotaxis models that a proper balance between PIP3 and PIP2 is essential for cell polarization.

One downstream target of Akt is GSK3β (glycogen synthase kinase 3β), a negative regulator of axon specification (Jiang et al., 2005). GSK3β is normally constitutively active and its inhibition at the tip of the developing axon by Akt-dependent phosphorylation is important for axonal outgrowth. Over-expression of a GSK3β variant that cannot be phosphorylated by Akt interferes with axon formation whereas knockdown of GSK3β level by siRNA or pharmacological inhibitors results in multiple axon formation (Jiang et al., 2005; Yoshimura et al., 2005). Interestingly, inhibiting GSK3β function in stage 4 neurons can convert pre-existing dendrites into axons (Jiang et al., 2005), highlighting the role of GSK3β in both establishing and maintaining neuronal polarity. However, the functional relationship between GSK3β and Akt is less clear in vivo as knock-in mice bearing non-phosphorylatable alleles of GSK3α/β do not display neuronal polarity defects (Gartner et al., 2006). Nevertheless, the observation that pharmacological inhibition of GSK3β induces multiple axon formation in vivo suggests that GSK3β negatively regulates neuronal polarity both in vitro and in vivo, although how GSK3β is regulated in vivo is unclear.

One way GSK3β inhibits axonogenesis is by phosphorylating and inactivating a panel of proteins known to be involved in microtubule dynamics. One such substrate is CRMP-2 (collapsin response mediator protein 2), which when unphosphorylated binds to tubulin dimers to promote microtubule assembly and axon outgrowth (Fukata et al., 2002). Phosphorylated CRMP-2 exhibits reduced affinity for tubulin. Consistent with this finding, non-phosphorylated CRMP-2 localizes to the tip of the developing axon where inactivated GSK3β (phosphorylated by Akt) is also found (Jiang et al., 2005; Yoshimura et al., 2005). Over-expression of a non-phosphorylatable CRMP-2 induces formation of multiple axon-like neurites and can suppress the growth-inhibitory effect of GSK3β over-expression (Yoshimura et al., 2005). These data suggest GSK3β regulates neuronal polarity through phosphorylation of CRMP2.

Other GSK3β substrates include microtubule-associated proteins (MAP) such as tau, MAP1B and APC. MAPs bind to microtubules and promote their stability when they are unphosphorylated (Mandelkow and Mandelkow, 1995). The critical role of MAPs in neuronal development is evidenced by the observation that mice doubly mutant for tau and MAP1B show severe neurite elongation defects (Takei et al., 2000). As axons tend to accumulate stable microtubules, they are marked by unphosphorylated tau. By contrast, phosphorylated tau is enriched in the dendrites (Kishi et al., 2005). Consistent with an inhibitory role of GSK3β in microtubule organization, depletion of GSK3β leads to accumulation of axonal microtubules (Zhou et al., 2004). APC (adenomatous polyposis coli), a microtubule plus end-binding protein, normally localizes to the tip of the developing axon but becomes uniformly distributed in the axon when GSK3β is depleted. The depletion of GSK3β causes a disruption in axonal outgrowth and highlights the
importance of spatial regulation of microtubule polymerization (Zhou et al., 2004). Taken together, the data on GSK3β regulation point to a model in which PI3K activation at the tip of the developing axon leads to the local inhibition of GSK3β, which in turn allows downstream effectors such as CRMP-2 and APC to stabilize microtubules and promote axonogenesis.

Another regulator of PI3K has been recently identified using a proteomics approach. shootin1, described in a previous section as one of the earliest markers of neuronal polarization, is found to co-localize with phosphorylated Akt at the tip of the developing axon (Toriyama et al., 2006). RNAi knockdown of shootin1 abrogates axon formation and leads to a loss of phosphorylated Akt signal. Conversely, over-expression of shootin1 results in supernumerary axons, a phenotype that can be suppressed by inhibition of PI3K activity. These data suggest that shootin1 is required to spatially localize PI3K activity important for neuronal polarization.

Ras and Rho family of small GTPases
Small GTPases are crucial regulators of membrane dynamics and vesicular trafficking. They switch from an inactive GDP-bound state to an active GTP-bound state via guanine-nucleotide exchange factors (GEFs) and to complete the cycle, GTPase-activating proteins (GAPs) help increase the endogenous GTPase activity of GTPases (Luo, 2000). Several members of the Ras family of GTPases have been implicated in neuronal polarity, including H-Ras. Over-expression of H-Ras causes multiple axons to form and this phenotype is suppressed by inhibition of PI3K activity, suggesting H-Ras is upstream of PI3K (Fivaz et al., 2008). Interestingly, PI3K inhibition also reduces the activity level of H-Ras, consistent with H-Ras and PI3K functioning in a positive feedback loop to promote axon outgrowth (Fivaz et al., 2008).

Rap1B belongs to Ras superfamily of GTPases and is localized to the tip of the prospective axon in unpolarized stage 2 neurons, preceding the accumulation of another small GTPase Cdc42 (Schwamborn and Puschel, 2004). Over-expression of either Rap1B or Cdc42 induces multiple axon formation whereas reducing their levels by siRNA suppresses formation of axons. Over-expression of Cdc42 rescues the no-axon phenotype in Rap1B siRNA knockdown but not vice versa, indicating that Cdc42 acts genetically downstream of Rap1B. Furthermore, axon loss resulting from PI3K inhibition can be reversed by over-expression of either Rap1B or Cdc42, placing both GTPases downstream of PI3K signaling in axon specification (Schwamborn and Puschel, 2004). In contrast to Rap1B and Cdc42, which are required to specify a single neurite to become the axon and extend rapidly, Rho and Rac GTPases antagonistically regulate the extension of both axons and minor neurites. Increasing Rho or decreasing Rac activity causes a reduction of neurite length, whereas decreasing Rho or increasing Rac activity has the opposite phenotype (Luo, 2000; Ng et al., 2002). While the role of Rac in regulating outgrowth has been demonstrated, it is less clear how it regulates polarity. A definitive role has been described for Tiam1, a GEF for Rac1, discussed in the next section.

Par proteins
Pioneering work by Kemphues and colleagues identified six par (partitioning defective) genes that are essential for the asymmetric cell division of the one-cell C. elegans embryo. In particular, the Par3/Par6/aPKC complex has since been shown to regulate cell polarization in other contexts such as the establishment of apical/basal polarity of epithelial cells and neuroblasts in Drosophila (Goldstein and Macara, 2007). In stage 3 hippocampal neurons, PAR-3, PAR-6 and phosphorylated (active) aPKC concentrate at the tip of the developing axon (Schwamborn and Puschel, 2004; Shi et al., 2003). In addition, pharmacological inhibition of aPKC disrupts axon formation (Shi et al., 2003), arguing that the PAR complex is important for axon specification in vitro. Given this role for the PAR complex in hippocampal neuron polarity, it is surprising that Drosophila mutant for PAR-3, PAR-6 or aPKC do not show defects in neuronal polarity (Rolls and Doe, 2004). Other polarization pathways may exist to compensate for the loss of PAR proteins in Drosophila.

In hippocampal neurons, the PAR complex integrates signaling from many pathways including the small GTPases Cdc42 and Rac, regulators of microtubules and kinesin (Arimura and Kaibuchi, 2007). PAR-6 and PAR-3 have been shown to physically interact with the GTP-bound Cdc42 and Tiam1, a GEF for Rac1, respectively (Lin et al., 2000; Nishimura et al., 2005). The observation that depletion of PAR-3 in cell culture can suppress lamellipodial protrusion induced by constitutively-active Cdc42 but not by Rac1 indicates that the PAR complex is genetically downstream of Cdc42 and upstream of Rac signaling (Nishimura et al., 2005). Tiam1 is found to co-localize with PAR-3 at the tip of the developing axon. Furthermore, over-expression of Tiam1 induces formation of multiple axon-like neurites whereas depletion of Tiam1 blocks axon formation (Nishimura et al., 2005). These data together suggest the PAR complex regulates neuronal polarity by mediating Cdc42-induced Rac activation. As GTP-bound Rac1 can activate PI3K (Keely et al., 1997), this forms a positive feedback loop which can sustain local activation of PI3K and downstream molecules to promote axon outgrowth (Arimura and Kaibuchi, 2007).

Another function of the PAR complex in neuronal polarity is through negative regulation of MARK2 (microtubule affinity-regulating kinase 2). This family of kinases phosphorylates microtubule-associated proteins such as tau, resulting in their dissociation from microtubules and increased microtubule dynamics (Drewes et al., 1997). Similar to the loss of GSK3β, depletion of MARK2 leads to reduced tau phosphorylation and supernumerary axons. By contrast, over-expression of MARK2 blocks axon formation (Chen et al., 2006). Interestingly, this phenotype can be rescued by ectopic expression of the PAR3/PAR6/aPKC complex as aPKC can phosphorylate and inactivate MARK2. This rescue is abolished if over-expressing a MARK2 mutant that cannot be phosphorylated by aPKC (Chen et al., 2006). Taken together, these data suggest that aPKC, in complex with PAR3 and PAR6, negatively regulates MARK2, thereby promoting microtubule stabilization and axon elongation.

Recently a class of secreted glycoproteins called Wnts (detailed in a later section) have been shown to act upstream of aPKC and MARK2 (Zhang et al., 2007). The binding of Wnt5a to the Frizzled receptor activates the cytoplasmic protein Dishevelled, which can
physically interact with aPKC and stimulates its kinase activity. Inhibition of aPKC function can suppress the multiple axon phenotype induced by over-expression of Wnt5a or Dishevelled, suggesting that Wnt signaling can promote axon differentiation by activating the PAR complex.

In addition to the PAR complex, LKB1, the mammalian homolog of *C. elegans* PAR-4, has recently been shown to be a critical regulator in axon specification both in vitro and in vivo (Barnes et al., 2007; Shelly et al., 2007). LKB1 kinase and its cofactor STRAD are found to co-localize in a single undifferentiated neurite prior to neuronal polarization and this localization predicts axonal fate. Over-expression of STRAD induces formation of multiple axons whereas siRNA-mediated knockdown of STRAD blocks axon formation. The activation of LKB1 requires PKA phosphorylation at S431 and over-expression of non-phosphorylatable LKB1$^{S431A}$ abolishes axon formation of developing cortical neurons in vivo, a phenotype also observed when LKB1 is inactivated by conditional knockout or RNAi (Barnes et al., 2007; Shelly et al., 2007). LKB1 has been shown to be a master kinase in vitro and can phosphorylate and activate at least 13 kinases in the AMPK subfamily, including SAD-A and SAD-B (Lizcano et al., 2004). Notably, neurons from double knockout mice for SAD-A and SAD-B fail to grow axons (Kishi et al., 2005). Together with the finding that RNAi knockdown of SAD-A/B largely abrogates the multiple axon phenotype caused by LKB1 over-expression (Barnes et al., 2007), these data suggest a kinase cascade (PKA $\rightarrow$ LKB1 $\rightarrow$ SAD-A/B) is essential for neuronal polarization in vivo. SAD kinases are structurally related to MARK and have also been shown to alter microtubule organization through tau phosphorylation (Kishi et al., 2005). However, unlike MARK2, SAD kinases are not inhibited by aPKC (Chen et al., 2006).

In addition to SAD-A/B, another kinase Stk25 has recently been reported to mediate the effect of LKB1 in neuronal polarization (Matsuki et al., 2010). Stk25 has previously been shown to interact with Golgi matrix protein GM130 to regulate Golgi morphology (Preisinger et al., 2004). Matsuki et al. show that LKB1, Stk25 and GM130 act in the same pathway to promote axon initiation through Golgi condensation. Interestingly, the Reelin-Dab1 pathway, well known for its role in radial migration of cortical neurons (Rice and Curran, 2001), antagonizes the LKB1 pathway by facilitating Golgi deployment and dendrite outgrowth. This study echoes earlier findings that positioning of the centrosome and Golgi predict the sites of axon emergence (de Anda et al., 2005; de Anda et al., 2010) and highlights the dual roles of Golgi in neuronal polarity. In axon specification, Golgi probably provide a signaling function such as nucleating microtubules instead of supplying membranes because manipulation of Golgi morphology changes axon number but not axon length. By contrast, Golgi likely serves as a supplier of membrane and materials in dendritic development. The selective trafficking of Golgi into dendrites may facilitate formation of Golgi outposts that are essential for dendritic branching and growth (Horton et al., 2005).

**The role of selective transport and degradation in neuronal polarization**

The asymmetric localization of axon determinants to a single neurite is essential for proper neuronal polarization. This can theoretically be achieved by many mechanisms
including selective protein synthesis, transport and degradation. The role of selective protein synthesis has been well established in axon guidance. For example, guidance receptors and polarity regulators can be locally synthesized in the growth cone of the axon in response to an extracellular cue (Lin and Holt, 2008). Whether selective protein synthesis is essential to polarize neurons remains to be determined. On the other hand, the roles of selective transport and degradation in neuronal polarization are well documented, as summarized below.

Selective transport
The axons of vertebrate neurons have uniformly plus-end-distal microtubules (Baas and Lin, 2010), and this feature allows kinesins to transport axon determinants and other cargos to the tip of the axon. It has been reported that guanylate kinase–associated kinesin (GAKIN) can mediate transport of PIP3 to the tip of the prospective axon (Horiguchi et al., 2006). Similarly, PAR-3 has been observed to accumulate at the tip of axon via binding to kinesin KIF3A (Nishimura et al., 2004; Shi et al., 2004).

Selective degradation
Selective protein degradation has been shown to be important for neuronal polarization as pharmacological inhibition of ubiquitin-proteasome system (UPS) leads to formation of multiple axons (Schwamborn et al., 2007; Yan et al., 2006). Conceptually, degradation of axon-promoting factors in minor neurites or degradation of axon-inhibitory factors in the prospective axon both can restrict axon-growing potential to a single neurite. Indeed, the growth-promoting Akt has been shown to be selectively degraded in the dendrites but not in the axons, resulting in a net enrichment of Akt in the axon (Yan et al., 2006). Furthermore, the small GTPase Rap1B in its inactive (GDP-bound) form becomes ubiquitinated by E3 ubiquitin ligase Smurf2, so only activated Rap1B (GTP-bound) at the tip of the nascent axon will be spared from degradation (Schwamborn et al., 2007). Intriguingly, another ubiquitin ligase, Smurf1, has recently been shown to regulate abundance of two proteins that have opposing functions in neuronal polarity: axon-promoting PAR-6 and growth inhibitory RhoA (Cheng et al., 2011). In the future axon, Smurf1 is phosphorylated by PKA and preferentially ubiquitinates RhoA. By contrast, the unphosphorylated Smurf1 found in minor neurites preferentially ubiquitinates PAR-6. This selective protein degradation results in asymmetric localization of factors that are essential for neuronal polarization.

The role of extracellular cues during neuronal polarization
In vitro
While hippocampal neurons grown in culture exhibit intrinsic polarity, it is clear that they can also respond to extracellular signals. To test whether growth-promoting molecules such as laminin and neuron-glial cell adhesion molecule (NgCAM) facilitate axon specification, Banker and colleagues (Esch et al., 1999) cultured neurons on substrates patterned with alternating stripes of poly-L-lysine and either laminin or NgCAM. They found that axons preferentially formed on laminin or NgCAM stripes, which supported their hypothesis. Recently Poo and colleagues performed a similar stripe assay and showed that brain-derived neurotrophic factor (BDNF) plays an important role in axon specification (Shelly et al., 2007). BDNF triggers a rise in cAMP level which results in
PKA activation in the prospective axon. Activated PKA can phosphorylate and activate at least two key substrates described in previous sections: (1) E3 ubiquitin ligase Smurf1, which leads to stabilization of axon determinant PAR-6; and (2) LKB1 kinase, which in turn activates SAD-A/B kinases to re-organize microtubules to promote axon outgrowth. This illustrates how extracellular signaling can regulate neuronal polarity through modulation of cytoskeleton.

Polleux and colleagues devised another elegant assay, the slice overlay assay, to address the role of extrinsic factors in neuronal polarization (Polleux et al., 1998; Polleux et al., 2000). In this assay, they plated fluorescently labeled dissociated pyramidal neurons onto neonatal cortical slices in culture. They found that the diffusible ligand semaphorin 3A (Sema3A) secreted near the marginal zone act as a chemo-attractant for apical dendrites and a chemo-repellent for pyramidal axons (Polleux et al., 2000). How can one signal elicit opposite responses from two compartments of the same neuron? It turned out that soluble guanylate cyclase was asymmetrically localized to the apical dendrite and this asymmetric cGMP signaling provided the basis for the differential response of axons and dendrites. This kind of regulation is not without precedents: it has been shown that artificially raising cGMP levels in *Xenopus* spinal cord neurons can convert Sema3A from a repellent to an attractant in steering of the growth cones (Song et al., 1998).

**In vivo**
Netrin is a conserved guidance cue important for the migration of cells and growth cones along the dorsal-ventral axis (Dickson, 2002). In the nematode *C. elegans*, UNC-6/netrin has been shown to direct the ventral polarization of the motor neuron HSN (Adler et al., 2006). In the third larval stage, multiple neurites can be observed extending from the ventral side but only one neurite remains at the beginning of the fourth larval stage. UNC-6 secreted from the ventral side of the worm induces asymmetric localization of its cognate receptor UNC-40/DCC and an actin regulator MIG-10/lamellipodin to the ventral side of HSN. This UNC-6 orientation pathway also requires small GTPase CED-10/Rac (Quinn et al., 2008) and components of the PI3K signaling such as AGE-1/PI3K and DAF-18/PTEN (Adler et al., 2006).

In contrast to the role of UNC-6/netrin in D/V guidance, Wnts has also been implicated in the migration of cells and growth cones along the anterior-posterior axis in diverse organisms (as detailed below). In *C. elegans*, Wnts are also critical regulators of neuronal polarity as Wnt mutants exhibit a reversed polarity phenotype. The signaling from Wnt LIN-44 to the Frizzled receptor LIN-17 is important for the polarization of mechanosensory neuron PLM. On the other hand, three Wnts CWN-1, CWN-2 and EGL-20 coordinately regulate the polarity of mechanosensory neurons ALM, but it is unclear which Frizzled receptor mediates Wnt signaling in these neurons (Hilliard and Bargmann, 2006; Prasad and Clark, 2006).

**Wnt signaling: an overview**
Wnts are a family of secreted glycoproteins conserved among metazoans. They can regulate a variety of developmental processes including cell fate specification, directed cell motility, body axis formation, organogenesis and even stem cell renewal (Komiya
and Habas, 2008). Defective Wnt signaling has been implicated in a number of human diseases such as cancers of the breast and colon, skeletal defects and neural tube closure defects called spina bifida (Logan and Nusse, 2004).

Wnts are generally thought to signal through a β-catenin dependent canonical pathway or a β-catenin independent non-canonical pathway. In both cases, Wnts function by binding to the extracellular cysteine-rich domain of seven-pass transmembrane receptor Frizzled, which in turn transduces the signal to the cytoplasmic protein Dishevelled. Downstream from Dishevelled the pathways bifurcate. In the case of the canonical pathway, β-catenin translocates into the nucleus and binds to lymphoid enhancer-binding factor 1/T-cell specific transcription factor (LEF/TCF) to activate transcription of Wnt target genes. By contrast, regulators of actin dynamics lead to modification of cytoskeleton in the non-canonical pathway (Komiya and Habas, 2008). How is signaling specificity achieved if Frizzled and Dishevelled are shared between canonical and non-canonical pathways? Part of the answer comes from the observation that Frizzled can bind to different co-receptors, and the resulting receptor complexes have different signaling specificities. For instance, the low-density-lipoprotein-related protein 5/6 (LRP5/6) and the receptor tyrosine kinase Ror can bias Frizzled signaling toward canonical and non-canonical pathways, respectively (Komiya and Habas, 2008). Moreover, a secreted co-factor called Cthrc1 can stabilize Wnt/Fz/Ror complex at the expense of the Wnt/Fz/LRP5/6 complex, thereby promoting non-canonical signaling and antagonizing canonical signaling (Yamamoto et al., 2008). As for Dishevelled, it has been shown that different domains of the protein can mediate different aspects of Wnt signaling. For instance, the N-terminal DIX domain is associated with canonical signaling whereas the C-terminal DEP domain is primarily responsible for non-canonical signaling (Wallingford and Habas, 2005).

**Wnt Signaling: canonical and non-canonical pathways**

The canonical signaling usually leads to transcription of cell fate specification genes, while the non-canonical signaling regulates various polarity contexts via modulation of cytoskeleton. Notably, there is one case where the transcriptional outcome of the canonical signaling affects *C. elegans* cell migration, as discussed below.

**Canonical Wnt signaling**

A hallmark of the canonical pathway is the stabilization and subsequent nuclear translocation of β-catenin. In the absence of Wnt signaling, β-catenin is targeted to the proteasome for degradation by the destruction complex comprised of Axin, glycogen synthase kinase 3β (GSK3β) and adenomatous polyposis coli (APC). However, the binding of Wnts to Frizzled/LRP5/6 receptor complex triggers the phosphorylation of cytoplasmic tail of LRP5/6, creating a docking site for Axin. This leads to the membrane recruitment of the destruction complex, which is subsequently internalized and sequestered into multi-vesicular body (Taelman et al., 2010). This frees up β-catenin to translocate into the nucleus where it binds to TCF/LEF transcription factor and converts it from a repressor to an activator, resulting in the transcriptional activation of Wnt target genes (Komiya and Habas, 2008).
The canonical Wnt signaling plays an essential role in tissue patterning and cell fate determination, and studies in *Xenopus* early embryos have provided a lot of insight in this process. It has been known for some time that the dorsal localization of Wnt components such as Dishevelled and β-catenin in the early *Xenopus* embryo is necessary for dorsal axis specification (De Robertis et al., 2000). The canonical Wnt components are also sufficient for axis formation as micro-injection of Dishevelled or β-catenin mRNA on the ventral side of the embryo induces the formation of secondary dorsal axis (De Robertis et al., 2000). However, how these components are asymmetrically localized remained a mystery until a later study identified maternal Wnt11 mRNA enriched at the dorsal side of the embryo as the critical upstream signal for axis formation (Tao et al., 2005).

In addition to specifying the dorsal axis, canonical Wnt signaling regulates patterning of the neural tube in the anterior-posterior axis later in embryogenesis. Wnts secreted from the posterior of the neural tube are essential to establish the fates of posterior structures. However, the observation that over-expression of Wnts causes expansion of posterior structures at the expense of anterior structures (Kiecker and Niehrs, 2001) suggests Wnt signaling has to be down-regulated in the anterior for the anterior structures to form. Indeed, several Wnt antagonists such as Cerberus and Dickkopf are found to be expressed near the anterior and they help shape a posterior-high, anterior-low Wnt gradient necessary for the proper fate specification of the neural tube (Ciani and Salinas, 2005).

Canonical signaling is generally involved in cell fate specification so it is noteworthy that this pathway also regulates the posterior migration of QL neuroblast in *C. elegans* (Korswagen, 2002). Mutations in positive regulators of the pathway such as Frizzled, Dishevelled, β-catenin and TCF all lead to anterior migration of QL (Korswagen, 2002). In contrast to vertebrates and *Drosophila*, worms don’t have a clear LRP5/6 homolog. However, worms have four β-catenin homologs as opposed to most other organisms that have only one. β-catenin has dual roles inside the cell: it can function in adhesion by binding to cadherins at the adherens junction and can mediate the effects of Wnt signaling (MacDonald et al., 2009). *C. elegans* has HMP-2 as the junctional β-catenin and BAR-1, WRM-1, and SYS-1 as the signaling β-catenins (Korswagen et al., 2000; Mizumoto and Sawa, 2007b). BAR-1 is the β-catenin involved in the canonical pathway to regulate QL migration. The roles of WRM-1 and SYS-1 will be discussed in a later section.

*Non-canonical Wnt signaling*

Wnt-related pathways that do not require the activation of β-catenin are collectively called non-canonical Wnt pathways. These pathways control a variety of developmental processes such as cell polarity, cell migrations, axon guidance, dendrite morphogenesis and synapse formation (Ciani and Salinas, 2005).

1.1 Planar cell polarity (PCP) in epithelia
In addition to polarizing in the apical-basal axis, epithelial cells organize in a plane of epithelium through the planar cell polarity (PCP) pathway. In *Drosophila*, PCP is important for the proper orientation of wing hairs and ommatidia in the eye. In vertebrates, PCP is evident in external features such as the fur of the mammals and internal organs such as the inner ear (Wu and Mlodzik, 2009). The first PCP mutants were identified in genetic screens in *Drosophila* and are have names such as *frizzled* and *prickle* for their disorganized hair orientation (Gubb and Garcia-Bellido, 1982). The core PCP proteins are evolutionarily conserved and are required for establishment of PCP in both flies and mammals. An emerging model from the field proposes that both intracellular signaling (cell-autonomous) and intercellular communication (cell non-autonomous) are important to establish PCP in response to a global directional cue propagated along the epithelium. Cell-autonomous PCP signaling induces asymmetric localization of factors to opposite poles of the cell. For instance in the *Drosophila* wing epithelium, Frizzled (Fz), Dishevelled (Dsh) and a cytoplasmic protein called Diego (Dgo) are found at the distal pole of the cell whereas a novel four-pass transmembrane protein Van Gogh (Vang) and another cytoplasmic protein called Prickle (Pk) localize to the proximal pole of the cell (Wu and Mlodzik, 2009). Interestingly, the atypical cadherin Flamingo (Fmi) is found at both ends of the cell (Usui et al., 1999). This molecular asymmetry is further reinforced through antagonism between the two complexes: for instance, the binding of Dgo to Dsh can protect Dsh from binding to Pk (Jenny et al., 2005). Fz/Dsh signaling on the distal side activates regulators of actin dynamics such as RhoA and Rho-associated kinase (Strutt et al., 1997; Winter et al., 2001). As a result, actin-rich prehair forms on the distal edge of the wing cell (Strutt and Warrington, 2008).

The observation that loss-of-function clones of *fz* and *vang* disrupt hair orientation in mutant cells as well as neighboring wildtype cells suggest Fz and Vang have additional roles in non-autonomous PCP signaling (Adler et al., 2000). It has recently been shown that the cysteine-rich domain of Fz and extracellular domain of Vang can bind to each other in trans (Wu and Mlodzik, 2008), and this interaction may be further stabilized by homophilic Fmi binding between neighboring cells (Chen et al., 2008). Although controversies exist as to whether Fz/Vang interaction or Fmi interaction in trans has a more prominent role in propagating the polarity information along the wing epithelium (Chen et al., 2008; Wu and Mlodzik, 2009), it is generally agreed that the non-autonomous signaling precedes the asymmetric localization of core PCP proteins (Wu and Mlodzik, 2009). Notably, Wnts are involved in mammalian PCP signaling but have not been implicated in PCP in *Drosophila* so far (Wu and Mlodzik, 2009). It remains to be seen whether other extrinsic polarizing cues are involved in *Drosophila* PCP.

1.2 PCP pathway in directed cell movement and division
Like PCP, many developmental processes polarize cells along a common axis. Therefore, it is not surprising that PCP proteins also play important roles in other polarity contexts. Below I will highlight recent findings concerning the role of PCP in two systems, a morphogenetic process called convergent extension and asymmetric cell division.

1.2.1 Convergent extension
Convergent extension (CE) is a morphogenetic process in vertebrates during which cells converge towards the midline and intercalate, resulting in the narrowing and extension of a tissue along the anterior-posterior axis (Wallingford et al., 2002). Prior to the onset of CE, cells randomly extend and retract lamellipodia. At the onset of CE, cells align mediolaterally and stabilize their lamellipodia toward medial and lateral ends, which allows them to crawl between their neighbors toward the midline (Wallingford et al., 2002). CE is critical for gastrulation and neural tube formation as mutations in PCP genes such as Fz7, Dvl, Vang2, Pk and Fmi lead to shortened embryos and open neural tubes (Vladar et al., 2009).

Similar to mammalian PCP, a requirement for Wnts have been demonstrated in CE. Interestingly, the two non-canonical Wnts, Wnt5a and Wnt11, display fundamentally different phenotypes when their levels are knocked down: Wnt11-depleted cells show little protrusive activity whereas Wnt5a-depleted cells fail to align mediolaterally and therefore exhibit random movements (Schambony and Wedlich, 2007). This observation suggests Wnt5a and Wnt11 may regulate CE through distinct pathways. Indeed, Wnt11 signals through Fz7 and Dvl to activate a formin-homology protein Daam1 and RhoA, which induces formation of lamellipodia (Djiane et al., 2000; Habas et al., 2001; Tahinci and Symes, 2003). By contrast, Wnt5a signaling in *Xenopus* is mediated by the alternative Wnt receptor Ror2 and JNK cascade. This pathway eventually leads to transcriptional upregulation of paraxial protocadherin XPAPC (Schambony and Wedlich, 2007).

1.2.2 PCP in asymmetric cell division

*SOP division in Drosophila*

Asymmetric cell division is a process in which the mother cell generates two daughter cells with distinct fates. This is usually achieved through a tight coordination between asymmetric distribution of cell fate determinants and mitotic spindle orientation (Knoblich, 2010). In sensory organ precursor (SOP) division of *Drosophila*, pl cell divides and generates an anterior pIIb cell, which is a neuronal precursor, and a posterior pIIa cell, a precursor for external support cells. In the prophase of pl cell, Fz and Dvl are enriched in the posterior cortex whereas Vang and Pk localize to the anterior cortex (Bellaiche et al., 2004). This asymmetric segregation of PCP proteins in turn directs the anterior localization of Dlg/Pins/Gui complex and posterior localization of PAR-3/PAR-6/aPKC complex through mechanisms that are still not well understood (Segalen and Bellaiche, 2009). aPKC at the posterior end of pl cell can phosphorylate cell fate determinant Numb and prevent it from associating with the posterior membrane, therefore restricting Numb localization to the anterior half of the cell (Bellaiche et al., 2001; Smith et al., 2007). As a result, Numb is selectively inherited by pIIb after the cell division. By contrast, Numb is abnormally partitioned into both pIIa and pIIb in PCP mutants such as *fz-/-*, leading to cell fate specification defects (Bellaiche et al., 2001).

In addition to promoting asymmetric distribution of determinants, Fz/Dsh pathway has recently been shown to regulate spindle orientation of pl cell along the anterior-posterior axis via the dynein-binding protein NuMA (David et al., 2005; Segalen et al., 2010). A similar pathway has also been reported to regulate cell division during zebrafish
gastrulation, suggesting this spindle orientation pathway is evolutionarily conserved (Segalen et al., 2010).

**B cell polarity in C. elegans**

In *C. elegans*, a PCP-like pathway has been implicated in regulating the polarity of the male-specific B cell, which divides asymmetrically to generate a larger anterior daughter and a smaller posterior daughter (Wu and Herman, 2006). Mutations in *lin-44/Wnt, lin-17/Fz* and *mig-5/Dsh* cause this size asymmetry to be lost or reversed. Consistent with this, the asymmetric localization of MIG-5 in the dividing cell is disrupted in *lin-44* or *lin-17* mutant (Wu and Herman, 2007). Notably, the observation that depleting PCP components such as RHO-1/RhoA and LET-502/RhoA-associated kinase, but not canonical pathway components PRY-1/Axin or BAR-1/β-catenin, leads to cell size asymmetry defects suggests a PCP-like mechanism (Wu and Herman, 2006).

1.3 *C. elegans* Wnt/β-catenin asymmetry pathway

In addition to the PCP-like pathway, another pathway known as the Wnt/β-catenin asymmetry pathway regulates many asymmetric cell divisions during *C. elegans* development. For instance, this pathway is required for the division of EMS blastomere during embryogenesis and that of hypodermal seam cells during larval stages (Mizumoto and Sawa, 2007b). The Wnt/β-catenin asymmetry pathway shares some of its components with the canonical Wnt/β-catenin pathway but it is regulated in a way that leads to the asymmetric localization and activities of these components within the cell, a feature reminiscent of the non-canonical PCP pathway.

1.3.1 EMS division

In the 4-cell *C. elegans* embryo, EMS divides asymmetrically to generate an anterior MS cell, which produces mesoderm, and a posterior E cell, which produces endoderm. The EMS specification requires MOM-2/Wnt signaling from the P2 blastomere posterior to the EMS (Rocheleau et al., 1997; Thorpe et al., 1997), and elegant experiments with isolated blastomeres in culture have shown that MOM-2 is instructive (meaning the distribution of Wnt is important) in this process (Goldstein et al., 2006). In the absence of Wnt signaling, POP-1/TCF acts as a transcriptional repressor of endoderm-specific genes. When Wnt signaling is activated in the posterior cell, the POP-1 level in the nucleus is down-regulated so that the posterior cell can adopt the endodermal fate. Conversely, a high POP-1 level in the anterior cell promotes mesodermal fate (Korswagen 2002). Mutation in Wnt pathway components such as *mom-5/Fz, sgg-1/GSK3β* and *apr-1/APC* results in incompletely penetrant transformation of E to MS fate (Bei et al., 2002). On the other hand, mutation in *wrm-1/β-catenin* and *lit-1/MAP kinase* abolishes the POP-1 asymmetry and leads to a completely penetrant gut-less phenotype (Kalla et al., 1997; Meneghini et al., 1999; Rocheleau et al., 1997; Rocheleau et al., 1999). It turns out that LIT-1, when bound to WRM-1, exhibits higher kinase activity and can phosphorylate POP-1 *in vitro* (Rocheleau et al., 1999). The phosphorylated POP-1 is then transported out of the nucleus via binding to a 14-3-3 protein (Lo et al., 2004). Taken together, these data point to a model in which the Wnt/MAPK pathway promotes endoderm specification by facilitating nuclear export of POP-1 in the posterior E cell.
In contrast to the role of WRM-1 in regulating POP-1 localization, another β-catenin
SYS-1 regulates asymmetric cell division by converting POP-1 from a repressor to an
activator in the Wnt-responsive cell (Mizumoto and Sawa, 2007b). The mode of SYS-1
action is reminiscent of BAR-1, the β-catenin in the canonical pathway. SYS-1 and POP-
1 are found to exhibit reciprocal nuclear asymmetry following EMS division: that is,
POP-1 level is high and SYS-1 level is low in the anterior MS cell and vice versa in the
posterior E cell (Huang et al., 2007). Over-expression of SYS-1 results in a MS to E
transformation (Huang et al., 2007), a phenotype also observed in pop-1 mutant (Lin et
al., 1995). These data along with experiments in other cell divisions suggest that
asymmetrically dividing cells use SYS-1 to POP-1 ratio as a general mechanism to
specify binary fates (Phillips and Kimble, 2009). In EMS division, a high SYS-1 to POP-
1 ratio in the posterior cell specifies the endodermal fate whereas a low SYS-1 to POP-1
ratio in the anterior cell specifies the mesodermal fate (Huang et al., 2007).

1.3.2 T cell polarity
The Wnt/β-catenin asymmetry pathway also plays a prominent role in the polarity of the
T cell, the most posterior hypodermal seam cell. T cell divides asymmetrically to
generate an anterior hypodermal cell and a posterior neuron. Similar to EMS division, the
T cell fate specification depends on Wnt/MAPK signaling to down-regulate POP-1 levels
in the posterior cell. A high SYS-1 to POP-1 ratio in this context leads to a neuronal fate
whereas a low SYS-1 to POP-1 ratio leads to a hypodermal fate. LIN-44 secreted from
the posterior side of the T cell recruits LIN-17/Fz and Dishevelleds MIG-5 and DSH-2 to
the posterior cortex (Mizumoto and Sawa, 2007a). This signaling is instructive as ectopic
expression of LIN-44 anterior of T cell can re-orient LIN-17 to the anterior side and
invert the division (Goldstein et al., 2006). Interestingly, WRM-1 and LIT-1 are initially
localized to the anterior cortex but accumulate in the posterior daughter nucleus at
telophase (Mizumoto and Sawa, 2007a; Takeshita and Sawa, 2005). A model emerging
from the study by Mizumoto and Sawa (2007a) is that WRM-1, functioning both at the
cortex and in the nucleus, has antagonistic roles in T cell polarity. WRM-1 localized to
the anterior cortex causes increased export of WRM-1 from the anterior nucleus,
preventing WRM-1/LIT-1 complex to phosphorylate POP-1 and trigger its nuclear export.
POP-1 levels consequently stay high in the anterior nucleus. On the other hand, the
absence of WRM-1 from the posterior cortex allows WRM-1 to accumulate in the
posterior nucleus to drive export of POP-1. Thus, the asymmetry in WRM-1 cortical
localization is translated into an asymmetry of POP-1 activity needed for T cell
specification.

1.4 Wnt/Calcium pathway
It was initially discovered that over-expression of Wnt5A or Fz2 led to the release of
intracellular calcium from ER in zebrafish embryos (Slusarski et al., 1997b; Slusarski et
al., 1997a) and this calcium influx requires heterotrimeric G proteins and
phosphatidylinositol signaling (Slusarski et al., 1997b). The release of calcium activates
several proteins including protein kinase C (PKC) (Sheldahl et al., 1999), calcium/
calmodulin-dependent kinase II (CamKII) (Kuhl et al., 2000) and phosphatase calcineurin
(Saneyoshi et al., 2002). The Wnt/calcium pathway regulates a number of development
processes including a gastrulation movement called tissue separation in Xenopus
(Winklbauer et al., 2001). It has recently been implicated in establishing an electric gradient in the developing heart (Panakova et al., 2010). However in most cases, how this pathway functions remains poorly understood. One interesting mechanistic insight comes from a study in the fate determination of the *Xenopus* embryo, as summarized below.

In response to the calcium influx, calcineurin activates the transcription factor NFAT to promote ventral cell fates in the *Xenopus* embryo (Saneyoshi et al., 2002). A constitutively active NFAT blocks the axis duplication phenotype induced by Wnt8, suggesting NFAT antagonizes the canonical Wnt signaling pathway. By contrast, a dominant negative NFAT produces a Wnt8-like phenotype by stabilizing β-catenin and allowing expression of Wnt target genes. This ectopic induction of Wnt targets can be suppressed by GSK3β or dominant negative TCF but not dominant negative Dsh, suggesting that NFAT interacts with the canonical pathway downstream of Dsh and upstream of β-catenin. Taken together, the Wnt/calcium pathway promotes ventral cell fates by inhibiting the canonical Wnt pathway in the *Xenopus* embryo.

**Wnt signaling: an integrated picture**

It was once thought that canonical and non-canonical pathways are more or less separate and parallel to each other with the former leading to transcription and the latter to changes in cytoskeletal dynamics. The distinction between the two pathways is more blurred now, as new evidence suggests that canonical signaling can modulate the cytoskeleton (Zimmerman et al., 2010) and non-canonical signaling can affect transcription (Schambony and Wedlich, 2007). Moreover, the Wnts traditionally associated with non-canonical Wnt signaling, Wnt5a and Wnt11, have also been shown to stabilize β-catenin (Mikels and Nusse, 2006; Tao et al., 2005). LRP5/6 (Tahinci et al., 2007) and ROR (Li et al., 2008; Winkel et al., 2008) are no longer confined to the canonical and non-canonical signaling pathway, respectively. With the recent discovery that Rac1 and JNK mediate canonical Wnt signaling in mouse limb bud development (Wu et al., 2008), it seems that signaling specificity is context-dependent instead of arising from intrinsic properties of Wnts, receptors, or cytoplasmic effectors. It may be helpful for us to discuss Wnt signaling as separate pathways, but the emerging picture is that Wnt signaling is highly dynamic and complex with extensive crosstalk among different components (van Amerongen and Nusse, 2009). As a result, the outcome of a given Wnt signal may be hard to predict. More advanced experimental and bioinformatics tools should help address this complexity.

**Goals of Current Project**

Cellular polarization is critical for many stages of neuronal development, including the asymmetric division of the neuroblast and the axon-dendrite specification of the mature neuron. In *C. elegans*, Wnt glycoproteins control the polarization of the ALM mechanosensory neurons, but the molecules that mediate Wnt signaling are unclear. The goal of my first project was to identify and characterize the potential downstream Wnt effectors in ALM polarity. First, I found that the Ror kinase CAM-1, which has been shown to bind to Wnts, is required for ALM polarity. CAM-1 acts in the ALM to promote its polarity and CAM-1 may also have a non-autonomous function in
sequestering Wnts. Second, I implicated actin regulators \textit{unc-34} and two known components of the Rac pathway, \textit{ced-10}/Rac and \textit{mig-10}/lamellipodin, in ALM polarity. Expression of \textit{unc-34} and \textit{mig-10} from a neuron-specific promoter \textit{unc-86} significantly rescued the ALM polarity defect, indicating these genes can function in the neurons. Third, I identified that the MIG-15 kinase, a member of the Ste20 kinase superfamily, and potential components of a MIG-15 signaling pathway that might be novel Wnt effectors in neuronal polarity.

Wnts also regulate the asymmetric cell divisions (ACD) of many non-neuronal cell types and the Q neuroblast lineage (Teuliere & Garriga, unpublished observation). The molecules that mediate Wnt signaling in ACD in the Q lineage are unclear. One possible candidate is PIG-1, which is orthologous to vertebrate MELK and belongs to a family of serine/threonine kinases including PAR-1, SAD-1 and AMPK (Cordes et al., 2006). This group of kinases can be phosphorylated and activated by the polarity-regulating kinase LKB1. LKB1 kinase, along with its binding partners STRAD and MO25, have been shown to be master regulators of polarity in many different contexts, and I find that \textit{C. elegans} orthologs of LKB1, STRAD and MO25 regulate the asymmetric cell division of the Q.p lineage and are potential regulators of PIG-1 in this process.
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CHAPTER TWO

THE C. ELEGANS ROR KINASE CAM-1 FUNCTIONS IN WNT-REGULATED NEURONAL POLARITY

Contributions to this Chapter:
Chun-Liang Pan characterized double Frizzled mutants mig-1 lin-17; mig-1 mom-5 and lin-17 mom-5. Julie Oppermann performed RNAi against mom-5 and Dishevelled (mig-5, dsh-1 and dsh-2) and analyzed interactions between Wnts and Dishevelled. Mark Gurling characterized the triple Frizzled mutant mig-1 lin-17 mom-5 and interactions between Wnts and mom-5.
SUMMARY
Wnts belong to a conserved family of secreted glycoproteins that are important for various developmental processes. Three Wnts, CWN-1, CWN-2 and EGL-20, coordinately regulate the anterior polarization of the mechanosensory neuron ALM (Fleming et al., 2010; Hilliard and Bargmann, 2006; Prasad and Clark, 2006), but it is unclear which receptor mediates Wnt signaling. We found that CAM-1/Ror and MOM-5/Frizzled act in parallel pathways in this process. We also showed that CAM-1 has dual and antagonistic functions in this process: CAM-1 promotes Wnt signaling within ALM but antagonizes Wnt signaling by binding to Wnts.

INTRODUCTION
Wnts belong to a conserved family of secreted glycoproteins that are important for various developmental processes including differentiation, cell fate specification, directed cell motility, organogenesis and even stem cell renewal (Komiya and Habas, 2008). Wnts can bind to seven-pass trans-membrane Frizzled receptors and signal through a “canonical” pathway that leads to the stabilization of β-catenin and through β-catenin-independent “non-canonical” pathways. (Komiya and Habas, 2008). Numerous positive and negative regulators of Wnt signaling have been identified and the observation that deregulated Wnt signaling can lead to cancer (Logan and Nusse, 2004) underscores the need for a precise control of opposing factors.

RORs are conserved receptor tyrosine kinases (RTK) with an extracellular immunoglobulin (Ig) domain, cysteine-rich domain (CRD) and kringle domain (Green et al., 2008b). Mutations of ROR genes in human and mice lead to defects in skeletal and cardiac development (Forrester, 2002). Similar to the CRD of the Frizzled receptors, the CRD of vertebrate RORs has been shown to bind to Wnts (Billiard et al., 2005; Hikasa et al., 2002; Kani et al., 2004; Mikels and Nusse, 2006; Oishi et al., 2003). Ror2 can function as a genuine RTK in cultured cells (Liu et al., 2007; Liu et al., 2008). However, Ror2 is best characterized as a positive regulator of the non-canonical pathway. Examples include migration of mouse embryonic fibroblasts (MEFs) (Nishita et al., 2006), orientation of mouse hair cells (Yamamoto et al., 2008) and activation of JNK in Xenopus convergent extension (Hikasa et al., 2002; Schambony and Wedlich, 2007). On the other hand, the role of Ror2 in canonical Wnt signaling is more complex, with earlier reports showing that Ror2 can attenuate the expression of a canonical Wnt reporter (Billiard et al., 2005; Mikels and Nusse, 2006) and more recent reports arguing for a stimulatory function of Ror2 (Li et al., 2008; Winkel et al., 2008).

C. elegans sole ROR ortholog is cam-1, originally identified in a genetic screen for defective migration of the CAN neuron (Forrester et al., 1999). cam-1 was shown to function autonomously in CAN migration (Forrester et al., 1999) and other aspects of neuronal development including positioning of an axon-rich structure called the nerve ring (Kennerdell et al., 2009), neurite elimination (Hayashi et al., 2009) and neurite outgrowth (Koga et al., 1999; Song et al., 2010). CAM-1 also has non-autonomous functions. In migration of the motor neuron HSN, egl-20/Wnt and cam-1 mutants exhibit reciprocal phenotypes (Forrester et al., 2004; Kim and Forrester, 2003). In addition, over-
expression of CAM-1 CRD mimics the egl-20 mutant phenotype, consistent with CAM-1 antagonizing EGL-20 through sequestration (Forrester et al., 2004; Kim and Forrester, 2003). While this antagonism is thought to represent a non-autonomous function of CAM-1, the site of CAM-1 function in this process is yet to be determined. In vulval development, loss of canonical Wnt signaling leads to a similar phenotype to the over-expression of the extracellular domain of CAM-1 in non-vulval tissues (Green et al., 2007). This suggests that CAM-1 can inhibit Wnt signaling non-autonomously by restricting the amount of Wnts that reach the target tissue.

In C. elegans, Wnts are also critical regulators of neuronal polarity along the anterior-posterior axis (Fleming et al., 2010; Hilliard and Bargmann, 2006; Prasad and Clark, 2006). The signaling from Wnt LIN-44 to the Frizzled receptor LIN-17 is important for the polarization of mechanosensory neuron PLM. On the other hand, the three Wnts CWN-1, CWN-2 and EGL-20 coordinately regulate the anterior polarization of mechanosensory neuron ALM but it is unclear which receptor mediates Wnt signaling. Here we present evidence that the Ror kinase CAM-1 and at least one Frizzled receptor MOM-5 regulate ALM polarity. We also show that CAM-1 has dual and antagonistic functions in this process: an autonomous signaling function that promotes anterior polarization and a Wnt-sequestering function that inhibits anterior polarization.

MATERIALS AND METHODS

Nematode Strains and Genetics

Strains were maintained at 20°C as described (Brenner, 1974). The following alleles are used in this study:

LG I: lin-17(n671), lin-17(n677) (Sawa et al., 1996), mig-1(n687), mig-1(e1787) (Pan et al., 2006), mom-5(ne12) (Rocheleau et al., 1997)

LG II: cwn-1(ok546) (Zinovyeva and Forrester, 2005), mig-5 rh147) (Walston et al., 2006), dsh-1(ok1445) (Klassen and Shen, 2007), cam-1 alleles include gm105, gm122 (Forrester et al., 1999), ks52 (Koga et al., 1999), xd13 (Song et al., 2010), sa692 (Ailion and Thomas, 2003), ak37 (Francis et al., 2005)

LG IV: egl-1(mg366) (Kennedy et al., 2004), cwn-2(ok895) (Zinovyeva and Forrester, 2005), egl-20(n585) (Maloof et al., 1999)

LG V: cfz-2(ok1201) (Zinovyeva and Forrester, 2005)

LG X: lin-18(e620) (Inoue et al., 2004), vang-1(tm1422) (Hoffmann et al., 2010)

Transgenic Animals

The following transgenic strains were used in this study: zdIs5[Pmec-4::gfp, lin-15(+)] (Clark and Chiu, 2003); gmEx191[Pcam-1::cam-1::gfp; pRF4[rol-6(su1006)]] (Forrester et al., 1999); xdEx636[Punc-86::cam-1(b)::yfp; Podr-1::dsRed] (Song et al., 2010); cwEx34[Pcam-1::cam-1(ΔCRD)::gfp; pRF4[rol-6(su1006)]] and cwEx152[Pcam-1::cam-1(ΔIkriIntra)::gfp; pRF4[rol-6(su1006)]] (Kim and Forrester, 2003)
Molecular Biology and Germline Transformation

Punc-86::cam-1(ΔIntra)::yfp was generated by modifying Punc-86::cam-1(b)::yfp (Song et al., 2010) with PCR-based mutagenesis (Invitrogen). The following primers are used: GTCGAGCAAGAAGAAGTACCCAGCTTTCTTG and its reverse complement. gmEx632, gmEx633, gmEx634, gmEx635 and gmEx636 were generated by injecting Punc-86::cam-1 (ΔIntra)::yfp into N2 hermaphrodites at 10 ng/µl with 3ng/µl Pmyo-2::mcherry (pCFJ90). Germline transformation was performed by direct injection of various plasmid DNAs into the gonads of adult wild-type animals as described (Mello et al., 1991).

RNA interference

RNAi was performed using the bacterial feeding method as (Kamath et al., 2001; Timmons and Fire, 1998). In all experiments, worms were grown on plates supplemented with 25 mM Carbenicillin and 1 mM IPTG at 20 °C. The RNAi cultures were prepared by inoculating bacterial strains in LB with 25 mM Carbenicillin for 15 hours at 37° C, followed by addition of 6 mM IPTG and incubation for another hour at 37° C. Bacterial strains used to inactivate genes by feeding were obtained from the library designed by the Ahringer lab (Fraser et al., 2000).

Scoring of the ALM polarity phenotype

Neuronal polarity of ALM was scored using the integrated array zdIs5 [Pmec-4::gfp], which expresses GFP in the ALM, PLM, AVM and PVM mechanosensory neurons. For ALM, the bipolar phenotype was defined as a normal anterior process and a posterior process that is longer than five ALM cell diameters in length.

Fluorescence Microscopy

For fluorescence microscopy, L4 to young adult hermaphrodite animals were anesthetized with 1% sodium azide, mounted on agar pad, and observed with a Zeiss Axioskop2 microscope.

RESULTS

CAM-1/Ror, MOM-5/Frizzled and all three Dishevelled are required for ALM neuronal polarity

C. elegans ALMs are a pair of bilaterally symmetric neurons located in the midbody. Each ALM extends a single anterior process toward the head. While ALM development is largely normal in cwn-1, cwn-2 and egl-20 single Wnt mutants, the polarity of the ALM is often disrupted in cwn-1; cwn-2 and cwn-1; egl-20 double mutants (Hilliard and Bargmann, 2006; Prasad and Clark, 2006). In Wnt double mutants, the ALMs can be bipolar or their polarity can be reversed, extending a single process toward the tail (Fig.1B, C, E, F).

The receptors that mediate Wnt signaling in ALM polarity are unknown. To determine which receptor is involved in this process, we examined single mutants of all CRD domain-containing receptors in C. elegans. The list included four Frizzled receptors (mig-1, cfz-2, lin-17, and mom-5) and cam-1/Ror, all of which have previously been shown to
function in Wnt-regulated cell polarity, migration and/or axon guidance (Silhankova and Korswagen, 2007). We also examined lin-18/Ryk, which encodes a receptor tyrosine kinase with an extracellular Wnt-binding WIF (Wnt-inhibitory factor) domain (Inoue et al., 2004). Using an integrated Pmech-4::gfp to visualize mechanosensory neurons, we found that, with the exception of cam-1 which showed a modest bipolar phenotype (21.3%), all other single mutants had no obvious ALM polarity defects (Fig. 1G and data not shown). Dishevelleds are cytoplasmic proteins that transduce Frizzled signaling to downstream effectors. Mutations in any one of the three Dishevelled genes, mig-5, dsh-1 or dsh-2, had no effect on ALM polarity (data not shown). We reasoned that a lack of phenotype might be due to redundancy or in the case of mom-5 and dsh-2, the presence of maternally supplied gene products. This is because mom-5 and dsh-2 are maternal effect lethal, so we analyzed the mutants from heterozygous mothers. Since cwn-1 is the only Wnt mutant that has a weak bipolar phenotype, we reasoned that it might provide a sensitized background to identify additional components in ALM polarity.

We performed RNAi against Wnt receptors and Dishevelleds using a strain containing cwn-1 and a mutation in eri-1 that sensitizes animals to the effects of RNAi (Kennedy et al., 2004). We found that RNAi against cam-1, mom-5 and all three Dishevelleds showed a significant enhancement of the ALM polarity defects compared to control RNAi with bacteria containing the empty vector L4440 (Fig. 1G and data not shown). We next tried to confirm the RNAi results by constructing double mutants with putative null alleles. cwn-1 in combination with either cam-1, dsh-1, or mig-5 generated an ALM polarity defect (Fig. 1G) comparable to the RNAi results, indicating the RNAi was effective in eliminating function of these genes. On the other hand, cwn-1; mom-5 double mutant generated a much weaker phenotype (Fig. 1G) compared to cwn-1; mom-5(RNAi), presumably due to a maternal contribution of mom-5.

CAM-1 and MOM-5 act in parallel pathways in ALM polarity
We observed a significant increase in ALM polarity defect when we removed cam-1 and mom-5 simultaneously (Fig. 2A), suggesting the two receptors act in parallel pathways. However, we did not detect an enhancement of polarity phenotype in other cam-1; Fz double mutants: namely cam-1; lin-17, cam-1; mig-1, and cam-1; cfz-2 (data not shown). lin-18/Ryk also did not enhance the polarity defect of cam-1 (data not shown).

Considering Frizzleds MIG-1 and CFZ-2 have been implicated as CAM-1 co-receptors (Kennerdell et al., 2009; Song et al., 2010), we wondered whether there are CAM-1 co-receptors functioning in parallel to MOM-5 in ALM polarity. Although mig-1 lin-17 did not have an effect on ALM polarity, mig-1 mom-5 and lin-17 mom-5 displayed a weak synthetic polarity phenotype (Fig. 2A). This phenotype was not further enhanced in the triple Frizzled mutant mig-1 lin-17 mom-5 (Fig. 2A). These observations suggest that MIG-1 and LIN-17 act as co-receptors to CAM-1 in ALM polarity.

To determine whether CAM-1 and MOM-5 mediate effects of specific Wnts (CWN-1, CWN-2 or EGL-20) in ALM polarity, we analyzed the polarity phenotype when a Wnt is removed from a receptor mutant background. The prediction is that if a receptor is specific for a Wnt ligand, then the removal of the Wnt in the receptor mutant background
should not enhance the polarity defect. On the other hand, if the Wnt and the receptor act in parallel pathways, this strain may display an enhanced polarity phenotype. Double mutant analysis with cam-1 and the Wnts showed that while all three Wnt mutants enhanced the polarity defect of cam-1, cwn-1 enhanced the most while egl-20 enhanced the least (Fig. 2B). Similarly, cwn-1 enhanced mom-5 more than egl-20 did (Fig. 2B).

We took an analogous genetic approach to address whether CAM-1 signals through dedicated Dishevelled proteins. Since cam-1 and all three Dishevelled genes are linked, we performed RNAi against the Dishevelleds in a cam-1; eri-1 background. RNAi inactivation of dsh-1, but not mig-5 or dsh-2, significantly enhances polarity defect of cam-1 (Fig. 2B). These data suggest mig-5 and dsh-2 acts in the cam-1 pathway and dsh-1 acts in a parallel pathway.

**CAM-1 acts cell autonomously and its intracellular domain is partially dispensable in ALM polarity**

A GFP-tagged CAM-1 driven from its endogenous promoter is broadly expressed during early embryogenesis and is highly expressed near the nerve ring when ALM polarizes around the 2 ½ fold stage of embryogenesis (Forrester et al., 1999). Curiously, this construct failed to rescue the polarity defect of a cam-1 single mutant, but did partially rescue a cwn-1; cam-1 double mutant (Fig. 3A). To test whether CAM-functions in the ALM, we expressed CAM-1 under the unc-86 promoter, which is expressed in many neurons including the ALM, but not in non-neuronal cells (Baumeister et al., 1996). This construct did not rescue the polarity defect in cam-1, but partially rescued the polarity defect in cwn-1; cam-1 (Fig. 3A), suggesting that CAM-1 acts autonomously for ALM polarity.

We next addressed whether the intracellular domain of CAM-1 is required for ALM polarity. Several studies have shown that this domain is at least partially, if not completely, dispensable for CAM-1 function through analysis of mutants or transgenes that lack the intracellular domain (Francis et al., 2005; Kennerdell et al., 2009; Song et al., 2010). We examined the available five cam-1 alleles alone or in combination with cwn-1 (Fig. 3B & 3C). The three cam-1 alleles that caused a bipolar phenotype are gm122 (Forrester et al., 1999), a non-sense mutation that truncates CAM-1 in the CRD; sa692 (Ailion and Thomas, 2003), a missense mutation in the CRD; and ak37 (Francis et al., 2005), a large deletion spanning from the kringle domain to the C-terminus. These three alleles also behave like strong loss-of-function, if not null, mutations in other contexts (Forrester et al., 1999; Kennerdell et al., 2009; Kim and Forrester, 2003; Song et al., 2010). By contrast, the two hypomorphic alleles that specifically disrupt the intracellular domain did not have an effect on ALM polarity. gm105 (Forrester et al., 1999) is a non-sense mutation which results in a CAM-1 that lacks the intracellular domain, whereas ks52 (Koga et al., 1999) is an in-frame deletion that removes the kinase domain. Nevertheless, these putative hypomorphs interacted synergistically with cwn-1. The observation that cwn-1 enhanced the putative null alleles of cam-1 more strongly than it did the hypomorphs suggests that CAM-1 intracellular domain is important but not essential for ALM polarity. Interestingly, a CAM-1 transgene lacking the intracellular domain (driven from its endogenous promoter) failed to significantly rescue the polarity
defect in cam-1 (Fig. 3A). We also found that cwn-1 cam-1 animals bearing this transgene have slightly lowered penetrance of defects compared to their non-transgenic counterparts (Fig. 3A), but the suppression is not statistically significant (p=0.08).

**CAM-1 CRD is both necessary and sufficient to sequester Wnts in ALM polarity**

Given that CAM-1 is highly expressed near the nerve ring when ALM polarizes, we wondered whether CAM-1 has a non-autonomous function by binding to Wnts. The observation that over-expression of full length CAM-1 from its endogenous promoter induced ALM polarity defects in a cwn-1 sensitized background (Fig. 4) suggests that excess CAM-1 may sequester Wnts (i.e. CWN-2 or EGL-20) to cause polarity phenotypes. Consistent with this, over-expression of a membrane-tethered CAM-1 CRD (a transgene with deletions of Ig, Kringle and intracellular domain) led to a polarity phenotype that is indistinguishable from that of the full-length CAM-1 in a cwn-1 background (Fig. 4). In addition, a CAM-1 transgene that lacks the CRD failed to induce polarity defects above background (Fig. 4). Taken together, these data suggest CAM-1 CRD is both necessary and sufficient for Wnt sequestration.

**DISCUSSION**

The outcome of Wnt signaling is intricately regulated by an ensemble of positive and negative factors acting both within and outside of the cell. Here we show that the Ror kinase CAM-1 has dual and antagonistic functions in ALM neuronal polarity in *C. elegans*: CAM-1 promotes Wnt signaling within ALM but antagonizes Wnt signaling by binding to Wnts. This conclusion is supported by the following observations. First, CAM-1 expressed from a neuron-specific promoter rescued the ALM polarity defect in *cwn-1; cam-1* double mutant (Fig. 3A). Second, the CAM-1 CRD induced polarity defect in a cwn-1 sensitized background (Fig. 4).

**CAM-1 acts cell autonomously and may signal through a novel non-canonical pathway to regulate ALM polarity**

Three Wnts, CWN-1, CWN-2 and EGL-20, coordinately regulate the anterior polarization of the mechanosensory neuron ALM (Fleming et al., 2010; Hilliard and Bargmann, 2006; Prasad and Clark, 2006), but it is unclear which receptor mediates Wnt signaling. We found that CAM-1/Ror and MOM-5/Frizzled act in parallel pathways in this process (Fig. 2A). CAM-1 expressed from a neuron-specific promoter rescues the ALM polarity defect in *cwn-1; cam-1* double mutant (Fig. 3A), suggesting that CAM-1 can function in the neurons. Genetic analysis of *cam-1* mutants in the absence of Wnts showed that egl-20 did not enhance the polarity phenotype of *cam-1* as much as *cwn-1* and *cwn-2* did (Fig. 2B). Moreover, RNAi against *dsh-1*, but not the other two Dishevelled genes *mig-5* and *dsh-2*, significantly enhanced the *cam-1* phenotype (Fig. 2C). However, we had to dilute *dsh-2* RNAi culture by 20 fold to partially bypass embryonic lethality, so that a lack of a *dsh-2* phenotype may be due to incomplete knockdown of this gene. Taken together, these data suggest that CAM-1 mainly transduces signals from Wnt EGL-20 to Dishevelled MIG-5 (and maybe also DSH-2) to promote anterior polarization of ALM. In the outgrowth of motor neurons, CAM-1 and DSH-1 were shown to act genetically in the same pathway and yeast-2-hybrid
experiments indicate that the intracellular domain of CAM-1 can physically interact with domains of DSH-1 (PDZ and DEP) that are traditionally associated with non-canonical Wnt signaling (Song et al., 2010). Consistent with this idea, mutations in β-catenins (bar-1, wrm-1, and sys-1) did not have an effect on motor neuron outgrowth (Song et al., 2010).

Our genetic analysis also suggests Wnts signal through a β-catenin-independent pathway to regulate ALM polarity. For instance, RNAi inactivation of canonical pathway components such as bar-1/β-catenin, pry-1/Axin, pop-1/TCF did not generate a significant phenotype in a cwn-1; eri-1 background (Oppermann and Garriga, unpublished observations). The planar cell polarity (PCP) pathway in Drosophila and vertebrates has been shown to be important to align cells along a common axis in response to polarizing signals (Vladar et al., 2009) but its role in C. elegans is not well understood. We tested mutants of C. elegans orthologs of PCP genes such as Van Gogh, Prickle and Flamingo (called vang-1, prkl-1 and fmi-1, respectively), but did not find any obvious defects either by themselves or in a cwn-1 sensitized background (data not shown). Instead, we previously implicated the Rac CED-10 and an actin-binding protein of Ena/VASP family, UNC-34, in ALM polarity (Fleming et al., 2010). Moreover, some C. elegans orthologs of the yeast RAM polarity pathway genes are involved in this process (Chapter 3). Taken together, these observations suggest that novel effectors mediate the effects of Wnts in ALM polarity.

**CAM-1 intracellular domain is important but not essential for ALM polarity**

Several studies have shown that the CAM-1 intracellular domain is at least partially, if not completely, dispensable for CAM-1 function through analysis of mutants or transgenes that lack this domain (Francis et al., 2005; Kennerdell et al., 2009; Kim and Forrester, 2003; Song et al., 2010). Mutations in gm105 and ks52, which specifically disrupt the intracellular domain, did not cause a polarity defect by themselves, but did so in a cwn-1 sensitized background. This is in contrast to other putative null alleles of cam-1 (gm122, sa692 and ak37), which generated a polarity phenotype by themselves. These data suggest that the intracellular domain is important but not essential for ALM polarity.

To further assess the functional role of this domain, we used a CAM-1 transgene (driven from its endogenous promoter) that lacks the intracellular domain (Pcam-1::cam-1(ΔIntra)). This construct has previously been shown to rescue the cell migration defects of the CAN and HSN (Kim and Forrester, 2003). We found that cwn-1 cam-1 animals bearing this transgene have slightly lowered penetrance of defects compared to their non-transgenic counterparts, but the suppression is not statistically significant (p=0.08).

Similarly, expressing this truncated CAM-1 transgene from the neuron-specific unc-86 promoter (Punc-86::cam-1(ΔIntra)) failed to significantly rescue the polarity defect in cwn-1 cam-1 (data not shown). A more sensitized background may be needed to observe rescuing activity from these CAM-1 transgenes.

The apparent dispensability of CAM-1 intracellular domain in some cases may be due to the ability of CAM-1 to form co-receptor complexes. Genetic experiments suggest that CAM-1 can form co-receptors with the Frizzled receptors MIG-1 and CFZ-2 (Kennerdell et al., 2009; Song et al., 2010) and VANG-1/Van Gogh (Green et al., 2008a; Hayashi et
al., 2009). Interestingly, the intracellular domain of CAM-1 is required in contexts where CAM-1 acts with VANG-1 (Green et al., 2008a; Hayashi et al., 2009) but partially dispensable when CAM-1 acts with Frizzled receptors (Kennerdell et al., 2009; Song et al., 2010). Frizzled and ROR kinases can physically interact via their CRD domains (Oishi et al., 2003). The discrepancy in the requirement of the ROR intracellular domain could be further explained by a recent observation that a mutation in the C-terminus of Vangl2 abolishes its interaction with Ror2 (Gao et al., 2011). We found that both mig-1 and lin-17 generated a weak synthetic polarity phenotype in a mom-5 background, suggesting that they are potential CAM-1 co-receptors functioning in parallel to MOM-5 in ALM polarity. However, cam-1, but not mig-1 lin-17, produced a polarity phenotype and mom-5; cam-1 had a much more severe defect than did mig-1 lin-17 mom-5. This suggests that there are additional CAM-1 co-receptors that function with MIG-1 and LIN-17. Alternatively, MIG-1 and LIN-17 could act in parallel to both MOM-5 and CAM-1 in ALM polarity.

**CAM-1 has a Wnt-sequestering function to inhibit anterior ALM polarization**

In addition to promoting Wnt signaling cell autonomously, CAM-1 has been shown to antagonize Wnt signaling cell non-autonomously. In vulval development as well as in the migration of the neurons HSN and PVM, CAM-1 is thought to sequester Wnts cell non-autonomously, thereby restricting the amount of Wnts that reach the target tissue (Forrester et al., 2004; Green et al., 2007; Kim and Forrester, 2003). Three Wnts, CWN-1, EGL-20 and CWN-2, coordinately promote the anterior polarization of ALM. Whereas CWN-1 and EGL-20 are expressed near the tail (Pan et al., 2006; Whangbo and Kenyon, 1999), CWN-2 is expressed near the anterior half of the worm during embryogenesis (Kennerdell et al., 2009; Song et al., 2010). Given that CAM-1 is broadly expressed during early embryogenesis and is highly expressed near the nerve ring when ALM polarizes around the 2 ½ fold stage of embryogenesis, we tested whether CAM-1 also has a non-autonomous function in sequestering Wnts. Consistent with this idea, expression of CAM-1 CRD led to a polarity phenotype that was indistinguishable from that of the full-length CAM-1 in a cwn-1 background (Fig. 4). In addition, a CAM-1 transgene that lacks the CRD failed to induce polarity defects above background (Fig. 4). Taken together, these data suggest that the CAM-1 CRD is both necessary and sufficient for Wnt sequestration. We will use CAM-1 transgenes expressed from a muscle-specific promoter myo-3 to further address the site of its function. We will also take a complementary approach by performing tissue-specific RNAi of cam-1.

The observations that CAM-1 has dual and antagonistic functions in ALM polarity may help explain some paradoxical results in the transgene rescue experiments. For instance, full-length CAM-1 expressed from either its endogenous promoter or a neuron-specific promoter unc-86 failed to rescue the polarity defect in cam-1 but did rescue cwn-1; cam-1. One possibility is that cam-1 and unc-86 promoters are expressed in many cells in addition to ALM. The ability of CAM-1 to sequester Wnts non-autonomously may mask the autonomous signaling function of CAM-1, preventing us rescuing the polarity phenotype of cam-1 mutants. Therefore, the rescuing activity of these transgenes is only revealed in a cwn-1; cam-1 sensitized background. This model predicts that CAM-1
expressed from an ALM-specific promoter should completely rescue the polarity defect of \textit{cam-1}.

The finding that CAM-1 can simultaneously function as a positive and negative regulator of Wnt signaling to polarize the ALM raises the intriguing prospect that CAM-1 may have dual roles in other Wnt-regulated processes. For instance, CAM-1 is thought to act cell non-autonomously in HSN and PVM migrations (Forrester et al., 2004; Kim and Forrester, 2003), but whether CAM-1 also functions in these neurons has not been addressed. It also raises the possibility that Wnt-binding receptors that are broadly expressed may have a previously unrecognized function in antagonizing Wnt signaling cell non-autonomously. For example, the Frizzled receptors LIN-17 and MIG-1 antagonize each other in HSN migration with MIG-1 acting autonomously to promote this migration (Pan et al., 2006). One possible mechanism for LIN-17’s role in this process is to sequester Wnts that act through MIG-1. It is tempting to speculate that a fine balance of autonomous and non-autonomous activities of CAM-1 and possibly other Wnt receptors determines the outcome of Wnt signaling in different contexts that require their function.
REFERENCES:


Figure legends:

**Figure 1: CAM-1/Ror, MOM-5/Frizzled and all three Dishevelled are required for ALM neuronal polarity.** (A–F) Photomicrographs of L4 zdIs5 (pmece-4::gfp) animals showing ALM neuronal morphology. The ALM cell bodies are labeled. Arrows indicate anterior processes, and arrowheads indicate posterior processes. Anterior is to the left, dorsal is up. The boxed regions of A–C are shown in D–F. (A, D) A wild-type ALM extends a single, anterior process. (B, E) A cwn-1; egl-20 ALM is bipolar, extending both a normal anterior process and an ectopic posterior process. (C, F) A cwn-1; egl-20 ALM extends a single posterior process, indicating a reversal of polarity. (G) Graph shows the percentage of ALM neurons with defective polarity for each genotype. Gray bars indicate the bipolar phenotype and black bars indicate reversed polarity. Mutations in *cam-1/Ror*, *mom-5/Frizzled* and the *Dishevelled* genes *mig-5, dsh-1* and *dsh-2* generated defects in ALM neuronal polarity in a *cwn-1* sensitized background. Numbers for each genotype are provided.
Figure 2: CAM-1 and MOM-5 act in parallel pathways in ALM neuronal polarity. (A) A mutation in mom-5 significantly enhanced the polarity defect in cam-1. Mutations in mig-1 and lin-17 weakly enhanced the polarity defect in mom-5. (B) cam-1 and mom-5 genetically interacted with Wnts. (C) cam-1 genetically interacted with Dishevelleds. Numbers for each genotype are provided. N.S., not significant; *p < 0.0001 (Fisher’s exact test).
Figure 3: CAM-1 acts cell-autonomously and its intracellular domain is partially dispensable in ALM polarity. (A) A full-length CAM-1 expressed from either its endogenous promoter or a neuron-specific promoter unc-86 failed to rescue the polarity defect in cam-1 but did rescue cwn-1; cam-1. A CAM-1 transgene lacking its intracellular domain (ΔIntra) failed to rescue the polarity defect in either cam-1 or cwn-1; cam-1. The cam-1 allele used is gm122. (B) Molecular lesions of cam-1 alleles. See text for details. The figure is adopted from Green et al., 2007. (C) Putative null alleles of cam-1 (gm122, sa692 and ak37) generated a polarity phenotype, but putative hypomorphic alleles (gm105 and ks52) did not. Numbers for each genotype are provided. N.S., not significant; *p < 0.0001 (Fisher’s exact test).
Figure 4: CAM-1 CRD is both necessary and sufficient to sequester Wnts in ALM polarity. Over-expression of CAM-1 CRD led to a polarity phenotype that is indistinguishable from that of the full-length CAM-1 in a cwn-1 background. A CAM-1 transgene that lacks the CRD failed to induce polarity defects above background. Numbers for each genotype are provided. N.S., not significant.
CHAPTER TWO APPENDIX ONE

THE ROLE OF C. ELEGANS ENA/VASP HOMOLOG UNC-34 IN NEURONAL POLARITY

Contributions to this Appendix:

This appendix is a section taken from a published manuscript (Tinya Fleming, Shih-Chieh Chien, Pamela J. Vanderzalm, Megan Dell, Megan K. Gavin, Wayne C. Forrester & Gian Garriga, Dev. Biol. 2010). The data presented is my contribution to the manuscript.
INTRODUCTION
The generation of neuronal polarity and the ability of neuronal growth cones to navigate to their synaptic targets contribute to the connectivity of the metazoan nervous system. When cultured, hippocampal and cortical neurons polarize with a single axon and multiple dendrites, indicating that these cells have an intrinsic polarity when they are cultured (Barnes and Polleux, 2009). In the developing nervous system, however, neuronal polarity is oriented by cues. The *C. elegans* HSN neuron, for example, extends a single axon ventrally and this extension is initiated by the guidance cue UNC-6/netrin (Adler et al., 2006). Cells and growth cones also migrate in culture in the absence of cues, but to reach their destinations, migrating cells and growth cones respond to both attractive and repulsive cues. In the past two decades, investigators have identified many conserved guidance cues and their receptors, and more recently, these molecules have also been shown to regulate neuronal polarity. How these receptors transduce extracellular cues to orient polarity and to guide migrating cells and growth cones, however, is not as well understood.

One important class of molecules thought to convey signals from guidance receptors to the actin cytoskeleton are members of the Ena/VASP family of proteins. Enabled (Ena) was first defined in *Drosophila* by mutations that dominantly suppress the lethality caused by mutations in the homolog of the Abelson oncogene (Gertler et al., 1990). Later, Ena mutants were found to have defects in axon morphology (Gertler et al., 1995). Vasodilator-Stimulated Phosphoprotein (VASP) was isolated as a protein in platelets that was phosphorylated in response to high levels of cAMP or cGMP (Haffner et al., 1995; Halbrugge et al., 1990; Waldmann et al., 1987). Mena, VASP and Ena-VASP like protein (Evl) are the three mammalian Ena/VASP family proteins (Gertler et al., 1996). These paralogs provide overlapping functions in the formation of filopodia and endothelial junctions that are only revealed in mouse mutants lacking function of all three genes (Furman et al., 2007; Kwiatkowski et al., 2007). *C. elegans* contains a single Enabled homolog known as UNC-34. Ena/VASP members all share three domains: an N terminal Ena/VASP homology 1 (EVH1) domain that can interact with several proteins, a central proline rich region (PRR) that can interact with Profilin and proteins containing SH3 domains, and a C-terminal EVH2 domain that mediates the formation of tetramers and interactions with actin (Krause et al., 2003).

In order to study the role of the Ena/VASP family in neuronal development, we performed a genetic analysis of *unc-34*, the single *C. elegans* Ena/VASP homolog. We discovered a role for UNC-34 in ALM neuronal polarity, a process that also requires Wnts, suggesting that Ena/VASP family members could function in Wnt signaling.

MATERIALS AND METHODS
Strains and genetics
Strains were grown at 20°C unless stated otherwise, and were maintained as described by Brenner (1974). In addition to the wild-type strain N2, strains with the following mutations or transgenes were used in this work:

*GLI: lin-44(n1792 and n2111) (Herman and Horvitz, 1994), zdIs5 [Pmec-4::gfp]*
(Prasad and Clark, 2006)

LG II: cwn-1(ok546) (Zinovyeva and Forrester, 2005),
LG III: mig-10(ct41) (Manser and Wood, 1990);
LG IV: egl-20(n585) (Maloof et al., 1999), cwn-2(ok895) (Zinovyeva and Forrester, 2005), ced-10(n1993) (Ellis et al., 1991), ced-10(n3246) (Reddien and Horvitz, 2000), ced-10(tm597) (National Bioresource Project of Japan);
LG V: unc-34 alleles included gm104, gm114, (Forrester and Garriga, 1997), ev561 and ev562 (Colavita and Culotti, 1998), mom-2(or309) (Thorpe et al., 1997), mom-2(ne874ts) (Zinovyeva et al., 2008),
Rearrangements and extrachromosomal arrays: nT1[qIs50] and dnT2[qIs50] (Belfiore et al., 2002), kyEx710 [Punc-86::GFP::unc-34, Podr-1::dsRed] and kyEx926 [Punc-86::mig-10::YFP, Podr-1::dsRed] (Adler et al., 2006).

Scoring of the ALM polarity phenotype
Neuronal polarity of ALM was scored using the integrated array zdIs5 [Pmec-4::gfp], which expresses GFP in ALM, PLM, AVM and PVM mechanosensory neurons.
For ALM, the bipolar phenotype was defined as a normal anterior process and a posterior process that is longer than five ALM cell diameters in length.

RESULTS
UNC-34 functions in Wnt-regulated neuronal polarity
The three Wnt genes cwn-1, cwn-2 and egl-20 regulate the polarity of the ALM neuron (Hilliard and Bargmann, 2006; Prasad and Clark, 2006). While ALM development is normal in cwn-1, cwn-2 and egl-20 single mutants, the polarity of the ALMs is often disrupted in cwn-1; cwn-2 and cwn-1; egl-20 double mutants. The ALMs normally extend a long single process to the head (Fig. 6A, D). In Wnt double mutants, the ALMs can be bipolar or their polarity can be reversed, extending a single process toward the tail (Fig. 6B, C, E, F). We found similar ALM polarity defects in cwn-1; cwn-2 and cwn-1; egl-20 mutants to those reported previously (Fig. 6G). Because the cwn-2 and egl-20 genes are linked, the egl-20 cwn-2 double mutant was not constructed and analyzed previously. We find that an egl-20 cwn-2 double mutant has a weak bipolar phenotype but that a cwn-1; egl-20 cwn-2 mutant shows a significantly stronger defect than any of the double mutants (Fig. 6G). These findings indicate that each of these three Wnts provide overlapping functions in ALM polarity. C. elegans has two additional Wnt genes: lin-44 and mom-2. Because a mom-2 mutation does not enhance the cwn-1 ALM defect, this Wnt gene may not have a role in ALM polarity (Fig. 6G). One caveat of this interpretation is that we could only analyze homozygous mom-2 mutants coming from heterozygous mothers since mom-2 loss leads to a completely penetrant maternal effect embryonic lethality. Thus, a maternally provided gene product could mask a role for mom-2 in this process. A lin-44 mutation suppresses the ALM polarity defects of the cwn-1; egl-20 double mutant (Hilliard and Bargmann, 2006; Prasad and Clark, 2006). We confirmed this result and also showed that a strain containing mutations in all five Wnt genes is less severely affected than the cwn-1; egl-20 cwn-2 triple mutant (Fig. 6G), supporting the hypothesis that LIN-44 antagonizes the effects of one or more of the other Wnts in this process.
By screening existing mutations for an effect on ALM polarity, we found that mutations in \textit{unc-34} produced a synthetic. The strong alleles \textit{gm104} and \textit{gm114} resulted in a higher penetrance of ALM defects than the weaker \textit{ev561} and \textit{ev562} alleles (Fig. 7A). We were also able to rescue partially the synthetic ALM defect of \textit{cwn-1; unc-34(gm104)} mutants by expressing \textit{unc-34} in the ALM from the \textit{unc-86} promoter, which is expressed in many neurons including the ALM, but not in non-neuronal cells (Baumeister et al., 1996) (Fig. 7A). This finding suggests that \textit{UNC-34} functions in the ALM to establish its polarity.

\textit{UNC-34} and the Rac \textit{CED-10} were found to act in parallel in \textit{UNC-6}-mediated ventral guidance of the AVM axon, and these two pathways were shown to act downstream of the \textit{UNC-6} receptor \textit{UNC-40} (Gitai et al., 2003). To address whether \textit{CED-10} also acts in ALM polarity, we scored \textit{ced-10, cwn-1; ced-10 and ced-10; unc-34} mutants (Fig. 7B). None of the three \textit{ced-10} alleles tested had much of an effect on ALM polarity, but the \textit{cwn-1; ced-10} double mutants displayed significant ALM polarity defects. The strongest \textit{ced-10} allele, \textit{tm597}, is a maternal effect lethal, and unlike the other \textit{ced-10} mutants that were analyzed, animals homozygous for \textit{tm597} came from heterozygous mothers. The weaker effect of the \textit{tm597} allele presumably reflects rescue by maternally supplied gene product.

Because mutations in both \textit{unc-34} and \textit{ced-10} generate a synthetic polarity defect in combination with the \textit{cwn-1} mutation, we attempted to construct a \textit{ced-10; unc-34} double mutant, but were unable to maintain the strains as homozygous stocks because of lethality. Instead, we balanced both mutations over the \textit{nT1} balancers. Because the balancers are marked by a transgene that expresses GFP in the pharynx, we scored animals that lacked the GFP marker. Very few double homozygous larvae are generated from the balanced strain and those that were produced arrested development as larvae. The \textit{ced-10; unc-34} double mutants lacked significant ALM polarity defects. The lack of a phenotype could result from the presence of maternally supplied \textit{ced-10, unc-34} or both.

The lamellopodin/RIAM homolog \textit{MIG-10} physically interacts with both \textit{UNC-34} and \textit{CED-10} (Quinn et al., 2006; Quinn et al., 2008). We found that like mutations in \textit{unc-34} and \textit{ced-10}, a \textit{mig-10} mutation did not cause an ALM polarity defect but did generate a synthetic phenotype with \textit{cwn-1} (Fig. 7B). The ability of a \textit{Punc-86::mig-10::gfp} transgene to partially rescue the synthetic phenotype suggests that \textit{mig-10} also acts in the ALM to regulate its polarity.

\textbf{unc-34 mutants are heat sensitive for neuronal polarity}

The locomotion phenotype of \textit{unc-34} mutants has been reported to be heat sensitive (Bloom, 1993). We confirmed that the Unc phenotype of all \textit{unc-34} mutants, including the null mutant \textit{gm104}, increased in severity at elevated temperatures. We tested whether the ALM polarity defects of \textit{unc-34} mutants were also affected by temperature. Whereas the penetrance of ALM polarity defects in \textit{cwn-1; egl-20} double mutants was not elevated significantly at higher temperatures, the penetrance of the \textit{cwn-1; unc-34(gm104)} double

mutant increased significantly with increasing temperature (Fig. 7C). Because the neuronal polarity in wild-type animals was largely unaffected in this same temperature range, these phenotypic differences reveal a temperature-sensitive process that is normally masked by wild-type unc-34 function.

**DISCUSSION**

**UNC-34 and neuronal polarity**

Ena/VASP family members are essential for polarization of cortical neurons grown in culture (Kwiatkowski et al., 2007). These neurons usually go through several stages to polarize, generating a single axon and multiple dendrites. In the first stage, neurons extend filopodia that develop into neurites, and these filopodia fail to form in cortical neurons derived from mice lacking all three Ena/VASP family members (Kwiatkowski et al., 2007). The finding that these triple mutant mice are almost devoid of cortical axon tracts is consistent with Ena/VASP family members playing an essential role in the early stages of neuronal polarization. Unlike the cortical neurons of the mouse triple mutants, C. elegans unc-34 neurons lacking all Eva/VASP function polarize normally. One explanation for this difference is that cortical neurons lack redundant pathways that can generate polarity in the absence of Ena/VASP family members. The finding that either expression of the actin nucleating protein mDia in the mutant cortical neurons or growing the neurons on laminin can rescue the defect in neuritogenesis is consistent with this hypothesis (Dent et al., 2007).

UNC-34 was implicated in the initial ventral polarization of the HSN motor neuron and the AVM mechanosensory neuron (Adler et al., 2006; Quinn et al., 2006). While unc-34 mutants rarely have defects in the ventral growth of the HSN and AVM neurons, genetic interaction studies show unc-34 mutations can interact synergistically with other mutations during ventral growth. For example, loss of UNC-34 and the Rac CED-10 leads to defects in AVM ventral guidance, and these molecules are thought to mediate the polarizing effects of the UNC-6-receptor UNC-40 (Gitai et al., 2003). We also tested whether CED-10 plays a role in ALM polarity and found that mutations in ced-10 produced a synthetic phenotype in a cwn-1 mutant background. The ced-10; unc-34 double mutants, however, did not have an ALM polarity defect. We balanced both the ced-10 and unc-34 mutations and scored the double homozygous animals coming from the heterozygous mothers, so the lack of a phenotype could reflect rescue by maternal products. Alternatively, additional pathways might function in ALM polarity. We do not believe that the presence of other Racs are responsible for the lack of a phenotype because reducing either mig-2 or rac-2 in a cwn-1 mutant background did not produce a significant ALM phenotype (data not shown). A recent report showed that CED-10 mediates the effects of Wnts in the engulfment of apoptotic cell corpses, the orientation of mitotic spindles during asymmetric cell divisions and the migration of the distal tip cells, somatic gonadal cells that shape the structure of the gonad (Cabello et al., 2010). Our results suggest that Wnts also act through CED-10 to control neuronal polarity. We also tested the role of MIG-10, a lamellipodin/RIAM homolog that physically interacts with both UNC-34 and CED-10 (Quinn et al., 2006; Quinn et al., 2008), and found that it plays a cell autonomous role in ALM polarity. Our findings indicate that the
same signal transduction molecules involved in polarizing the AVM along the dorsoventral axis also function in polarizing the ALM along the anterioposterior axis. The lack of interactions between \textit{unc-34} and either \textit{egl-20} or \textit{cwn-2} suggests that \textit{unc-34} could mediate the effects of these Wnts and act in parallel to CWN-1. Alternatively, the \textit{cwn-1} mutant background might provide a more sensitized background than that of the other Wnt mutants. In either case, UNC-34 might mediate the effects of these Wnts on ALM polarity. It is noteworthy that \textit{unc-34} and the three Wnts involved in ALM polarity also control HSN migration, consistent with a general role for UNC-34 in Wnt mediated events (Forrester and Garriga, 1997; Pan et al., 2006). How Wnts regulate cell motility and neuronal polarity is poorly understood. Recent papers have implicated an atypical protein kinase C, PI3 kinase (Wolf et al., 2008) and a Rac in Wnt regulated motility (Cabello et al., 2010). Our results support the role of Rac in this process and implicate Ena for the first time in the Wnt regulated neuronal polarity.
REFERENCES:


Figure legends:

**Figure 6: unc-34 and cwn-1 mutations synergize to produce defects in ALM neuronal polarity.** (A–F) Photomicrographs of L4 zdIs5 (pmec-4::gfp) animals showing ALM neuronal morphology. The ALM cell bodies are labeled. Arrows indicate anterior processes, and arrowheads indicate posterior processes. Anterior is to the left, dorsal is up. unc-34 mutants exhibit defects in ALM neuronal polarity. The boxed regions of A–C are shown in D–F. (A, D) A wild-type ALM extends a single, anterior process. (B, E) A cwn-1; egl-20 ALM is bipolar, extending both a normal anterior process and an ectopic posterior process. (C, F) A cwn-1; egl-20 ALM extends a single posterior process, indicating a reversal of polarity. (G and H): Graph shows the percentage of ALM neurons with defective polarity for each genotype. Gray bars indicate the bipolar phenotype and black bars indicate reversed polarity. In G, the lin-44 mutation was n2111 in the single wnt mutant strain and n1972 in the strains with multiple wnt mutations. The mom-2 mutation was or309 in the single wnt mutant and ne874 in the multiple wnt mutant strain. Numbers for each genotype are provided. N.S., not significant; *p < 0.01 (Fisher’s exact test).
Figure 7: Genetic interactions among genes involved in ALM polarity. (A-C) Graph shows the percentage of ALM neurons with defective polarity for each genotype. Gray bars indicate the bipolar phenotype and black bars indicate reversed polarity. (A) Multiple alleles of unc-34 interact with cwn-1 and unc-34 functions cell-autonomously in ALM polarity. (B) A mutation in cwn-1 significantly enhanced polarity defect of putative null alleles of unc-34 (gm104 and gm114) but not putative hypomorphic alleles of unc-34 (ev561 and ev562). Driving unc-34 expression from the neural promoter unc-86 significantly rescued polarity defect in cwn-1; unc-34(gm104). (B) The roles of ced-10 and mig-10 in ALM polarity. (C) unc-34(gm104) is temperature-sensitive for ALM polarity. Numbers for each genotype are provided. N.S., not significant; *p < 0.01 (Fisher’s exact test).
CHAPTER TWO APPENDIX TWO

THE IMMUNOGLOBULIN SUPER FAMILY PROTEIN RIG-3 PREVENTS SYNAPTIC POTENTIATION AND REGULATES WNT SIGNALING

Contributions to this Appendix:

This appendix is a result of collaboration with Kavita Babu and Joshua Kaplan from Harvard Medical School. This appendix is a section modified from a submitted manuscript (Kavita Babu, Zhitao Hu, Shih-Chieh Chien, Gian Garriga, & Joshua Kaplan, Neuron). The data presented in the figures is my contribution to the manuscript.
INTRODUCTION
Cell surface Ig superfamily proteins (IgSF) have been implicated in diverse aspects of neuronal development, including: cell and axon migration, target recognition, axon fasciculation, axon ensheathment by glia, synapse formation and synapse function (Rougon and Hobert, 2003). Many IgSF proteins act as either homo- or heterophilic cell adhesion molecules (CAMs), e.g. NCAM (Yamada and Nelson, 2007). Other IgSF proteins act as receptors for secreted ligands, or as auxiliary subunits of such receptors (Barrow and Trowsdale, 2008; Wang and Springer, 1998). IgSF proteins comprise a large family of proteins (765 in humans, 142 in flies, 80 in worms (Lander et al., 2001; Vogel et al., 2003) and mutations in the corresponding genes have been implicated in several human neurological disorders (Fransen et al., 1997; Sun et al., 2003; Uyemura et al., 1996).

Many aspects of neuron and synapse development are regulated by both positive and negative factors. Axon and cell migrations are shaped by gradients of secreted attractants and repellents (Dickson, 2002). Similarly, synapse formation is governed by both positive and negative factors (Klassen and Shen, 2007; Poon et al., 2008; Scheiffele, 2003; Washbourne et al., 2004). For instance, Wnts are a class of conserved secreted glycoproteins that can both promote and inhibit synaptic assembly (Ciani and Salinas, 2005; Klassen and Shen, 2007). At C. elegans neuromuscular junction, the Wnt receptor CAM-1/Ror kinase has previously been shown to regulate the postsynaptic distribution of acetylcholine receptors (Francis et al., 2005). Here we show that mutants lacking RIG-3, a cell surface IgSF molecule, have an exaggerated paralytic response to a cholinesterase inhibitor, aldicarb. Although RIG-3 is expressed in motor neurons, heightened drug responsiveness in rig-3 mutants was caused by an aldicarb-induced increase in postsynaptic acetylcholine receptor abundance, and a corresponding potentiation of postsynaptic responses. Mutants lacking RIG-3 also had defects in the polarity of ALM neurons. RIG-3’s effects on ACR-16 trafficking and ALM polarity were both mediated by altering Wnt signaling, and in particular by regulating the Wnt receptor CAM-1.

MATERIALS AND METHODS
Strains and genetics
Strains were grown at 20°C unless stated otherwise, and were maintained as described by Brenner (1974). In addition to the wild-type strain N2, strains with the following mutations or transgenes were used in this work:

LGI: zdIs5[Pmec-4::gfp, lin-15(+)] (Clark and Chiu, 2003);
LG II: cwn-1(ok546) (Zinovyeva and Forrester, 2005), cam-1(ak37) (Francis et al., 2005), mig-14(ga62) (Eisenmann and Kim, 2000);
LG IV: egl-20(n585) (Maloof et al., 1999);
LGX: rig-3(ok2156) (Caenorhabditis Genetics Center).

Scoring of the ALM polarity phenotype
Neuronal polarity of ALM was scored using the integrated array zdIs5 [Pmec-4::gfp],
which expresses GFP in the six mechanosensory neurons, ALMs, PLMs, AVM and PVM. For ALM, the bipolar phenotype was defined as a normal anterior process and a posterior process that is longer than five ALM cell diameters in length.

**Fluorescence Microscopy**

For fluorescence microscopy, L4 to young adult hermaphrodite animals were anesthetized with 1% sodium azide, mounted on agar pad, and observed with a Zeiss Axioskop2 microscope.

**RESULTS**

**Mutants lacking RIG-3 are hypersensitive to aldicarb**

To identify new molecules involved in neuromuscular signaling, we used RNAi to screen for cell adhesion molecules whose absence alters the responsiveness of *C. elegans* to the acetylcholinesterase inhibitor aldicarb. Aldicarb treatment causes acute paralysis due to the accumulation of acetylcholine (ACh) in the synaptic cleft at the neuromuscular junction (NMJ). Gene inactivations that alter synaptic function can cause either resistance or hypersensitivity to aldicarb (Sieburth et al., 2005; Vashlishan et al., 2008). For this screen, we selected a collection of 216 putative cell adhesion molecules, based on the presence of protein domains found in CAMs (data not shown).

A gene identified in this screen was *rig-3*, which encodes a GPI-anchored protein containing two Ig domains and a divergent fibronectin type III (FNIII) domain (Fig. 1A). RIG-3 has a pattern of protein domains that is similar to the *Drosophila* proteins Klingon and Wrapper, and to mammalian NCAMs (Cox et al., 2004; Yamagata et al., 2003). RIG-3 was previously implicated in axon guidance in *C. elegans*; however *rig-3* single mutants do not show guidance defects (Schwarz et al., 2009).

Inactivation of *rig-3* by RNAi caused significant hypersensitivity to aldicarb and a similar defect was observed in homozygous *rig-3(ok2156)* mutants (data not shown). The *ok2156* mutation deletes 1.5kb of the *rig-3* gene, spanning exons 2-5 (including most of the Ig domains and part of the FNIII domain); consequently, *ok2156* is likely to cause a severe loss of gene function (Fig. 1A).

**Aldicarb increases the synaptic abundance of ACR-16 receptors in *rig-3* mutants and this increase requires the Wnt receptor CAM-1.**

Increased acetylcholine responses could be caused by altered expression or activity of acetylcholine receptor ACR-16. Following aldicarb treatment, ACR-16::GFP puncta fluorescence was significantly increased in *rig-3* mutants (35%, p<0.001), while levels in wild type animals were unaltered (data not shown). A prior study showed that CAM-1 (a Ror-type receptor tyrosine kinase) promotes ACR-16 delivery to NMJs (Francis et al., 2005). Consistent with this study, we observed modestly reduced synaptic ACR-16::GFP fluorescence (78% wild type, p<0.01) in *cam-1* mutants (data not shown). Thus, CAM-1 and RIG-3 have opposite effects on synaptic ACR-16 levels. Prompted by these results, we tested the idea that RIG-3’s effects on ACR-16 are mediated by changes in CAM-1 activity. Consistent with this idea, the aldicarb hypersensitivity and the increased ACR-16::GFP levels following aldicarb treatment were all eliminated in *cam-1; rig-3* double...
mutants (data not shown). To determine if RIG-3 regulates CAM-1 levels, we analyzed GFP-tagged CAM-1 fluorescence in body muscles. Aldicarb treatment significantly increased CAM-1 synaptic fluorescence in \textit{rig-3} mutants, but had no effect on CAM-1 levels in wild type controls (data not shown). Taken together, these results suggest that RIG-3 negatively regulates CAM-1 levels at NMJs, and that increased CAM-1 activity is required for RIG-3’s effects on ACR-16.

Several prior studies showed that CAM-1 acts as a Wnt receptor, mediating the effects of Wnt ligands on several tissues (Green et al., 2008). This implies that RIG-3’s effects on synaptic transmission could result from changes in Wnt signaling at the NMJ. Consistent with this idea, we found that a \textit{mig-14} Wntless mutation, which reduces Wnt secretion (Myers and Greenwald, 2007; Pan et al., 2008; Yang et al., 2008), confers resistance to aldicarb-induced paralysis, and eliminates the \textit{rig-3} aldicarb hypersensitivity defect in \textit{mig-14; rig-3} double mutants (data not shown). These results support the idea that the \textit{rig-3} synaptic defects are caused by increased Wnt signaling at the NMJ.

**RIG-3 alters Wnt regulation of ALM polarity**

RIG-3’s effects on CAM-1 at NMJs suggest that RIG-3 might also regulate Wnt signaling in other tissues. To test this idea, we analyzed the anteroposterior polarity of the ALM mechanosensory neurons. Several prior studies showed that ALM polarity is regulated by Wnt signaling (Hilliard and Bargmann, 2006; Pan et al., 2006; Prasad and Clark, 2006). The mCherry-tagged \textit{rig-3} genomic construct was expressed in ALM neurons (Fig. 8A), suggesting that RIG-3 could play a role in Wnt mediated control of ALM polarity. In wild type animals, ALM neurons have a single anteriorly directed process (Fig. 8B-C). In mutants with decreased Wnt signaling, ALM neurons exhibit either of two defects, with some neurons having both an anterior and a posterior process (bipolar ALMs) while others have a single posteriorly directed process (reversed ALMs) (Fig. 8B-C) (Fleming et al., 2010; Hilliard and Bargmann, 2006; Pan et al., 2006; Prasad and Clark, 2006). The prevalence of bipolar and reversed ALM neurons differs among Wnt mutants. These differences in ALM defects likely result from the fact that \textit{C. elegans} has five Wnt ligands, which have distinct effects on ALM polarity (Fleming et al., 2010; Prasad and Clark, 2006). For example, two prior studies showed that the effects of two Wnts (CWN-1 and EGL-20) on ALM polarity are antagonized by a third Wnt (LIN-44) (Fleming et al., 2010; Prasad and Clark, 2006). Thus, the precise ALM phenotype observed is determined by how each mutation alters signaling by the different Wnt ligands.

To further investigate if RIG-3 plays a role in Wnt signaling, we analyzed the effect RIG-3 inactivation on ALM polarity in several genetic backgrounds. Although ALM polarity was unaltered in \textit{rig-3} single mutants, the \textit{rig-3} mutation significantly altered ALM polarity defects caused by other Wnt pathway mutations in double and triple mutants. Inactivating RIG-3 in \textit{cwn-1; egl-20} double mutants decreased the severity of ALM polarity defects: ALM reversals were significantly reduced in \textit{cwn-1; egl-20; rig-3} triple mutants ($p < 0.01$, Fishers exact test), while the number of bipolar ALMs was unaffected ($p = 0.21$). The effects of RIG-3 on ALM polarity were distinctly different in mutants
with reduced Wnt secretion (mig-14 Wntless mutants). Inactivating RIG-3 in mig-14 mutants decreased ALM reversals and increased bipolar ALMs (Fig. 8B-C). The different outcome in mig-14 mutants likely results from the fact that MIG-14 is required for secretion of all Wnt ligands. By contrast, the rig-3 mutation had no effect on ALM polarity in two strains lacking CAM-1, i.e. cam-1; rig-3 double mutants and cam-1 mig-14; rig-3 triple mutants (Fig. 8C). These results lead to three conclusions. First, RIG-3 plays an important role in Wnt regulation of ALM polarity. Second, CAM-1 is absolutely required for RIG-3’s effects on ALM polarity. Third, RIG-3’s effects on ALM polarity and on ACR-16 regulation at the NMJ can both be explained by changes in Wnt signaling.

DISCUSSION

Here we define a novel function for an IgSF protein, RIG-3. Our results lead to three primary conclusions. First, the synaptic potentiation observed in rig-3 mutants is mediated by aldicarb-induced accumulation of post-synaptic ACR-16 nAChR receptors. Second, inactivating RIG-3 also alters the polarity of ALM neurons. And third, RIG-3’s effects on cholinergic transmission and on ALM polarity are both mediated by changes in Wnt signaling, and in particular by inhibiting the activity of a Wnt-binding protein (CAM-1). Below, we discuss the significance of these findings.

RIG-3 is a novel regulator of Wnt signaling

Prior studies have shown that CAM-1 can play either of two alternative functions in Wnt signaling. In many cases, CAM-1 functions as a receptor mediating the effects of Wnt ligands; however, CAM-1 can also act as an antagonist inhibiting Wnt binding to other Wnt receptors (Green et al., 2008). Despite this ambiguity, all of CAM-1’s known effects on development are mediated by changes in Wnt signaling (Green et al., 2008). Thus, RIG-3’s absolute requirement for CAM-1 suggests that RIG-3’s effects on synaptic transmission and on ALM polarity are both mediated by changes in Wnt signaling. RIG-3 inhibition of CAM-1 could potentially promote or inhibit Wnt signaling, depending on whether CAM-1 functions as a receptor or an antagonist. Consequently, to assess how RIG-3 affects Wnt signaling, we compared the effect of rig-3 mutations to those caused by mutations inactivating Wnt ligands or decreasing Wnt secretion. At the NMJ, a mig-14 Wntless mutation and a rig-3 mutation had opposite effects on aldicarb-induced paralysis and the effect of RIG-3 on aldicarb-responsiveness was eliminated in mig-14; rig-3 double mutants. These results suggest that RIG-3 regulates aldicarb responses by inhibiting Wnt signaling at the NMJ.

For ALM polarity, the results are more complicated. Prior studies showed that four of five Wnt ligands play a role in dictating ALM polarity but that distinct ALM defects (i.e. bipolar versus reversed ALM neurons) are observed when different combinations of Wnt ligands are inactivated (Fleming et al., 2010; Prasad and Clark, 2006). Two results suggest that a global reduction in Wnt signaling primarily leads to reversed ALM neurons: quintuple mutants containing mutations in all five Wnt ligands (55% reversed, 5% bipolar) (Fleming et al., 2010) and mig-14 mutants (which reduce secretion of all Wnt ligands) (69% reversed, 12% bipolar) (Fig. 8). These data suggest that the different ALM phenotypes observed in Wnt mutants comprise a graded series whereby more extreme
Wnt defects cause primarily ALM reversals while less severe defects cause fewer reversals and increased bipolar ALMs. Inactivating RIG-3 in cwn-1; egl-20 double mutants significantly decreased reversed ALMs and had no effect on bipolar ALMs, indicating that RIG-3 and these two Wnt ligands have opposite effects on ALM polarity. Inactivating RIG-3 in mig-14 mutants also resulted in a less severe ALM phenotype (with decreased ALM reversals and increased bipolar ALMs). In both experiments, rig-3 mutations and mutations inactivating Wnt signaling had opposite effects on ALM polarity. Thus, our analysis of RIG-3’s effects on both the NMJ and on ALM polarity is most consistent with the idea that RIG-3 normally inhibits Wnt signaling. These results do not exclude the possibility that RIG-3 promotes Wnt signaling in other contexts. In particular, in cases where CAM-1 functions as a Wnt antagonist, RIG-3 inhibition of CAM-1 could enhance Wnt signaling.

A RIG-3 translational reporter is expressed in neurons but not body wall muscles (Schwarz et al., 2009 and data not shown). The observation that RIG-3 is also expressed in ALM (Fig. 8A) where CAM-1 functions (Chapter 2) suggests that RIG-3 acts cell-autonomously. RIG-3 and CAM-1 both contain Ig domains, which could mediate direct binding interactions between these proteins. Alternatively, RIG-3 could inhibit Wnt secretion or Wnt binding to CAM-1 or other Wnt receptors. Further experiments will be required to distinguish between these possibilities.

Several other Wnt antagonists have been described (Kawano and Kypta, 2003). For example, several genes encode secreted frizzled-related proteins (SFRPs), which bind to Wnt ligands to inhibit their signaling. By contrast, secreted Dickkopf proteins bind to low density lipoprotein receptor related proteins (LRPs), which are accessory subunits for Frizzled receptors, thereby inhibiting Wnt signaling. Our results identify RIG-3 as a novel Wnt regulator, and show that RIG-3 acts by inhibiting the Wnt receptor CAM-1. These results suggest that different inhibitors are utilized to regulate Wnt signaling in different contexts.
REFERENCES:


Figure legends:

**Figure 1: RIG-3 is a cell surface Ig superfamily protein (IgSF).** (A) A schematic of the RIG-3 protein is shown, indicating the signal sequence (ss), Ig, FNIII, and GPI-anchoring domains, and the site utilized for mCherry tagging. The domains deleted in *rig-3(ok2156)* mutants are indicated by the bar.
Figure 8: RIG-3 antagonizes the effects of Wnt on ALM polarity.

(A) Expression of the mCherry-tagged rig-3 genomic construct is shown in ALM neurons. The ALM neurons were visualized with the zdIs5 transgene, which expresses GFP in the touch neurons (with the mec-4 promoter). (B) Representative images and schematic drawings are shown illustrating wild type, bipolar (less severe), and reversed (more severe) ALM defects. (C) Summary data for ALM polarity defects are shown for the indicated genotypes. The number of animals analyzed is indicated for each genotype. All strains contain the zdIs5 transgene, to allow visualization of ALM neurons. Values that differ significantly from wild type controls are indicated (***, $p<0.001$; **, $p<0.01$).
CHAPTER THREE

THE C. ELEGANS MIG-15 KINASE AND ITS POTENTIAL EFFECTORS
FUNCTION IN WNT-REGULATED NEURONAL POLARITY
SUMMARY
Nck-interacting kinase (NIK) and its orthologs share a common function in regulating cell shape and polarity. In C. elegans, three Wnts, CWN-1, CWN-2 and EGL-20, coordinately regulate the polarity of mechanosensory neuron ALM, but it is unclear what molecules mediate Wnt signaling. Here we report that the C. elegans NIK ortholog, MIG-15, may be a novel effector of Wnts in ALM polarity. A distant MIG-15 homolog is the Ste20 kinase Kic1p of S. cerevisiae, which is one of the six proteins in the RAM pathway that regulates polarized morphogenesis. We showed that two C. elegans orthologs of the RAM pathway genes, sax-2 and mop-25.2, are required for ALM polarity.

INTRODUCTION
Wnts are a class of conserved secreted glycoproteins that regulate many aspects of neuronal development including neuronal migration, neuronal polarity, axon guidance, dendritic morphogenesis and synaptic development (Salinas and Zou, 2008). The binding of Wnt to the seven-pass transmembrane receptor Frizzled (Fz) activates the cytoplasmic protein Dishevelled (Dsh), but how Dsh activation leads to changes in cytoskeletal dynamics is not well understood. NIK (Nck-interacting kinase) and its orthologs are serine/threonine kinases that can mediate the effects of Fz/Dsh signaling in planar cell polarity (Paricio et al., 1999) and regulate both cell shape (Chapman et al., 2008; Koppen et al., 2006; Su et al., 1998; Treisman et al., 1997) and migration (Chapman et al., 2008; Cobreros-Reguera et al., 2010; Shakir et al., 2006; Xue et al., 2001). In this study, we characterize the role of C. elegans ortholog of NIK, MIG-15, in Wnt-regulated neuronal polarity.

MIG-15, along with its murine ortholog NIK and Drosophila ortholog Misshapen, belong to the Ste20 kinase superfamily (Su et al., 1998). The Sacchromyces cerevisiae Ste20 is a MAP4K that transduces signals to a downstream triple kinase module in the pheromone response pathway (Dan et al., 2001). NIK and Misshapen have been shown to activate the downstream kinase JNK (Becker et al., 2000; Paricio et al., 1999; Su et al., 1997; Su et al., 1998). The failure to activate JNK results in defects in dorsal closure, a convergent extension-like process that zippers up the dorsal side of Drosophila embryo (Su et al., 1998). NIK and Misshapen have also been directly linked to the actin cytoskeleton through phosphorylation of cytoskeletal regulators (Baumgartner et al., 2006; Ruan et al., 2002). In cultured cells, NIK phosphorylates ERM proteins to promote growth factor-induced lamellipodial protrusion (Baumgartner et al., 2006). In Drosophila, the phosphorylation of actin regulator Bifocal by Misshapen causes the growth cones of photoreceptor axons to collapse (Ruan et al., 2002).

NIK and its orthologs have a conserved N-terminal kinase domain and a C-terminal regulatory domain (Su et al., 1998) that shares homology with Citron, an effector for Rac GTPases (Madaule et al., 1995). NIK does not physically interact with activated Rac (Su et al., 1998), but does bind to β-integrins (Poinat et al., 2002). This binding increases the affinity of the integrin toward the extracellular matrix, which may promote migration of cells and growth cones (Becker et al., 2000). The region between the kinase and the regulatory domains of NIK is less conserved but it contains a number of proline-rich
motifs (PxxP) that can bind to the SH3 domain-containing adaptor protein Nck (Su et al., 1997). The Nck ortholog of *Drosophila*, Dock, is required for photoreceptor axon targeting but not dorsal closure (Su et al., 2000), suggesting that Misshapen employs distinct factors to regulate different pathways.

The ability to regulate morphogenesis and cytoskeletal dynamics is a general feature of Ste20 group kinases (Dan et al., 2001). A distant Ste20p homolog in *S. cerevisiae*, Kic1p, has been implicated in polarized cell growth as part of a signaling network called RAM (regulator of Ace2p activity and cellular morphogenesis) (Nelson et al., 2003). The RAM pathway consists of six proteins: two kinases (a Ste20 kinase Kic1p and a NDR kinase Cbk1p), two adaptors (Hym1p and Mob2p), a scaffold (Tao3p) and a leucine-rich repeat-containing protein (Sog2p). Genetic and biochemical studies suggest that Kic1p, Sog2p, Hym1p and Tao3p act upstream of Cbk1p and Mob2p to control the catalytic activity of Cbk1p (Nelson et al., 2003). An analogous morphogenesis pathway has also been described in other yeast species such as *S. pombe* and *C. albicans* (Kanai et al., 2005; Song et al., 2008). An emerging picture from these studies is that these components localize to the site of polarized growth to promote F-actin assembly (Huang et al., 2005; Nelson et al., 2003)

In *C. elegans*, three Wnts, CWN-1, CWN-2 and EGL-20, coordinately regulate the polarity of mechanosensory neuron ALM (Fleming et al., 2010; Hilliard and Bargmann, 2006; Prasad and Clark, 2006). However, it is unclear what molecules mediate Wnt signaling. Here we show that the *C. elegans* NIK ortholog, MIG-15, and two orthologs of RAM genes, SAX-2/Tao3p and MOP-25.2/Hym1p, may be novel effectors of Wnt signaling in ALM polarity.

MATERIALS AND METHODS

Nematode Strains and Genetics

Strains were maintained at 20°C as described (Brenner, 1974). The following alleles and transgenes are used in this study:

**LGI:** *zdIs5 [Pmec-4::gfp, lin-15(+)]* (Clark and Chiu, 2003)

**LG II:** *cwn-1(ok546)* (Zinovyeva and Forrester, 2005)

**LGIII:** *sax-2(bx130)* (Jia and Emmons, 2006), *sax-2(ky166)* (Zallen et al., 1999), *sax-2(ot10)* (Altun-Gultekin et al., 2001)

**LGIV:** *eri-1(mg366)* (Kennedy et al., 2004)

**LGV:** *egl-20(n1993)* (Ellis et al., 1991)

**LGX:** *sax-1(ky211)* (Zallen et al., 1999), *sax-1(ky491)* (Zallen et al., 2000), *mig-15* alleles used include *rh148* (Poinat et al., 2002), *rh80* and *rh326* (Shakir et al., 2006)

Extrachromosomal array: *kyEx700 [Pmec-4::sax-2::gfp; Podr-1::dsRed]* (Gallegos and Bargmann, 2004)

RNA interference

RNAi was performed using the bacterial feeding method as described (Kamath et al., 2001; Timmons and Fire, 1998). In all experiments, worms were grown on plates supplemented with 25 mM Carbenicillin and 1 mM IPTG at 20°C. The RNAi cultures
were prepared by inoculating bacterial strains in LB with 25 mM Carbenicillin for 15 hours at 37 °C, followed by addition of 6 mM IPTG and incubation for another hour at 37 °C. Bacterial strains used to inactivate genes by feeding were obtained from the library designed by the Ahringer lab (Fraser et al., 2000).

**Scoring of the ALM polarity phenotype**

Neuronal polarity of ALM was scored using the integrated array zdIs5 [Pmec-4::gfp], which expresses GFP in the ALM, PLM, AVM and PVM mechanosensory neurons. For ALM, the bipolar phenotype was defined as a normal anterior process and a posterior process that is longer than five ALM cell diameters in length.

**Fluorescence Microscopy**

For fluorescence microscopy, L4 to young adult hermaphrodite animals were anesthetized with 1% sodium azide, mounted on agar pad, and observed with a Zeiss Axioskop2 microscope. Animals were raised at 20°C except for the temperature-sensitive alleles of *sax-1* and *sax-2*, which were raised at 25°C.

**RESULTS**

**Mutations in mig-15, the *C. elegans* NIK ortholog, cause defects in ALM polarity**

*C. elegans* ALMs are a pair of bilaterally symmetric neurons located in the midbody. Each ALM extends a single anterior process toward the head. While ALM development is largely normal in *cwn-1, cwn-2* and *egl-20* single Wnt mutants, the polarity of the ALM is often disrupted in *cwn-1; cwn-2* and *cwn-1; egl-20* double mutants (Hilliard and Bargmann, 2006; Prasad and Clark, 2006). In Wnt double mutants, the ALMs can be bipolar or their polarity can be reversed, extending a single process toward the tail. Considering NIK and its orthologs share a common function in regulating cell morphology and polarization, we asked whether *mig-15*, the *C. elegans* NIK ortholog, plays a role in ALM polarity. Using an integrated array *Pmec-4::gfp* to visualize the mechanosensory neurons, we observed that three alleles of *mig-15* have graded effects on ALM polarity. The hypomorphic allele *rh148*, which is a missense mutation in the ATP-binding-pocket of the kinase domain (Shakir et al., 2006), had little effect on ALM polarity (1.5% bipolar; Fig. 1). By contrast, we observed 15.9% bipolar ALMs in animals homozygous for the stronger allele *rh80*, which encodes a premature stop codon in the middle of the C-terminal regulatory domain (Shakir et al., 2006). Both *rh148* and *rh80* likely retain residual *mig-15* activity. The strongest allele *rh326* also encodes a premature stop codon, but it truncates the protein in the middle of the proline-rich domain and thus is likely a null (Shakir et al., 2006). We observed 29.1% bipolar ALMs in *rh326*. On the basis of ALM polarity defects, the three *mig-15* alleles form an allelic series from least severe to most severe: *rh148* < *rh80* < *rh326*, a trend consistent with the effects of these mutations on viability, cell migration and axon guidance (Chapman et al., 2008; Shakir et al., 2006). We next investigated the genetic interaction between *mig-15* and the Wnts. We found that *cwn-1*, but not *egl-20*, significantly enhanced the ALM polarity defect in all three *mig-15* alleles (Fig. 1).

*C. elegans* orthologs of the JNK pathway genes are not implicated in ALM polarity
MIG-15 belongs to the Ste20 kinase superfamily, in which the prototypical member Ste20p is a MAP4K that can phosphorylate and activate the MAP3K Ste11p (Dan et al., 2001). In addition, MIG-15 orthologs NIK and Missha pen have both been shown to activate the downstream JNK pathway (Becker et al., 2000; Paricio et al., 1999; Su et al., 1997; Su et al., 1998). To determine whether MIG-15 has a conserved function in activating JNK pathway in ALM polarity, we analyzed existing mutants of JNK pathway genes either by themselves or in a cwn-1 sensitized background. To knock down expression of target genes, we also took a complementary RNAi approach by using a strain containing cwn-1 and a mutation in eri-1 that sensitizes animals to the effects of RNAi (Kennedy et al., 2004).

In C. elegans, JNK-1/MAPK and its direct upstream activator JKK-1/MAP2K have been implicated in several processes including stress resistance, lifespan extension and transport of synaptic vesicle (SV) components in neurons (Byrd et al., 2001; Kawasaki et al., 1999; Oh et al., 2005). The last function is shared by UNC-16, which serves as a conserved molecular tether between motor proteins and their cargos (Byrd et al., 2001; Sakaguchi et al., 2004). UNC-16 has also been shown to directly bind to both JNK-1 and JKK-1, thus acting as a scaffold protein in the JNK pathway (Byrd et al., 2001). lrk-1 is homologous to the human LRK2, the causative gene in the familial Parkinson’s disease (Sakaguchi-Nakashima et al., 2007). lrk-1 encodes a protein that has a MAP3K-like domain and is required for proper targeting of SV components similar to other JNK pathway genes, suggesting that it can act as a MAP3K in the JNK pathway (Sakaguchi-Nakashima et al., 2007). We examined the deletion mutants of jnk-1, jkk-1, and lrk-1, as well as hypomorphic mutants of unc-16 (e1089, e109 and ju146) because unc-16 null mutants arrest as larvae (Byrd et al., 2001). None of these mutations have an effect on ALM polarity (data not shown). In addition, mutation of jkk-1 or RNAi against lrk-1 in a cwn-1 sensitized background failed to generate a synthetic phenotype (data not shown). We also analyzed additional JNK pathway genes including a MAP3K (mom-4), two MAP2Ks (sek-1 and mek-1) and two MAPKs (kgb-1 and kgb-2) (Sakaguchi et al., 2004). We did not observe a significant ALM phenotype in these strains (data not shown).

Taken together, we were unable to implicate a single JNK pathway gene in ALM polarity, possibly due to redundancy between different kinases or insufficient knockdown of genes by RNAi. It is also possible that MIG-15 regulates neuronal polarity independent of activation of the JNK pathway.

C. elegans orthologs of the RAM pathway genes, sax-2 and mop-25.2, are required for ALM polarity

Given that perturbation of the JNK pathway did not have an effect on ALM polarity, we considered the possibility that mig-15 may regulate this process in novel ways. A distant MIG-15 homolog is the Ste20 kinase Kic1p of S. cerevisiae, which is one of the six proteins in the RAM pathway that regulates polarized morphogenesis (Nelson et al., 2003). All proteins with the exception of the leucine rich repeat-containing Sog2p have homologs in C. elegans (Table 1), and we wondered whether these homologs mediate the effects of Wnt signaling in ALM polarity.
One key output of the RAM pathway is the activation of the NDR kinase Cbk1p. NDR kinases are conserved serine/threonine kinases that regulate various developmental processes such as mitosis, cell growth and morphogenesis (Hergovich et al., 2006). They are activated upon (1) binding to co-activators called Mobs; and (2) upstream phosphorylation by Ste20 kinases, an activity probably facilitated by binding to HEAT/Armadillo repeats-containing scaffold proteins (Hergovich et al., 2006). There are two NDR kinases in C. elegans: WTS-1 and SAX-1. WTS-1 has an essential function in intestinal morphogenesis (Kang et al., 2009) whereas SAX-1, along with the scaffold protein SAX-2 (orthologous to Tao3p), has been implicated in the regulation of cell shape, neurite outgrowth and termination (Gallegos and Bargmann, 2004; Zallen et al., 1999; Zallen et al., 2000). Two alleles of sax-1, ky211 and ky491, did not exhibit a significant ALM polarity phenotype either by themselves or in a cwn-1 background (Fig. 2B). RNAi against wts-1 in a cwn-1 background also failed to generate an additive phenotype (data not shown). However, combined reduction of NDR functions by RNAi against wts-1 in a cwn-1; sax-1 background generated a weak synthetic reversal phenotype (three reversed ALMs out of a total of 294 scored or 1%; Fig. 2B). We considered the presence of reversed ALMs to be significant because they were never observed in cwn-1 or sax-1 alone (n \geq 100). Therefore, NDR kinases might be involved in ALM polarity. We also observed a significant enhancement of polarity defect when we compared cwn-1; sax-2 double mutants to cwn-1 and sax-2 single mutants (Fig. 2B). In addition, a sax-2 cDNA expressed from the mechanosensory neuron-specific promoter mec-4 (Gallegos and Bargmann, 2004) partially but significantly rescued the ALM polarity defect in cwn-1; sax-2 animals (Fig. 2B), indicating that sax-2 can function in the neurons.

HYM1 of the RAM pathway encodes the MO25 adaptor protein. C. elegans have three MO25 homologs called mop-25.1, mop-25.2 and mop-25.3. Based on sequence homology, MOP-25.1 is paralogous to MOP-25.2 (69% identity and 84% similarity) and is more distantly related to MOP-25.3 (20% identity and 45% similarity) (Wormbase Blast Search). Two alleles of mop-25.2, ok2073 and m3694, and one allele of mop-25.3, ok2350, did not produce a significant ALM polarity phenotype either by themselves or in a cwn-1 background (data not shown). However, given that both alleles of mop-25.2 are maternal effect lethal, and we analyzed mutants from heterozygous mothers, we wondered whether a maternal contribution of mop-25.2 may mask a role of this gene in ALM polarity. Indeed, RNAi against mop-25.2 in a cwn-1; eri-1 background produced a significant ALM polarity phenotype (Fig. 2B). By contrast, RNAi against either mop-25.1 or mop-25.3 did not generate an ALM polarity phenotype (data not shown), suggesting mop-25.2 is the principal MO25 homolog that regulates ALM polarity.

There are four C. elegans proteins with sequence homology to Mob2p (Table 1). We tested the two Mob-like genes (F09A5.4 & C30A5.3) for which the RNAi clones were available in a cwn-1; eri-1 background. We did not observe any significant phenotype (data not shown), suggesting that the untested Mob genes are involved, that Mob genes function redundantly in ALM polarity, or that they do not function in this process. We also considered the possibility that other Ste20 homologs in addition to mig-15 are...
involved in ALM polarity. We tested gck-1, gck-4, cst-1 and kin-18 by RNAi as well as pak-1, pak-2 and max-2 by mutant analysis, but did not observe any obvious defects (data not shown).

**MIG-15 may regulate Rac GTPase and SAX-2 in ALM polarity**

Previously we implicated a Rac, CED-10, as a regulator of ALM polarity (Fleming et al., 2010). Given that mig-15 has been shown to act in the Rac pathway in axon guidance (Shakir et al., 2006), we wondered whether this is also true in neuronal polarity. We were also interested in determining how the RAM pathway gene sax-2 genetically interacts with ced-10 and mig-15 in this process. Since ced-10 null mutants are maternal effect lethal and maternal contribution may mask a role of this gene in ALM polarity (Fleming et al., 2010), we used a hypomorphic allele, n1993. We also chose to work with the hypomorphic mig-15 allele, rh148, because strains containing the stronger rh80 or rh326 alleles were often very sick. We found that ced-10 and sax-2 mutations interacted synergistically to produce a bipolar phenotype that is as penetrant as the putative mig-15 null mutant, rh326, suggesting that ced-10 and sax-2 act in separate pathways. A similar synthetic bipolar phenotype was observed in sax-2; mig-15(rh148) but not in ced-10; mig-15(rh148). In addition, cwn-1; mig-15(rh326) generated a polarity phenotype that is stronger than cwn-1; sax-2 but is indistinguishable from cwn-1; sax-2; ced-10, suggesting that ced-10 and sax-2 mediate the effects of mig-15 in ALM polarity.

**DISCUSSION**

**MIG-15 in Wnt-regulated ALM polarity**

Nck-interacting kinase (NIK) and its orthologs share a common function in regulating cell shape and polarity. In *C. elegans*, three Wnts, CWN-1, CWN-2 and EGL-20, coordinately regulate the polarity of mechanosensory neuron ALM, but it is unclear what molecules mediate Wnt signaling. Here we report that the *C. elegans* NIK ortholog, MIG-15, may be a novel effector of Wnts in ALM polarity. The observation that cwn-1, but not egl-20, significantly enhanced the ALM polarity defect of mig-15 (Fig. 1) suggests that mig-15 mediates the effects of egl-20 in parallel to cwn-1. Another interpretation of the data is that egl-20 may simply not provide a sensitized enough background to enhance mig-15 mutants.

Misshapen, the NIK ortholog in *Drosophila*, regulates a number of developmental processes including photoreceptor axon targeting, dorsal closure and planar cell polarity (Paricio et al., 1999; Su et al., 2000). The adaptor protein Dock, the Nck ortholog, is required for axon targeting but dispensable for both dorsal closure and planar cell polarity. On the other hand, JNK signaling is important for dorsal closure and planar cell polarity but not for axon targeting (Paricio et al., 1999; Su et al., 2000). Several observations suggest that MIG-15 does not function the same way as Misshapen in these processes. First, a mutation in the *C. elegans* Nck ortholog, nck-1, did not have an effect on ALM polarity either by itself or in a cwn-1 sensitized background (data not shown). Second, mutations in genes of the JNK pathway did not produce a phenotype similar to mig-15 mutants (data not shown), although we cannot rule out the possibility that multiple kinases act redundantly in this process.
**C. elegans** orthologs of RAM pathway genes, *sax-2* and *mop-25.2*, are required for ALM polarity

In a quest to identify additional components that may function with MIG-15 in ALM polarity, we tested *C. elegans* orthologs of the yeast RAM pathway genes. In this 6-component signaling network, all proteins with the exception of the leucine-rich repeat-containing protein Sog2p have evolutionarily conserved roles in morphogenesis and cell polarity. For instance, the HEAT/Armadillo repeats-containing scaffold protein Tao3p physically interacts with the NDR kinase Cbk1p and activates its kinase activity (Du and Novick, 2002; Nelson et al., 2003). Similarly in *Drosophila*, the NDR kinase Tricornered (Trc) and scaffold protein Furry (Fry) coordinately regulate wing hair morphology and patterning of dendritic fields (Cong et al., 2001; Emoto et al., 2004; Geng et al., 2000). The trc; fry double mutant resembles trc or fry single mutants, indicating that trc and fry act in the same pathway. Furthermore, Fry binds to Trc and is necessary for the kinase activity of Trc (Emoto et al., 2004; He et al., 2005), suggesting that Fry-like scaffold proteins have a conserved function in binding to and activating the NDR kinases.

The *C. elegans* orthologs of Trc and Fry are SAX-1 and SAX-2, respectively. SAX-1 and SAX-2 have been implicated in the regulation of cell shape, neurite outgrowth and termination with *sax-1* mutants typically exhibiting a phenotype slightly less severe or just as severe as *sax-2* mutants (Gallegos and Bargmann, 2004; Zallen et al., 1999; Zallen et al., 2000). To our surprise, *sax-2*, but not *sax-1*, displayed a polarity phenotype in a *cwn-1* background (Fig. 2). *wts-1* (orthologous to *Drosophila* Lats/Warts or Wts) encodes the other NDR kinase in *C. elegans*, so WTS-1 and SAX-1 may provide overlapping functions in neuronal polarity. Consistent with this idea, RNAi against *wts-1* in a *cwn-1*; *sax-1* background generated a low percentage of ALM reversals that were never observed in *cwn-1* or *sax-1* single mutants. Nevertheless, reducing function of NDR kinases did not have the same effect as removing *sax-2* in a *cwn-1* background, possibly due to incomplete knockdown of *wts-1*. *wts-1* null animals arrest as larvae due to defects in intestinal morphogenesis (Kang et al., 2009). A recently acquired strain with an array carrying intestinally-expressed *wts-1* that rescues the lethal phenotype of the *wts-1* mutant should help us further address the role of NDR kinases in ALM polarity.

In *Drosophila*, the two NDR kinases Trc and Wts are both phosphorylated and activated by the Ste20 kinase Hippo (Emoto et al., 2006), a homolog of MIG-15. This is reminiscent of the Cbk1p activation by the Ste20 kinase Kic1p. Mammalian NDRs can also be activated by upstream Ste20 kinases (Hergovich et al., 2006). RNAi against multiple *C. elegans* Ste20 homologs, including the Hippo homologs *cst-1* and *cst-2*, failed to generate a polarity phenotype in a *cwn-1* background (data not shown). Therefore, *mig-15* may be the relevant Ste20 homolog in ALM polarity. In the yeast RAM pathway, the small protein Mob2p physically interacts with Cbk1p and is required for the activity of Cbk1p. Similarly, *Drosophila* Mob protein Mats binds to Wts and activates its kinase activity (Lai et al., 2005). There are four Mob-like proteins in *C. elegans*, and RNAi against two of them did not generate an obvious phenotype (data not shown). Deletion alleles of these Mob genes are now available so we can address more carefully the role of Mob in ALM polarity. A regulatory paradigm emerging from studies...
in *Drosophila* and cultured human cells is that the activation of NDR kinases requires phosphorylation by upstream Ste20 kinases and the binding of Mob coactivators and Fry-like scaffold proteins. More experiments need to be done to see whether this also applies for neuronal polarity in *C. elegans*.

In the yeast RAM pathway, *HYM1* encodes the MO25 adaptor protein. In addition to the RAM complex, MO25 is found in the same complex with LKB1 kinase and STRAD pseudokinase in metazoans. The resulting heterotrimeric complex is a master regulator of polarity in diverse cell types, underscoring an ancient and evolutionarily conserved role of MO25 in cell polarization (Jansen et al., 2009). *C. elegans* has three MO25 homologs, *mop-25.1*, *mop-25.2* and *mop-25.3*. RNAi against *mop-25.2*, but not *mop-25.1* or *mop-25.3*, generated a synthetic phenotype in a *cwn-1* background, suggesting that MOP-25.2 is the principal homolog that regulates ALM polarity. *C. elegans* orthologs of LKB1 and STRAD (*par-4* and *strd-1*, respectively) did not have much effect on ALM polarity either by themselves or in a *cwn-1* background (data not shown). Therefore, it seems that MOP-25.2 functions with other RAM components, namely MIG-15 and SAX-2, in ALM polarity.

Previously we implicated the Rac CED-10 and an actin-binding protein of Ena/VASP family, UNC-34, in ALM polarity (Fleming et al., 2010). In guidance of the AVM axon, CED-10 and UNC-34 have been shown to act in parallel pathways downstream of UNC-6/Netrin and UNC-40/DCC receptor (Gitai et al., 2003). *mig-15* has also been implicated in the Rac pathway in axon guidance (Shakir et al., 2006). Our preliminary mutant analysis suggests *ced-10* and *sax-2* mediate the effects of *mig-15* in ALM polarity. However, the results are qualified because we did not use null alleles of *ced-10* and *mig-15*. By using a balancer for the null allele of *mig-15* to bypass its lethality, we should be able to further address the genetic interactions between *ced-10*, *unc-34*, *mig-15* and *sax-2* in ALM polarity.

**Future directions**

*mig-15* has been shown to act cell autonomously in axon pathfinding and cell migration (Chapman et al., 2008; Poinat et al., 2002). Previous attempts to address MIG-15 autonomy in ALM polarity have been unsuccessful. A *mig-15* cDNA expressed from the mechanosensory neuron-specific promoter *mec-7* did not rescue axon guidance defects in *mig-15* mutants (data not shown), possibly because the introns contain regulatory elements necessary for MIG-15 expression. However, full-length *mig-15* genomic DNA expressed from the neural-specific *unc-86* promoter also had no rescuing activity (data not shown), presumably due to low expression levels. Re-injection of this construct at a higher concentration should address this issue. Alternatively, *mig-15* may have non-autonomous functions. If this is the case, expression of *mig-15* from Wnt-secreting cells, hypodermis or muscle should rescue *mig-15* mutants.

The observation that egl-20/Wnt driven from a heat-shock promoter (*muIs53*) can induce ALM polarity defects in a wildtype background (Hilliard & Bargmann, 2006) raises the possibility that mutations in egl-20 effector genes might suppress this phenotype. If
MIG-15 mediates the effect of EGL-20 in ALM polarity, *muIs53; mig-15* should have a less severe polarity defect compared to *muIs53* alone. On the other hand, if *mig-15* acts in parallel to the *egl-20* pathway, this strain might display an enhanced polarity phenotype. This analysis should determine whether MIG-15 and orthologs of RAM pathway components mediate effects of Wnts in ALM polarity.
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Figure legends:

**Figure 1:** Mutations in *mig-15*, the *C. elegans* NIK ortholog, cause defects in ALM polarity. *cwn-1*, but not *egl-20*, significantly enhanced the ALM polarity defect in all three *mig-15* alleles. Numbers for each genotype are provided. N.S., not significant; *

*p* < 0.0001 (Fisher’s exact test).
Table 1: A list of yeast RAM pathway genes and *C. elegans* homologs

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<td>Cbk1p</td>
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<tr>
<td>Mob2p</td>
<td>Kinase coactivator</td>
<td>F38H4.10, F09A5.4, T12B3.4 &amp; C30A5.3</td>
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Figure 2: *C. elegans* orthologs of yeast RAM pathway genes, *sax-2* and *mop-25.2* are required for ALM polarity. (A) A cartoon diagram of the components of the RAM pathway in *S. cerevisiae*. (B) RNAi against *mop-25.2* and mutations in *sax-2*, but not *sax-1*, led to defects in ALM polarity in a *cwn-1* background. A *sax-2* cDNA expressed from the mechanosensory neuron-specific promoter *mec-4* (Gallegos and Bargmann, 2004) partially rescued the ALM polarity defect in *cwn-1; sax-2* animals. Combined reduction of NDR functions by RNAi against *wts-1* in a *cwn-1; sax-1* background generated a weak synthetic reversal phenotype. Numbers for each genotype are provided. *p < 0.0001* (Fisher’s exact test).
Figure 3: Genetic interactions between Rac GTPase ced-10, mig-15 and sax-2. ced-10 and sax-2 mutations interacted synergistically to produce a bipolar phenotype. A similar synthetic bipolar phenotype was observed in sax-2; mig-15(rh148) but not in ced-10; mig-15(rh148). In addition, cwn-1; mig-15(rh326) generated a polarity phenotype that is stronger than cwn-1; sax-2 but is indistinguishable from cwn-1; sax-2; ced-10. Numbers for each genotype are provided. N.S., not significant.
CHAPTER THREE APPENDIX

THE C. ELEGANS MIG-15 KINASE FUNCTIONS IN WNT-REGULATED NEURONAL MIGRATION

Contributions to this Appendix:
Gian Garriga performed scoring of cell positions by Nomarski microscopy.
INTRODUCTION

Wnts are a class of conserved secreted glycoproteins that regulate many aspects of neuronal development including neuronal migration, neuronal polarity, axon guidance, dendritic morphogenesis and synaptic development (Salinas and Zou, 2008). However, in most cases, the signaling pathways downstream of Wnts remain incompletely understood. A well-defined canonical Wnt signaling pathway has been implicated in Q neuroblast migration in *C. elegans*. Q neuroblasts are present at identical antero-posterior positions on either side of the body posterior to the presumptive gonad, and they produce the same set of daughter cells through a series of asymmetric cell divisions. However, while the Q neuroblast on the right side (QR) and its descendents migrate anteriorly, the left Q neuroblast (QL) and its descendents migrate posteriorly. The model that has emerged from work over the last 20 years suggests that the canonical, β-catenin-dependent Wnt signaling pathway activates a posterior migration program specifically on the left side by turning on a homeobox transcription factor gene called *mab-5*, which in turn regulates genes that guide the QL descendents posteriorly (Korswagen, 2002).

Specifically, the signaling from EGL-20/Wnt through the MIG-1 Frizzled receptor activates Dishevelled MIG-5. MIG-5 in turn inhibits the function of the destruction complex composed of PRY-1/Axin, SGG-1/GSK3β and APR-1/adenomatous polyposis coli (APC), sparing BAR-1/β-catenin from proteasomal degradation. This allows BAR-1 to translocate into the nucleus and to bind the TCF/LEF transcription factor POP-1. The interaction converts POP-1 from a repressor into an activator, leading to the activation of *mab-5*. Mutations in the genes involved in canonical Wnt signaling cause a reduction in *mab-5* expression in QL, and as a result, the cells migrate anteriorly instead of posteriorly (Korswagen, 2002).

EGL-20 also regulates the anterior migration of QR neuroblasts. However, unlike QL migration, *mab-5* activation is not required for QR migration. Here we show that mutations in *mig-15*, the ortholog of murine NIK (Nck-interacting kinase) and *Drosophila* Misshapen, lead to QR and QL migration defects reminiscent of the *egl-20* mutant. In addition, *mig-15* is required for the transcriptional activation of *mab-5*. Finally, *mig-15* acts genetically upstream of both *pry-1/Axin* and *mab-5/Hox* in the canonical pathway to regulate QL migration.

MATERIALS AND METHODS

Nematode Strains and Genetics

Strains were maintained at 20°C as described. The following alleles and transgenes were used:

**LGI:** *pry-1(mu38)* (Maloof et al., 1999), *zdIs5[Pmec-4::gfp, lin-15(+)]* (Clark and Chiu, 2003)

**LGII:** *mulIs32 [Pmec7::gfp, lin-15(+)]* (Ch'ng et al., 2003)

**LGIII:** *mab-5(e1751gf)* (Salser and Kenyon, 1992)

**LGV:** *egl-20(n585)* (Maloof et al., 1999), *mulIs3[Pmab-5::lacZ; pRF4[rol-6(su1006)]]* (Cowing and Kenyon, 1992)

**LGX:** *mig-15* alleles used include *rh148* (Poinat et al., 2002), *rh80* and *rh326* (Shakir et al., 2006)
Nomarski Microscopy for Q Neuroblast Migrations

The positions of Q descendents were scored in L1s 4-6 hours after hatching. Their positions were scored relative to the invariant positions of the non-migrating hypodermal nuclei. In wild-type animals, QL descendents migrate to a position posterior to the V4 hypodermal cell, and the QR descendents migrate to a position anterior to the V2 hypodermal cell. The QL descendents are positioned anterior to the V4 cell in animals with mutations that disrupt their migration.

β-Galactosidase Immunohistochemistry

Animals containing the integrated array \textit{mu}ls3[\textit{Pmab-5::lacZ}] were synchronized by hatching in M9, fed for 4 hours, and fixed in cold acetone. β-galactosidase activity was assayed according to an established protocol (Maloof et al., 1999). Animals were counterstained with DAPI for better cell recognition. Worms were examined using a Zeiss Axioskop2 microscope. Images were acquired using an ORCA-ER CCD camera (Hammamatsu) and Openlab imaging software (Improvision).

Fluorescence Microscopy

For fluorescence microscopy, L4 to young adult hermaphrodite animals were anesthetized with 1% sodium azide, mounted on agar pad, and observed with a Zeiss Axioskop2 microscope.

RESULTS

\textit{mig-15} functions upstream of \textit{pry-1} and \textit{mig-15} in Wnt-regulated QL migration

The \textit{C. elegans} Wnt homolog EGL-20 regulates the migration of several neurons including the Q neuroblasts. In the first larval stage, the QR neuroblast and its descendents migrate anteriorly, while the QL neuroblast and its descendents migrate posteriorly. We found that the QR descendents stopped anterior migration prematurely (Fig. 1A), while QL descendents reversed their migratory directions and migrated anteriorly in both \textit{egl-20} and \textit{mig-15} mutants (Fig. 1B). The severity of Q migration defects was well correlated with the strength of the alleles: \textit{rh}326 > \textit{rh}80 > \textit{rh}148, as reported for other cell migration and axon guidance phenotypes (Chapman et al., 2008; Shakir et al., 2006). The similar migration patterns between \textit{mig-15} and \textit{egl-20} mutants suggested that \textit{mig-15} might function in Wnt signaling to control Q cell migration. To test this hypothesis, we asked whether expression of the homeobox transcription factor gene \textit{mab-5} in QL was affected by \textit{mig-15} mutations. Using a \textit{Pmab-5::lacZ} reporter (Maloof et al., 1999), we found that \textit{mab-5} expression was greatly attenuated in both \textit{rh}148 and \textit{rh}80 mutants, with up to 75% of worms showing weak or undetectable β-galactosidase activity (Fig. 2). This observation is consistent with MIG-15 playing an important role in Wnt signaling. We also found that a gain-of-function \textit{mab-5} mutation, \textit{e1751}, completely suppressed the QL migration defect of \textit{mig-15} mutants (Fig. 3), providing genetic evidence that \textit{mig-15} acts upstream of \textit{mab-5} in the Wnt signaling pathway.

To further dissect how \textit{mig-15} genetically interacts with the canonical pathway genes, we asked whether \textit{pry-1} can suppress the QL migration defect of \textit{mig-15}. Previously Maloof et al. showed that \textit{pry-1} suppressed the QL migration phenotype of \textit{egl-20/Wnt} but not
bar-1/β-catenin, placing pry-1 downstream of egl-20 and upstream of bar-1 genetically (Maloof et al., 1999). We found that pry-1 completely suppressed QL migration defect of mig-15, suggesting that mig-15 acts upstream of, or in parallel to, pry-1.

DISCUSSION
MIG-15 in Wnt-regulated neuronal migration
MIG-15, along with its murine ortholog NIK and Drosophila ortholog Misshapen, share a common function in regulating cell migration (Chapman et al., 2008; Cobreros-Reguera et al., 2010; Shakir et al., 2006; Xue et al., 2001). Here we report that MIG-15 functions in Wnt-regulated migration of QR and QL neuroblasts. In mig-15 mutants, QR descendents stopped anterior migration prematurely (Fig. 1a), while QL descendents reversed their migratory directions and migrated anteriorly (Fig. 1b), a phenotype reminiscent of egl-20 mutants. The activation of the homeobox transcription factor mab-5 has previously been shown to be both necessary and sufficient for the posterior migration of Q descendents (Korswagen, 2002). We found that mab-5 expression was often greatly attenuated in the QL lineage of mig-15 mutants, a finding consistent with previous reports (Chapman et al., 2008). Shakir et al. showed that mig-15 genetically acts upstream of mab-5 by using a gain-of-function allele, e1751 (Shakir et al., 2006). We confirmed this observation and also showed that mig-15 acts upstream of, or in parallel to, pry-1.

The migration pattern of Q neuroblasts is complex and can be divided into two phases: the Wnt-independent initial polarization and migration phase followed by the Wnt-dependent migration phase. The Q neuroblasts are born along equivalent positions between the seam cells V4 and V5. Shortly after hatching, the Q neuroblast on the right side (QR) and the one on the left side (QL) extend their lamellipodia anteriorly and posteriorly, respectively. Following the initial polarization is a short migration after which QR sits on top of V4R and QL sits on top of V5L. Approximately four hours after hatching, QR divides over V4R to produce QR.a and QR.p while QL divides over V5L to produce QL.a and QL.p. Mutation in components of the canonical signaling pathway such as bar-1/β-catenin has no effect on the initial polarization and migration of Q neuroblasts (Chapman et al., 2008). On the other hand, loss of the Netrin receptor UNC-40/DCC or a novel transmembrane protein DPY-19 causes Q cells to randomize their initial polarization (Honigberg and Kenyon, 2000). Interestingly, UNC-6/Netrin is not involved in the Q polarization (Honigberg and Kenyon, 2000). Chapman et al. observed that in mig-15(rh80) mutants, the Q neuroblasts initially did not extend robust lamellipodia and later failed to restrict the protrusive activity of lamellipodia to the appropriate end of the cell (i.e. QL polarizing anteriorly or in both directions instead of posteriorly) (Chapman et al., 2008). Coupled with a defect in the initial migration toward the V cells, approximately 40–45% of Q neuroblasts divided between V4 and V5 in rh80 mutants.

The ability of the QR and QL neuroblasts to polarize and migrate in opposite directions is important for the subsequent migration of their descendents. Mutations that disrupt Q polarizations, such as dpy-19 and mig-15 showed occasional aberrant activation of mab-5 in QR descendents, leading to their posterior migration (Chapman et al., 2008; Honigberg
and Kenyon, 2000). In line with this, loss of mab-5 significantly suppresses the QR under-migration defects of dpy-19 and mig-15 mutants (Chapman et al., 2008; Honigberg and Kenyon, 2000). The ability of mab-5 loss-of-function mutation to partially suppress mig-15 led Chapman et al. to conclude that mig-15 is unlikely to directly activate mab-5 in the canonical Wnt pathway (Chapman et al., 2008). However, given that the penetrance of the migration defect of Q descendents is higher (60–70%) compared to that of the polarization defect (40–45%), it seems possible that mig-15 also plays a role in Wnt-dependent Q migration.

**Hypothesis on how MIG-15 may act in the canonical pathway to control QL migration**

Using a proteomics approach, TNIK, a closely related NIK homolog, was recently identified as an interacting partner of the TCF/LEF transcription factor Tcf4 in the crypt of the mouse small intestine (Mahmoudi et al., 2009). TNIK has been shown to be recruited to promoters of Wnt target genes in a β-catenin-dependent manner and acts as an essential transcriptional activator of these genes. Nevertheless, our genetic analysis places mig-15 upstream of, or in parallel to, pry-1, suggesting MIG-15 does not act in the same way as TNIK.

Several homologs of MIG-15 have been implicated in membrane trafficking. HGK, the human ortholog of MIG-15, has been shown in vitro to physically interact with components of the exocyst complex, which regulate vesicular trafficking to the plasma membrane (Balakireva et al., 2006). Furthermore, inactivation of XMINK, a MIG-15 homolog in Xenopus, blocks the translocation of Dishevelled from cytoplasm to the plasma membrane in animal cap cells (Lee et al., 2007). Taken together, it is tempting to speculate that MIG-15 is involved in the membrane targeting of Wnt pathway components such as MIG-1/Frizzled or MIG-5/Dishevelled to control migration of QL descendents.
REFERENCES:


Figure legends:

Figure 1A: QR migration in mig-15 mutants.
The positions of QR descendants were scored as described in the Methods. Numbers in boxes indicate the percentages of QR descendants in defined positions, with gray scales corresponding to the percentages. N indicates the number of QR descendants scored for each genotype. In wild-type animals, QR descendants migrate to a position anterior to the V2 hypodermal cell, the position of which is marked by a vertical line.
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Figure 1B: QL migration in *mig-15* mutants.
The positions of QL descendents were scored as described in the Methods. Numbers in boxes indicate the percentages of QL descendents in defined positions, with gray scales corresponding to the percentages. N indicates the number of QL descendents scored for each genotype. In wild-type animals, QL descendents migrate to a position posterior to the V4 hypodermal cell, the position of which is marked by a vertical line.
Figure 2: *mig-15* activates a transcriptional reporter of *mab-5*.

(A-D) β-galactosidase activity in animals with the integrated array *muIs3[Pmab-5::lacZ]*. Dotted circles indicate the positions of the QL neuroblast daughters; arrowheads indicate the positions of motor neurons that express β-galactosidase. Wild-type animals show moderate (C) to intense (D) staining in the QL neuroblast daughters. However in *mig-15* mutants, the staining is mostly undetectable (A) or weak (B) in the QL lineage. (E) Graph shows quantification on *mab-5* transcriptional activities with *muIs3[Pmab-5::lacZ]*. Numbers of animals scored for each genotype are provided.
**Figure 3: mig-15 acts upstream of pry-1 and mab-5 in QL migration.**
Graph shows the percentage of QL descendents that migrate in the wrong direction toward the head of the animal. *mab-5(e1751gf)* and *pry-1* completely suppressed the migration defects of QL descendents in *mig-15* mutants. Numbers of animals scored for each genotype are provided.
CHAPTER FOUR

*C. ELEGANS* ORTHOLOGS OF LKB1, STRAD AND MO25 REGULATE ASYMMETRIC CELL DIVISION OF THE Q NEUROBLAST LINEAGE
SUMMARY
The heterotrimeric complex composed of LKB1 kinase, STRAD pseudokinase and MO25 adaptor is a conserved polarity regulator in epithelial cells and neurons. Many C. elegans neuroblasts, including the Q.p neuroblasts, divide to produce a larger neuronal precursor and a smaller cell that dies, but how this fate and size asymmetry is generated in these divisions is not understood. In this study, we present evidence that C. elegans orthologs of LKB1 (PAR-4), STRAD (STRD-1) and MO25 (MOP-25.2) regulate the asymmetric cell division (ACD) of the Q.p neuroblast. pig-1 was previously identified as a crucial regulator of multiple ACDs in C. elegans (Cordes et al., 2006). The gene encodes a protein orthologous to vertebrate MELK and belongs to a family of kinases (AMPK-related kinase family) that can be phosphorylated and activated by LKB1 on a conserved threonine residue in their activation loops (Lizcano et al., 2004). We showed that PAR-4 and STRD-1 function in the same pathway as PIG-1 in the Q.p lineage. We also demonstrated that the conserved threonine residue in the activation loop is essential for PIG-1 activity.

INTRODUCTION
Loss of the tumor suppressor LKB1 causes the Peutz-Jeghers syndrome (PJS) in humans, which is characterized by polyp formation in the gastrointestinal tract and predisposition for certain types of cancer (Giardiello et al., 2000; Hemminki et al., 1998; Jeghers et al., 1949; Jenne et al., 1998; Peutz, 1921). How loss of LKB1 leads to PJS is unclear. LKB1 encodes a highly conserved serine/threonine kinase that activates several downstream kinases by phosphorylating a conserved threonine residue in their activation loops (Lizcano et al., 2004). One key substrate of LKB1 is adenosine monophosphate–activated protein kinase (AMPK), a master regulator of metabolism. When cellular energy level is low, AMPK is activated to inhibit ATP-consuming pathways and activate ATP-generating pathways, thereby restoring energy homeostasis in the cell (Steinberg and Kemp, 2009). However, recent reports (Gan et al., 2010; Gurumurthy et al., 2010; Nakada et al., 2010) have shown that LKB1 regulates energy homeostasis in hematopoietic stem cells largely independent of AMPK. Interestingly, loss of AMPK leads to defects in epithelial polarity in Drosophila under energy starvation conditions, and this novel function of AMPK requires LKB1 (Lee et al., 2007; Mirouse et al., 2007). LKB1 and its orthologs (called PAR-4 in C. elegans) can also regulate polarity through other AMPK-related kinases such as SAD and MARK kinases. SAD-A and SAD-B mediate the effects of LKB1 in axon specification of cultured rat hippocampal neurons (Barnes et al., 2007). In early divisions of the C. elegans embryo, PAR-4 dependent phosphorylation of PAR-1 (a MARK ortholog) leads to asymmetric segregation of cell fate determinants (Narbonne et al., 2010; Watts et al., 2000). By contrast, PAR-1 acts upstream of LKB1 in Drosophila oocyte polarity (Martin and St Johnston, 2003). In vitro, LKB1 is found in a complex with pseudokinase STRAD and adaptor MO25. The association of these two co-factors to LKB1 has been shown to promote its kinase activity, stability and nuclear-to-cytoplasmic translocation (Baas et al., 2003; Boudeau et al., 2003; Dorfman and Macara, 2008). Indeed, the recently solved crystal structure of the heterotrimeric complex suggests the binding of MO25 and STRAD locks LKB1 in the active conformation (Zeqiraj et al., 2009). Co-overexpression of LKB1 and STRAD leads to cell-autonomous polarization of single isolated epithelial cell (Baas et al., 2004).
and axon specification in developing neurons (Shelly et al., 2007). However, LKB1 has also been shown to have STRAD-independent functions in C. elegans (Kim et al., 2010; Narbonne et al., 2010).

An in vitro study found that although most AMPK family kinases tested can be phosphorylated and activated by LKB1, one notable exception is Maternal Embryonic Leucine zipper Kinase or MELK (Lizcano et al., 2004). MELK exhibits a high basal activity and the addition of LKB1 does not enhance its kinase activity (Lizcano et al., 2004). Nevertheless, the conserved threonine residue in the activation loop is essential as mutating this residue to alanine abolishes kinase activity of MELK (Beullens et al., 2005; Lizcano et al., 2004). These data suggest MELK is activated through autophosphorylation of its activation loop residue in vitro. MELK has been implicated in many developmental processes including stem cell renewal, cell cycle progression and spliceosome assembly (Davezac et al., 2002; Nakano et al., 2005; Vulsteke et al., 2004).

Many C. elegans neuroblasts, including the Q.p neuroblasts, divide to produce a neuronal precursor and a cell that dies, but how this fate asymmetry is generated in these divisions is not well understood. The sole C. elegans MELK ortholog PIG-1 (encoded by par-1-like gene) has recently been shown to regulate asymmetric cell division by controlling spindle positioning and actomyosin contractility (Cordes et al., 2006; Ou et al., 2010). Here we provide evidence that C. elegans orthologs of LKB1, STRAD and MO25 are involved in the asymmetric cell division of the Q.p neuroblast lineage and are potential regulators of PIG-1 in this process.

MATERIALS AND METHODS
Nematode Strains and Genetics
Nematodes were cultured as previously described (Brenner, 1974). N2 Bristol was the wild-type strain used in this study, and strains were maintained at 20° C except for strains containing par-4 or par-1, which were maintained at 15° C. The following alleles and transgenes are used in this study:

LGI: zdIs5[Pmec-4::gfp, lin-15(+)] (Clark and Chiu, 2003)
LGII: mop-25.2(ok2073) (Caenorhabditis Genetics Center), mop-25.2(tm3694) (National Bioresource Project of Japan), rrf-3(pk1426) (Simmer et al., 2002)
LGIII: strd-1(ok2283), strd-1(rr91) (Narbonne and Roy, 2009)
LGIV: pig-1(gm280), pig-1(gm301) (Cordes et al., 2006) ced-3(n717), ced-3(n2436) (Shaham et al., 1999)
LGV: par-4(it57ts) (Watts et al., 2000), par-1(zu310ts) (Kemphues et al., 1988)
LGX: sad-1(ky289) (Crump et al., 2001), aak-2(gt33) (Lee et al., 2008)

For phenotypic analyses, L4-stage hermaphrodites from it57ts and zu310ts, and control strains were cultured at 15° C and transferred to 25° C 24 hours later when they carried embryos. Their progenies were examined at L3~L4 stage.

RNA interference
RNAi was performed using the bacterial feeding method as (Kamath et al., 2001; Timmons and Fire, 1998). In all experiments, worms were grown on plates supplemented
with 25 mM Carbenicillin and 1 mM IPTG at 20°C. The RNAi cultures were prepared by inoculating bacterial strains in LB with 25 mM Carbenicillin for 15 hours at 37°C, followed by addition of 6 mM IPTG and incubation for another hour at 37°C. Bacterial strains used to inactivate genes by feeding were obtained from the library designed by the Ahringer lab (Fraser et al., 2000).

Molecular biology and germline transformation

The Pmab-5::pig-1(T169A)::gfp and Pmab-5::pig-1(T169D)::gfp constructs were generated by modifying Pmab-5::pig-1::gfp (Cordes et al., 2006) with PCR-based mutagenesis (Invitrogen). The following primers are used: for T169A, GATAAGCACAATTTGGATGCGTGTTGTGGATCTCCG and its reverse complement; for T169D, GTATTGATAAGCACAATTTGGATGACTGTTGTGGATCTCCG and its reverse complement. gmEx610, gmEx611 and gmEx612 were generated by injecting Pmab-5::pig-1(T169A)::gfp into N2 hermaphrodites at 10 ng/µl with 3ng/µl Pmyo-2::mcherry (pCFJ90). gmEx613, gmEx614 and gmEx615 were generated by injecting Pmab-5::pig-1(T169D)::gfp into N2 hermaphrodites at 10 ng/µl with 3ng/µl Pmyo-2::mcherry (pCFJ90). Germline transformation was performed by direct injection of various plasmid DNAs into the gonads of adult wild-type animals as described (Mello et al., 1991).

Analysis of neuroblast daughter size

The daughters of the Q.p neuroblast were identified using ayIs9 (Pegl-17::gfp) (Branda and Stern, 2000). We measured cell area in a single plane of focus. These cells are extremely flat, and thus measurements of cell area are good estimates of cell size. We calculated cell area by circumscribing the cell and measuring its interior area with Openlab software (Improvision). We averaged two measurements per cell.

Fluorescence Microscopy

For fluorescence microscopy, L4 to young adult hermaphrodite animals were anesthetized with 1% sodium azide, mounted on agar pad, and observed with a Zeiss Axioskop2 microscope.

RESULTS

C. elegans orthologs of LKB1, STRAD and MO25 regulate asymmetric cell division of the Q.p neuroblast lineage

All 302 neurons in C. elegans arise from neuroblast lineages that divide asymmetrically. Given that LKB1 kinase, along with its binding partners STRAD and MO25, have been shown to be master regulators of polarity in different contexts (Jansen et al., 2009), we wondered whether C. elegans orthologs of these genes are involved in regulating asymmetric cell division (ACD) of the Q.p neuroblast lineage. The Q.p neuroblasts divide to produce a neuronal precursor and a cell that is fated to die (Fig. 1A). The neuronal precursor can then divide to generate the mechanosensory neuron A/PVM and the interneuron SDQR/L (Fig. 1A & 1B). Mutations in genes that regulate these divisions
transform the fate of the apoptotic cell to that of its sister, resulting in the production of extra neurons.

There is one C. elegans ortholog of LKB1 called par-4, one STRAD ortholog called strd-1 and three MO25 homologs called mop-25.1, mop-25.2 and mop-25.3. Based on sequence homology, MOP-25.1 is paralogous to MOP-25.2 (69% identity and 84% similarity) and is more distantly related to MOP-25.3 (20% identity and 45% similarity) (Wormbase Blast Search). Using an integrated Pmec-4::gfp to visualize mechanosensory neurons, we observed a small but significant number of extra A/PVMs in a strong temperature-sensitive mutant of par-4, it57ts, and two putative null strd-1 mutants, ok2283 and rr91 (Fig. 1C). We reasoned that the cell fate mutants may display a weak phenotype because the underlying cell fate transformation is masked by cell death (Cordes et al., 2006), so we removed ced-3, which encodes a caspase in the canonical programmed cell death pathway (Yuan et al., 1993), from the par-4 or strd-1 mutant background. Consistent with this idea, ced-3 interacted synergistically with either par-4 or strd-1 (Fig. 1). Two existing alleles of mop-25.2 (ok2073 & tm3694) did not produce a significant extra neuron phenotype either by themselves or in a ced-3 sensitized background (data not shown). However, given that both alleles of mop-25.2 are maternal effect lethal and we analyzed mutants from heterozygous mothers, we wondered whether a maternal contribution of mop-25.2 may mask a role of this gene in ACD. Indeed, RNAi against mop-25.2 in an RNAi-sensitive rrf-3 background (Simmer et al., 2002) produced a mild extra neuron phenotype and this phenotype was further enhanced in a weak ced-3 (n2436); rrf-3 background (Fig. 1C). We used a weaker ced-3 allele as opposed to the putative null ced-3(n717) allele in the double mutant analysis because n2436 produced no extra cells by itself (data not shown), thereby providing a clean background for screening candidate genes. The rrf-3 mutation sensitizes the background to the effects of RNAi (Simmer et al., 2002). In contrast to mop-25.2, RNAi against either mop-25.1 or mop-25.3 in a ced-3; rrf-3 background did not generate a phenotype (data not shown), suggesting mop-25.2 is the principal MO25 homolog that functions in ACD of the Q.p lineage.

PAR-4 and STRD-1 regulate daughter cell size asymmetry in the Q.p division
Asymmetric cell division generates daughter cells with different fates and often, different sizes. In the Q.p division, the mitotic precursor is four times larger than its apoptotic sister due to asymmetric spindle positioning (Cordes et al., 2006; Ou et al., 2010). Previous studies have shown that mutations in cell fate regulators such as pig-1, which encodes a MELK kinase, and cnt-2, which encodes a GTPase-activating protein (GAP) of Arf GTPases, result in daughter cells that are more equivalent in size (Cordes et al., 2006; Singhvi et al., in press). We also observed defects in cell size asymmetry in par-4 and strd-1 mutant (with a ratio of 2.7 to 1 and 1.7 to 1, respectively) (Fig. 2).

PAR-4 and STRD-1 function in the same pathway as pig-1 in the Q.p lineage
The observation that the strd-1(rr91); par-4(it57) double mutant died during embryogenesis even at the permissive temperature (15°C) for par-4(it57) (Kim et al., 2010; Narbonne et al., 2010) precluded us from addressing whether these two genes act genetically in the same pathway. pig-1 was previously identified as a crucial regulator of
multiple ACDs in *C. elegans* (Cordes et al., 2006). The gene encodes a protein orthologous to vertebrate MELK and belongs to a family of kinases (AMPK-related kinase family) that can be phosphorylated and activated by LKB1 (Lizcano et al., 2004). We tested whether *par-4* and *strd-1* function in the same pathway as *pig-1*. A *par-4* mutation enhanced the extra neuron phenotype of a *pig-1* hypomorph (gm280) but not a *pig-1* null (gm301) (Fig. 3), consistent with *par-4* and *pig-1* acting in the same pathway. Similarly, a *strd-1* mutation did not enhance *pig-1*(gm301) (Fig. 3).

LKB1 and its orthologs regulate polarity by activating both AMPK and AMPK-related kinases (Jansen et al., 2009). In particular, SAD and MARK kinases have both been implicated in neuronal polarization of cultured hippocampal neurons (Chen et al., 2006; Kishi et al., 2005). We wondered whether *C. elegans* orthologs of these AMPK family kinases work in concert with PIG-1 to regulate the ACD of Q.p. *C. elegans* has two orthologs of AMPK catalytic α subunits called *aak-1* and *aak-2*, one ortholog of SAD called *sad-1* and one ortholog of MARK called *par-1*. RNAi against *aak-1* or mutation in *aak-2*, *sad-1*, or *par-1* failed to generate a significant extra neuron phenotype in a *ced-3* sensitized background (data not shown). Furthermore, *sad-1* and *par-1* did not enhance the *pig-1* null mutant gm301 (Fig. 3), suggesting SAD and MARK kinases are not involved in the ACD of Q.p.

The conserved threonine residue in the activation loop is essential for PIG-1 activity

LKB1 encodes a highly conserved serine/threonine kinase that activates AMPK family kinases by phosphorylating a conserved threonine residue in their activation loops (Lizcano et al., 2004). However, MELK is autophosphorylated at that residue (Beullens et al., 2005), and mutating this residue to alanine abolishes kinase activity (Beullens et al., 2005; Lizcano et al., 2004). To test whether the threonine residue in the activation loop (T169) of PIG-1 is equally essential for its activity, we generated two variants of PIG-1 by introducing point mutations into a transgene containing a *pig-1* cDNA: a non-phosphorylatable form, *pig-1*(T169A), and a phosphomimetic form, *pig-1* (T169D). These transgenes were expressed from the *mab-5* promoter, which is expressed in the left Q.p lineage (PVM) but not in the right Q.p lineage (AVM) (Salser and Kenyon, 1992). Using *Pmab-5::pig-1::gfp* to address cell autonomy, Cordes et al. observed no rescue of the extra AVM, but an almost complete rescue of the extra PVM phenotype of a *pig-1* mutant, indicating that *pig-1* acts in the Q lineage (Cordes et al., 2006). As expected, transgenes expressing either *PIG-1*(T169A) or *PIG-1*(T169D) failed to rescue the extra AVM phenotypes of *pig-1* (data not shown). *Pmab-5::pig-1(T169A):::gfp* also failed to rescue the PVM phenotype of a *pig-1* mutant (Fig. 4A), indicating that the threonine residue is important for PIG-1 activity. We observed a partial but significant rescue of the extra PVM phenotype from transgenes expressing *PIG-1*(T169D) (Fig. 4B), which could be due to up-regulated or un-regulated PIG-1 activity. Consistent with this idea, *PIG-1*(T169D) induced extra PVMs in the wildtype background and this phenotype was further enhanced in a *ced-3* sensitized background (Fig. 4C). To our surprise, *PIG-1*(T169A) also produced an extra PVM phenotype in the wildtype background even when it showed no activity in our rescue assay. Given that over-expression of the full length *PIG-1* did not generate an extra neuron phenotype by itself (Cordes and Garriga,
unpublished observation), this finding suggests that the transgene expressing PIG-1(T169A) acts as a dominant negative.

**DISCUSSION**

*C. elegans* orthologs of LKB1, STRAD and MO25 regulate asymmetric cell division of the Q.p neuroblast lineage

The heterotrimeric complex composed of LKB1 kinase, STRAD pseudokinase and MO25 adaptor is a conserved polarity regulator in epithelial cells and neurons (Jansen et al., 2009). In this study, we present evidence that *C. elegans* orthologs of LKB1 (PAR-4), STRAD (STRD-1) and MO25 (MOP-25.2) regulate the ACD of the Q.p neuroblast.

Many *C. elegans* neuroblasts, including the Q.p neuroblasts, divide to produce a larger neuronal precursor and a smaller cell that dies, but how this fate and size asymmetry is generated in these divisions is not understood. In the Q.p division, previous studies have shown that mutations in *pig-1*, which encodes a MELK kinase, and *cnt-2*, which encodes a GTPase-activating protein (GAP) of Arf GTPases, result in daughter cells that are more equivalent in size and transform the fate of the apoptotic cell to that of its sister, resulting in the production of extra neurons (Cordes et al., 2006; Singhvi et al., in press). We found that the daughter cells of the Q.p neuroblast are more symmetric in size in *par-4* and *strd-1* mutants (Fig. 2). Furthermore, mutations in *par-4*, *strd-1* and RNAi against *mop-25.2* generated a significant extra neuron phenotype in a *ced-3* sensitized background (Fig. 1). RNAi against *mop-25.2* did not have as strong of a phenotype as mutations in *par-4* or *strd-1* in a *ced-3* background, possibly due to incomplete knockdown of *mop-25.2*. Alternatively, the other two *C. elegans* MO25 homologs *mop-25.1* and *mop-25.3* may provide overlapping functions with *mop-25.2*. Taken together, our observations indicate that PAR-4, STRD-1 and MOP-25.2 are new regulators of ACD of the Q.p lineage.

**PAR-4 and STRD-1 function in the same pathway as PIG-1 in the Q.p lineage**

Cell fate regulators PIG-1 and CNT-2 have previously been shown to act in the same pathway in the Q.p lineage (Singhvi et al., in press). Given that MELK, the vertebrate ortholog of PIG-1, belongs to the family of kinases (AMPK-related kinase family) that can be phosphorylated and activated by LKB1 (Lizcano et al., 2004), we asked whether PAR-4 and STRD-1 function in the same pathway as PIG-1. Consistent with this hypothesis, *par-4* and *strd-1* mutations enhanced the extra-neuron phenotype of a weak but not a null *pig-1* mutant (Fig. 3).

*par-4* was one of the six *par* (*partitioning defective*) genes essential for the ACD of the one-cell *C. elegans* embryo (Kemphues et al., 1988; Watts et al., 2000). PAR-4 was thought to be at the top of the PAR-signaling cascade because its localization is not affected by the loss of other PAR proteins (Watts et al., 2000). More recently, it was shown that PAR-4 promotes phosphorylation of another PAR protein, PAR-1, which is orthologous to the vertebrate MARK and a member of the AMPK family kinase. PAR-4 and PAR-1 are required for the phosphorylation of the cell fate determinant MEX-5 in the early embryo (Narbonne et al., 2010; Tenlen et al., 2008). Interestingly, STRD-1 is dispensable in this process (Narbonne et al., 2010). SAD-1 is another AMPK-related kinase that regulates polarity (Crump et al., 2001; Hung et al., 2007). Mutations of *sad-1*, *par-4* and *strd-1* lead to mis-localization of pre-synaptic markers onto the dendrites of
DD neurons, indicating that the three genes are important for axonal-dendritic polarity (Kim et al., 2010). Intriguingly, STRD-1 is found in a complex with SAD-1 and acts genetically in parallel to PAR-4 (Kim et al., 2010). Considering PAR-1 and SAD-1 are both polarity-regulating AMPK family kinases, we wondered whether they function in concert with PIG-1 (which is also an AMPK-related kinase) in the ACD of Q.p. Mutation of par-1 or sad-1 did not generate a significant extra neuron phenotype in a ced-3 background (data not shown) and failed to further enhance the phenotype of a pig-1 null mutant (Fig. 3), suggesting PAR-1 and SAD-1 are not involved in the ACD of Q.p.

LKB1 has traditionally been linked to the regulation of energy metabolism through activation of AMPK. In response to low cellular ATP levels, LKB1 and AMPK inhibit anabolic processes and stimulate catabolic processes, thereby restoring energy homeostasis within a cell (Steinberg and Kemp, 2009). Nevertheless, it has recently been shown that LKB1 activates AMPK under energy starvation conditions to polarize single epithelial cells in culture (Lee et al., 2007). In addition, Drosophila AMPK null mutants display defects in epithelial polarity (Lee et al., 2007; Mirouse et al., 2007). These data suggest that the regulation of LKB1 of metabolism and polarity are intricately linked. C. elegans have two orthologs of AMPK catalytic α subunits called aak-1 and aak-2. RNAi against aak-1 or mutation in aak-2 did not have an effect on the extra neuron phenotype in a ced-3 background (data not shown). It remains to be tested whether aak-1 and aak-2 act redundantly to regulate the ACD of Q.p.

The conserved threonine residue in the activation loop is essential for PIG-1 activity
LKB1 activates AMPK family kinases by phosphorylating a conserved threonine residue in their activation loops (Lizcano et al., 2004). By contrast, MELK is autophosphorylated at this residue (Beullens et al., 2005), and mutating this residue to alanine abolishes kinase activity (Beullens et al., 2005; Lizcano et al., 2004). To test whether the threonine residue in the activation loop (T169) of PIG-1 is equally essential for its activity, we generated a non-phosphorylatable form, PIG-1(T169A), and a phosphomimetic form, PIG-1 (T169D). Transgenes expressing PIG-1(T169A) failed to rescue the extra-neuron phenotype of a pig-1 mutant, indicating that the threonine residue is important for PIG-1 activity. We observed a partial rescue of the extra-neuron phenotype from transgenes expressing PIG-1(T169D). PIG-1(T169D) also induced extra neurons in the wildtype background, suggesting that the phosphomimetic form possessed deregulated PIG-1 activity. To our surprise, PIG-1(T169A) also produced an extra PVM phenotype in the wildtype background even when it showed no rescue activity, suggesting this transgene acts as a dominant negative. T169A may act as a dominant negative by sequestering a limiting co-factor needed for PIG-1 activation. The observation that PIG-1(T169D) may have deregulated activity should allow us to test whether this transgene can suppress the loss of potential upstream regulators, namely par-4 and strd-1.

Over-expression of the full length PIG-1 did not generate an extra neuron phenotype either by itself or in a ced-3 background (Cordes and Garriga, unpublished observations), suggesting that PIG-1’s activity is normally regulated. Indeed, MELK is intricately regulated: the C-terminal kinase-associated domain can bind to its N-terminal kinase domain, and this interaction may affect both the kinase activity as well as the localization.
of the protein (Beullens et al., 2005; Chartrain et al., 2006). Truncation of the C-terminus enhances the kinase activity of MELK in vitro (Beullens et al., 2005). A similar auto-inhibitory mechanism was observed in yeast PAR-1 homologs, Kin1p and Kin2p (Elbert et al., 2005). It has also recently been reported that the conserved basic residues in the kinase-associated domain of AMPK family kinases, including MELK, can bind to anionic phospholipids in yeast cells (Moravecic et al., 2010), but the physiological relevance of this interaction remains to be elucidated. Interestingly, Xenopus MELK (called pEg3) is normally broadly distributed in the cytoplasm during interphase, but a portion of the protein becomes enriched near the cortex during anaphase and telophase of mitosis (Chartrain et al., 2006). The deletion of N-terminus of pEg3 causes the protein to localize to the cell periphery independent of cell cycle progression, indicating that the N-terminus plays an inhibitory role in pEg3 cortical localization (Chartrain et al., 2006). An emerging model from these observations suggests that the mutual inhibition between the N-terminal kinase domain and the C-terminal kinase-associated domain keeps MELK in an inactive state until some yet unidentified upstream signals relieve its auto-inhibition. Consistent with this, a PIG-1 transgene lacking the kinase-associated domain induced extra neurons in the wildtype background (Brinkmann and Garriga, unpublished observations). Currently, we are unable to observe PIG-1 localization at the cortex since existing GFP-tagged PIG-1 expressed from multi-copy extra-chromosomal arrays all show a diffuse cytoplasmic localization (Cordes et al., 2006; data not shown). One way to further address this issue would be to localize PIG-1 at different stages in the cell cycle using time-lapse confocal microscopy. Another possibility is to integrate a single-copy of the PIG-1 transgene to observe its expression at a more physiological level.

**Future directions**

Previous attempts to address PAR-4 and STRD-1 autonomy in ACD of the Q.p lineage have been unsuccessful. A par-4 or strd-1 cDNA expressed from either an egl-17 or mab-5 promoter that is expressed in many cells including the Q.p neuroblast did not rescue the extra neuron phenotype in ced-3; par-4 or ced-3; strd-1, respectively (data not shown). This could be due to low expression levels because little or no fluorescence is observed in these GFP or mcherry-tagged transgenes. Re-injection of these constructs at a higher concentration might address this issue.

PIG-1 has recently been shown to regulate the ACD of the Q lineage via two distinct mechanisms (Ou et al., 2010). In the Q.p division, PIG-1 controls the posterior displacement of the spindle to generate a larger anterior daughter and a smaller posterior daughter. However, the target of PIG-1 in the Q.p division is unknown. On the other hand, PIG-1 regulates the asymmetric distribution of the non-muscle myosin NMY-2 in the Q.a division. NMY-2 is found to concentrate in the anterior cortex of Q.a neuroblast and the resulting actomyosin contractility forces the cytoplasm to flow into the posterior half of the cell, leading to the production of a smaller anterior daughter and a larger posterior daughter. This novel myosin-dependent, spindle-independent mode of ACD has also been recently reported for neuroblast divisions in Drosophila (Cabernard et al., 2010). It will be interesting to see whether par-4 and strd-1 are involved in regulating ACD of the Q.a lineage. It is tempting to speculate that given PAR-4 and STRD-1 localization to the
cortex in early embryos (Narbonne et al., 2010), these proteins may also localize to the
cortex of the Q neuroblasts to regulate PIG-1 activity in these lineages.
REFERENCES:


Figure legends:

**Figure 1: C. elegans orthologs of LKB1, STRAD and MO25 regulate asymmetric cell division of the Q.p neuroblast lineage.** (A) A schematic diagram of the Q.p lineage. In wildtype, the Q.p neuroblast divides asymmetrically to produce Q.pa, a neuronal precursor, and Q.pp, which is destined for apoptosis. (B) A schematic diagram of a hermaphrodite: anterior is to the left, dorsal is up. Colored circles indicate the neurons produced from the Q.p lineage. (C) Mutations in *C. elegans* orthologs of LKB1 (*par-4*), STRAD (*strd-1*) and MO25 (*mop-25.2*) interacted synergistically with *ced-3* to produce extra A/PVMs, which are visualized with an integrated transgene *zds5 (Pmec-4::gfp)*. Numbers for each genotype are provided.
Figure 2: PAR-4 and STRD-1 regulate daughter cell size asymmetry in the Q.p division. In the Q.p division, the mitotic precursor Q.pa is four times larger than its apoptotic sister Q.pp (Cordes et al., 2006; Singhvi et al., in press). The transcriptional reporter ayIs9 (Pegl-17::gfp) was used to identify and measure the size of the daughters of the Q.p neuroblast. A mutation in par-4 and strd-1 disrupted the cell size asymmetry of daughter cells of Q.p neuroblast. *p < 0.001 (Student’s t-test).
Figure 3: PAR-4 and STRD-1 function in the same pathway as PIG-1 in Q,p lineage. Mutation of *par-4* and *strd-1* enhanced the extra neuron phenotype of a *pig-1* hypomorph, *gm280*, but not of a *pig-1* null, *gm301*. Mutation of *sad-1* and *par-1* failed to enhance *pig-1(gm301)*. Numbers for each genotype are provided. N.S., not significant; *p* < 0.005 (Fisher’s exact test).
Figure 4: The conserved threonine residue in the activation loop of PIG-1 is essential for its activity. (A) Transgenes expressing PIG-1(T169A) failed to rescue the extra neuron phenotype of pig-1(gm301). (B) Transgenes expressing PIG-1(T169D) partially rescued the extra neuron phenotype of pig-1(gm301). (C) Transgenes expressing PIG-1(T169A) and PIG-1(T169D) induced extra neurons in a wildtype background. A ced-3 mutation enhanced the extra neuron phenotype of a transgene expressing PIG-1(T169D). Numbers for each genotype are provided. N.S., not significant; *p < 0.005 (Fisher’s exact test).