A Statistically-Oriented Asymmetric Localization (SOAL) Model for Neuronal Outgrowth Patterning by *Caenorhabditis elegans* UNC-5 (UNC5) and UNC-40 (DCC) Netrin Receptors

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ABSTRACT Neurons extend processes that vary in number, length, and direction of “outgrowth”. Extracellular cues help determine outgrowth patterns. In *Caenorhabditis elegans*, neurons respond to the extracellular UNC-6 (netrin) cue via UNC-40 (DCC) and UNC-5 (UNC5) receptors. Previously, we presented evidence that UNC-40 asymmetric localization at the plasma membrane is self-organizing, and that UNC-40 can localize and mediate outgrowth at randomly selected sites. Here, we provide further evidence for a statistically-oriented asymmetric localization (SOAL) model in which UNC-5 receptor activity affects patterns of axon outgrowth by regulating UNC-40 asymmetric localization. According to the SOAL model, the direction of outgrowth activity fluctuates across the membrane over time. Random walk modeling predicts that increasing the degree to which the direction of outgrowth fluctuates will decrease the outward displacement of the membrane. By differentially affecting the degree to which the direction of outgrowth activity fluctuates over time, extracellular cues can produce different rates of outgrowth along the surface and create patterns of “extension”. Consistent with the SOAL model, we show that *unc-5* mutations alter UNC-40 asymmetric localization, increase the degree to which the direction of outgrowth fluctuates, and reduce the extent of outgrowth in multiple directions relative to the source of UNC-6. These results are inconsistent with current models, which predict that UNC-5 mediates a “repulsive” response to UNC-6. Genetic interactions suggest that UNC-5 acts through the UNC-53 (NAV2) cytoplasmic protein to regulate UNC-40 asymmetric localization in response to both the UNC-6 and EGL-20 (Wnt) extracellular cues.

KEYWORDS neuronal development; axon guidance; asymmetric localization; *Caenorhabditis elegans*; netrin and Wnt signaling

DURING development, an intricate network of neuronal connections is established. As processes extend from the neuronal cell bodies, distinct extension patterns emerge. Some extensions remain as a single process, whereas others branch and form multiple processes. If they branch, the extensions can travel in the same or in different directions. Extensions vary in length. Extracellular cues are known to influence this patterning, but the underlying logic that governs the formation of patterns remains a mystery.

The secreted extracellular UNC-6 (netrin) molecule and its receptors, UNC-5 (UNC5) and UNC-40 (DCC), are highly

conserved in invertebrates and vertebrates, and are known to play key roles in cell and axon migrations. In *Caenorhabditis elegans*, UNC-6 is produced by ventral cells in the midbody and by glia cells at the nerve ring in the head (Wadsworth and Hedgecock 1996; Wadsworth *et al.* 1996; Asakura *et al.* 2007). It has been observed that neurons that express the receptor UNC-40 extend axons ventrally, toward the UNC-6 sources, whereas neurons that express the receptor UNC-5 alone or in combination with UNC-40 extend axons dorsally, away from the UNC-6 sources (Hedgecock *et al.* 1990; Leung-Hagesteijn *et al.* 1992; Chan *et al.* 1996; Wadsworth *et al.* 1996).

It is commonly proposed that axons are guided by attractive and repulsive mechanisms (Tessier-Lavigne and Goodman 1996). According to this model, an extracellular cue acts as an attractant or repellant to direct neuronal outgrowth toward or away from the source of a cue. UNC-5 (UNC5) has been described as a repulsive netrin receptor because it mediates guidance away from netrin sources (Leung-Hagesteijn *et al.* 1992; Hong *et al.* 1999;

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Keleman and Dickson 2001; Moore *et al.* 2007). The attraction and repulsion model is deterministic. That is, given the same conditions, the response of the neuron, attractive or repulsive, will always be the same. This idea forms the bases of the analysis and interpretation of experimental results. Axonal growth cone movement toward or away from the source of a cue is considered to be mediated by attractive or repulsive responses to the cue. In genetic studies, a mutation that disrupts movement toward the cue source denotes gene function within an attractive pathway, whereas a mutation that disrupts movement away from a source denotes gene function within a repulsive pathway. If an axonal growth cone is observed to move toward and then away from the source of a cue, the responsiveness of a neuron is thought to switch from attractive to repulsive. However, it is important to note that attraction or repulsion is not an intrinsic property of the interaction between the receptor and ligand. In fact, the interaction only promotes or inhibits outward movement of the membrane. Attraction and repulsion refers to a direction, which is an extrinsic property of the cellular response that varies depending on the physical positions of the ligands. Movement toward or away from a cue source is caused by attractive and repulsive *effects*, such as chemoattraction and chemorepulsion, which is movement that is directed by chemical gradients of ligands. We argue that classifying gene function as attractive or repulsive is problematic, since attraction and repulsion are not intrinsic properties of cellular mechanisms.

We have proposed an alternative model in which the movement of neuronal outgrowth is not considered in terms of attraction and repulsion. This model comprises three concepts. The first concept is that receptors along the surface of the membrane change position. This is important since the spatial distribution of receptors can influence the movement that a neuron has in response to the extracellular ligands (Nguyen *et al.* 2014, 2015). We hypothesize that the spatial distribution of UNC-40 can influence the manner through which force is applied to the membrane and thereby affect the outward movement of the membrane. It is known that the surface localization of the UNC-40 receptor undergoes dramatic changes during the development of the HSN axon (Adler *et al.* 2006; Xu *et al.* 2009; Kulkarni *et al.* 2013). As HSN axon formation begins, UNC-40 becomes asymmetrically localized to the ventral surface of the cell body, which is nearest to the ventral sources of the secreted UNC-6 ligand. Live imaging of the developing leading edge reveals a dynamic pattern of UNC-40 localization, with areas of concentrated UNC-40 localization shifting positions along the surface (Kulkarni *et al.* 2013). Dynamic UNC-40::GFP localization patterns have also been reported during anchor cell extension (Wang *et al.* 2014). Similar to axon extension, the anchor cell also sends an extension through the extracellular matrix, and this extension is also regulated by UNC-40 and UNC-6 (Ziel *et al.* 2009; Hagedorn *et al.* 2013). Live imaging of the anchor cell reveals that UNC-40::GFP “clusters” form, disassemble, and reform along the anchor cell’s plasma membrane (Wang *et al.* 2014).

The second concept is that the asymmetric localization of the receptor, and the subsequent outgrowth activity that it mediates, are stochastically oriented. It was observed that UNC-40 can asymmetrically localize to a randomly selected surface if the UNC-6 ligand is not present to provide a pre-established asymmetric cue (Xu *et al.* 2009). We noted that the self-organizing nature of UNC-40 localization is reminiscent of a self-organizing process observed in single-cell yeast, *Dictyostelium discoideum*, and neutrophils, where cell movement will occur in a random direction if the chemotactic cue is absent or is uniformly presented (Fraser *et al.* 2000; Arriemerlou and Meyer 2005; Mortimer *et al.* 2008). The process through which outgrowth activity becomes asymmetrically organized is thought to utilize positive and negative feedback loops (Bourne and Weiner 2002; Graziano and Weiner 2014). Such loops might also drive the asymmetric localization of UNC-40 (Xu *et al.* 2009; Wang *et al.* 2014). Positive and negative feedback are considered to be complementary mechanisms; positive feedback amplifies the polarized response to an extracellular cue, while negative feedback limits the response and can confine the positive feedback to the leading edge (Bourne and Weiner 2002). The biological nature of feedback loops controlling UNC-40 activity is unclear. However, they may involve the differential transport of receptors and effectors to the plasma membrane surface. Imaging experiments of cells in culture suggest that netrin-1 (UNC-6) regulates the distribution of DCC (UNC-40) and UNC5B (UNC-5) at the plasma membrane (Gopal *et al.* 2016). In these studies, netrin-1 (UNC-6) was shown to stimulate translocation of DCC (UNC-40) and UNC5B (UNC-5) receptors from intracellular vesicles to the plasma membrane and, further, the transported receptors were shown to localize at the plasma membrane (Gopal *et al.* 2016).

We argue that the process that localizes UNC-40 to a site on the plasma membrane possesses inherent randomness (Figure 1). Evidence suggests that the conformation of the UNC-40 molecule controls whether the process will cause UNC-40 localization to the site of UNC-6 interaction or to another site (Xu *et al.* 2009). We observed that a single amino acid substitution in UNC-40 will allow UNC-40 to asymmetrically localize to different surfaces in the absence of UNC-6. The binding of UNC-6 to this UNC-40 molecule causes localization to the surface nearest the UNC-6 source. However, the binding of UNC-6 with a single amino acid substitution will enhance the asymmetric localization to different surfaces. A second-site UNC-6 amino acid substitution will suppress this enhancement and increase UNC-40 asymmetric localization at the surface toward the UNC-6 source. These results indicate that UNC-40 conformational changes differentially influence each activity. In the context of feedback loops, UNC-40 activity regulates both the positive and negative loops that control the asymmetric localization of UNC-40 to the plasma membrane. Because the system is controlled by the conformation of the molecule, randomness will be introduced in the system by stochastic

“fluctuations” in ligand–receptor binding and by stochastic conformational changes.

The outcome of an **UNC-40** receptor’s activity is to either cause an **UNC-40** receptor to localize to the site of **UNC-6** interaction or to a different site (Figure 1). This is an important discovery because it means that the asymmetric localization events are mutually exclusive and, therefore, there is statistical dependence. We refer to this process as statistically-oriented asymmetric localization (SOAL). This model states that the probability of **UNC-40** localizing and mediating outgrowth at the site of **UNC-6** interaction affects the probability of **UNC-40** localizing and mediating outgrowth at another site, and vice versa. We have found that other extracellular cues can also affect **UNC-40** asymmetric localization, and thus can influence the probability of **UNC-40**-mediated outgrowth from different sites (Tang and Wadsworth 2014; Yang *et al.* 2014).

The development of an extension can be considered as a stochastic process. At any one instance of time at innumerable sites along the neuron’s surface, **UNC-40** interacts with **UNC-6** to mediate **UNC-40** asymmetric localization and the outgrowth response. At the next instance of time, other **UNC-40** receptors, including any just transported to the surface, may interact with **UNC-6**. Because the plasma membrane is a fluid, the forces generated by the outgrowth response are not always acting in parallel and the direction of outward force can fluctuate (Figure 2A). It is the collective impact of all outgrowth events over a period of time that allows the extension to form. The outward movement of an extension could be precisely described if the effect of each outgrowth event were known. However, it is extremely difficult to measure the effect of each single event, since there are innumerable events happening at each instance of time. We also argue that the pattern of **UNC-40** localization and outgrowth across the surface of the membrane evolves over time through a random process. Therefore, the outgrowth events can only be described probabilistically and, as such, the time evolution of extension is also probabilistic in nature.

Understanding the role that a gene plays in controlling outgrowth movement might require knowledge of its role in regulating this stochastic process. To do this, we use the direction of HSN extension. We reason that the collective impact of all the outgrowth events over a period of time cause the development of the HSN axon. The direction of extension from the cell body has a probability of being orientated in one direction (Kulkarni *et al.* 2013; Tang and Wadsworth 2014; Yang *et al.* 2014). Mathematically, the direction of HSN extension is a variable that takes on different values; “anterior,” “posterior,” “ventral,” and “dorsal.” A probability is associated with each outcome, thus creating a probability distribution. This distribution describes the effect that all the outgrowth events had over a period of time. During normal development, the probability of each **UNC-40**-mediated outgrowth event being ventrally oriented is very high and a ventral extension develops. We have shown that certain gene mutations affect the probability distribution,

thus revealing that a gene plays a role in the stochastic process. We can compare wild-type animals and mutants to gauge the degree to which a mutation causes the direction of extension to fluctuate. This reflects the degree to which the mutation has caused the direction of outgrowth activity, and the outward force it creates, to fluctuate over the course of extension development.

We argue that understanding the function of a gene in terms of a stochastic model of membrane movement is useful. Often, the goal of a genetic analysis of axon guidance is to uncover a molecular mechanism. Frequently, a deterministic model is made that describes some molecular event that the gene affects. Because the mutation affects axon guidance, the molecular event plays a role in causing directed movement. However, these models tend to reduce a complex biological process to an isolated component. In reality, understanding how a molecular event is able to cause directed movement requires knowledge of all the many ways in which the event influences, and is influenced by, the other molecular events of directed movement. A stochastic model is a useful tool to explore how a gene affects the overall behavior of the system. To make an analogy, a roulette wheel can be described deterministically; if every force acting on the ball at every instance of time is known, then the number on which the ball stops can be precisely determined. The role of a component of the roulette wheel could be described by the effect that it has on the forces that act on the ball at every instance of time. However, understanding how the effect of this component causes a particular outcome requires an understanding of the effects of all the other components. Because this is so complex, the outcome of a roulette wheel is studied using a stochastic model. That is, how does the component affect the probability of the ball stopping on a particular number. A roulette wheel must be exactly leveled to have an equal probability for each number. Removing a component of the wheel can cause the wheel to tilt in a particular manner. This will result in a new outcome, *i.e.*, the ball will have a higher probability of stopping on certain numbers. Although this does not reveal the precise event that occurs between the component and the ball, it will reveal the effect that the component has in determining an outcome. Further, by studying the effect of removing multiple components, relationships that lead to particular outcomes can be revealed.

The third concept of our model is that neuronal membrane outgrowth is a mass transport phenomenon that can be described as advection and diffusion (Figure 2). Signaling by **UNC-40** receptors along a surface of the neuron can lead to cytoskeletal changes that create force and membrane movement (Figure 2A). As a result, there is a mean flow of membrane mass in an outward direction (Figure 2B). This motion is advection, which is mass transport by a mean velocity field. In addition to advection, membrane mass transport also occurs through random movement, *i.e.*, diffusion. Because the cell membrane is fluid, membrane mass will move in different directions as the membrane is subjected to forces that change its shape (Figure 2C). The degree to which the membrane

Figure 1 Statistically-oriented asymmetric localization (SOAL). At sites along the plasma membrane, UNC-40 interacts with the UNC-6 extracellular cue. A self-organizing process is triggered that utilizes positive and negative feedback loops. Positive feedback (yellow arrows) amplifies the polarized response to an extracellular cue, while negative feedback (yellow lines) limits the response and can confine the positive feedback to the site of UNC-40 and

UNC-6 interaction. The outcome of an UNC-40 receptor's activity is to either cause an UNC-40 receptor to localize and mediate outgrowth at the site of UNC-6 interaction or at a different site. Randomness is considered inherent in this process and each localization event is mutually exclusive. The statistical dependence means that the probability of UNC-40 localizing and mediating outgrowth at the site of UNC-6 interaction affects the probability of UNC-40 localizing and mediating outgrowth at another site, and vice versa. As time passes, this process causes randomly directed outgrowth activity (force) that drives the outward movement of the membrane.

mass undergoes random movement is important because diffusion processes and advection processes have different effects on the extent to which mass will be displaced outward in a given amount of time. The random movements can be mathematically described using random walks. A random walk is a succession of randomly directed steps (Figure 2D). Random walk models are used to describe many diverse types of behavior, including the movement of a particle through fluid, the search pattern of a foraging animal, and the fluctuating price of a stock. The behavior of neuronal growth cone movement during chemotaxis has also been modeled using random walks (Katz *et al.* 1984; Buettnner *et al.* 1994; Odde and Buettnner 1995; Wang *et al.* 2003; Maskery *et al.* 2004). However, rather than using a random walk model to describe the gross morphological changes observed during growth cone movement, in this study, the random walk is used to model the random movement of membrane mass to understand how gene activity influences the outward displacement of the membrane. A property of random motion is that the mean square displacement (MSD) grows proportionate to the time traveled. This means that the more the direction of movement fluctuates, the shorter the distance of travel in a given amount of time (Figure 2E). The model predicts that if force is applied to the membrane in a manner that increases random movement, then the outward displacement of the membrane's mass will decrease.

Because of the effect that random movement has on displacement, the SOAL model makes predictions about how UNC-40 activity affects the rate of extension. In a deterministic model, outgrowth activity causes straight-line outward motion from the site of interaction. The SOAL model predicts that the interaction between UNC-40 and UNC-6 increases the probability that UNC-40 asymmetric localization and UNC-40-mediated outgrowth will be oriented at the site of interaction. It also decreases the probability that localization and outgrowth will be oriented elsewhere. Therefore, the interaction influences the spatial distribution of UNC-40 along the surface and, in doing so, will change the way forces are applied to the membrane. As

this process continues over time, the direction of the forces acting on the fluid membrane fluctuates. This will alter the advective and diffusive transport of membrane mass. As an example, if the probability of ventral outgrowth is 0.33, of anterior outgrowth is 0.33, and posterior outgrowth is 0.33, then there will be a high degree of random movement. Interactions between UNC-40 and UNC-6 at the leading ventral surface could shift the probabilities for ventral outgrowth to 0.8, for anterior outgrowth to 0.1, and for posterior outgrowth to 0.1. This change will decrease the degree to which the direction of outgrowth fluctuates. As modeled in Figure 2E, this will increase displacement, meaning that the membrane mass will now be able to travel further outward over a given amount of time. It is worth noting that fluctuations in the direction of outgrowth activity could occur as very rapid minute movements of membrane mass. When observed at the macroscale, these microscale fluctuations might not be seen. Instead, the outward movement of an extension will appear as linear, straight-line, movement. In this paper, fluctuation refers to variation in the direction of outgrowth activity. Outgrowth refers to the movement of membrane mass at the microscale, whereas extension refers to the movement of the axon that is observed at the macroscale.

The SOAL model makes distinctive predictions about UNC-40-mediated outgrowth activity *in vivo* and the direction of extension. In a deterministic model, the direction of extension is determined by the outward movement of the membrane from the site where UNC-6 and UNC-40 interact. Positional information is encoded by gradients so that UNC-6 guides extension toward the UNC-6 source. In the SOAL model, UNC-6 and other extracellular cues govern the probability of UNC-40 asymmetric localization, and subsequent UNC-40-mediated outgrowth, at each surface of the membrane (Xu *et al.* 2009; Kulkarni *et al.* 2013). The direction of outgrowth is determined by a directional bias that is created over time by the combined effect of extracellular cues. If, for example, the probability of outgrowth toward a ventral UNC-6 source is 0.3, the probability of dorsal outgrowth is 0.3, and that of anterior outgrowth is 0.4, the direction of outgrowth will be in the anterior direction. That is, at any

Figure 2 Model for outgrowth movement. (A) The outward movement of the neuronal membrane is depicted as a mass transport phenomenon. The cell membrane is fluid and membrane mass will move in different directions as the membrane is subjected to forces (arrows) that change its shape. Receptors mediate cellular responses, which creates the outward force. The force causes movement of the lipids and proteins of the plasma membrane. A unit of this mass is shown within a box. Membrane mass is represented by a box in subsequent schematic diagrams. (B) The mean flow of membrane mass (box) can be described as advection and diffusion. The probability density function of the position of mass as a function of space and time is described mathematically by an advection–diffusion equation. Mass transport by a mean velocity field is advection. Because of the statistically-oriented asymmetric localization (SOAL) process and the fluid nature of the membrane, mass transport also occurs through random movement, which is diffusion. (C) During outward movement of the leading edge (times 1–4), membrane molecules move in the direction of advection as well as randomly in other directions. (D) The path that the membrane molecules take during outgrowth can be described as a random walk, which is a succession of randomly directed steps. Depicted are the positions of mass after each step of a succession of four steps, as shown in (C). Each step corresponds to a time point. (E) For two examples, 50 simulated random walks of 500 steps were plotted from an origin (0,0). For each step, the probability of moving to the right, left, or down is given below the plots. The plots illustrate that increasing the degree to which the direction of movement fluctuates decreases the outward distance that mass can travel. We predict that the SOAL process influences the degree of random membrane movement and, consequently, the outward displacement of the membrane.

instance of time, there is a chance that outgrowth movement will be directed ventrally toward the **UNC-6** source; however, over a longer period of time the outgrowth will travel anteriorly because there is always a greater likelihood that outgrowth will be anterior instead of ventral or dorsal. To reiterate, the direction of extension is a product of a stochastic process, in which the outcome evolves over time. The probability of ventral outgrowth created by the **UNC-40**-mediated response to **UNC-6** is required for the anterior bias. Without the ventrally directed outgrowth in response to **UNC-6**, the probability of ventral outgrowth would decrease, shifting the directional bias. Thus, when considered as a stochastic process, the observed directional response to the interactions between **UNC-40** and **UNC-6** is not necessarily an extension toward the **UNC-6** source. Because of SOAL, positional information is encoded by the location and level of the extracellular cues along the surface of the neuron.

The SOAL model suggested that **UNC-40** activity could affect extension movement in ways that had not been obvious. The first insight is that forward movement of an exten-

sion could be inhibited as it moves toward a source of a cue that promotes outgrowth (Figure 3A). At the leading edge of an outgrowth, a strong directional basis for movement toward an **UNC-6** source occurs only as long as the probability of **UNC-40** localization at surfaces facing toward the source are greater than the probability of **UNC-40** localization at surfaces facing other directions. As an extension moves toward an **UNC-6** source, a higher proportion of the **UNC-40** receptors that flank the leading edge can become ligated (Figure 3B). Because of the SOAL process, this will increase the probability of localization and outgrowth at the flanking sites while decreasing the probability of localization and outgrowth at the leading edge. The result will be an increase in random movement and a decrease in the outward displacement of the membrane's mass. Paradoxically, the rate of extension will decrease as the extension moves toward the **UNC-6** source (Figure 3C). It is worth noting that even if the probability of outgrowth in each direction becomes equal, there will still be a directional bias. For example, if the probability of outgrowth toward a ventral **UNC-6** source is 0.33, of anterior outgrowth is 0.33, and of posterior outgrowth is

Figure 3 Model for outgrowth movement toward extracellular cues that promote or inhibit outgrowth activity. (A) Schematic diagram of the outgrowth of a neuron through an environment of multiple extracellular cues. These cues may be molecules present at the surfaces of surrounding cells and extracellular matrix, or they may be physical interactions that influence outgrowth activity. The extracellular cues are represented as color gradients of blue, orange, and red. The neuron's response to cues arranged along the anterior-posterior axis (orange and red),

create an equal probability for UNC-40 asymmetric localization and outgrowth in the anterior and posterior directions. The extension transverses three different positions (1–3) as it develops toward a ventral source of a cue (blue). (B) The statistically-oriented asymmetric localization process is illustrated as in Figure 1 for the three positions shown in (A). Shown are scenarios for movement toward a cue [(A), blue] that promotes outgrowth (blue +) or that inhibits outgrowth (blue –). The cues act to increase (promote) and decrease (inhibit) the probability of outgrowth from a surface. At positions 1 and 2, cues along the anterior-posterior axis (orange – and red –) inhibit outgrowth in the anterior or posterior directions. At position 3, the cue from the ventral source predominates. (C) Random walk modeling as described in Figure 2E. At each position [(A), 1–3], cues alter the probability distribution for the direction of localization and outgrowth. Below each plot is the probability distribution used to create the random walk (see *Materials and Methods*). Probability distributions were selected to represent how different levels of the ventral cue might change the probability distribution at each position. The plots illustrate the probability density function of the position of mass as a function of space and time if movement occurred according to that probability distribution. For both scenarios, an equal probability of anterior and posterior outgrowth can allow a ventral directional bias at position 1. Movement toward a promoting cue source can allow a greater probability for ventral outgrowth and, correspondingly, a lower probability for anterior and posterior outgrowth (position 2). Movement toward an inhibiting cue source can allow a lower probability for ventral outgrowth and, correspondingly, a greater probability for anterior and posterior outgrowth (position 2'). The ventral directional bias is maintained. In either scenario, an equal probability for outgrowth in all directions may occur as the receptors become saturated because of the high level of the cue from the ventral source (position 3). The modeling predicts that in both scenarios, changing levels of the ventral cue will not alter the direction of outward movement, although it may alter the outward displacement of the membrane's mass. See text for details.

0.33, the directional bias is ventral [the probability of movement in the direction of the axon shaft (backward) is low].

A second insight is that an extension could move toward the source of a cue that inhibits outgrowth (Figure 3A). For example, if together the extracellular cues create a probability for ventral outgrowth of 0.7, of anterior outgrowth is 0.15, and of posterior outgrowth is 0.15, a directional bias for ventral outgrowth is created (Figure 3B). This can occur even if there is a ventral source of an inhibitory cue. The extension can move ventrally toward this inhibitory cue source. Eventually, the probability for ventral outgrowth might change to 0.33, anterior to 0.33, and posterior to 0.33 (Figure 3C); but even in this case, there is still a directional bias for ventral outgrowth and extension will continue to move toward the source of the inhibitory cue.

The model predicts that movement toward a source of a cue causes the system to trend toward a state where the probabilities of outgrowth in different directions become equal. Axons often change their trajectory near the source of a cue. It is possible that the state is important because the equilibrium might allow cues to be more effectual at reorienting outgrowth (Figure 4).

The third insight is that multiple extensions from a neuron could move in the same direction without having to follow prepatterned extracellular pathways. Some neurons send out multiple extensions that run in parallel toward a target. It is commonly proposed that these patterns form because exten-

sions follow parallel pathways that were previously formed by extracellular guidance cues. The SOAL model suggests that multiple UNC-40-mediated outgrowths can be initiated at a leading surface and that multiple extensions can maintain their positions without having to follow prepatterned extracellular pathways. In this model, a separate extension begins to form at the leading edge because the directional bias at one site becomes greater than that at flanking sites. We propose that along the leading edge, the self-organizing UNC-40 localization process can create multiple sites that have a greater directional bias (Figure 5A). The positive and negative feedback loops of the SOAL process can allow spatial patterns of outgrowth to develop autonomously. Once these sites are established, outgrowth can proceed from each site in the same direction (Figure 5B). The strongest directional bias is created when the probabilities for outgrowth are equal in the directions perpendicular to the direction of extension. The actual value of the perpendicular probabilities is not crucial for establishing a directional bias. Even though the value of the perpendicular probabilities may vary depending on the position of outgrowth along the perpendicular axis, the direction of outgrowth will be the same. If a perpendicular equilibrium is maintained, then cues that affect UNC-40 localization and outgrowth, and which are distributed along the perpendicular axis, will have little effect on the direction of outgrowth. Such an equilibrium can be established if the outgrowth effects of cues distributed along the perpendicular

Figure 4 Model for outgrowth movement that changes direction. (A) Schematic diagram of the outgrowth of a neuron through an environment of multiple extracellular cues as described in Figure 3A. Positions 1'–3' represent the position after a change from ventral to anterior outgrowth. (B) At each position [(A) 1–3], the probability distribution for the direction of localization and outgrowth is given as in Figure 3C. In order for the direction of outgrowth to shift anteriorly at each position, the probability distribution must shift to create a bias for anteriorly directed outgrowth. This is depicted by the probability distribution of 0.4 anterior, 0.3 dorsal, and 0.3 ventral for positions 1'–3'. Numbers in red indicate the degree to which the probabilities must change between the positions. The model predicts that as the system trends toward a state where the probabilities of outgrowth in different directions become equal, cues that could shift the direction bias become more effectual.

axis balance out each other. Such a condition could be established by cues that effect outgrowth equally at surfaces facing the perpendicular axis. Even if cues are distributed in a gradient, an equilibrium could exist. Studies indicate that gradient steepness, rather than the concentration of cues, is important for growth cone turning and guidance (Baier and Bonhoeffer 1992; Rosoff *et al.* 2004; Mortimer *et al.* 2010; Sloan *et al.* 2015). Therefore, cues may create a perpendicular equilibrium if they are distributed in a shallow gradient along the perpendicular axis.

A fourth insight is that cues can direct movement without being in a concentration gradient. The SOAL activity within the cell initiates random walk movement. As long as an equilibrium along the perpendicular axis exists, a directional bias along the other axis can be created. In Figure 5B, outgrowth is toward a ventral cue source, and as outgrowth moves up the concentration gradient of this cue the probability of outgrowth in each direction changes. However, movement toward the source would still occur if the concentration of extracellular cues remained constant and the probabilities never changed. Because of the SOAL process, a directional bias can be maintained along a track of a uniformly distributed cue.

The last insight is that extracellular cues could affect **UNC-40** localization and outgrowth, but not affect the direction of outgrowth. However, these cues could have an effect on the

morphology and patterning of an extension. A candidate for such a cue is **EGL-20** (Wnt). The *egl-20* gene is one of several Wnt genes in *C. elegans*. These genes are expressed in a series of partially overlapping domains along the anterior–posterior axis of the animal (Sawa and Korswagen 2013). **EGL-20** is expressed in cells posterior to the HSN (Whangbo and Kenyon 1999; Pan *et al.* 2006; Harterink *et al.* 2011). The sources of **UNC-6** and **EGL-20** are roughly perpendicular to each other. We have observed that loss of **EGL-20** function causes **UNC-40** asymmetrical localization to orient to randomly selected surfaces of the HSN and causes the axon to initially extend from the HSN cell body in different directions (Kulkarni *et al.* 2013; Tang and Wadsworth 2014). **UNC-6** and **EGL-20** signaling could both impinge on the feedback loops that regulate **UNC-40** SOAL. In doing so, these cues would act together to influence the pattern of extension. In this paper, we provide further genetic evidence that the downstream signals from both cues converge to regulate the **UNC-40** SOAL process.

We suggest that **UNC-5** plays an important role in coordinating the **UNC-40** SOAL process with non-**UNC-40**-mediated responses that affect outgrowth. Previously, we reported that loss of **UNC-5** causes **UNC-40** asymmetrical localization to orient to randomly selected surfaces of the HSN, causing the axon to initially extend in different directions (Kulkarni *et al.* 2013). This suggests that **UNC-5** functions to increase the probability of **UNC-40** asymmetric localization being oriented to the site of **UNC-6** and **UNC-40** interaction. That is, **UNC-5** promotes straight-line motion by inhibiting the degree to which the direction of **UNC-40**-mediated outgrowth fluctuates. **UNC-5** has other functions as well. **UNC-5** is primarily known for its role in mediating movement away from **UNC-6** sources. For example, **UNC-5** is required for the dorsal migration of DA and DB motor neuron axons away from ventral **UNC-6** sources (Hedgecock *et al.* 1990). DA and DB guidance utilizes both **UNC-40**-dependent and **UNC-40**-independent pathways, although guidance is significantly less disrupted by loss of **UNC-40** than by loss of **UNC-5** (Hedgecock *et al.* 1990; MacNeil *et al.* 2009). We hypothesize that **UNC-5** increases the probability of non-**UNC-40**-mediated outgrowth being oriented toward sites where there are no interactions between **UNC-5** and **UNC-6**. This increases the probability of outgrowth movement in directions that are not toward **UNC-6** sources. Finally, we predict that **UNC-5** function can also increase the probability that non-**UNC-40**-mediated outgrowth will orient to the site of interaction between non-**UNC-40** receptors and non-**UNC-6** extracellular cues. Evidence for this comes from the observation that in *rpm-1* mutants, the overextension of the PLM axon can be suppressed by loss of **UNC-5**, but not by the loss of **UNC-40** or **UNC-6** (Li *et al.* 2008). In summary, we believe that **UNC-5** can: (1) increase the probability of **UNC-40**-mediated outgrowth at the sites of **UNC-6** and **UNC-40** interactions; (2) decrease the probability of **UNC-40**-mediated outgrowth at sites where **UNC-6** is not present; (3) decrease the probability of non-**UNC-40**-mediated outgrowth at the site of **UNC-5** and

Figure 5 Model for the development of multiple outgrowths that extend in the same direction. (A) The statistically-oriented asymmetric localization (SOAL) process is illustrated for sites along a surface of a neuron as in Figure 1. The positive and negative feedback loops of the SOAL process allow spatial patterns of outgrowth to develop autonomously. The number of sites where a strong directional bias is ultimately created is dictated by the relative effectiveness of the positive and negative feedback loops. (B) Schematic diagram of the outgrowth of a neuron through an environment of multiple extracellular cues, as described in Figure 3A. The flow of membrane mass (box) at different sites depends on the probability distribution for the direction of outgrowth created at regions along the surface. Random walk modeling, as described in Figure 2E, is shown below the schematic diagram. At time X, two sites that have a greater directional bias (two and four) are established by the SOAL process, as depicted in (A). Cues may not be present in steep gradients along the axis perpendicular to

the direction of extension. The response to these cues creates probabilities for outgrowth that are equal in the perpendicular directions. The greatest directional bias is created when there is an equilibrium for the probability of outgrowth in perpendicular directions. Because cue levels may vary gradually along the perpendicular axis, the strength of the directional bias at sites may differ; however, the bias will be oriented in the same direction. At time $x + 1$, positions 2 and 4 have proceeded further outward because of greater membrane displacement. This effect is magnified by increasing levels of the outgrowth-promoting cue (blue). This activity together, when averaged over time across a surface, is predicted to cause the dynamic development of multiple extensions.

UNC-6 interactions; and (4) increase the probability of non-UNC-40-mediated outgrowth at sites where there are no UNC-6 interactions. These functions can be considered in terms of the positive and negative feedback loops of the SOAL model (Figure 6), where UNC-5 helps to regulate the feedback loops associated with UNC-40 activity.

Because of these ideas, we reasoned that UNC-5 activity could affect extension movement in ways that had not been obvious to us. First, UNC-5 might affect the patterning of extension that travels toward an UNC-6 source. As discussed above, previous evidence suggests that UNC-5 regulates the asymmetric localization of UNC-40. UNC-5 interactions with UNC-6 and UNC-40 could influence the feedback loops (Figure 1 and Figure 6). By regulating the degree to which the direction of UNC-40-mediated outgrowth fluctuates, UNC-5 could affect random movement and the outward displacement of membrane mass. This could affect the rate of extension toward an UNC-6 source or whether extension can occur. In cases where multiple extensions form from a surface, the effect that UNC-5 has on the loops could influence whether sites with a predominant directional bias can be established.

Second, UNC-5 could play a role in determining whether an extension changes direction. As discussed earlier, if the UNC-40 receptors become saturated near an UNC-6 source then the probability of UNC-40-mediated outgrowth toward the source and along the perpendicular axis tends to become equal. At this point, even a small increase in the probability of non-UNC-40-mediated outgrowth to the site of non-UNC-6 and non-UNC-40 interactions could alter the directional bias (Figure 4). A change in UNC-5 activity could help promote a shift from a directional bias determined by UNC-40 and UNC-6 interactions to one determined by non-UNC-40 and non-UNC-6 interaction.

Because of the predictions that the UNC-40 SOAL model makes, we decided to reexamine the *unc-5* loss-of-function phenotypes and to investigate genetic interactions among

unc-5, *unc-6*, *unc-40*, and *egl-20* that regulate the asymmetric localization of UNC-40. We find evidence that UNC-5 regulates the length and number of processes that extend toward an UNC-6 source and that UNC-5 helps control the ability of axons to extend in different directions. In addition, we find genetic interactions that suggest UNC-5, together with UNC-53 (NAV2), functions to regulate UNC-40 SOAL in response to the UNC-6 and EGL-20 (Wnt) extracellular cues. In the *Results* section of this paper, we describe phenotypes caused by mutations, and in the *Discussion* section, we describe how these phenotypes could be predicted by the SOAL model. We suggest that the SOAL model is useful for understanding how genes regulate the patterning of axon extensions.

Materials and Methods

Strains

Strains were handled at 20° using standard methods (Brenner 1974) unless stated otherwise. A Bristol strain N2 was used as wild-type. The following alleles were used: LGI, *unc-40(e1430)*, *unc-40(ur304)*, and *zdis5[mec-4::GFP]*; LGII, *unc-53(n152)*; LGIV, *unc-5(e152)*, *unc-5(e53)*, *unc-5(ev480)*, *unc-5(ev585)*, *egl-20(n585)*, and *kyIs262[unc-86::myr-GFP;odr-1::dsRed]*; LGIV, *madd-2(ky592)* and *madd-2(tr103)*; and LGX, *mig-15(rh148)*, *unc-6(ev400)*, *sax-3(ky123)*, and *sax-3(ky200)*.

Transgenes maintained as extrachromosomal arrays included *kyEx1212 [unc-86::unc-40-GFP;odr-1::dsRed]*.

Analysis of axon outgrowth and cell body position

HSN neurons were visualized using expression of the transgene *kyIs262[unc-86::myr-GFP]*. The mechanosensory neurons, AVM, ALM, and PLM, were visualized using the expression of the transgene *zdis5[Pmec-4::GFP]*. Synchronized worms were obtained by allowing eggs to hatch overnight in M9 buffer without food. The

Figure 6 Model for the control of outgrowth activity by statistically-oriented asymmetric localization (SOAL). Schematic diagram of the control of UNC-40- and non-UNC-40-mediated activity. Neuronal surfaces of the neuron are subjected to different levels of UNC-6 (blue) as well as non-UNC-6 extracellular cues (orange). The SOAL process regulates both UNC-40- and non-UNC-40-mediated activity. Positive feedback (arrows) amplifies the polarized response to an extracellular cue, while negative feedback (lines) limits the response and can confine the positive feedback to the site of ligand interaction. The long-range negative feedback mediated by UNC-40 inhibits UNC-40 activity, as well as non-UNC-40 activity. Similarly, long-range negative feedback mediated by non-UNC-40 activity inhibits non-UNC-40 activity, as well as UNC-40 activity.

larval stage was determined by using differential interference contrast (DIC) microscopy to examine the gonad cell number and the gonad size. Staged larvae were mounted on a 5% agarose pad with 10 mM levamisole buffer. Images were taken using epifluorescent microscopy with a Zeiss ([Carl Zeiss], Thornwood, NY) 63× water immersion objective.

The number of processes during the early L1 larval stage was scored by counting the number of processes that extended for a distance greater than the length of one cell body. We report instances in which there were no such processes, one process, or more than one process. In the L2 larval stage, a single early process was scored if there was only one major extension from the ventral leading edge. The HSN cell body in L2 stage larvae was scored as dorsal if the cell body had failed to migrate ventrally and was not positioned near the PLM axon. In L4 stage larvae, a multiple ventral processes phenotype was scored if more than one major extension protruded from the ventral side of cell body.

Extension into the nerve ring was scored as defective if the axon did not extend further than approximately half the width of the nerve ring. Anterior extension was scored as defective if the axon did not extend further anteriorly than the nerve ring. PLM axons were scored as overextending if they extended further anterior than the position of the ALM cell body.

Analysis of the direction of HSN outgrowth

The HSN was visualized using the transgene *kyIs262[unc-86::myr-GFP]*. L4 stage larvae were mounted on a 5% agarose pad with 10 mM levamisole buffer. An anterior protrusion was scored if the axon extended from the anterior side of

the cell body for a distance greater than the length of three cell bodies. A dorsal or posterior protrusion was scored if the axon extended dorsally or posteriorly for a distance greater than two cell body lengths. The HSN was considered multipolar if more than one process extended a length longer than one cell body. Images were taken using epifluorescent microscopy with a Zeiss 40× objective.

Analysis of UNC-40::GFP localization in L2 stage animals

For analysis of UNC-40::GFP localization, L2 stage larvae with the transgenic marker *kyEx1212[unc-86::unc-40::GFP; odr-1::dsRed]* were mounted on a 5% agarose pad with 10 mM levamisole buffer. Staging was determined by examining the gonad cell number and the gonad size under DIC microscopy. Images were taken using epifluorescent microscopy with a Zeiss 63× water immersion objective. The UNC-40::GFP localization was determined by measuring the average intensity under lines drawn along the dorsal and ventral edges of each HSN cell body using ImageJ software. For analysis of the anterior–posterior orientation of UNC-40::GFP, the dorsal segment was geometrically divided into three equal lengths (dorsal anterior, dorsal central, and dorsal posterior segments). The line-scan intensity plots of each of these segments were recorded. An ANOVA test was used to determine if there is a significant difference between the intensities of the three segments. The dorsal distribution was considered uniform if $P \geq 0.05$ and was considered asymmetrical if $P \leq 0.05$. Within an asymmetric population, the highest percent intensity was considered to localize UNC-40::GFP to either the anterior, posterior, or central domain of the dorsal surface.

Computations

A program to simulate a two-dimensional lattice random walk based on the probability of dorsal, ventral, anterior, and posterior outgrowth for a mutant (Table 1) was created using MATLAB (the directions of the axons from multipolar neurons were not scored; these axons appear to behave in the same manner as the axons from monopolar neurons, but this has not yet been tested). The probability of dorsal, ventral, anterior, or posterior outgrowth was assigned for the direction of each step of a random walk moving up, down, left, or right, respectively (Figure 9). Each variable was considered independent and identically distributed. Simulations of 500 equal size steps (size = 1) were plotted for 50 tracks. A Gaussian distribution for the final positions of the tracks was generated using MATLAB's random function.

The MSD is used to provide a quantitative characteristic of the motion that would be created by the outgrowth activity undergoing the random walk. Using the random walks generated for a mutant, the MSD can be calculated: Here, $r(t)$ is the position at time t and τ is the lag time between two positions used to calculate the displacement, $\Delta r(\tau) = r(t + \tau) - r(t)$. The time-average over t and the ensemble-average over the 50 trajectories were calculated. This yields the MSD as a function of the lag time. A coefficient giving the relative rate of diffusion was derived from a linear fit of the curve. The first

Table 1 Direction of axon formation from the HSN cell body

	Direction of axon protrusion					<i>n</i>	Reference
	Dorsal %	Ventral %	Anterior %	Posterior %	Multipolar %		
Wild-type	0	96 ± 2	3 ± 2	0	1 ± 1	221	Kulkarni <i>et al.</i> (2013)
<i>unc-6(ev400)</i>	2 ± 2	3 ± 2	81 ± 2	8 ± 2	6 ± 1	218	Kulkarni <i>et al.</i> (2013)
<i>unc-40(e1430)</i>	2 ± 1	6 ± 2	67 ± 2	19 ± 1	6 ± 1	183	Kulkarni <i>et al.</i> (2013)
<i>unc-5(e53)</i>	0	75 ± 3	19 ± 2	1 ± 1	5 ± 1	245	Yang <i>et al.</i> (2014)
<i>unc-53(n152)</i>	0	67 ± 3	22 ± 2	5 ± 1	6 ± 1	238	Kulkarni <i>et al.</i> (2013)
<i>sax-3(ky123)</i>	2 ± 1	31 ± 1	21 ± 1	37 ± 2	9 ± 2	232	Tang and Wadsworth (2014)
<i>sax-3(ky200)^a</i>	2 ± 1	32 ± 1	19 ± 2	42 ± 3	5 ± 2	198	Tang and Wadsworth (2014)
<i>unc-5(e53);sax-3(ky200)</i>	2 ± 1	40 ± 3	24 ± 2	28 ± 2	6 ± 1	120	
<i>unc-5(e53);unc-6(ev400)</i>	4 ± 2	5 ± 3	59 ± 4	22 ± 4	9 ± 1	201	
<i>unc-5(e53);egl-20(n585)</i>	3 ± 1	28 ± 4	22 ± 4	35 ± 5	11 ± 2	114	
<i>unc-53(n152);unc-5(e53)</i>	0	19 ± 1	62 ± 2	17 ± 1	3 ± 1	224	Kulkarni <i>et al.</i> (2013)
<i>unc-53(n152);unc-6(ev400)</i>	24 ± 2	0	19 ± 2	22 ± 2	34 ± 3	144	Kulkarni <i>et al.</i> (2013)
<i>unc-53(n152);sax-3(ky123)</i>	1 ± 1	47 ± 3	24 ± 2	23 ± 5	6 ± 3	207	Tang and Wadsworth (2014)
<i>unc-40(e1430);unc-5(e53)</i>	5 ± 1	6 ± 1	55 ± 2	19 ± 2	14 ± 1	196	Kulkarni <i>et al.</i> (2013)
<i>unc-40(e1430);sax-3(ky200)^a</i>	14 ± 3	2 ± 1	40 ± 2	35 ± 3	9 ± 4	191	Tang and Wadsworth (2014)
<i>sax-3(ky200)^a; unc-6(ev400)</i>	8 ± 1	8 ± 2	49 ± 3	20 ± 5	14 ± 2	211	Tang and Wadsworth (2014)
<i>unc-53(n152);unc-5(e53);unc-6(ev400)</i>	23 ± 2	0	34 ± 2	15 ± 2	28 ± 2	148	Kulkarni <i>et al.</i> (2013)
<i>unc-53(n152);sax-3(ky200)^a;unc-6(ev400)</i>	11 ± 2	2 ± 1	33 ± 4	30 ± 3	25 ± 5	189	
<i>egl-20(n585)</i>	0	64 ± 2	21 ± 2	7 ± 1	8 ± 1	304	Tang and Wadsworth (2014)
<i>egl-20(n585); unc-6(ev400)</i>	18 ± 2	0	43 ± 2	15 ± 2	24 ± 2	205	Tang and Wadsworth (2014)
<i>unc-40(e1430); egl-20(n585)</i>	6 ± 2	17 ± 2	45 ± 5	15 ± 2	16 ± 2	173	Tang and Wadsworth (2014)
<i>egl-20(n585);sax-3(ky123)</i>	1 ± 1	12 ± 2	39 ± 2	39 ± 1	8 ± 3	177	Tang and Wadsworth (2014)
<i>madd-2(tr103)</i>	0	19 ± 2	55 ± 5	17 ± 4	8 ± 2	179	
<i>madd-2(ky592)</i>	0	52 ± 2	43 ± 2	5 ± 1	0	95	
<i>unc-5(e53);madd-2(tr103)</i>	3 ± 1	15 ± 2	52 ± 4	17 ± 4	13 ± 1	197	
<i>madd-2(tr103);sax-3(ky123)</i>	2	24 ± 3	19 ± 4	47 ± 1	7 ± 2	171	
<i>unc-53(n152);madd-2(tr103)</i>	1 ± 1	15 ± 2	43 ± 2	17 ± 1	24 ± 4	148	
<i>mig-15(rh326)</i>	2 ± 1	15 ± 1	24 ± 3	11 ± 3	48 ± 8	131	Yang <i>et al.</i> (2014)

Numbers represent percentage value ± SEM.

^a Animals grown at the *sax-3(ky200)* restrictive temperature (25°).

two lag time points were not considered, as the paths often approximate a straight line at short intervals.

Data availability

All strains not provided by the *Caenorhabditis* Genetics Center are available upon request. The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

Results

UNC-5 regulates the pattern of outgrowth from the HSN neuron

To investigate whether UNC-5 activity can regulate the length or number of processes that a neuron can develop when outgrowth is toward an UNC-6 source, we examined the development of the HSN axon in *unc-5* mutations. The HSN neuron sends a single axon to the ventral nerve cord, which is a source of the UNC-6 cue (Wadsworth *et al.* 1996; Adler *et al.* 2006; Asakura *et al.* 2007). Axon formation is dynamic (Adler *et al.* 2006). Shortly after hatching, the HSN extends short neurites in different directions. These neurites, which dynamically extend and retract filopodia, become restricted to the ventral side of the neuron where a leading edge forms. Multiple neurites extend

from this surface until one develops into a single axon extending to the ventral nerve cord. Measurements of growth cone size, maximal length, and duration of growth cone filopodia indicate that UNC-6, UNC-40, and UNC-5 control the dynamics of protrusion (Norris and Lundquist 2011).

We observe that in *unc-5* mutants, the patterns of extension are altered. In wild-type animals at the L1 stage of development, most HSN neurons extends more than one short neurite; however, in *unc-5(e53)* mutants, nearly half the neurons do not extend a process (Figure 7, A and B). During the L2 stage in wild-type animals, a prominent ventral leading edge forms and the cell body undergoes a short ventral migration that is completed by the L3 stage. By comparison, in *unc-5* mutants, the cell body may fail to migrate and instead a single large ventral process may form early during the L2 stage (Figure 7, A, C, and E). It may be that the ventral migration of the HSN cell body requires the development of a large leading edge with multiple extensions. Together, the observations indicate that loss of *unc-5* function affects the patterning of outgrowth, *i.e.*, the timing, length, and number of extensions that form. Loss of *unc-5* function does not prevent movement; in fact, a single large ventral extension can form in the mutant at a time that is even earlier than when a single ventral extension can be observed in wild-type animals. The earlier appearance of a single ventral extension

Figure 7 UNC-5 regulates the patterning of outgrowth extensions from the HSN. (A) Photomicrographs of HSN at the L1, L2, and adult stages in wild-type and *unc-5(e53)* mutants. In L1 and L2 animals, neurite extensions (arrows) are often observed in wild-type animals but are rarer in *unc-5* mutants. The short ventral migration of the cell body that occurs in wild-type animal sometimes fails in *unc-5* mutants, leaving the cell body farther from the PLM axon (arrowhead) with a single longer ventral extension. The position of the cell body remains dorsal. Bar, 10 μ m. (B) The percentage of HSN neuron with no, one, or more than one neurite extension at the L1 stage. In *unc-5* mutants, nearly half of the neurons do not extend a process. Error bars indicate the SEM; *n* values are indicated above each column. Significant differences (two-tailed Student's *t*-test), * *P* < 0.001. (C) The percentage of HSN neurons with a single long extension at the L2 stage. Several *unc-5* alleles were tested, as described in the text. In mutants with loss-of-function, there is more often a single extension from the cell body and the cell body is dorsally mispositioned. (D) Photomicrographs of the HSN at the early L4, late L4, and adult stages in wild-type animals and in animals expressing UNC-6 Δ C. The expression of UNC-6 Δ C induces multiple processes, most often two major extensions, that are guided ventrally. Bar, 10 μ m. (E) The percentage of HSN neurons with a cell body mispositioned dorsally at the L2 stage. In loss-of-function mutants, the cell body often fails to undertake a short ventral migration during the L2 stage. The migration is not delayed, but rather it remains dorsal. (F) The percentage of HSN neurons with multiple ventral extensions at the L4 stage. The additional processes induced by UNC-6 Δ C can be suppressed by *unc-5* and *mig-10* mutations. Additional processes induced by *mig-15(rh148)* can also be suppressed by the *unc-5* mutation. (G) Photomicrographs of the HSN at adult stages in a *mig-15* mutant. Similar to UNC-6 Δ C expression, *mig-15* mutations can also cause additional processes that are guided ventrally (Yang *et al.* 2014). Bar, 10 μ m.

in *unc-5* mutants appears to be the result of a difference in morphology, rather than of developmental timing. The failure of the HSN cell body to migrate ventrally and the different pattern of outgrowth at the leading edge causes an earlier discernable single extension.

We tested four different *unc-5* alleles in these experiments. The *unc-5(e53)* allele is a putative molecular null allele, *unc-5(ev480)* is predicted to truncate UNC-5 after the cytoplasmic ZU-5 domain and before the death domain,

unc-5(e152) is predicted to truncate UNC-5 before the ZU-5 domain and death domain, and *unc-5(ev585)* is a missense allele that affects a predicted disulfide bond in the extracellular Ig(C) domain (Killeen *et al.* 2002). Although both the *unc-5(ev480)* and *unc-5(e152)* alleles are predicted to cause premature termination of protein translation in the cytoplasm, the *unc-5(e152)* product retains the signaling activity that prevents these phenotypes. Based on other phenotypes, previous studies have reported that the *unc-5(e152)* allele

Figure 8 UNC-5 regulates the patterning of extension from the ALM, AVM, and PLM. (A) Photomicrographs of the ALM, AVM, and PLM neurons at the L4 stage in wild-type animals and *mig-15* mutants. In wild-type animals, (top) a single PLM axon travels anteriorly from the posterior cell body (not shown). Near the vulva (arrow), the axon branches; one branch extends to the ventral nerve cord and another extends anteriorly. The anterior extension terminates before reaching the area of the ALM cell body. In *mig-15* mutants, the PLM can extend anteriorly past the ALM cell body (bottom). Bar, 10 μ m. (B) The percentage of PLM neurons where the PLM neuron extend anteriorly past the ALM cell body. The anterior extension often overextends in *mig-15* mutants. Loss of *unc-5* or *unc-40* function can suppress this phenotype. (C) Photomicrographs of the ALM and AVM neurons at the L4 stage in wild-type animals and mutants showing different patterns of outgrowth extension. In wild-type animals (top), a single axon travels anteriorly to the nerve ring (arrowheads). At the nerve ring, the axon branches; one branch extends further anteriorly and the other extends into the nerve ring. In mutants, one or both axons may only extend anteriorly and will not extend into the nerve ring (second from top), one or both axons will only extend into the nerve ring and will not extend anteriorly (third from top), or one or both axons will fail to extend into either the nerve ring or anteriorly (bottom). Bar, 20 μ m. (D) The percentage of AVM neurons where the AVM neuron failed to extend into the nerve ring. The neuron often fails to extend in the *unc-40* and *mig-15;unc-5* mutants, whereas it does extend in the *mig-15*, *unc-5*, and *mig-15;unc-40* mutants. Error bars indicated the SEM; *n* values are indicated above each column. Significant differences (two-tailed Student's *t*-test), * *P* < 0.001. (E) The percentage of AVM neurons where the AVM neuron failed to extend anteriorly, past the nerve ring. The neuron often fails to extend anteriorly in the *mig-15;unc-5* mutants, whereas it does extend in the *mig-15*, *unc-5*, *unc-40*, and *unc-40;mig-15* mutants. There is a significant difference between the *unc-40* and *unc-40;mig-15* mutants.

retains UNC-40-dependent signaling functions (Merz *et al.* 2001; Killeen *et al.* 2002).

UNC-5 is required for the induction of multiple HSN axons by UNC-6 Δ C and a *mig-15* mutation

The results above suggest that UNC-5 activity can regulate the number of HSN extensions that form. To further test this hypothesis, we checked whether loss of UNC-5 function can suppress the development of additional processes that can be induced. Previously, we reported that expression of the N-terminal fragment of UNC-6, UNC-6 Δ C, induces excessive branching of ventral nerve cord motor neurons and that loss of UNC-5 function can suppress this branching (Lim *et al.* 1999). We now report that the HSN develops an extra process in

response to UNC-6 Δ C and that loss of UNC-5 function suppresses the development of this extra process (Figure 7, D and F).

To investigate whether this UNC-5 activity might involve known effectors of asymmetric neuronal outgrowth, we tested for genetic interactions between *unc-5* and both *mig-10* and *mig-15*. MIG-10 (lamellipodin) is a cytoplasmic adaptor protein that can act cell-autonomously to promote UNC-40-mediated asymmetric outgrowth (Adler *et al.* 2006; Chang *et al.* 2006; Quinn *et al.* 2006, 2008; McShea *et al.* 2013). MIG-15 (NIK kinase) is a cytoplasmic protein and evidence indicates that *mig-15* functions cell-autonomously to mediate a response to UNC-6 (Poinat *et al.* 2002; Teulière *et al.* 2011). It is proposed that *mig-15* acts with *unc-5* to polarize the

Figure 9 Assay to measure the effects that a mutation has on movement. (A) The direction of outgrowth extension from the HSN cell body can vary, and whether the axon developed in the dorsal (d), anterior (a), posterior (p), or ventral (v) direction in L4 stage animals is scored (left panel). This creates a probability distribution in which the direction (X) is a random variable (center panel). A simple random walk is generated by using the same probability distribution for a succession of steps with an equal time interval (right panel). (B) For wild-type animals and two mutants, 50 simulated random walks of 500 steps were plotted from an origin (0,0). The results graphically indicate the directional bias for movement. For random walk movement created in mutant A [red, results from *unc-5(e53)*], the directional bias is shifted anteriorly (left) relative to wild-type animals. The results also graphically show the displacement of movement. For random walk movement created in mutant B [blue, results from *egl-20(n585);sax-3(ky123)*], the average of the final position (displacement) from the origin is a much shorter distance than that of wild-type animals. (C) Plots of the normal distribution of the final position along the x-axis of the random walk tracks shown in (B). The mean position for each is set at 0. The plots graphically illustrate how random walks constructed from the probability distribution for the direction of outgrowth extensions can reveal a diffusion process.

growth cone's response, and that it controls the asymmetric localization of **MIG-10** and **UNC-40** (Teulière *et al.* 2011; Yang *et al.* 2014). We previously noted that HSN neurons often become bipolar in *mig-15* mutants and frequently **UNC-40::GFP** is localized to multiple surfaces in a single neuron, suggesting that loss of **MIG-15** enhances the ability of **UNC-40::GFP** to cluster (Yang *et al.* 2014). In our experiments, we used the *mig-10* (*ct141*) loss-of-function allele (Manser and Wood 1990; Manser *et al.* 1997) and the *mig-15* (*rh148*) allele, which causes a missense mutation in the ATP-binding pocket of the kinase domain and is a weak allele of *mig-15* (Shakir *et al.* 2006; Chapman *et al.* 2008).

We find that the extra processes induced by **UNC-6ΔC** expression are suppressed by *mig-10*(*ct141*) (Figure 7F). We also find that the *mig-15* mutation causes extra HSN processes and that the loss of **UNC-5** function suppresses these extra HSN processes (Figure 7, F and G). These results support the hypothesis that the ability of **UNC-5** to regulate the development of multiple protrusions involves the mo-

lecular machinery that controls **UNC-40**-mediated asymmetric neuronal outgrowth.

UNC-5 is required for PLM overextension

The SOAL model predicts that the ability of **UNC-5** to regulate the length and number of neural protrusions is independent of the direction of outgrowth. The HSN sends a single axon ventrally, while PLM sends an axon anteriorly from a posteriorly positioned cell body. The HSN axon travels toward **UNC-6** sources, whereas the PLM axon pathway is perpendicular to **UNC-6** sources. To investigate whether **UNC-5** activity can regulate the length or number of processes that develop perpendicular to **UNC-6** sources, we examined the development of the PLM axon. We also chose PLM because **UNC-5** was already known to affect the length of the PLM axon (Li *et al.* 2008).

Given that **UNC-5** activity is involved in the overextension of the PLM axon, and that the *mig-15* mutation affects HSN outgrowth in an **UNC-40**-dependent fashion, we decided to test whether PLM overextension might be induced by the

Figure 10 Mutations have different effects on movement. Examples of random walk analyses using the direction of axon development from the HSN neuron in different mutants (Table 1). The graphs were created as described in the figure legend of Figure 9. For each panel, plots are shown for the normal distribution of the final position along the x-axis for the random walk tracks plotted in the inserts. The inserts depict the random walk movement that would be produced by the probability distribution for the direction of outgrowth in the mutant. Plots derived from the same data are colored alike. Each panel depicts the analyses of four different mutants and wild-type animals. Three different distribution patterns are observed: (1) the wild-type distribution, which has the distribution curve with the highest peak; (2) the *unc-5*, *egl-20*, *unc-53*, and *unc-6* (data not shown) distribution, which is flatter than the wild-type curve; and (3) the *madd-2*, *sax-3*, *mig-15*, and double combinations, which have the flattest distribution curve.

mig-15 mutation in an *UNC-40*-dependent fashion. The HSN results suggest that altering *mig-15* function creates a sensitized genetic background. That is, the *unc-5(ev480)* mutation suppresses HSN outgrowth extension in both the wild-type and *mig-15(rh148)* backgrounds, but the *mig-15* mutation creates a stronger patterning phenotype. This idea is supported by the evidence that the *mig-15* mutation enhances the ability of *UNC-40* to localize at surfaces (Yang *et al.* 2014).

We find that in *mig-15(rh148)* mutants, the PLM axon often fails to terminate at its normal position and instead extends beyond the ALM cell body. This overextension is suppressed in *unc-5(ev480);mig-15(rh148)* and *unc-40(e1430);mig-15(rh148)* mutants (Figure 8, A and B). The results are consistent with the idea that *UNC-5* is required for the *UNC-40*-mediated outgrowth activity that causes overextension in *mig-15(rh148)* mutants.

***UNC-5* is required for ALM and AVM branching and extension**

We also investigated the effect of *UNC-5* activity on patterning where sources of *UNC-6* and other cues are in a more complex arrangement. Specifically, we examined whether *UNC-5* plays

a role in the outgrowth of AVM and ALM processes at the nerve ring. During larval development, processes from the AVM neuron and the two ALM neurons (one on each side of the animal) migrate anteriorly to the nerve ring at dorsal and ventral positions, respectively (Figure 8C). At the nerve ring, each axon branches; one branch extends further anteriorly and the other extends into the nerve ring. Evidence suggests that at the mid-body of the animal, the positioning of these axons along the dorsal-ventral axis requires *UNC-6*, *UNC-40*, and *UNC-5* activity. In *unc-6*, *unc-40*, and *unc-5* null mutants, or when the *UNC-6* expression pattern is altered, the longitudinal nerves are mispositioned (Ren *et al.* 1999). Glia cells and neurons at the nerve ring are sources of *UNC-6* (Wadsworth *et al.* 1996). The guidance of some axons in the nerve ring are disrupted in *unc-6* and *unc-40* mutants (Hao *et al.* 2001; Yoshimura *et al.* 2008). The precise spatial and temporal arrangement of the *UNC-6* cue in relationship to the position of the migrating growth cones is not fully understood. Nevertheless, the anteriorly migrating growth cones appear to use the *UNC-6* cue from the ventral sources to help maintain the correct dorsal-ventral position, even while moving toward the nerve ring,

Figure 11 Mutations alter the spatial extent of movement. (A) Plotted are the mean squared displacement (MSD) curves as a function of time interval (dt). The values are in arbitrary units, since the timescale was arbitrarily set at 1. The curves show the extent to which different mutations can alter the MSD relative to wild-type animals and the MSD caused by an unbiased random walk. For each time interval, mean and SEM are plotted. (B) From the slope of MSD curves, a coefficient can be derived that gives the relative rate of diffusion. Colored bars correspond to the like-colored curves given in (A). The coefficients for *unc-5*, *egl-20*, *unc-53*, and *unc-6* form a class that is distinct from that derived from wild-type animals and from the double mutants.

which is a new source of *UNC-6* that is perpendicular to the ventral source. At the nerve ring the axons branch. One process continues anteriorly, moving past the new *UNC-6* source, whereas the other projects at a right angle and moves parallel to the new source.

We find genetic interactions involving *unc-5*, *unc-40*, and *mig-15* that affect outgrowth patterning of the ALM and AVM extensions at the nerve ring (Figure 8, C–E). In *mig-15(rh148)*; *unc-5(e53)* mutants, the AVM axon often fails to extend anteriorly from the branch point and only extends into the nerve ring,

Figure 12 Models for the relationship between UNC-40-mediated outgrowth activity and UNC-40 receptor clustering. (A) The statistically-oriented asymmetric localization (SOAL) process is illustrated as in Figure 1. In this model, the self-organizing UNC-40 SOAL process causes observable UNC-40 receptor clustering. UNC-6 stabilizes receptor clustering at a site and the outgrowth machinery is then recruited to cause outgrowth at the site. Although the initial direction of asymmetric receptor localization is determined stochastically, the direction of outgrowth is determined by the site of stabilization. (B) In this model, the self-organizing UNC-40 SOAL process is coupled to the outgrowth machinery. The direction of both asymmetric receptor localization and outgrowth activity are stochastically determined. Observable receptor clustering arises as the result of the process, because receptor localization can become successively concentrated to a smaller area over time. Cluster formation is an observable phenomenon of the process, not a prerequisite for outgrowth activity. This model postulates innumerable fluctuating sites that generate force in various directions along the membrane.

fails to extend into the nerve ring and only extends anteriorly, or fails to do both and terminates at this point. In *unc-40(e1430)* mutants, the axon often fails to branch into the nerve ring, although it extends anteriorly. In comparison, in *unc-40(e1430); mig-15(rh148)* mutants, more axons extend into the nerve ring. These results suggest that *UNC-5* (and *MIG-15*) helps regulate UNC-40-mediated outgrowth to pattern the outgrowth at the nerve ring.

Interactions between *unc-5* and other genes affect a probability distribution for the direction of extension

We hypothesize that there are interactions between *unc-5* and other genes that control the degree to which the direction of outgrowth fluctuates. Probability distributions for the direction of extension are used to study how genes affect the fluctuation of outgrowth activity. By comparing the distributions created from wild-type and mutant animals, the relative effect that genes have on the fluctuation can be determined. To accomplish this, the direction that the HSN axon initially extends from the cell body is scored (Figure 9A).

Using this assay, we examined genetic interactions between *unc-5* and four other genes; *egl-20*, *sax-3*, *madd-2*, or *unc-6*. We have chosen these particular genes because previous observations suggest interactions. (1) *EGL-20* (Wnt) is a secreted cue expressed from posterior sources (Pan *et al.* 2006) and it affects the surface of the HSN to which the *UNC-40* receptor localizes and mediates outgrowth (Kulkarni *et al.* 2013). Based on a directional phenotype, a synergistic interaction between *unc-5* and *egl-20* has been observed. In either *unc-5* or *egl-20*

A Dorsal-ventral orientation of UNC-40 asymmetric localization

B Anterior-posterior orientation of UNC-40 asymmetric localization

Figure 13 Mutations affect asymmetric intracellular UNC-40::GFP localization. (A) Graph indicating the dorsal–ventral localization of UNC-40::GFP in the HSN. The graph shows the average ratio of dorsal-to-ventral intensity from line-scan intensity plots of the UNC-40::GFP signal around the periphery of the HSN cell. UNC-40::GFP is ventrally localized in wild-type animals, but the ratio is different in the mutants. Error bars represent SEM. Below is a graphic representation of the possible UNC-40 localization patterns when the intensity ratio is ≥ 1 or < 1 . (B) Graph indicating the anterior–posterior localization of UNC-40::GFP. To determine orientation, line-scan intensity plots of the UNC-40::GFP signal across the dorsal periphery of the HSN cell were taken, the dorsal surface was geometrically divided into three equal segments, and the total intensity of each was recorded. The percent intensity was calculated for each segment and ANOVA was used to determine if there is a significant difference between the three segments (see *Materials and Methods*). The graph shows the percent of animals that had significant localization in the indicated segments or that had uniform distribution. Whereas in the *unc-5* and *egl-20* mutants there is a bias for anterior or posterior localization, there is a uniform distribution in *unc-5;egl-20* double mutants. Uniform distribution is also observed in strong loss-of-function *sax-3* and *madd-2* mutants. (*) Animals grown at the *sax-3(ky200)* restrictive temperature (25°). Below each graph is a representation of the possible UNC-40 localization patterns. The orientation of UNC-40 localization is color-coded as in (B).

mutants, the ventral extension of the AVM and PVM axons is only slightly impaired, whereas in the double mutants there is much greater penetrance (Levy-Strumpf and Culotti 2014). (2) *SAX-3* (Robo) is a receptor that regulates axon guidance and is required for the asymmetric localization of UNC-40 in the HSN (Tang and Wadsworth 2014). Based on a directional phenotype, *SAX-3* and UNC-40 appear to act in parallel to guide the HSN toward the ventral nerve cord (Xu *et al.* 2015). (3) *MADD-2* is a cytoplasmic protein of the tripartite motif family that potentiates UNC-40 activity in response to UNC-6 (Alexander *et al.* 2009, 2010; Hao *et al.* 2010; Morikawa *et al.* 2011; Song *et al.* 2011; Wang *et al.* 2014). *MADD-2*::GFP and F-actin colocalize with UNC-40::GFP clusters in the anchor cell

(Wang *et al.* 2014). (4) Of course, UNC-6 is an UNC-5 ligand. DCC (UNC-40) and UNC5 (UNC-5) are thought to act independently or in a complex to mediate responses to netrin (UNC-6) (Colavita and Culotti 1998; Hong *et al.* 1999; MacNeil *et al.* 2009; Lai Wing Sun *et al.* 2011).

In a test for interaction with *egl-20*, we found that, in comparison to *unc-5(e53)* or *egl-20(n585)* mutants, the *unc-5(e53);egl-20(n585)* double mutant had a lower probability for ventral outgrowth and higher probability for outgrowth in other directions (Table 1). This suggests that *unc-5* and *egl-20* may act in parallel to achieve the highest probability for HSN ventral outgrowth, *i.e.*, they act to prevent UNC-40-mediated outgrowth from fluctuating in other directions.

Figure 14 Model for the outgrowth movement of the PLM. Schematic diagrams of the anteriorly directed outgrowth of the PLM. The features of the schematic are presented in Figure 3. (A) An extension encounters different levels of extracellular cues at each of three positions (1–3). Cues dorsal and ventral to the pathway maintain an equal probability of outgrowth in both directions. The extension encounters increasing levels of an extracellular cue(s) as it moves toward position 3. At position 3, a cue(s) prevent further anterior extension in wild-type animals. (B) Table showing the positive feedback (arrows) and negative feedback (lines) for each of the positions depicted in (A), associated with statistically-oriented asymmetric localization (SOAL) activity (see Figure 6) and the predicted effect that the SOAL activity has on outgrowth activity. At position 1, strong UNC-40 activity along the dorsal- and ventral-facing (not shown) surfaces of the leading edge inhibit non-UNC-40 activity. Because of the statistically-oriented asymmetric localization (SOAL) process, this inhibition increases non-UNC-40 activity at the anterior surface of the leading edge. At positions 2 and 3, increasing levels of the non-UNC-40 activity at the dorsal and ventral surfaces inhibits UNC-40 activity. The increase in non-UNC-40 activity at the dorsal and ventral surfaces causes a decrease in non-UNC-40 activity at the anterior surface. As a result, the degree to which the direction of non-UNC-40-mediated outgrowth activity fluctuates is greatest at position 3. (C) For each position in (A), random walk modeling is shown as described in Figure 2E. The response to the extracellular cues progressively increases the degree to which the direction of outgrowth activity fluctuates. By position 3, the degree of fluctuation causes a much lower displacement of membrane mass. The low rate of outward movement causes extension to stall.

In a test for interaction with *sax-3*, we found that the probability of outgrowth in each direction in *unc-5(e53); sax-3(ky200)* mutants was similar to the probabilities in *sax-3(ky200)* or *sax-3(ky123)* mutants (Table 1). Given the results with *unc-5* and *egl-20*, we further tested the probability of outgrowth in each direction in *egl-20(n585); sax-3(ky123)* mutants. We found that it is similar to the probabilities in *sax-3(ky200)* or *sax-3(ky123)* mutants (Table 1). The *sax-3(ky123)* allele results in a deletion of the signal sequence and first exon of the gene, whereas *sax-3(ky200)* contains a missense mutation that is thought to cause protein misfolding and mislocalization at the restrictive temperature (25°) (Zallen *et al.* 1998; Wang *et al.* 2013). The *egl-20(n585); sax-3(ky123)* mutants do not grow well and so it is easier to use the temperature-sensitive *sax-3* allele. Together, the results suggest that *sax-3* may be required for both the *unc-5*- and the *egl-20*-mediated activities that allow the highest probability for HSN ventral outgrowth.

In a test for interaction with *madd-2*, we found that the probability of outgrowth in each direction in *unc-5(e53); madd-2(tr103)* mutants was similar to the probabilities in *madd-2(tr103)* mutants (Table 1). There was a higher probability for anterior HSN outgrowth, similar to what is observed in *unc-40(e1430)* mutants. These results suggest that *madd-2* might be required for

the *unc-40* outgrowth activity. The probability of outgrowth in each direction in *madd-2(tr103); sax-3(ky123)* mutants is similar to the probabilities in *sax-3(ky200)* or *sax-3(ky123)* mutants (Table 1). The *madd-2(tr103)* allele appears to act as a genetic null (Alexander *et al.* 2010).

In a test for interaction with *unc-6*, we found that the probability of outgrowth in each direction in *unc-5(e53); unc-6(ev400)* and *unc-40(e1430); unc-5(e53)* mutants was similar to the probabilities in *unc-6(ev400)* mutants, insofar as there is a lower probability for ventral outgrowth and a higher probability for anterior outgrowth (Table 1). However, the probabilities in each direction are closer to those obtained from the *unc-40(e1430)* mutants because the probability of anterior outgrowth is lower in these mutants than in *unc-6* mutants. This suggests that *UNC-5* and *UNC-40* might help increase the probability of anterior outgrowth in the absence of *UNC-6*.

***unc-5* is a member of a class of genes that has a similar effect on the spatial extent of movement**

The results above show that *unc-5* and its interactions with other genes affect the degree to which the direction of outgrowth fluctuates. The degree of fluctuation differs depending on the genes involved. A property of random movement is that the more the direction of movement fluctuates, the shorter the

Figure 15 Model for the effects that mutations have on the outgrowth movement of the PLM. Table showing the effects of different mutations on the outgrowth of the PLM at position 3, Figure 14. PLM outgrowth stalls in wild-type animals, and *unc-40*, and *unc-5;mig-15* mutants, at position 3, but overextends in *mig-15* mutants. In this model, the *mig-15* mutation represses the ability of non-UNC-40 statistically-oriented asymmetric localization (SOAL) activity to suppress UNC-40 activity at the dorsal and ventral surfaces. Increased UNC-40 SOAL activity suppresses non-UNC-40 SOAL activity at these surfaces. Because of the statistical dependence of the localization process, decreasing non-UNC-40 SOAL activity at the dorsal and ventral surfaces increases non-UNC-40 SOAL activity at the anterior surface. As compared to wild-type animals, the degree to which the direction of non-UNC-40 outgrowth activity fluctuates is less and, therefore, outward displacement is greater. This allows further anterior outgrowth at position 3. Loss of UNC-5 function in the *mig-15* mutant decreases the ability of UNC-40 SOAL activity to suppress non-UNC-40 SOAL activity at the dorsal and ventral surfaces, thereby increasing the degree to which the direction of non-UNC-40 outgrowth activity fluctuates. This reduces outward displacement and suppresses the overextension phenotype.

distance of travel is in a given amount of time (Figure 2E). To depict how *unc-5* and other genes differentially regulate the spatial extent of movement, we use random walk modeling. Random walks describe movement that occurs as a series of steps in which the angles and the distances between each step are decided according to some probability distribution. By using the probability distribution obtained from a mutant for each step of a random walk, and by keeping the distance of each step equal, a random walk can be constructed (Figure 9A). In effect, this method applies the probability distribution to discrete particles having idealized random walk movement on a lattice. By plotting random walks derived from wild-type animals and different mutants, the relative effect that mutations have on random walk movement can be visualized. For example, Figure 9B shows 50 tracks of 500 steps for wild-type animals and two mutants [mutant A is *unc-5(e53)* and mutant B is *egl-20(n585);sax-3(ky123)*]. This reveals the effect that a mutation has on the displacement of movement. After 500 steps, the displacement from the origin (0,0) is on average less for mutant A than for wild-type animals, and less for mutant B than for wild-type animals or mutant A.

The random walk models show the relative effect that a mutation has on a property of outgrowth movement. It is

worth noting that this is not modeling the actual trajectory of migrating axons. As discussed in the *Introduction*, neuronal outgrowth is essentially a mass transport process in which mass (the molecular species of the membrane) is sustained at the leading edge and moves outward. Our assay compares the effect that different mutations would have on the movement of mass at the leading edge of an extension if the conditions of the system were kept constant. Of course, the conditions are not constant *in vivo*. For one, as an extension moves, it will encounter new environments where the cues may be new or at different concentrations, all of which affect the probability distribution. The actual patterns of outgrowth observed are the result of all the probabilities for outgrowth that occur at each instance of time. It has recently been suggested that our description might be more accurately described as neuro-percolation, a superposition of random-walks (Aiello 2016).

Our random walk analysis compares the effect that different mutations have on the properties of movement. In wild-type animals, there is a high probability for outgrowth in the ventral direction. The analysis shows that conditions in wild-type animals create nearly straight-line movement, *i.e.*, if the same random walk is repeatedly done for the same number of steps, starting at the same origin, the final position of the walk along the x-axis does not vary a great amount. In comparison, we find that a mutation can create random walk movement in which the final position is more varied. This variation occurs because the mutation increases the probability of outgrowth in other directions. For each mutation, we simulate 50 random walks of 500 steps and derive the mean and SD of the final position along the x-axis. To compare strains, we plot the normal distribution, setting the mean at the same value for each. The difference between the curve for a mutant and wild-type animal shows the degree to which the mutation causes the direction of outgrowth to fluctuate (Figure 9C).

The results reveal four different distribution patterns (Figure 10). The first class is the wild-type distribution, which has the distribution curve with the highest peak. The second class comprises *unc-5*, *egl-20*, *unc-53*, and *unc-6*, in which the distribution curve is flatter than the wild-type curve. We included *unc-53* because our previous study showed that it has genetic interactions with *unc-5* and *unc-6* (Kulkarni *et al.* 2013). The *unc-53* gene encodes a cytoskeletal regulator related to the mammalian NAV proteins and *unc-53* mutations cause guidance defects (Maes *et al.* 2002; Stringham *et al.* 2002; Stringham and Schmidt 2009). The third class has a distribution curve that is flatter than the second and comprises *sax-3*, *mig-15*, and several double-mutation combinations (Figure 10). The fourth class has the flattest distribution curve and comprises *egl-20;sax-3*, *unc-40;sax-3*, and *unc-53;sax-3;unc-6*. This class indicates the greatest degree of fluctuation. The ability to cause the direction of movement to fluctuate is not associated with a specific direction of HSN movement. For example, *unc-5;sax-3*, *unc-53;unc-6*, *unc-40;egl-20*, and *madd-2;sax-3* each show a widely dispersed pattern, but the direction is ventral, dorsal, anterior, and posterior, respectively (Figure 10).

A position

B leading edge activity

C mass displacement

lowers cues that are arranged dorsally and anteriorly to the projection to effectively create a bias for outgrowth in the respective direction (see Figure 4). A dorsal directional bias will develop because UNC-40 and other receptors mediate an outgrowth response to UNC-6 and other cues along the nerve ring. An anterior directional bias will develop because non-UNC-40 mediates an outgrowth response to cues along an anterior pathway.

The distribution patterns indicate that genes have different effects on the extent to which outgrowth movement can travel through the environment. MSD is a measure of the spatial extent of random motion. The MSD can be calculated from the random walk data. Plotting MSD as a function of the time interval shows how much an object displaces, on average, in a given interval of time, squared (Figure 11A). For normal molecular diffusion, the slope of the MSD curve is directly related to the diffusion coefficient. In cell migration models, this value is referred to as the random motility coefficient. Coefficients are experimentally determined; they describe how long it takes a particular substance to move through a particular medium. We determine this value to numerically and graphically compare how mutations can alter displacement relative to wild-type animals (Figure 11B). The four classes of genes are apparent by comparing the height of the bars in Figure 11B. Results for the *unc-40* mutation are also shown. The random walk pattern is published (Tang and Wadsworth 2014).

The results of this modeling suggest that the activities of certain genes, and combinations of genes, have distinct effects on the rate of outgrowth movement. In theory, these differences could be an important means by which genes cause different outgrowth patterns.

UNC-40 receptor clustering is coupled to the SOAL process

We investigated the relationship between UNC-40::GFP localization and outgrowth movement. Beginning in the early L2 stage, UNC-40::GFP becomes localized to the ventral side of the HSN in wild-type animals (Adler *et al.* 2006; Kulkarni *et al.* 2013). Reflecting the dynamic morphological changes that occur as the HSN axon forms, the site of asymmetric UNC-40::GFP localization alternates in the neurites and along

Figure 16 Model for the outgrowth movement of the AVM at the nerve ring. Schematic diagrams of the outgrowth of AVM at the nerve ring. The features of the schematic are described in Figure 3. (A) At position 1, all surfaces experience high levels of UNC-6. At position 2, the extension encounters new cue(s) at the anterior surface. (B) Table showing, for each of the positions depicted in (A), the positive (arrows) and negative feedback (lines) associated with statistically-oriented asymmetric localization (SOAL) activity (see Figure 6), and the predicted effect that the SOAL process has on outgrowth activity. At position 1, strong UNC-40 SOAL activity along anterior- and dorsal-facing surfaces of the leading edge inhibits non-UNC-40 SOAL activity. There is strong UNC-40-mediated outgrowth from all surfaces. At position 2, non-UNC-40 SOAL activity at the anterior surface inhibits UNC-40 SOAL activity, whereas UNC-40 SOAL activity at the dorsal surface inhibits non-UNC-40 SOAL activity. As a result, UNC-40 outgrowth activity is limited to the dorsal surface and non-UNC-40 outgrowth activity is limited to the anterior surface. (C) Random walk modeling is shown as described in Figure 2E. Because of the large degree to which the direction of outgrowth fluctuates, outward movement stalls. This al-

the ventral surface of the neuron (Kulkarni *et al.* 2013). Dynamic UNC-40::GFP localization patterns have also been reported for the anchor cell, in which UNC-40 and UNC-6 are also key regulators of extension (Ziel *et al.* 2009; Hagedorn *et al.* 2013). Live imaging of the anchor cell reveals that UNC-40::GFP clusters form, disassemble, and reform along the membrane (Wang *et al.* 2014). However, live imaging cannot directly ascertain whether the position of a cluster is randomly determined, since a movement event cannot be repeatedly observed to determine a probability distribution. Mathematical modeling of cluster movement as a stochastic process has not been done.

The UNC-40::GFP clustering phenomenon raises questions about the relationship between robust UNC-40 clustering (*i.e.*, sites of distinct UNC-40 localization observable by UNC-40::GFP) and UNC-40-mediated outgrowth activity. Two models are presented in Figure 12. In the first model, the output of the SOAL process is receptor clustering (Figure 12A). After a cluster becomes stabilized at a site, the machinery required for outgrowth is recruited and outgrowth occurs. In our model, the SOAL process and UNC-40-mediated outgrowth activity are coupled, and are part of the same stochastic process that occurs at the microscale (Figure 12B). UNC-40::GFP clustering is a macroscale event that can be observed. It is a consequence of the microscale events.

The models make specific predictions that can be tested. In the first model, UNC-40-mediated outgrowth will not happen if UNC-40 does not cluster. In our model, the loss of UNC-40 clustering does not lead to a loss of UNC-40-mediated outgrowth. In the *sax-3* mutant, there is a large fluctuation in the direction of outgrowth; it is in the third class of mutants (Figure 10 and Figure 11). We previously reported that *sax-3* is required for robust UNC-40::GFP asymmetric localization; in *sax-3*

Figure 17 Model for the effects that mutations have on the outgrowth movement of AVM at the nerve ring. Table showing the effects of different mutations on the outgrowth of AVM at position 2, Figure 16. Whereas in wild-type animals, UNC-40 statistically-oriented asymmetric localization (SOAL) activity suppresses non-UNC-40 SOAL activity at the dorsal surface, in *unc-40* mutants there is no suppression and non-UNC-40 activity may occur. However, non-UNC-40 activity is greater at the anterior surface because of the response to anterior cues and this depresses non-UNC-40 activity at the dorsal surface because of the SOAL process. As a result, there is often anterior extension from the nerve ring area but no dorsal extension into the nerve ring. In *unc-5* mutants, UNC-40 SOAL activity is reduced but it is still dorsally oriented. This

allows dorsal extension in the mutants. In *mig-15* mutants, non-UNC-40 SOAL activity is repressed. This results in lower non-UNC-40 activity at the anterior surface. However, anterior extension still occurs, as does dorsal extension because of UNC-40 activity. Loss of UNC-40 in the *mig-10* background allows extension in both directions. In comparison to the single *unc-40* mutant, the reduced non-UNC-40 SOAL activity at the anterior surface in the double *unc-40;mig-15* mutant does not depress dorsal non-UNC-40 outgrowth activity as much, allowing more extension into the nerve ring. Loss of UNC-5 in the *mig-10* background causes the most abnormal outgrowth morphologies, presumably because the repression of both UNC-40 and non-UNC-40 SOAL activities does not allow UNC-40 and non-UNC-40 outgrowth activities to be well sorted to different surfaces.

mutants, UNC-40::GFP remains uniformly dispersed around the periphery of the HSN (Tang and Wadsworth 2014; Figure 13). Whereas in the *sax-3* mutant there is a ventral bias for outgrowth, in the *unc-40;sax-3* mutant there is not (Figure 10). This suggests that in the *sax-3* mutant there is UNC-40-mediated outgrowth activity that helps create a ventral bias. This is consistent with our model because UNC-40-mediated outgrowth activity is occurring even when robust UNC-40::GFP is not observed.

We hypothesize that a consequence of the microscale SOAL process over time is macroscale UNC-40 clustering. If so, then *unc-5* activity should affect UNC-40::GFP clustering because it affects the degree to which the direction of UNC-40 receptor localization fluctuates. However, even though there is a higher probability that localization occurs at surfaces other than at the ventral surface, we observe robust asymmetrically localized UNC-40::GFP clustering in *unc-5(e53)* mutants (Kulkarni *et al.* 2013). We speculate that *unc-5(e53)*, as well as other gene mutations, do not cause the direction of UNC-40 localization to fluctuate enough to prevent observable UNC-40::GFP clustering. Therefore, we decided to examine UNC-40::GFP clustering in double mutants to determine whether the ability to observe UNC-40::GFP clustering is correlated with the degree of fluctuation.

We made double-mutant combinations between *unc-5* and *egl-20* or *unc-53*. In *egl-20* and *unc-53* single mutants, there was fluctuation in the direction of outgrowth (Figure 10 and Figure 11) and robust asymmetrical UNC-40::GFP localization [Figure 13; *unc-53* results were previously reported (Kulkarni *et al.* 2013)]. In comparison to the single mutants, the double mutants all showed an increase in the degree to which the direction of outgrowth fluctuated (Figure 10 and Figure 11). Further, in contrast to the single mutants, UNC-40::GFP remained uniformly dispersed around the periphery of the HSN in the double mutants (Figure 13). The results suggest a correlation between increased fluctuation of UNC-40-mediated outgrowth activity and the ability to detect UNC-40::GFP clustering. This is

consistent with our model (Figure 12B). We also observed that in *madd-2(tr103)* mutants the direction of outgrowth fluctuates (Table 1), but unlike *egl-20* and *unc-53* single mutants, there was not robust asymmetrical UNC-40::GFP localization and UNC-40::GFP remained uniformly dispersed (Figure 13). The double mutant *unc-5;madd-2* was similar to the single *madd-2* mutant. Similar results are observed with *sax-3* and *unc-5;sax-3* mutants (Figure 13). We hypothesize that in the *madd-2* and *sax-3* mutations, the degree to which the direction of UNC-40 localization fluctuates is so great that the *unc-5* mutation makes no difference to the UNC-40::GFP clustering phenotype.

Discussion

We have proposed a model of neuronal outgrowth movement that is based on SOAL. This model states that the probability of UNC-40 localizing and mediating outgrowth at one site affects the probability of localization and outgrowth at other sites as well. By regulating this process, genes control the degree to which the direction of outgrowth fluctuates and, consequently, the outward movement of the plasma membrane. UNC-5 is a receptor for UNC-6 and can form a complex with UNC-40. UNC-5 is commonly proposed to direct outgrowth by mediating a repulsive response to UNC-6. In contrast, our model is not based on the concept of repulsion and it predicts that UNC-5 can control the rate of outward movement that is directed toward, away from, or perpendicular to UNC-6 sources. We report that *unc-5* loss-of-function mutations affect the development of multiple neurites that develop from the HSN and extend toward UNC-6 sources. They also suppress the development of extra HSN processes that are induced by a *mig-15* mutation or by expression of the N-terminal fragment of UNC-6, and which extend toward UNC-6 sources. We also observe that *unc-5* mutations suppress the anterior overextension of the PLM axon that occurs in the *mig-15* mutant. This axon extends perpendicular to UNC-6 sources.

Figure 18 Model for the outgrowth movement of the HSN. Schematic diagrams of the ventral outgrowth of the HSN. The features of the schematic are described in Figure 3. (A) As the leading edge of the extension moves ventrally it encounters higher levels of UNC-6. At position 3, all surfaces experience high levels of UNC-6. Cues anterior and posterior to the pathway maintain an equal probability of outgrowth in both directions. (B) Table showing, for each of the positions depicted in (A), the positive feedback (arrows) and negative feedback (lines) associated with statistically-oriented asymmetric localization (SOAL) activity (see Figure 6) and the predicted effect that the SOAL process has on outgrowth activity. At position 1, strong UNC-40 SOAL activity along the ventral-facing surfaces of the leading edge inhibits non-UNC-40 SOAL activity. Because of the SOAL process, this inhibition increases non-UNC-40 activity at the anterior and posterior (data not shown) surfaces of the leading edge. Non-UNC-40 SOAL activity along the anterior and posterior surfaces suppresses UNC-40 SOAL activity at these surfaces. At positions 2 and 3, increasing levels of the UNC-40 SOAL activity at the anterior and posterior surfaces inhibit non-UNC-40 SOAL activity. The increase in UNC-40 activity at the anterior and posterior surfaces cause a decrease in non-UNC-40 SOAL activity. As a result, the degree to which the direction of UNC-40-mediated outgrowth activity fluctuates is greatest at position 3. (C) For each position in (A), random walk modeling is shown as described in Figure 2E. At first, the response to the extracellular cues may progressively decrease the degree to

which the direction of UNC-40 and non-UNC-40 outgrowth activity fluctuates. However, as UNC-40 SOAL activity predominates at all surfaces, the degree by which the direction of UNC-40 outgrowth fluctuates increases. By position 3, the degree of fluctuation causes a much lower displacement of membrane mass.

Finally, *unc-5* loss-of-function mutations affect the branching and extension of the ALM and AVM axons at the nerve ring, where the sources of UNC-6 are in a more complex arrangement. Below, we discuss how the SOAL model can be used to interpret *unc-5* mutant phenotypes. We argue that in each case, phenotypes can be explained by the ability of UNC-5 to affect UNC-40 asymmetric localization, which in turn controls the degree to which the direction of outgrowth activity fluctuates and the extent of outward movement. Our model also suggests that genes that were previously classified as regulating attraction or repulsion might act with *unc-5* to regulate neuronal outgrowth by controlling the degree to which the direction of UNC-40-mediated outgrowth fluctuates. We show that UNC-5 acts together with the cytoplasmic protein UNC-53 to regulate UNC-40 asymmetric localization in response to the UNC-6 and EGL-20 extracellular cues.

PLM extension phenotype

We hypothesize that cues present around the PLM cell body create a strong bias for anterior outgrowth activity (Figure 14A). These include UNC-6 and other cues that flank the longitudinal pathway and cause an equal probability of outgrowth in the dorsal and ventral directions. UNC-40 SOAL activity acts to suppress non-UNC-40 SOAL activity (Figure 6). As the extension moves toward more anterior positions (Figure 14A, positions 2 and 3), it encounters higher levels of a cue(s) that promotes outgrowth through non-UNC-40

receptors. As a result, the probability of non-UNC-40 SOAL activity at the dorsal and ventral surfaces of the leading edge increases. Because the asymmetric localization of a receptor is statistically dependent, the probability of non-UNC-40 SOAL activity at the anterior surface of the leading edge must decrease as the localization elsewhere increases. While this effect does not necessarily change the anterior bias for outgrowth, it significantly increases the degree to which the direction of non-UNC-40 outgrowth activity fluctuates, which consequently decreases the extent of outward movement (Figure 14C). This effect stalls forward movement.

We hypothesize that mutations affect the degree to which the direction of non-UNC-40 outgrowth activity fluctuates at position 3 (Figure 15). *MIG-15* appears to promote non-UNC-40 SOAL activity, whereas UNC-5 promotes UNC-40 SOAL activity. Because each activity can suppress the other, different domains of non-UNC-40 and UNC-40 SOAL activity can be established along the surface of the leading edge. As the extension moves toward position 3, there is higher non-UNC-40 activity at the more anterior surface and higher UNC-40 activity at the dorsal and ventral surfaces. By suppressing non-UNC-40 activity, the *mig-15* mutation increases, relative to wild-type animals, the UNC-40 activity at the dorsal and ventral surfaces at position 3. This UNC-40 activity decreases the probability of non-UNC-40 activity at these surfaces and increases the probability of non-UNC-40 activity at the anterior surface. By reducing the degree to which non-UNC-40

Figure 19 Model for the effects that mutations have on the outgrowth movement of the HSN. Table showing the effects of different mutations on the outgrowth of the HSN at position 1, Figure 18. Mutations can alter the rate of outgrowth and the number of extensions. In this model, the relative levels of UNC-40 and non-UNC-40 statistically-oriented asymmetric localization (SOAL) activity controls this patterning. In wild-type animals, the ability of UNC-40 SOAL activity to predominate at one site along the membrane is enhanced by non-UNC-40 SOAL activity, which may increase the threshold at which UNC-40 positive feedback becomes effective. Over time, the area where UNC-40 SOAL predominates causes greater UNC-40 outgrowth activity. As outward movement occurs, higher levels of UNC-6 are encountered. This enhances and localizes the process to that area. In *mig-15* mutants, the suppression of non-UNC-40 SOAL activity reduces the threshold at which UNC-40 SOAL activity may predominate at a site. This allows multiple sites along the membrane where UNC-40 SOAL activity may predominate. Multiple extensions may develop as shown in Figure 5. Loss of UNC-5, which suppresses UNC-40 SOAL activity, retards the ability to enhance and localize the process. This causes greater fluctuation in the direction of outgrowth activity across the entire ventral surface of the neuron, which uniformly decreases the displacement of membrane.

outgrowth fluctuates, a greater anterior directional bias is created in the *mig-15* mutants. This results in overextension. The *unc-5* mutation represses the UNC-40 activity at the dorsal and ventral surfaces, increasing the degree to which the direction of the non-UNC-40 outgrowth activity fluctuates. This suppresses the overextension caused by the *mig-15* mutation.

AVM nerve ring branching and extension phenotype

Similar to what is proposed for PLM at position 3 in Figure 14, all the surfaces of the leading edge of the AVM become exposed to high levels of a cue(s) (Figure 16A, position 1). The degree to which the direction of outgrowth activity fluctuates greatly increases and outward movement stalls. For the AVM, this occurs at the nerve ring, which is a source of UNC-6. However, for the AVM there are cues at the nerve ring that are arranged perpendicular to one another. We propose that the high level of UNC-6 at all surfaces allows UNC-40 SOAL activity to become more uniformly distributed along all surfaces of the leading edge (Figure 16B), and creates a state where the probabilities of outgrowth in every direction become equal. Both anterior and dorsal outward movement stalls (Figure 16C). This state allows any new cues encountered to effectively create a directional bias (Figure 4). UNC-6 and other cues are arranged along the

nerve ring, whereas non-UNC-6 cues are arranged at the anterior of the nerve ring. As some outgrowth ventures anteriorly and dorsally, these cues stimulate the development of non-UNC-40 and UNC-40 SOAL activity domains (Figure 16B, position 2). Even slight outward movement in the anterior or dorsal directions may reinforce movement in that direction if cues arranged along the axis perpendicular to the direction bias suppress the UNC-40 or non-UNC-40 SOAL activity along the surfaces perpendicular to the directional bias (as depicted in Figure 14B, position 1).

We hypothesize that mutations affect the degree to which the direction of UNC-40 and non-UNC-40 outgrowth activity fluctuates at position 2 (Figure 17). In *unc-40* mutants, the lack of dorsal UNC-40 activity allows the direction of non-UNC-40 outgrowth to fluctuate more. However, the anterior cues increase the probability of anteriorly directed non-UNC-40 outgrowth and, thereby, decrease the probability of dorsally directed activity. This suppresses dorsal extension, while still allowing anterior extension. Loss of MIG-15 activity in the *unc-40* mutant background suppresses the non-UNC-40 SOAL. In comparison to the single *unc-40* mutant, in *unc-40;mig-15* mutants, the probability of anterior outgrowth in response to the anterior cues is lower. Consequently, the probability of dorsal non-UNC-40 outgrowth is higher. We speculate that this allows some dorsal extension.

Figure 20 Genetic pathways for self-organizing UNC-40 asymmetric localization. (A) Table summarizing the results of experiments previously reported and described in Figure 13 of this paper. (B) The genetic data support a model whereby the UNC-6 and EGL-20 extracellular cues regulate at least three pathways, leading to robust asymmetric UNC-40 localization. Robust asymmetric UNC-40 localization refers to the ability to observe UNC-40::GFP clustering at the surface of the neuron. Arrows represent activation; bars represent repression. See text for the logic used to construct the pathways.

In *unc-5* mutants, UNC-40 SOAL activity is reduced, but the activity is still sufficient to allow dorsal extension. Loss of both UNC-5 and MIG-15 function most severely hampers the ability to specifically direct the receptors to one surface. The *unc-5*; *mig-15* mutants have the most abnormal outgrowth patterns.

HSN extension phenotypes

We hypothesize that there is high probability for ventrally directed outgrowth from the HSN cell body because of the strong outgrowth-promoting effect of the UNC-40-mediated response to the UNC-6 cue, which is at a higher concentration ventral to the cell body (Figure 18A). We hypothesize that the same process takes place in the HSN neuron as in the PLM neuron, except that the movement is toward the UNC-6 source. In Figure 8B, we depict that at position 1 there is some non-UNC-40 SOAL activity at the ventral surface of the leading edge. By position 2, higher levels of UNC-6 increase UNC-40 SOAL and suppress ventral non-UNC-40 SOAL activity. At position 3, UNC-6 is present at high levels along all surfaces and the direction of UNC-40 outgrowth greatly fluctuates. Possibly, the degree to which the direction of UNC-40 and non-UNC-40 outgrowth activity fluctuates is greater at position 1 than at position 2 (Figure 18C). However, at position 3 the fluctuation is greatest.

We hypothesize that the interplay between UNC-40 and non-UNC-40 SOAL activity allows multiple extensions to develop in the same direction. In fact, UNC-40, UNC-5, MIG-15, and UNC-6 (netrin) activities may function as a type of reaction–diffusion system (Turing 1952; Gierer and Meinhardt 1972; Meinhardt and Gierer 2000; Kondo and Miura 2010; Goehring and Grill 2013). Along the ventral surface, there is competition between UNC-40 receptors to direct further UNC-40 localization to that site and to inhibit flanking receptors from doing the same. Over time, the SOAL activity that began at one site predominates,

leading to an area of higher outgrowth activity (Figure 19). We speculate that by helping to suppress UNC-40 SOAL activity, non-UNC-40 activity increases the threshold at which the SOAL activity at one site can begin to predominate. The *mig-15* mutation suppresses the non-UNC-40 SOAL activity and decreases the threshold. This may allow more sites along the membrane where UNC-40 SOAL activity can predominate. The sites do not overlap because of the long-range negative feedback that inhibits neighboring UNC-40 activity. The *unc-5* mutation suppresses UNC-40 SOAL activity, both by positive and negative feedback loops. This retards the ability to enhance and localize the process to one area of the surface. This causes greater fluctuation in the direction of outgrowth activity across the entire ventral surface of the neuron. As a result, the rate of initial outgrowth is even less than that which occurs in wild-type animals and the area of outward growth is broader.

A genetic pathway for UNC-40 asymmetric localization:

We present a genetic pathway for the asymmetrical localization of UNC-40 based on the phenotype of robust UNC-40::GFP clustering in the HSN. A full understanding of the molecular mechanisms underlying the SOAL process is an important long-term goal. Since we believe that UNC-40::GFP clustering is a readout of that process, constructing genetic pathways for the clustering of UNC-40::GFP is a step toward this goal. We wish to know how UNC-5 mediates signaling within the HSN that controls the UNC-40 asymmetric localization process. However, a role for UNC-5 in the HSN is paradoxical given the widespread idea that UNC-5 mediates a repulsive response to UNC-6 and that HSN outgrowth is toward the source of UNC-6. All the same, we suggest that a cell-autonomous role for UNC-5 in the HSN is the most parsimonious model. First, UNC-5 is an UNC-6 receptor that

Figure 21 Model for HSN membrane movement mediated by UNC-5 activity. (A) Schematic diagrams of statistically-oriented asymmetric localization (SOAL) activities mediated by UNC-5, UNC-40, and an unknown non-UNC-40 receptor. Receptor SOAL activity occurs dynamically along the membrane at the microscale (Figure 1 and Figure 2), and can create a net directional bias for outgrowth over time (green arrow). UNC-5 functions together with UNC-40 to enhance UNC-40 SOAL activity and UNC-40-mediated outgrowth at the site of UNC-6 interaction (1). If UNC-5 activity is reduced or lost, the probability of UNC-40 SOAL activity at sites where UNC-40 is not UNC-6-ligated increases, and this increases the degree to which the direction of UNC-40-mediated outgrowth fluctuates over time (2). In the absence of UNC-40, UNC-5 inhibits outgrowth activity at the sites of UNC-6 interaction. UNC-5 SOAL activity at the site of UNC-6 interaction prevents non-UNC-40 SOAL activity and non-UNC-40-mediated outgrowth at this location, increasing the probability of non-UNC-40 SOAL activity and non-UNC-40-mediated outgrowth at other locations. Over time, this creates a directional bias away from the sites where UNC-6 interacts with UNC-5 (3). If UNC-5 activity is reduced, the inhibition of non-UNC-40-mediated outgrowth at sites where UNC-6 interacts with UNC-5 decreases. This increases the degree to which the direction of non-UNC-40-mediated outgrowth fluctuates over time (4). (B) Random walk modeling, as described in Figure 2E, is shown for each of the depictions shown in (A). The directional bias that evolves over time directs the flow of membrane mass (box). The displacement of membrane mass depends on the probability distribution for the direction of outgrowth. Displacement toward the UNC-6 source is greatest when UNC-40 and UNC-5 activities decrease the degree to which the direction of outgrowth fluctuates (1), whereas displacement decreases as UNC-5 activity is reduced and the degree to which the direction of UNC-40-mediated outgrowth fluctuates increases (2). In the absence of UNC-40, UNC-5 helps increase non-UNC-40-mediated membrane displacement at sites where there is less UNC-5 and UNC-6 interaction (3). If the strength of UNC-5 inhibition decreases at the sites of UNC-5 and UNC-6 interaction, then the degree to which the direction of non-UNC-40-mediated outgrowth fluctuates will increase, thereby decreasing membrane displacement (4).

can mediate neuronal responses when in complex with UNC-40 (Hong *et al.* 1999; Geisbrecht *et al.* 2003; Kruger *et al.* 2004; Finci *et al.* 2014). We previously showed that UNC-40 conformational changes regulate HSN asymmetric localization in the HSN (Xu *et al.* 2009), and we now show that UNC-5 regulates UNC-40 asymmetric localization in the HSN. It is therefore plausible that UNC-5 affects UNC-40 conformational changes that regulate UNC-40 asymmetric localization. Second, UNC-5 can alter the number of HSN outgrowths in response to UNC-6 and to the UNC-6ΔC ligand. Directional guidance by UNC-6 and UNC-6ΔC is generally normal in an *unc-5* mutant, suggesting that the ability of UNC-5 to regulate the number of outgrowths is not due to an alteration in

the extracellular distribution of its UNC-6 ligand. Further, the UNC-6ΔC ligand and the *mig-15* mutation create the same outgrowth phenotype, which can be suppressed by loss of UNC-5 function, and we have shown that MIG-15 acts cell-autonomously in the HSN to regulate UNC-40 asymmetric localization (Yang *et al.* 2014). Further, we have shown that the UNC-5-mediated response that regulates UNC-40 asymmetric localization also depends on UNC-53 (NAV2) (Kulkarni *et al.* 2013), a cytoplasmic protein that functions cell-autonomously for cell migration and axon guidance (Stringham *et al.* 2002). Together, these observations strongly suggest that UNC-5 directly regulates signaling within the HSN. Third, a role for UNC-5 in the guidance of AVM and PVM axons toward UNC-6 sources has also been suggested. A synergistic interaction between *unc-5* and *egl-20* is observed; in either *unc-5* or *egl-20* mutants, the ventral extension of AVM and PVM axons is only slightly impaired, whereas in the double mutants there is a much greater penetrance (Levy-Strumpf and Culotti 2014). The expression of an *unc-5* transgene in the AVM and PVM can rescue the AVM and PVM axon guidance defects of the *unc-5;egl-20* double mutant (Levy-Strumpf and Culotti 2014). We note that for the HSN, transgenic rescue using *unc-5* constructs has not been successful, and that in wild-type animals UNC-5 expression in the HSN has not been reported. In addition, expression has not been reported in AVM, PVM, and PLM wild-type neurons. We suspect that there may be technical difficulties or that UNC-5 expression might be low in these cells. UNC-5 is detected in PLM in *rpm-1* mutants, which is consistent with evidence that UNC-5 activity is required for PLM overextension in these mutants (Li *et al.* 2008).

To construct genetic pathways, we use the readout of whether UNC-40::GFP is clearly and consistently localized to any side of the HSN neuron in different mutants (Figure 13). A summary of the results is presented (Figure 20A). UNC-6 is required for robust asymmetric UNC-40 localization; in the absence of UNC-6 function, UNC-40 remains uniformly distributed along the surface of the plasma membrane. The loss of both UNC-53 and UNC-5 function also results in a uniform distribution; however, loss of either one alone does not. This suggests that UNC-53 and UNC-5 pathways act redundantly downstream of UNC-6 (Figure 20B). Moreover, we observe that there is robust asymmetric UNC-40 localization when there is a loss of UNC-6 activity in addition to the loss of UNC-53 and UNC-5. This suggests a third pathway that is suppressed by UNC-6 when UNC-53 and UNC-5 activity are missing. Loss of both UNC-5 and UNC-6 does not allow UNC-40 localization, whereas loss of both UNC-53 and UNC-6 does; therefore, UNC-53, rather than UNC-5, acts with UNC-6 to suppress the third pathway.

UNC-40 becomes localized when EGL-20 activity is lost. In addition, UNC-40 becomes localized when both EGL-20 and UNC-53 activities are lost. This is consistent with UNC-6 promoting UNC-40 localization via the UNC-5 pathway. Loss of EGL-20 and UNC-5 prevents UNC-40 localization. In these animals, the UNC-5 pathway is absent and UNC-6 is present to block the third pathway; therefore, the UNC-53 pathway

Figure 22 Model for the overriding logic of how multiple receptor statistically-oriented asymmetric localization (SOAL) activities direct the initial outgrowth of the HSN. Schematic diagrams as described in Figure 22 for the SOAL activities mediated by UNC-5, UNC-40, SAX-3, an EGL-20 receptor, and an unknown non-UNC-40 receptor (top). As described below each diagram, the activities can occur in concert with each other and are interdependent. Random walk analyses using the direction of axon development from the HSN neuron in different mutants are shown as described in Figure 10 (bottom). The graphs were created as described in the figure legend of Figure 9. The graphs indicate the relative effects that the mutations have on the directional bias and displacement

of movement during the initial outgrowth from the HSN. Lines connecting the random walk analysis of a mutant to SOAL activities depict the logic underlying the directional bias. For example, in *unc-40(-);sax-3(-)*, there is a dorsal directional bias (random walk analysis in green). As described in Figure 21, UNC-5 SOAL activity can orient non-UNC-40-mediated outgrowth dorsally in the absence of UNC-40 (green connecting line). However, this requires the loss of dorsal SAX-3 activity, which would otherwise inhibit UNC-5 and non-UNC-40 activity (green connecting lines with X). See text for further details about the logic underlying each mutant.

that leads to UNC-40 localization must require EGL-20, as well as UNC-6.

Loss of UNC-6 activity, or loss of both UNC-6 and EGL-20 activity, prevents localization, whereas loss of only EGL-20 does not. To explain this, we propose that when UNC-6 is lost, the third pathway, which would otherwise be activated by the loss of UNC-6, remains suppressed because EGL-20 activity promotes suppression via UNC-53 activity. This suppression also explains why loss of UNC-6 and UNC-5 activity does not cause localization.

The genetic pathways are consistent with the models proposed in Figure 1 and Figure 6. In the models, positive feedback loops amplify the polarized responses to extracellular cues, whereas negative feedback limits the responses and confines the positive feedback to the sites of interaction. We hypothesize that the UNC-5 and UNC-53 genetic pathways shown at the top and bottom of Figure 20B correspond to the positive feedback loops depicted in Figure 1 and Figure 6 by the arrows. The “?” genetic pathway corresponds to UNC-40 asymmetric localization and outgrowth activity in the absence of UNC-6. The UNC-53 genetic pathways in Figure 20B that block the ? pathway correspond to the negative feedback loops (lines) in Figure 1 and Figure 6, which prevent UNC-40 asymmetric localization and outgrowth in the absence of UNC-6. Loss of both UNC-6 and EGL-20 prevents robust asymmetric UNC-40 localization because both UNC-6- and EGL-20-mediated positive feedback loops are disrupted. A positive feedback loop may be necessary to establish a negative feedback loop. Therefore, the ? pathway is not active when both UNC-6 and EGL-20 are absent.

Model for how multiple receptor SOAL activities determine the direction and displacement of membrane movement: We hypothesize that UNC-5 activity regulates the SOAL activities

of both the UNC-40 and non-UNC-40 receptors. UNC-5 functions with UNC-40 to increase the probability of UNC-40 localizing and mediating outgrowth at sites where UNC-6 interacts with UNC-40 (Figure 21A, 1). If UNC-5 activity is reduced or lost, UNC-40 still mediates outgrowth, but there is a higher probability of localization and outgrowth at sites where UNC-40 does not interact with UNC-6 (Figure 21A, 2). In either case, outgrowth occurs in the same direction. However, the effect on membrane displacement is different (Figure 21B, 1 and 2). In the absence of UNC-40, UNC-5 can mediate an outgrowth response to UNC-6. We hypothesize that in response to UNC-6, UNC-5 inhibits outgrowth activity. This inhibition lowers the probability of non-UNC-40 receptors localizing and mediating outgrowth at the sites of UNC-5 and UNC-6 interaction and, consequently, increases the probability of non-UNC-40-mediated outgrowth at sites without UNC-6 (Figure 21A, 3). By affecting the probabilities of non-UNC-40 localization, UNC-5 activity influences the degree to which the direction of non-UNC-40-mediated outgrowth fluctuates (Figure 21A, 4) and, therefore, the level of UNC-5 activity affects membrane displacement produced by non-UNC-40 outgrowth activity (Figure 21B, 3 and 4). The SOAL activities of these receptors is proposed to occur dynamically and concurrently across the membrane at the microscale (Figure 1 and Figure 2). This model predicts that the rate of outgrowth and the net directional bias are regulated by the relative activities of the UNC-5, UNC-40, and non-UNC-40 receptors.

For the initial outgrowth of the HSN, we propose that multiple receptor SOAL activities determine the direction and displacement of membrane movement. Figure 22 shows an overlay of the random walk analyses derived from different mutants that have lost receptor function (bottom schematic). This provides a picture of the relative effects that

these receptors have on the directional bias and displacement of membrane movement during the initial outgrowth from the HSN cell body. To explain the different patterns, we propose that the receptor SOAL activities act in concert with each other and are interdependent (Figure 22, top schematics). In wild-type animals, the *UNC-40* response to *UNC-6* at the ventral surface dominates (Figure 22, 1). However, *UNC-5* and *SAX-3* activities modify this response by regulating the degree to which the direction of *UNC-40*-mediated outgrowth fluctuates. Whereas *UNC-5* may interact directly with *UNC-40* to regulate the *UNC-40* response to *UNC-6*, *SAX-3* might inhibit dorsal outgrowth activity in response to the *SLT-1* extracellular cue, which is expressed by the dorsal body wall muscles during HSN axon development (Hao *et al.* 2001). This inhibition lowers the probability of *UNC-40* receptors localizing and mediating outgrowth at more dorsal sites and, consequently, increases the probability of *UNC-40*-mediated outgrowth at ventral sites (Figure 22, 4). By increasing the degree to which the direction of *UNC-40*-mediated outgrowth fluctuates, both *UNC-5* and *SAX-3* regulate membrane displacement; however, they have less influence on the initial ventral directional bias, which is primarily created by the *UNC-40* response to *UNC-6*.

Loss of *UNC-40* or of both *UNC-40* and *UNC-5* causes an anterior directional bias (Figure 22). We hypothesize that this bias is created by non-*UNC-40* receptors using the same process through which *UNC-5* can direct outgrowth away from the sites of *UNC-6* interaction. We speculate that an extracellular cue from the posterior tail region of the animal interacts with a receptor that inhibits outgrowth. This increases the probability of a non-*UNC-40* receptor to localize and mediate outgrowth at sites that are not suppressed (Figure 22, 5). As discussed earlier, *EGL-20* is a candidate for such an extracellular cue.

We observe that loss of *UNC-40* and *SAX-3* causes dorsal outgrowth. This suggests that *SAX-3*, in addition to inhibiting *UNC-40* localization, also inhibits the non-*UNC-40* outgrowth activity (Figure 22, 2). In the absence of *SAX-3* inhibition, *UNC-5* activity can orient the non-*UNC-40* outgrowth activity away from the ventral sites of *UNC-6* interaction (Figure 22, 6). Loss of *SAX-3* also affects the orientation of outgrowth activity in the single *sax-3* mutant. The bias toward the sites of *UNC-6* interaction that is induced along the ventral membrane by *UNC-40* activity (Figure 22, 1) may be shifted posteriorly due to the loss of the inhibition of *UNC-5* activity by *SAX-3* (Figure 22, 3). *UNC-5* inhibits non-*UNC-40* outgrowth activity (Figure 22, 6), which contributes to anteriorly directed bias. Similarly, loss of only *UNC-5* activity could shift the bias anteriorly, since the loss of *UNC-5* activity that inhibits non-*UNC-40* outgrowth activity would allow a greater anterior bias due to increased non-*UNC-40* outgrowth activity. This effect may be lost in the *unc-5;sax-3* double mutant, because the loss of *UNC-40* inhibition by *SAX-3* would allow greater inhibition of non-*UNC-40* activity by *UNC-40*.

A theory of outgrowth patterning: This paper describes a theory of outgrowth patterning. The theory is based on the underlying principles described in the *Introduction*. Clearly,

the theory needs to be further tested and refined. For example, to explain patterns of outgrowth caused by some mutations, we invoked molecular activities and states for which there is very little experimental evidence. Nevertheless, the theory may prove useful precisely because it presents new ideas that can be experimentally tested. Indeed, we reexamined *unc-5* mutants to look for phenotypes predicted by the theory. It is hoped that eventually this theory could lead to a more unifying framework to explain the development of outgrowth patterns.

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