Focus Review

Cellular responses to extracellular guidance cues

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Extracellular guidance cues have a key role in orchestrating cell behaviour. They can take many forms, including soluble and cell-bound ligands (proteins, lipids, peptides or small molecules) and insoluble matrix substrates, but to act as guidance cues, they must be presented to the cell in a spatially restricted manner. Cells that recognize such cues respond by activating intracellular signal transduction pathways in a spatially restricted manner and convert the extracellular information into intracellular polarity. Although extracellular cues influence a broad range of cell polarity decisions, such as mitotic spindle orientation during asymmetric cell division, or the establishment of apical–basal polarity in epithelia, this review will focus specifically on guidance cues that promote cell migration (chemotaxis), or localized cell shape changes (chemotropism).

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Introduction

Cell migration is a fundamental process seen in all multicellular organisms throughout embryonic development, but also in the adult during wound repair, immune responses and tissue regeneration (Jin *et al*, 2008; Laird *et al*, 2008; Friedl and Gilmour, 2009). The movement of cells is typically not random, but directed by extracellular guidance cues (chemotaxis). The interaction of these cues with plasma membrane receptors elicits spatially restricted intracellular signal transduction pathways that influence the assembly, disassembly and arrangement of the actin cytoskeleton in distinct ways at the front and the rear of the cell to drive directional migration. Guidance mechanisms are evolutionarily, highly conserved and have been widely studied in vertebrates (both *in vivo* and in tissue culture), *Caenorhabditis elegans* and *Drosophila*, as well as in the single cell organism, *Dictyostelium discoideum* (Montell, 1999; Janetopoulos and Firtel, 2008; Weijer, 2009; Aman and Piotrowski, 2010).

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Guidance cues have been extensively studied in the context of axon pathfinding during neural development (O'Donnell *et al*, 2009). The specialized tip of a growing axon, the growth cone, receives signals from a plethora of both attractive and repulsive guidance cues to direct the axon to its final destination, sometimes over remarkably long distances. Furthermore, localized changes to the actin cytoskeleton within the growth cone are thought to be the major driving force, in this case to orient a microtubule-dependent shape change, namely elongation of the growing axon. The ability of cells to change their morphology in response to guidance cues (chemotropism) is highly conserved evolutionarily. *Saccharomyces cerevisiae*, for example, undergoes polarized shape changes in response to a gradient of extracellular pheromone and this too involves localized re-organization of the actin cytoskeleton (Slaughter *et al*, 2009b). A related process in the same organism, spatially localized bud growth during cell division, is directed by an intrinsic cue (the bud scar) on the surface of the mother cell; however, this involves similar signalling pathways to those induced by pheromone. The relative simplicity of these responses in *S. cerevisiae* and the powerful genetics available have provided some of the most detailed insights into how intracellular signalling pathways can be initiated and maintained in a spatially restricted manner at the cell cortex.

Polarized shape changes in yeast

Cell division in *S. cerevisiae* involves the establishment of a bud site at the cortex of the mother cell, followed by bud growth and eventual cytokinesis to form a new daughter cell. Although no extracellular factor is required, a cortical cue is involved in determining the position of the bud site. The analysis of how a signalling platform is first generated at this site has provided general mechanistic insight into a step that is critical to all guidance cues—the establishment of a spatially restricted domain at the cortical surface.

Budding yeast use the previous bud site scar as the cortical cue for cell polarization during cell division, and genetic analysis has revealed much insight into this process, in particular, the importance of small GTPases (Park and Bi, 2007). In brief, Rsr1, a member of the Ras family of small GTPases, is first recruited to the bud scar to initiate bud formation. It recruits Cdc24, a guanine nucleotide exchange factor (GEF) for Cdc42, which is a member of the Rho family of small GTPases (Shimada *et al*, 2004). In its GTP-bound state, Cdc42 interacts with a scaffold protein, Bem1, in a complex with Cla4 (a PAK-like, ser/thr kinase) and Cdc24 (Yamaguchi *et al*, 2007; Kozubowski *et al*, 2008). As Cla4 is a target of Cdc42 and Cla4 is thought to phosphorylate and activate Cdc24, this creates a positive feedback loop leading to a cluster of Cdc42 activity that constitutes a spatially localized bud site (Figure 1A) (Gulli *et al*, 2000). Once the

Figure 1 Polarization in yeast. (A) Budding. Rsr1, a Ras-related small GTPase, localizes to the bud scar, a cortical remnant from the previous cell division. In its GTP-bound state, Rsr1 recruits Cdc24, an exchange factor for Cdc42. Activated Cdc42 captures the freely diffusing Bem1–Cdc24–Cla4 complex, through a direct interaction with Bem1, which serves to locally activate more Cdc42. There is evidence that Cla4 phosphorylates and activates Cdc24. This module serves as positive feedback loop creating a polarized patch of Cdc42 activity, which defines the new bud site. Subsequent recruitment of other Cdc42 targets and other Rho GTPases leads to the rearrangement of actin and microtubule cytoskeletons in the mother cell and to bud growth. (B) Mating. During mating, pheromone is recognized by surface receptors on cells of the opposite mating type. The pheromone gradient leads to the formation of a mating projection (shmoo) in the direction of the gradient source by first initiating receptor polarization, via an endocytosis and polarized recycling pathway. The subsequent release of Gβγ subunits from the associated heterotrimeric G protein recruits a scaffold protein, Far1, which is ordinarily resident in the nucleus in a complex with Cdc24. This forms the basis of a positive feedback loop. (C) Dynamics. The development of a single patch of active Cdc42 at the cortex is influenced by the rates of protein delivery to the membrane, either by free diffusion, or by transport along actin filaments, and by the rate of dissociation of proteins from the membrane, involving endocytosis, GDI-mediated solubilization of Cdc42 and GAP stimulated GTP hydrolysis on Cdc42. The balance of these activities determines the overall distribution of the patch at the cortex to produce a round bud or a pointed shmoo.

site is established, Cdc42 recruits additional target proteins to control a variety of cellular responses necessary for continued bud growth, including re-arrangements of the actin cytoskeleton, polarized vesicle trafficking and new cell wall synthesis (Park and Bi, 2007).

Interestingly, cells are capable of forming a single bud in the absence of Rsr1. In this case, spontaneous cell polarization, known as symmetry breaking, generates a randomly positioned bud on the cortex of the mother cell (Slaughter *et al*, 2009b). This scenario has uncovered special features of Cdc42, which influence the positive feedback loop and the biological response. Using a series of artificial fusion proteins between Bem1 and Cla4, Bem1 and Cdc24, or Cla4 and Cdc24, a recent study demonstrated the importance of the trimolecular Bem1–Cdc24–Cla4 complex in generating the positive feedback loop to promote a single, cortical cluster of active Cdc42, even in the absence of a cue (Kozubowski *et al*, 2008). The critical role of the exchange factor Cdc24 is adequately demonstrated by the inability of constitutively activated versions of Cdc42, which cannot interact with Cdc24, to induce symmetry breaking (Irazoqui *et al*, 2004). The positive feedback loop does not in itself explain why only one unique bud site is generated from the stochastic formation of small, cortical clusters of activated Cdc42. Mathematical modelling, however, indicates that when coupled to competition for a limiting cytoplasmic pool of a rapidly diffusing Bem1–Cdc24–Cla4 complex, and to Cdc42 turnover (e.g. through GTP hydrolysis), this will rapidly lead (<2 min) to a single dominant site of Cdc42 activity (Howell *et al*, 2009).

Extracellular cues, in the form of secreted pheromones, induce polarized morphogenetic changes in yeast cells, a process referred to as chemotropism. A pheromone gradient, generated by cells of the opposite mating phenotype, is

recognized by a seven-pass membrane receptor leading to the release of Gβγ from an associated heterotrimeric G protein (for review, see (Bardwell, 2004)). The subsequent signalling pathway leads to localized assembly of actin filaments at the cell cortex to produce a mating projection (a shmoo), which is required to establish cell–cell contact leading to cell fusion. Cdc42 is also a major player in interpreting the gradient and initiating localized shape changes. Although Bem1 is involved in this pathway, another scaffold protein, Far1, seems to be the major player (Wiget *et al*, 2004). In addition to inhibiting the cell cycle, Far1 participates in a positive feedback loop to promote Cdc42 activation, through its ability to interact directly with Cdc42–GTP and Gβγ/Cdc24 (Figure 1B; Nern and Arkowitz, 1999). In cells lacking Far1, Cdc24 clusters at random sites on the cortex (Wiget *et al*, 2004).

Many outstanding questions remain. Clearly, the Cdc24–Cdc42 positive feedback loop is the driving force for both bud site selection and mating projection formation, yet these cellular responses are morphologically distinct (round or pointed protrusions, respectively). A possible explanation for these differences follows from the observation that despite the apparent stability of Cdc42–Cdc24 clusters at the bud site and mating projection, they are in fact highly dynamic (Wedlich-Soldner *et al*, 2004). The delivery of these proteins to the membrane, through diffusion and directed transport along actin filaments, is balanced by recycling to the cytosol, through actin-dependent endocytosis and guanine nucleotide dissociation inhibitor (GDI)-dependent membrane dissociation of Cdc42 (Figure 1C; Wedlich-Soldner and Li, 2004; Marco *et al*, 2007; Howell *et al*, 2009). Experimental analysis and mathematical modelling indicate that fine tuning of the rate of internalization could give rise to different distributions of active Cdc42 at the cortex, to produce the different shapes

of buds and shmoo (Slaughter *et al*, 2009a). Additional regulators of Cdc42, in particular GTPase-activating proteins (GAPs), are likely to be important in defining its spatial distribution at the cortex, but their recruitment and regulation are not well understood (Wedlich-Soldner *et al*, 2004; Tong *et al*, 2007). Finally, yeast can polarize in response to a 1000-fold range of pheromone concentrations and recognize gradients as shallow as $0.1\% \mu\text{m}^{-1}$, but the mechanism by which pheromone engages a receptor that is initially delocalized around the cell, to generate a spatially restricted signalling platform under such diverse conditions, is still unclear. Surprisingly, after pheromone binding, the receptor and associated G protein are first internalized through the endocytic pathway before being recycled into a polarized patch at the cortex (Suchkov *et al*, 2010). This is in contrast to receptor polarization through membrane diffusion, as seen during axon guidance, or no receptor polarization, as seen during *Dictyostelium* chemotaxis (see below). It has been suggested that this may reflect the different time scales of the responses; shmoo formation being slow and stable (hours), migration and growth cone turning being rapid and flexible (minutes).

Polarized shape changes in neurons

The ability of a neuron to change shape in response to extracellular cues is the foundation on which the development of neural circuitry is built. The formation of a single axon leads to the establishment of neuronal cell polarity, and conceptually at least this is not unlike the formation of a bud or mating projection in yeast. A specialized structure at the end of the axon, the growth cone, integrates signals from the extracellular environment to affect axon guidance.

Axon formation

Cultured neurons have been used extensively to explore the process of axon formation. After attachment and spreading, hippocampal neurons develop several (typically 2–6) dynamic thin protrusions, or neurites, which extend and retract rapidly. After a further 12–24 h, one neurite continues to elongate and becomes the axon. Elongation is driven by microtubule polymerization taking place just behind the actin-rich growth cone located at the tip of the axon. Distinct vesicle trafficking pathways, to the axon or to the neurites, are established and a week or so later neurites take on the characteristics of dendrites. The neuron is now fully polarized (Dotti *et al*, 1988; Figure 2A).

The mechanism underlying the decision to form an axon from one of several neurites has attracted a lot of attention. It has been suggested that axon emergence is directed by an intrinsic cue, namely the position of the centrosome/Golgi, which remains apposed to the first neurite formed after mitosis (Zmuda and Rivas, 1998; de Anda *et al*, 2005). However, recent work using a laser ablation technique showed that the centrosome is not essential for axon outgrowth (Stiess *et al*, 2010). *In vivo* this decision is probably influenced by developmental determinants and extracellular cues (Gomez and Spitzer, 1999; Killeen and Sybingco, 2008; Barnes and Polleux, 2009). Nevertheless, classic experiments, in which initial outgrowth is curtailed by cutting the axon, have shown that the decision can be rendered stochastic, with any one of the other neurites capable of elongating to

form an axon (Dotti and Banker, 1987; Goslin and Banker, 1989). As with yeast, characterization of this albeit non-physiological, stochastic process has identified important mediators in this symmetry breaking decision. The microtubule and actin cytoskeletons, together with the secretory pathway are clearly important players. Localized stabilization of microtubules, with taxol, or destabilization of actin filaments, with cytochalasin, at the tip of one neurite is sufficient to induce axon formation, suggesting that the microtubule and actin cytoskeletons generate counteracting forces to inhibit neurite elongation (Bradke and Dotti, 1999; Witte *et al*, 2008). Stochastic imbalances (*in vitro*), or extracellular cues (*in vivo*) that affect one or more of these forces probably initiate a cell-intrinsic program for extension. This program must be such that only one axon forms, and perhaps similar to yeast, a positive feedback loop coupled to competition for a limited signalling component is involved. The observation that the centrosome and Golgi influence axon positioning indicates that the delivery of proteins through the secretory pathway makes an important contribution.

Several signalling molecules accumulate at the tip of a single neurite before elongation (Figure 2A). The second messenger PIP_3 (phosphatidylinositol 3,4,5 trisphosphate) is consistently observed here and as inhibition of PI3 kinase interferes with axon formation, it is believed to be an important player (Shi *et al*, 2003; Menager *et al*, 2004; Jiang *et al*, 2005). PIP_3 has been linked primarily to the activation of kinase Akt, which in turn phosphorylates and inactivates another kinase GSK-3 (Shi *et al*, 2004; Jiang *et al*, 2005). Despite similar conclusions being reached by several groups, hippocampal neurons isolated from knockin mice in which the Akt phosphorylation sites of GSK-3 have been removed, polarize normally in culture (Gartner *et al*, 2006). Nevertheless, the requirement for GSK-3 inhibition seems to be retained even in these knockin neurons, suggesting the existence of an alternative mechanism. The primary consequence of localized GSK-3 inhibition is thought to be dephosphorylation of the tumour suppressor protein APC (adenomatous polyposis coli). In its dephosphorylated state, APC interacts with and stabilizes microtubule plus ends, thereby promoting microtubule-dependent elongation of the neurite (Zumbrunn *et al*, 2001; Shi *et al*, 2004).

Numerous small GTPases localize to the neurite/axon tip, including members of the Ras (Ras, R-Ras and Rap1) and Rho (Cdc42, Rac and Rho) families (Figure 2A). Given its involvement in a wide range of cell polarity contexts, Cdc42 was an early focus for study. Inhibition of Cdc42 prevents axon formation, whereas expression of activated mutants induces multiple axons—results consistent with a determining role in axon formation (Schwamborn and Puschel, 2004). In agreement with this, tissue-specific ablation of Cdc42 in the mouse brain leads to numerous defects, including the absence of axon tracts, whereas neurons isolated from these mice have severe problems in establishing axons (Garvalov *et al*, 2007). If Cdc42 is the key initiator of axon formation, it might be expected to engage in a positive feedback loop, which in its simplest form would involve recruitment of a Cdc42 target coupled to a Cdc42 exchange factor. To date, no Cdc42 exchange factor has been described specific for axon initiation. However, the Par6–aPKC (atypical protein kinase C) complex, which mediates the effects of Cdc42 in many polarity pathways, accumulates at the tip of nascent axons

Figure 2 Polarization and guidance in neurons. **(A)** Axon formation. The elongation of a single neurite to form an axon establishes a polarized neuron. *In vivo* this decision is guided by extracellular cues, but *in vitro*, stochastic changes in signalling activities at neurite tips, amplified by positive feedback loops, initiate axon formation. Several small GTPases localize to the tip of a presumptive axon. The proposed Rap1–Cdc42–Rac pathway is reminiscent of Rsr1–Cdc42–Rho in yeast polarization, although in the neurite, PI3 kinase seems to be the key downstream effector of Cdc42. It is capable of initiating a feedback loop through the generation of PIP₃, a known activator of GEFs for Cdc42 and Rac. PI3 kinase is also a target of Ras and this too has been proposed to promote a feedback loop, probably through a Ras GEF. Changes in the dynamic behaviour of the actin and microtubule cytoskeletons at the neurite tip initiate axon extension. Rac directly regulates the actin and microtubule cytoskeletons through effector proteins such as a LIM kinase and PAK kinase, but in addition the Par6–aPKC polarity complex, a direct target of Cdc42, localizes to the presumptive axon tip to promote stabilization of microtubules. This involves inhibition of GSK-3 leading to accumulation of APC at microtubule plus ends, though there is some uncertainty whether GSK-3 inhibition is direct (i.e. by phosphorylation) or indirect. **(B)** Axon guidance. A large number of extracellular cues are involved in guiding axons to their final destination. Both repulsive and attractive cues interact with the growth cone to influence the direction of elongation of axonal microtubules. They are thought to act by modifying the actin cytoskeleton in a spatially restricted manner within the growth cone causing it to turn towards or away from the source. In its simplest form, localized activation of Rho might be expected to lead to actomyosin-dependent retraction of the plasma membrane and induce turning away from a repellent, whereas activation of Rac or Cdc42 (↑) and inactivation of Rho (↓) could promote membrane protrusion and turning towards an attractant. Activation of GTPases can be achieved through activation of GEFs (↑) or inactivation of GAPs (↓). The situation is, however, clearly more complicated. Rac, for example, is activated during plexin-mediated repulsion, and the response to some repulsive cues can be reversed if the balance of cAMP and cGMP present within the growth cone is altered. Changes in cytosolic calcium seem to be essential for guidance signalling. A detailed understanding of these responses is lacking, but probably is dependent on the GEFs, GAPs and targets present in the growth cone of different neurons and their responsiveness to the different guidance cues encountered.

(Shi *et al.*, 2003; Etienne-Manneville, 2004). It could provide an alternative route to GSK-3 inhibition, and it can interact with Stef, an exchange factor for Rac (Nishimura *et al.*, 2005; Schlessinger *et al.*, 2007). As Rac activates PI3 kinase, this provides the opportunity for a positive feedback loop through the recruitment of PIP₃-sensitive Rac or Cdc42 GEFs. In addition, through local activation of the ser/thr kinase PAK, Rac can stabilize the microtubule cytoskeleton (Daub *et al.*, 2001). Intriguingly, it has been reported that the localization of Cdc42 to the tip of the presumptive axon is dependent on Rap1, the mammalian orthologue of yeast Rsr1 involved in bud site selection (Schwamborn and Puschel, 2004).

Others have concluded that Ras is a major mediator in initiating axon formation. Using live FRET imaging, activated

Ras (GTP loaded) was detected at the tip of nascent axons (Fivaz *et al.*, 2008). Ras activation is dependent on PI3 kinase and, as PI3 kinase is a target of Ras, this again raises the possibility of a positive feedback loop (Yoshimura *et al.*, 2006). Furthermore, Ras is targeted to the axon through directed vesicle transport, providing a possible mechanism to ensure that only one axon is formed. Despite these compelling observations, the nervous system of knockin mice, in which the Ras-binding site on the p110 α subunit of PI3 kinase has been mutated, seems normal (Gupta *et al.*, 2007). These discrepancies could point to *in vivo* redundancy in signalling, or perhaps reflect the limitations of manipulating neurons in culture combined with the difficulties in distinguishing axon initiation from subsequent axon elongation.

Axon guidance

Axons elongate under the control of a wide variety of soluble or cell-bound ligands and matrix-coated surfaces acting as extracellular guidance cues. Axons are capable of extending over huge distances (a meter or more) and probably encounter different cues along the way. Some of the better-studied cues include Netrins, Slits, Semaphorins and Ephrins, but there are many others (O'Donnell *et al*, 2009).

Guidance cues interact with receptors located in the growth cone, a specialized structure at the tip of the elongating axon. The growth cone is actin-rich and can be morphologically divided into distinct regions: (i) a peripheral domain along the outer edge consisting of bundled (filopodia) and branched (lamellipodia) actin filaments, (ii) a central domain of bundled microtubules exiting the axon shaft, and (iii) a transitional zone, occupied by actin filament arcs (Figure 2B; Schaefer *et al*, 2008). Dynamic, pioneer microtubules transiently enter the peripheral region. Growth cone turning towards an attractant is thought to involve localized stabilization of the peripheral actin cytoskeleton proximal to the guidance gradient, coupled with destabilization of distal peripheral actin structures. These changes influence microtubule plus-end dynamics biasing elongation and stabilization towards the attractant. The mechanisms by which extracellular guidance cues effect growth cone turning have been studied extensively in culture using various types of neurons, and *in vivo* using primarily *C. elegans*, *Drosophila* and mice.

The non-polarized presentation of a repulsive cue leads to the complete collapse of growth cones in cultured neurons. These experiments have demonstrated the importance of Rho family GTPases in controlling the morphology of the growth cone. Activation of the Ephrin receptor EphA4, for example, induces collapse through activation of Rho and its target, the ser/thr kinase ROCK (Wahl *et al*, 2000). The kinase ROCK is a major regulator of myosin II leading to increased actomyosin contractility (Narumiya *et al*, 2009). An Eph4-interacting Rho exchange factor, ephexin-1, has been identified providing the outline of a signal transduction pathway affecting growth cone morphology (Shamah *et al*, 2001). Extrapolation of these observations leads to the idea that localized activation of EphA4 would induce spatially restricted retraction forces, causing the growth cone to steer away for the source of this repulsive cue. However, there are many other potential variations on this theme and with such complexity of neuronal cell types and guidance receptors; it is unlikely there is a single mechanism. Axon targeting in the corticospinal tract of mice, for example, depends on EphrinB3 acting as a repulsive cue through EphA4 and in this case a Rac GAP, α -chimerin, is required (Iwasato *et al*, 2007). Loss of α -chimerin leads to mis-targeted axons, suggesting that EphA4 induces activation of the GAP leading to local inhibition of Rac and attenuation of protrusive activity. The Semaphorins Sem3A and Sem4D, similar to Ephrins, induce growth cone collapse in cultured neurons, through their receptors PlexinA1 and PlexinB1, respectively. This effect also depends on Rho, but unexpectedly ROCK and actomyosin contractility are not involved (Arimura *et al*, 2000; Driessens *et al*, 2001; Perrot *et al*, 2002; Swierz *et al*, 2002; Barberis *et al*, 2004). Instead, Rac has a positive rather than a negative role in these responses and in fact Rac interacts with the cytoplasmic tail of PlexinA and PlexinB in a GTP-dependent manner (Rohm *et al*, 2000;

Driessens *et al*, 2001). It may be that Rac's effects here are actin independent and suggested possibilities include direct effects on Plexin receptor activity, or on endocytosis (Fournier *et al*, 2000; Turner *et al*, 2004). The latter is intriguing, as both the recruitment and the dissociation of signalling components is thought to be crucial for the establishment of localized, cortical signalling platforms (see yeast section).

Attractive cues also engage members of the Rho family to affect the actin cytoskeleton. Netrin-1, acting through the receptor DCC activates Cdc42 and Rac to promote neurite outgrowth in cultured neurons (Li *et al*, 2002). Two Rac GEFs, Trio and DOCK180, are involved and although growth cone collapse is clearly not growth cone guidance, study in mice, chick embryos and *C. elegans* has confirmed the importance of Trio and DOCK180 in directing several netrin-1-dependent axonal projections (Wu *et al*, 2002; Briancon-Marjollet *et al*, 2008; Li *et al*, 2008). Cdc42 and Rac both promote Arp2/3-dependent actin polymerization through N-WASP, leading to membrane protrusion, whereas another target, PAK, can stabilize both actin and microtubule filaments through LIM kinase-dependent phosphorylation of cofilin and phosphorylation of Op18/stathmin, respectively (Daub *et al*, 2001; Shekarabi *et al*, 2005).

In vitro growth cone-turning assays, while more challenging than collapse assays, confirm the importance of Rho GTPases and localized changes in the actin cytoskeleton for guidance. Growth cones of *Xenopus* spinal neurons, for example, are attracted to a variety of extracellular guidance cues (e.g. BDNF, netrin-1 and NT-3), when presented in the form of a gradient and both Cdc42 and Rac are required (Yuan *et al*, 2003). A gradient of a repulsive cue (e.g. LPA) on the other hand activates Rho/ROCK. One of the more dramatic results to emerge from these studies relates to the plasticity of the response. The attractants BDNF and netrin-1 (but not NT-3) were observed to act as repellants in the presence of a protein kinase A (PKA) inhibitor (Ming *et al*, 1997; Song *et al*, 1997). Further analysis suggests that it is the ratio of cAMP to cGMP, which determines the response. These cyclic nucleotides probably modulate responses through PKA and protein kinase G (PKG), respectively, though they also regulate channels in the plasma membrane leading to a calcium influx that activates PKC (Nishiyama *et al*, 2003). One report has suggested that calcium/PKC regulates the activity of Rac and Cdc42, probably through GEFs or GAPs, but the details are unclear (Jin *et al*, 2005). It is interesting that ryanodine (which releases Ca^{2+} from intracellular stores) can act as a chemoattractant when applied as an extracellular gradient, further supporting the importance of cytosolic calcium in growth cone guidance (Figure 2B). Phosphatidylinositol 3,4,5 trisphosphate, which as described earlier is a major player in initiating axon formation, is likely to be important for guidance and, in agreement with this, PI3 kinase is required for netrin responses in *Xenopus* and *C. elegans* neurons (Ming *et al*, 1999; Adler *et al*, 2006; Chang *et al*, 2006). Surprisingly, however, the role of PIP_3 in axon guidance has not been widely studied.

The use of turning assays allows detailed analysis of the spatial organization of signalling components within the growth cone. Using single quantum dot imaging on cultured rat neurons, a gradient of the chemoattractant GABA (γ -aminobutyric acid) was shown to induce the asymmetric

redistribution of GABA receptors laterally within the plane of the membrane (Bouzigués *et al*, 2007). The mechanism of redistribution seems to require microtubules and calcium, although a potential problem in interpreting these results is that nocodazole, which was used to indicate microtubule dependence, is a potent activator of Rho. Nevertheless, the authors proposed an interesting positive feedback loop, in which receptor activation leads to a localized calcium increase and stabilization of microtubules (perhaps via Cdc42 and Rac). Further recruitment of receptors by stabilized microtubules completes the loop (Bouzigués *et al*, 2010). Finally, studies in *C. elegans* have provided evidence that Rho GTPases can influence guidance receptor activity. The Rac-like GTPase MIG-2, together with VAB-8, a kinesin-like microtubule motor, and UNC-73, an MIG-2 exchange factor, are required for trafficking and localization of the netrin receptor to the growth cone (Levy-Strumpf and Culotti, 2007; Watari-Goshima *et al*, 2007). In rat spinal cord neurons, the inactivation of Rho by netrin receptor during a chemoattractive response, not only affects growth cone morphology through changes in actin dynamics, but also leads to an increase in netrin receptor at the plasma membrane (Moore *et al*, 2008).

Cell migration

Migration of single cells

Neutrophils and *Dictyostelium* are exquisitely sensitive to chemotactic gradients and are capable of rapidly responding and moving towards the source. Directed migration is accomplished through spatial orientation of intracellular signalling complexes downstream of surface receptors that recognize the external stimulus. During periods of starvation, *Dictyostelium* secrete cAMP as a guidance cue inducing neighbouring cells to migrate and form aggregates that eventually undergo differentiation and morphogenesis (Willard and Devreotes, 2006). Neutrophils, together with macrophages, are key cells of the innate immunity system. They patrol blood and host tissues, and are capable of highly efficient chemotaxis in response to infection, for example, in response to formyl peptides, such as fMLP, released by bacteria and recognized by the FRP2 receptor (Le *et al*, 2001; Cicchetti *et al*, 2002).

The morphological and intracellular responses to chemoattractive agents are remarkably similar in these two highly divergent cell types. The primary driving force for migration is remodelling of the actin cytoskeleton, seen as actin-rich protrusions at the front (i.e. in the direction of the gradient) and actomyosin filaments generating contractile forces at the sides and rear (Figure 3A). Interestingly, in shallow gradients, stable morphological polarization is not so clear in *Dictyostelium*, instead protrusions form randomly, but their relative stabilities are biased by the gradient (Andrew and Insall, 2007). In *Dictyostelium* and neutrophils, guidance cues interact with G protein-coupled receptors (GPCRs) to stimulate the release of G_{α} and $G_{\beta\gamma}$ subunits (Wu *et al*, 1995; Zhang *et al*, 2001; Jin *et al*, 2008). However, in contrast to yeast pheromone and axon guidance cues, this does not lead to significant asymmetric distributions of either the receptor, or the associated G protein, and so the generation of a highly polarized morphology must be due to the asymmetric

distribution of subsequent downstream signals (Jin *et al*, 2000; Servant *et al*, 2000).

One of the best characterized downstream signals in *Dictyostelium* is PIP₃, which has a central role in cell polarization towards an extracellular gradient. It localizes in a patch at the leading edge during chemotaxis, whereas the lipid phosphatase PTEN, a negative regulator of PIP₃ levels, is spatially restricted to the trailing and side edges (Figure 3A; Funamoto *et al*, 2002; Iijima and Devreotes, 2002). Interestingly, the membrane recruitment of PTEN is dependent on PIP₂, the precursor of PIP₃, and it has been suggested that this in itself could generate an intracellular PIP₂/PIP₃ gradient (Kortholt *et al*, 2007). Furthermore, localized accumulation of PIP₃ is seen in randomly migrating cells, strongly suggesting that it is an integral part of a feedback loop (Sasaki *et al*, 2007). Increased levels of PIP₃ coincide with the localized recruitment of pleckstrin homology (PH) domain-containing proteins, such as AKT, and regulators of actin polymerization, such as Rac, WASP, Scar/WAVE and the Arp2-Arp3 complex. The morphological changes induced by these effectors, however, are not required to maintain PIP₃ polarization, which still occurs in the presence of actin polymerization inhibitors, such as latrunculin A (Sasaki *et al*, 2004; Sasaki and Firtel, 2005).

Ras is thought to participate in a positive feedback loop leading to PIP₃ accumulation at the leading edge, as sustained activation of PI3 kinase is dependent on Ras and sustained activation of Ras is dependent on PI3 kinase (Figure 3A; Sasaki and Firtel, 2006). Ras protein remains evenly distributed around the plasma membrane, but studies using Ras-binding domain reporters have demonstrated that in response to chemoattractants it is rapidly activated in a spatially restricted manner at the leading edge (Kae *et al*, 2004; Sasaki *et al*, 2004). Furthermore, activation of Ras occurs 2–4 s before activation of PI3 kinase (Sasaki *et al*, 2004). In general agreement with these conclusions, mutants lacking two particular Ras genes, *RasG* and *RasC*, are unable to chemotax towards cAMP (Bolourani *et al*, 2006). Interestingly, in $G_{\beta\gamma}$ null cells, chemotaxis is lost, but the Ras-PI3 kinase circuit is stochastically activated leading to random migration (Sasaki *et al*, 2007). Despite these data, it is now clear that Ras and PIP₃ are not the only mediators required for generating cell polarity in a gradient. The most direct demonstration of this is seen in cells deleted of all five PI3 kinases and PTEN, which still chemotax (Hoeller and Kay, 2007). Clearly, other signals downstream of Ras must also have a part and one suggestion has been the TORC2 kinase complex (Charest *et al*, 2010). It is required for efficient chemotaxis and one of its components, R1P3, is a RasG target (Lee *et al*, 2005). In yeast, the TORC2 complex activates Rho1 to polarize actin filaments during cell division, whereas in mammalian cells it activates Rac and induces actin assembly, suggesting an evolutionary conserved role for this complex in organizing the actin cytoskeleton (Martin and Hall, 2005).

The genetic tractability of *Dictyostelium* has greatly facilitated the identification of other activities required for directional sensing. Inhibition of PLA₂ and PI3 kinase together, for example, blocks chemotaxis and both activities are up-regulated upon cAMP addition (Chen *et al*, 2007). Arachidonic acid metabolites are generated by PLA₂, but their role is not known. At later stages of *Dictyostelium* development soluble

guanylyl cyclase (sGC) is essential for cytokinesis (Veltman *et al*, 2008). Confusingly, sGC localizes at the leading edge, but the cGMP produced seems to act mostly at the rear of the cell to facilitate actomyosin contractility (Bosgraaf *et al*, 2002; Veltman *et al*, 2005). There is evidence that Ras is required for the activation of sGC, but it is not clear whether it acts upstream of PLA₂ (Bolourani *et al*, 2008). The overall take home message is that several partially redundant signalling pathways contribute to chemotaxis in this highly specialized organism, even in the context of a single chemotactic agent (Van Haastert and Devreotes, 2004).

Neutrophils become morphologically polarized during chemotaxis and although they have a more diverse set of surface G protein coupled receptors than *Dictyostelium*, the importance of localized accumulation of PIP₃ is conserved (Figure 3B; Servant *et al*, 2000). In addition, this is coupled to negative regulation of PIP₃ levels at the sides and rear of the cell, though this is mediated by SHIP1 phosphatase, not PTEN (Nishio *et al*, 2007). The importance of PIP₃ has been demonstrated directly by the localized delivery of this phospholipid to neutrophils, which promotes morphological, front-like changes (Weiner *et al*, 2002). In addition, this

elegant experiment revealed that PIP₃ participates in a positive feedback loop leading to the generation of more PIP₃, probably through the recruitment of PI3 kinase. The localized accumulation of PIP₃ in neutrophils is coupled to the extracellular gradient through a receptor and the heterotrimeric G protein, G_i, but in contrast to *Dictyostelium*, the feedback loop is dependent on Rac, not Ras (Wang *et al*, 2002; Weiner *et al*, 2002). In this case, Rac activation is mediated by the DOCK2 exchange factor, which is recruited to the plasma membrane in a PIP₃-dependent manner (Nishikimi *et al*, 2009). However, sustained activation of DOCK2 (and therefore Rac) depends on the localized generation of phosphatidic acid (PA), probably through the activation of phospholipase D (PLD). How this is achieved is not known, but interestingly, PLD is activated by Rho GTPases, including Rac (Jenkins and Frohman, 2005). A second heterotrimeric G protein, G_{12/13}, activates a Rho-ROCK pathway at the rear of the cell to promote actomyosin contractility (Xu *et al*, 2003; Wong *et al*, 2007).

Dictyostelium and neutrophils are two of the fastest migrating cells known, moving at speeds of 10 µm/min. However, many other cell types are capable of directed migration. Fibroblasts, for example, migrate in a gradient of platelet-derived growth factor (PDGF), though at much lower speeds (2 µm/min; von Philipsborn and Bastmeyer, 2007). Platelet-derived growth factor is recognized by a tyrosine kinase receptor and many of the same signalling molecules, such as Rac and PI3 kinase, are involved in generating polarized morphology, but it seems there are some significant differences (Haugh *et al*, 2000). Most notably, PIP₃ activity is not sustained by a positive feedback loop (Derman *et al*, 1997).

The use of new fluorescence biosensors for activated Rho family GTPases has provided some unexpected surprises when used in migrating cells. The morphology of a migrating cell, together with much functional analysis, has led to a general model in which localized actin polymerization at the front and actomyosin contractility at the rear are regulated by Rac and Rho, respectively (Raftopoulou and Hall, 2004). However, real-time assays using biosensors for active Rho and Rac expressed in the same migrating cell reveal co-localization of the two at the front (Machacek *et al*, 2009). Image analysis has gone

further and suggested that Rho activation at the front precedes Rac activation by approximately 40 s. The exact meaning of these results is not yet clear, but it has led to some re-thinking regarding actin dynamics at the front of a migrating cell. For example, Rho clearly regulates actomyosin contractility through ROCK, but it can also promote actin polymerization through another target, the formin-containing protein mDia1 (Nakano *et al*, 1999). Although it has been assumed that the branched actin network underlying lamellipodial extensions (regulated by Rac-Arp2-Arp3) drives forward movement during migration, an alternative possibility raised by the imaging work is that the assembly of linear actin filaments (regulated by Rho-mDia1) is required and may even precede Rac-Arp2-Arp3 (Machacek *et al*, 2009).

Collective migration

The migration of cells in groups or sheets is seen throughout animal development (Rorth, 2009). Cells respond to guidance cues and often a few cells at the front of the group act as leaders and are responsible for interpreting the extracellular guidance cues. Epithelial cells are particularly intriguing. In some cases, they undergo an EMT (epithelial-to-mesenchymal transition), and migrate similar to mesenchymal cells. However, they may also maintain their characteristic apical-basal polarity, while establishing the front-back polarity that drives migration. In this case, epithelial cells retain intimate contact through cell-cell junctions, yet are capable of rearranging with respect to each other and maintain an intact monolayer organization during migration.

Collective migration can be induced experimentally by scratching a monolayer of cultured cells and this has provided some important insights into the underlying molecular mechanisms. Using fibroblasts, astrocytes or endothelial cells, Cdc42 has been shown to have an essential role in directing actin polymerization to the leading edge and in orienting microtubules (Figure 3C; Etienne-Manneville and Hall, 2001; Tzima *et al*, 2003; Watanabe *et al*, 2004; Cau and Hall, 2005; Gomes *et al*, 2005). Cdc42 is activated specifically at the front of leading edge cells and although the trigger for GTP loading is not clear, it could be loss of cell-cell contact, or an activity released by damaged cells at the scratch edge. Cdc42 is responsible for the spatially localized activation

Figure 3 Directed cell migration. (A) *Dictyostelium* chemotaxis. Ras is activated in response to a gradient of cAMP interacting with a surface GPCR. The receptor and associated G protein, as well as Ras, remain evenly distributed around the cell, but active, GTP-bound Ras is localized specifically in the direction of the gradient. Ras activates PI3 kinase to produce PIP₃, which leads not only to the recruitment of proteins involved in modifying the actin cytoskeleton, such as SCAR/WAVE and Rac, but also a Ras GEF, which could potentially generate a positive feedback loop. PTEN localizes at the sides and rear of the cell to spatially restrict the distribution of PIP₃ within the plasma membrane. Two other second messenger systems have important roles in directional sensing, PLA₂ and soluble guanylyl cyclase (sGC), which produce arachidonic acid metabolites (AA) and cGMP, respectively. Contractile actomyosin forces at the sides and rear together with actin-driven protrusions at the front drive migration. (B) Neutrophil chemotaxis. An extracellular gradient of the bacterial-derived peptide fMLP, interacting with a GPCR (FMP2), leads to activation of an associated G_i and PI3 kinase. The PIP₃ produced recruits DOCK2 to activate Rac, which can activate PI3 kinase and set up a positive feedback loop. Sustained recruitment of DOCK2 also requires localized generation of phosphatidic acid (PA) through activation of PLD. Another G protein, G_{12/13}, activates Rho and ROCK at the rear of the cell to promote actomyosin contractility. The lipid phosphatase SHIP1 is required to maintain spatially localized PIP₃. (C) Collective migration *in vitro*. After scratching a monolayer of primary embryo-derived fibroblasts, cells migrate into the free space collectively as a sheet. Cell-cell contacts (N-cadherin) are maintained during migration. Cdc42 is activated at the leading edge of front row cells and, via PAK kinase, serves to localize Rac activity such that actin polymerization and protrusive activity are restricted to the front of the migrating cell. Through another effector, the Par6-aPKC complex, Cdc42 causes the microtubule cytoskeleton to reorganize such that the centrosome and Golgi face the direction of migration. Par6-aPKC leads to the inhibition of GSK-3 specifically at the front of the cell, through a pathway that involves Wnt5a and Dishevelled (data not shown), and causes the tumour suppressor protein APC to associate with and stabilize the plus ends of neighbouring microtubules. (D) Collective migration *in vivo*. Border cells (yellow) in the *Drosophila* ovary delaminate from the follicular epithelium (brown) and migrate as a group through nurse cells (white/grey) towards the oocyte. PVR and EGFR receptors in the border cells recognize a gradient of extracellular cues (PVF1 and Spitz) from the oocyte, and activate Rac through the Rac GEF, Mbc, to induce forward facing protrusion in cells at the front of the group. Cell-cell interactions (through E-cadherin) are required to be maintained throughout migration, though cells continuously reorganize within the group.

of at least two signalling pathways. One is mediated by the Par6-aPKC polarity complex, which is recruited to the leading edge to promote reorientation of the microtubule-associated centrosome/Golgi to face the direction of migration (Figure 3C; Etienne-Manneville and Hall, 2001). This has interesting similarities to axon initiation (see above) and involves the localized inhibition of GSK-3 coupled to APC accumulation at and stabilization of microtubule plus ends proximal to the leading edge. Further analysis has revealed greater complexity in this pathway, as inhibition of GSK-3 was observed to depend on the co-operation of Par6-aPKC with a constitutive Wnt5a/Dishevelled signalling pathway (Schlessinger *et al*, 2007; Zhang *et al*, 2007). The second Cdc42 pathway involves activation of PAK and is required to localize Rac activity at the front of the cells (Cau and Hall, 2005). Rac is essential for promoting actin polymerization, leading to cell protrusions and in the absence of Cdc42 this activity becomes de-localized around the cell periphery (Nobes and Hall, 1999). PI3 kinase does not have a major role in these migration events. Although primary fibroblasts lack strong cell-cell contacts, they nevertheless migrate collectively as a sheet in this assay. The mechanism by which cells communicate with each other, however, is not clear. Possibilities include, mechanical tension exerted on follower cells by first row cells, or biochemical signalling. Calcium increases, as well as MAP kinase activation, for example, have been observed in cells as far as 5–10 cells back from the scratch edge (Nobes and Hall, 1999).

In vivo, one of the best-studied examples of collective migration takes place in the *Drosophila* ovary, in which a group of 6–8 ‘border’ cells migrate through nurse cells to the oocyte at a speed of around 0.5 $\mu\text{m}/\text{min}$ (Figure 3D; Montell, 2003; Rorth, 2009). Genetic screens have identified a large number of gene products essential to this process, including two tyrosine kinase receptors expressed in the border cells, EGFR and PVR, which recognize EGF-like (Spitz) and PDGF-like (PVR) cues, respectively, derived from the oocyte (Duchek *et al*, 2001; McDonald *et al*, 2006). The PVR and EGFR receptors do not cluster during migration, however, their activity is spatially restricted at the leading edge of one or two leader cells in the group and interestingly, this is dependent on receptor endocytosis (Jekely *et al*, 2005; Janssens *et al*, 2010). Time-lapse imaging reveals extensive protrusive activity in cells at the leading edge of the group, though cells at the rear also show some activity (Prasad and Montell, 2007). Rac and GEF Mbc are activated downstream of these receptors and are required for migration, but PI3 kinase is reported not to be involved. However, it is possible that PIP₃ acts redundantly with another signal, as has been proposed for Ras and PLC γ , which are also required for border cell migration, but when depleted individually have no effect (Duchek *et al*, 2001; Bianco *et al*, 2007). In addition, the Par6-aPKC complex is required for migration (Pinheiro and Montell, 2004). Surprisingly, however, the protein localizes orthogonally to the direction of migration at early stages of migration, and at junctions between cells, at later times before arrival at the oocyte. Therefore, it seems unlikely that Par6-aPKC is involved directly in interpreting the guidance gradient, as was the case in collective fibroblast migration, rather it has been suggested it may be more important for maintaining polarity between interacting border cells. This is consistent with the observation that the maintenance of

E-cadherin-based cell-cell contacts is essential during migration and suggests that they retain at least some of their epithelial-like characteristics (Niewiadomska *et al*, 1999). In a recent report, a photo-activatable and genetically encoded version of Rac was introduced into border cells to explore how cells communicate with each other during migration. Activation of Rac in just one cell in the cluster promoted migration of all the cells, but more remarkably this occurred even in the presence of dominant negative guidance cue receptors (Wang *et al*, 2010). The mechanism by which the Rac-activated cell talks to other cells in the group is still unclear, though activation of the JNK pathway is required.

Conclusion

Cellular responses to extracellular guidance cues are highly dynamic and require coordination of many interconnected signalling pathways. Eukaryotic cells have devised sophisticated ways of spatially organizing these molecular networks to achieve directional sensing. Guidance cues stimulate the asymmetric distribution of signalling complexes, which direct the localized remodelling of the plasma membrane seen during chemotropism and chemotaxis. Initial asymmetry can take place at the level of the surface receptor itself (yeast mating, axon chemoattraction), or of intracellular signalling components (chemotaxis). This then promotes a cellular response through changes in the dynamic behaviour of the actin and microtubule cytoskeletons, in the endocytic and exocytic trafficking pathways, and in the adhesive forces through which cells interact with the external environment. Positive feedback loops are a prominent feature in the initiation of these responses—as experimental analysis and mathematical modelling have shown, they are capable of generating a discrete, localized cortical signalling platform from even a shallow gradient of an extracellular guidance cue. Molecular switches of the Ras and Rho GTPase families, together with PI3 kinase/PTEN lipid signalling seem to be particularly important in establishing these signalling loops, through the interplay between: (i) upstream activators and downstream targets of GTPases, (ii) lipid domains created by PI3 kinase and PTEN, and (iii) PI3 kinase and GTPases. It is perhaps no coincidence that these same molecules are key regulators of downstream responses, notably the actin cytoskeleton. There is still much to understand about the establishment of polarized signalling and the subsequent changes in cell behaviour initiated by guidance cues. New biosensors and sophisticated image analysis are beginning to provide insights into guidance mechanisms, previously unattainable with molecular and genetic approaches alone. Together with RNAi, which has opened up pseudo-genetic approaches in mammalian cells, these technologies will probably provide further greater insight into this fascinating area of cell biology.

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Conflict of interest

The authors declare that they have no conflict of interest.

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