Spring 5-16-2011

# SDF1 Antagonism of Axonal Repellents Requires Multiple G Protein Components and an Adam Metalloprotease

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**Abstract**
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Growth cones of developing axons navigate by interpreting signals from multiple cues. Some of these are the familiar guidance cues netrin, semaphorin, slit, and ephrin. Growth cones are also influenced by GPCR ligands, including neurotransmitters such as glutamate and chemokines such as SDF1. Previous work from our lab demonstrated that either glutamate or SDF1, acting through their receptors mGluR1 or CXCR4, respectively, can reduce growth cone responsiveness to repellent cues. This effect is pertussis toxin-sensitive, implicating $Ga_{i/o}$ proteins, yet dependent on increased cAMP, implicating $Ga_s$ proteins. The antirepellent effect of SDF1 could also be mimicked by inhibition of Rho, suggesting that inhibition of Rho is a component of the antirepellent pathway. Here, I demonstrate that SDF1 antirepellent activity is blocked by peptides or proteins targeting $Ga_i$, $Ga_q$, or Gbg. This suggests that multiple G protein components are required for SDF1 signaling. I also show that SDF1 antirepellent activity is mimicked by constitutively active forms of $Ga_q$, $Ga_i$, or $Ga_s$. This suggests that higher-than-physiological levels of individual G protein components can substitute for a combination of G protein components in antirepellent signaling. A role for $Ga_q$ in antirepellent signaling is further supported by the ability of a phospholipase C (PLC) inhibitor to block the SDF1 antirepellent effect, consistent with $Ga_q$'s canonical activation of PLC. My work also reveals an alternate mechanism for SDF1-induced antagonism of repellent signaling. I show that the metalloprotease ADAM10 can cleave the repellent receptor neuropilin-1. Further, SDF1 antirepellent activity is blocked by either the metalloprotease inhibitor TAPI-2 or a dominant-negative ADAM10. Thus, inhibitory shedding of repellent receptors may contribute to the antirepellent effect. Previous work has shown that the antirepellent effect is mimicked by pharmacologically increased cAMP or blocked by a cAMP antagonist. TAPI-2 does not block the antirepellent effects of a cAMP analogue, suggesting that ADAM activation belongs to a separate pathway not downstream of cAMP. This work supports a model wherein SDF1/CXCR4 activates multiple G protein components to both increase cAMP and activate ADAM10. This would reduce sensitivity to repellents through inactivation of Rho and clearing of repellent receptors from the growth cone surface.

**Degree Type**
Dissertation

**Degree Name**
Doctor of Philosophy (PhD)

**Graduate Group**
Neuroscience

**First Advisor**
Jonathan A. Raper

**Keywords**
SDF1, axon guidance, G protein, ADAM10, CXCR4, GPCR

**Subject Categories**
Developmental Neuroscience

# SDF1 ANTAGONISM OF AXONAL REPELLENTS REQUIRES MULTIPLE G PROTEIN COMPONENTS AND AN ADAM METALLOPROTEASE

Esther Naomi Twery

A DISSERTATION

in

Neuroscience

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2011

SUPERVISOR OF DISSERTATION

_____

Jonathan Raper, Professor of Neuroscience

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## ACKNOWLEDGMENTS

Many thanks to JAR and to my family and friends for their advice and support through this process. --ENT

**ABSTRACT.**

SDF1 ANTAGONISM OF AXONAL REPELLENTS REQUIRES MULTIPLE G PROTEIN COMPONENTS AND AN ADAM METALLOPROTEASE

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Growth cones of developing axons navigate by interpreting signals from multiple cues. Some of these are the familiar guidance cues netrin, semaphorin, slit, and ephrin. Growth cones are also influenced by GPCR ligands, including neurotransmitters such as glutamate and chemokines such as SDF1. Previous work from our lab demonstrated that either glutamate or SDF1, acting through their receptors mGluR1 or CXCR4, respectively, can reduce growth cone responsiveness to repellent cues. This effect is pertussis toxin-sensitive, implicating $G\alpha_{i/o}$ proteins, yet dependent on increased cAMP, implicating $G\alpha_s$ proteins. The antirepellent effect of SDF1 could also be mimicked by inhibition of Rho, suggesting that inhibition of Rho is a component of the antirepellent pathway. Here, I demonstrate that SDF1 antirepellent activity is blocked by peptides or proteins targeting $G\alpha_i$, $G\alpha_q$, or $G\beta\gamma$. This suggests that multiple G protein components are required for SDF1 signaling. I also show that SDF1 antirepellent activity is mimicked by constitutively active forms of $G\alpha_q$, $G\alpha_i$, or $G\alpha_s$. This suggests that higher-than-physiological levels of individual G protein components can substitute for a combination of G protein components in antirepellent signaling. A role for $G\alpha_q$ in antirepellent signaling is further supported by the ability of a phospholipase C (PLC)

inhibitor to block the SDF1 antirepellent effect, consistent with $G\alpha_q$'s canonical activation of PLC. My work also reveals an alternate mechanism for SDF1-induced antagonism of repellent signaling. I show that the metalloprotease ADAM10 can cleave the repellent receptor neuropilin-1. Further, SDF1 antirepellent activity is blocked by either the metalloprotease inhibitor TAPI-2 or a dominant-negative ADAM10. Thus, inhibitory shedding of repellent receptors may contribute to the antirepellent effect. Previous work has shown that the antirepellent effect is mimicked by pharmacologically increased cAMP or blocked by a cAMP antagonist. TAPI-2 does not block the antirepellent effects of a cAMP analogue, suggesting that ADAM activation belongs to a separate pathway not downstream of cAMP. This work supports a model wherein SDF1/CXCR4 activates multiple G protein components to both increase cAMP and activate ADAM10. This would reduce sensitivity to repellents through inactivation of Rho and clearing of repellent receptors from the growth cone surface.

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## Chapter 1: Introduction

Axon guidance is a complex process required for the formation of proper connections between neurons and their targets. Guidance is influenced by external cues, signaling initiated by those cues, and the state of the cell when it encounters those cues. In this chapter, I will discuss aspects of the classical guidance molecules, G protein signaling, and modulation of axon guidance.

### Guidance cues

### Basic properties of classical guidance cues

The first several axon guidance cues identified fall into four major gene families, the semaphorins, slits, ephrins, and netrins. Those four classes of proteins comprise what are sometimes called the classical guidance cues, to distinguish them from the morphogens, chemokines, neurotransmitters, and other molecules which have more recently been identified as helping to guide axons.

Semaphorins (semas) are a large family of proteins, divided into six classes in animals and a separate class V, composed of of viral semaphorins (see Fiore and Püschel, 2003). Classes 1, 4, 5, 6, and V are transmembrane, class 7 is GPI-linked, and classes 2 and 3 are secreted (see Fiore and Püschel, 2003). Though different classes of semas have different receptor complexes, plexins are receptors or coreceptors for all except class 2 semas (Yazdani and Terman, 2006). Neuropilins are the primary family of sema coreceptors,

particularly for class 3 semaphorins, for which they contribute to ligand-receptor specificity (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). However, neuropilins have short intracellular domains, leaving plexins to transduce most of the intracellular signaling.

Sema3A, the first identified semaphorin and the primary repellent which I will discuss in later chapters, was initially identified from chick brain and is a strong repellent for dorsal root ganglion axons (Luo et al., 1993). In some systems, the sema3A receptor complex has been shown to include the cell adhesion molecules L1 (Castellani et al., 2000; Castellani et al., 2002) and TAG-1 (Law et al., 2008) as well as neuropilin-1 and plexin-A1 (Kolodkin et al., 1997; Takahashi et al., 1999). Sema3A can also act as an attractant for dendrites from cortical neurons (Polleux et al., 2000), and other class 3 semas can attract certain populations of axons (Chauvet et al., 2007; Kolk et al., 2009) in addition to their better studied actions as repellents.

There is no widely established signaling pathway for repulsion induced by any semaphorin, and the intermediate steps identified differ across semas. Sema3A has been shown to quickly induce actin depolymerization (Fan et al., 1993), which is a likely component of all repellent signaling. Similarly, recent work in fly shows a requirement for the flavoprotein monooxygenase Mical in sema-induced actin rearrangement, and this appears to be a relatively direct pathway (Hung et al., 2010, Terman et al., 2002), but sema signaling in other contexts is more complex. Sema3A-induced growth cone collapse can be enhanced by constitutively active Rac1 or reduced by dominant-negative

Rac1, suggesting that activation of Rac1 is a component of the sema3A signaling pathway, but a similar dominant-negative RhoA had no effect on sema3A-induced collapse (Jin and Strittmatter, 1997). Similarly, Kuhn et al. (1999) found that dominant-negative Rac1 or dominant-negative Cdc42 could block collapse in response to sema3A, further supporting a role for small G proteins in sema signaling. However, collapse in response to sema4D, which signals through plexin-B1 rather than plexin-A1 (Tamagnone et al., 1999), can be blocked by a dominant-negative PDZ-Rho-GEF, which binds directly to plexin-B1 (Aurandt et al., 2002; Swiercz et al., 2002). In addition to their effects on small G proteins, semas have been shown to increase active GSK-3β (Eickholt et al., 2002), condense growth cone microtubules (Dent et al., 2004), and inactivate cofilin (Aizawa et al., 2001).

The slits are a smaller family of secreted repellents, with three main forms in vertebrates and one in invertebrates (see Dickson and Gilestro, 2006). They act through the robo family of receptors (Brose et al., 1999). Slit and robo were first identified in the midline of the fly (Rothberg et al., 1988; Seeger et al., 1993). Mutations in *robo* cause inappropriate crossing and recrossing of the midline and mutations in *slit* cause axons to collapse upon the midline. Slit/robo signaling is also important in the vertebrate midline, both at the spinal cord and the optic chiasm. At the chiasm, robo mutations in zebrafish (*astray*) lead to inappropriate ipsilateral and anterior projections from the optic nerve (Fricke et al., 2001). In the spinal cord, disruption of slit/robo signaling resembles the fly phenotype of axons failing to leave the ventral midline (Long et al., 2004). Slit/robo signaling is somewhat better understood than sema signaling, and it also appears to be

largely conserved across species. In fly, slit binding to robo induces recruitment of the adaptor protein Dock and the Rac GEF Sos (Fan et al., 2003; Yang and Bashaw, 2006). In vertebrate neurons, slit/robo signaling has been shown to activate the microtubule plus-end tracking protein CLASP (Lee et al., 2004), and robo has been shown to directly bind a family of Rho GAPs (Wong et al., 2001).

Netrins are the only classical guidance cues to have major functions as attractants. They were first identified in *C. elegans* as UNC-6, where mutations disrupted dorsal and ventral pathfinding (Ishii et al., 1992), and soon thereafter in chick, where they were shown to promote outgrowth *in vitro* (Serafini et al., 1994). There are three classes of netrin receptors, DCC/UNC-40/Frazzled, UNC-5, and DSCAM (Chan et al., 1996; Kolodziej et al., 1996; Leonardo et al., 1997; Ackerman et al., 1997; Ly et al., 2008). Of these, DCC and DSCAM can mediate attraction (Keino-Masu et al., 1996 ; Ly et al., 2008). UNC-5, however, mediates repulsion in both worms and vertebrates (Hedgecock et al., 1990; Colamarino and Tessier-Lavigne, 1995). Netrins are perhaps best known for their role in attracting axons to the midline, whether in worm (Ishii et al., 1992), fly (Harris et al., 1996) or vertebrates (Serafini et al., 1994; Serafini et al., 1996). Netrin signals through cAMP and PKA (Ming et al., 1997) and activation of the small G proteins Cdc42 and Rac (Shekarabi and Kennedy, 2002; Li et al., 2002).

The last major class of classical guidance molecules is the ephrins and Ephs. Ephs are receptor tyrosine kinases, and their activation by ephrins induces receptor dimerization and phosphorylation (Kalo and Pasquale, 1999). Ephrins come in two subtypes, the GPI-

linked ephrin As and the transmembrane ephrin Bs. Generally, the ephrin As bind EphA-type receptors and ephrin Bs bind EphB-type receptors, but ephrinA5 has also been shown to bind EphB2 (Himanen et al., 2004). Ephrin/Eph activity is classically required in topographical patterning in the optic tectum through gradients of A and B-type signals, (Cheng et al., 1995; Marcus et al., 1996) and in the mammalian optic chiasm, where EphB1 is required to send non-crossing RGC axons to the ipsilateral superior colliculus (Williams et al., 2003). Ephs are also involved in the formation of excitatory synapses. EphB2 recruits both NMDA- and AMPA-type glutamate receptors to developing dendritic spines (Dalva et al., 2000; Kayser et al., 2006). In repulsive axon guidance, in addition to Eph autophosphorylation, Ephs can bind SH2- and PDZ-containing proteins (Holland et al., 1997; Dodelet et al., 1999; Torres et al., 1998) as well as GEFs for Rho (Penzes et al., 2003; Sahin et al., 2005; Cowan et al., 2005). In some situations, such as the formation of the anterior commissure in mouse (Kullander et al., 2001), functional Eph signaling does not require kinase activity and must thus work through other Eph effectors.

**Morphogens as guidance cues**

Morphogens are proteins that act early in development to pattern the embryo and control cell fate decisions, but several classes of morphogen have also been shown to act as guidance cues later in development. Wnts, for example, are involved in early patterning, during which they provide a caudal signal in somitogenesis and within the neural plate

(McGrew et al., 1995). Later, Wnt-1 is required for the formation of the caudal midbrain (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). Gradients of Wnts also contribute to the anterior turn made by commissural axons after crossing the midline (Lyuksyutova et al., 2003). BMPs are a major dorsalizing factor in the spinal cord (Liem et al., 1997; Nguyen et al., 2000), where they promote sensory neuronal fates. Once neurons are specified and their axons are extending, BMPs repel commissural axons from the dorsal spinal cord, so they can cross at the floorplate (Augsburger et al., 1999; Butler and Dodd, 2003). Sonic hedgehog (Shh) is also active in early embryonic development, for example, acting as a ventralizing factor in the spinal cord in opposition to BMPs. In axon guidance, Shh acts along with netrin as a floorplate/midline attractant for commissural axons (Charron et al., 2003).

**Classical guidance cues in other systems**

Morphogens are not the only cues that function in multiple stages of development. Classical guidance cues also have other functions, both embryonically and in the adult. The most common additional function for guidance cues is guidance in angiogenesis. Slit/robo signaling promotes angiogenesis (Wang et al., 2003; Yang et al., 2010). Netrin, however, has been shown to negatively regulate branching of blood vessels (Lu et al., 2004) and to act as an anti-angiogenic factor (Baioni et al., 2010). Though class 3 semas do not act directly in angiogenesis, their receptors neuropilin-1 and neuropilin-2 are also receptors for vascular endothelial growth factor (VEGF). Neuropilins promote

angiogenesis upon VEGF stimulation (Gu et al., 2003), and sema/neuropilin signaling is required for development of the heart (Gu et al., 2003).

The promotion of angiogenesis, while clearly useful in the developing embryo, can later contribute to tumor angiogenesis and support cancer growth. Recent work has found that inhibition of slit signaling can reduce angiogenesis and tumor growth in melanoma (Wang et al., 2003). Cancer cells are also often motile and can thus be guided by classical axon guidance cues. Semaphorins and netrins have also been linked to several aspects of cancer progression (Flannery and Duman-Scheel, 2009; Duman-Scheel, 2009). Sema4D/Plexin-B1 signaling, in particular, contributes to invasive growth of epithelial cells (Giordano et al., 2002; Basile et al., 2005), and it contributes to the overall and vascular growth of carcinomal tumors in culture (Basile et al., 2006).

**GPCR signaling**

Though none of the classical guidance cues signal through GPCRs, neurotransmitters, chemokines, and other signaling molecules do activate GPCRs. This signaling contributes to migration of various cell types and to axon guidance, including the reduction in growth cone responsiveness to repellents which is the focus of my work.

## G protein signaling

The G protein alpha subunits are classified into four families based on their most common signaling properties. These families, named for the founding members, are $G\alpha_s$, $G\alpha_{q/11}$, $G\alpha_{i/o}$, and $G\alpha_{12/13}$. The $G\alpha_s$ family, comprising $G\alpha_s$ and $G\alpha_{olf}$, is best known for its ability to activate adenylate cyclases (Gilman, 1987). The $G\alpha_{i/o}$ family, which also includes $G\alpha_t$ (transducin) and $G\alpha_z$, was initially identified for its ability to inhibit adenylate cyclases (Gilman, 1987), but $G\alpha_{i/o}$ proteins also commonly activate src and Akt (Ram and Iyengar, 2001). The $G\alpha_{q/11}$ family includes $G\alpha_{14}$, $G\alpha_{15}$, and $G\alpha_{16}$ as well as $G\alpha_q$ and $G\alpha_{11}$, and it is best known for its activation of PLC and subsequent increases in cytosolic calcium (Birnbaumer 2006). The last family, $G\alpha_{12/13}$, typically activates the small G protein Rho (Buhl et al., 1995), but $G\alpha_{12/13}$ has recently been shown to also activate adenylate cyclase 7, thereby increasing cAMP (Li et al., 2008).

## Beta-arrestins

It is now clear that GPCRs can signal through mechanisms other than G protein activation. One such pathway uses beta-arrestins, which are scaffolding proteins that bind GPCRs. Beta-arrestins were first identified as negative regulators of GPCRs through ligand-dependent endocytosis and silencing (Lohse et al., 1990). More recently, however, beta-arrestins have been shown to mediate GPCR-dependent signaling, both

alongside G protein-dependent signaling and independent of G proteins (see DeFea, 2008). Interestingly, beta-arrestin-dependent endocytosis and signaling may be performed by separate beta-arrestins, so that the two processes are independent of each other (Zidar et al., 2009). The best established function of beta-arrestin signaling, whether G protein-dependent or –independent, is MAPK activation (Rakhit et al., 2001; Sun et al., 2002, among others). In G protein-dependent beta-arrestin signaling, G proteins typically activate MAPK away from the plasma membrane and beta-arrestin activates MAPK at the cell surface; G protein-independent beta-arrestin signaling includes activation of MAP kinases, inactivation of PI3K, and inactivation of Akt (Shenoy et al., 2006, Wang and DeFea, 2006; Beaulieu et al., 2005).

**Multiplicity of G proteins**

Although there are GPCRs which signal through a single type of G protein, many GPCRs work through multiple G proteins, spanning two or more classes. The G proteins activated in any given circumstance may vary by cell type, process, or ligand.

In osteoblasts, responses to parathyroid hormone receptor 1 (PTH1R) require $G\alpha_s$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$ for appropriate bone development (Wang et al., 2010). Interestingly, Wang et al. show that different G proteins regulate different subsets of PTH-controlled genes, with most genes being regulated by combinations of G proteins.

Another receptor that is known to signal through multiple G proteins is the class I metabotropic glutamate receptor mGluR1. Most studies focus on the activation of $G\alpha_{q/11}$-type receptors and increases in Ca2+, but activation of $G\alpha_i$ is also widely accepted (see Ferraguti et al., 2008). Some work (Francesconi and Duvoisin, 1998; Tateyama and Kubo, 2006) has suggested that mGluR1 can activate $G\alpha_s$ along with $G\alpha_q$, and others have shown mGluR1 activation of $G\alpha_q$, $G\alpha_i$, and $G\alpha_s$ in hippocampal cells (Berkeley and Levey, 2003). Previous work from our lab (Kreibich et al, 2004) found a cAMP increase from mGluR1, which we attribute to $G\alpha_{i/o}$ based on its sensitivity to PTX.

Chemokine receptors, including CXCR4, have been shown in various cell types to activate both $G\alpha_{i/o}$ and $G\alpha_{q/11}$ family G proteins (Hall, et al., 1999; Rosenkilde et al., 2004; Kawata et al., 2005; Ignatov et al., 2006; Shi et al., 2007; Tian et al., 2008; Ngai et al., 2009). Further, Soede et al. (2000) found that these G proteins were differentially required in myeloid leukemia cells depending on the type of chemotaxis being assayed. That is, both $G\alpha_i$ and $G\alpha_q$ were required for migration to the liver and spleen, but only $G\alpha_q$ was required for migration to the bone marrow. This suggests that SDF1-induced motility might depend on different processes, which vary depending upon destination or other cell surroundings.

**Efficacy and specificity of minigenes**

GPCRs activate specific G proteins, and the part of the G protein that enables specific receptor-G protein binding is the C-terminal tail of the alpha subunit. Over the last fifteen years, inhibitory peptides based on those sequences have been employed to study the receptor/G protein pairs involved in various signaling processes. Earlier work used longer peptides, of about 50 amino acids, to block G protein binding. Rasenick et al. (1994), using the longer peptides, found that targeting $G\alpha_s$ proteins blocked beta2-adrenergic-receptor-induced increases in cAMP in C6 glioma cells. However, much work over the last ten years has found 10-12aa peptides sufficient. These peptides have been shown in multiple systems to specifically block the targeted class of G protein.

Gilchrist et al. (1999), demonstrating the functionality of the shorter peptides in transfected HEK293 cells, could block the activity of the M2 acetylcholine receptor, which is known to activate $G\alpha_i$, with a $G\alpha_i$-targeting peptide but not with those based on $G\alpha_s$ or $G\alpha_q$ or a scrambled version of the $G\alpha_i$ peptide. Lin et al. (2005) found that short peptides based on $G\alpha_{12/13}$ caused the same gastrulation defects in zebrafish embryos as did morpholinos against these G proteins. Yao et al. (2010) showed that a short peptide targeting $G\alpha_q$ could reduce carbon dioxide sensitivity in *Drosophila* olfactory receptor neurons, but there was no change in response when they used peptides targeting $G\alpha_i$, $G\alpha_o$, or $G\alpha_s$, or a scrambled version of the $G\alpha_q$ peptide.

**Modulation in axon guidance**

Work from several labs has shown a role for cyclic nucleotides in controlling growth cones' responses to extracellular cues. The Poo lab has shown that using pharmacological reagents to change the levels of cAMP and cGMP in *Xenopus* spinal neurons can switch growth cone responses either from attractive to repulsive or from repulsive to attractive, depending on the guidance cue applied (Ming et al., 1997; Song et al., 1998). Their model is that this is due to the ratio of cAMP to cGMP. Similarly, previous work from our lab (Chalasani et al., 2003) demonstrated that SDF1's ability to reduce growth cone responsiveness to repellents could be blocked by inhibition of cAMP and PKA, and that pharmacologically increasing cAMP levels in chick explants could reduce growth cone responsiveness to repellents, thus mimicking SDF1. Comparable alterations of cGMP levels had no effect on growth cone behavior. In mouse, Imai et al. (2006) showed that the levels of cAMP in growth cones/axons changed the location to which mouse olfactory sensory neurons project in the olfactory bulb. Mutating olfactory receptors to decrease cAMP moved the projection anteriorly, while the addition of a constitutively active $G\alpha_s$ moved the projection posteriorly. Though such mutations in olfactory receptor signaling do not quite constitute modulation of extracellular cues, this supports a model for growth cone navigation that is strongly influenced by cAMP levels.

Growth cone responses can also be modulated by alterations in transcription or translation of receptors for various guidance cues. For example, Imai et al. (2006) found

that neurons expressing a dominant-negative PKA also expressed lower levels of the sema3A receptor neuropilin-1. This, presumably by reducing repulsive responses to sema3A, led to projections anterior to those of neurons expressing that olfactory receptor without the dominant-negative PKA.

Recent work from the Fournier lab (Kent et al., 2010) showed that scaffolding molecules other than beta-arrestins can modulate growth cone responses. Specifically, 14-3-3, by binding PKA, promotes a repulsive response to NGF. Since PKA is a major effector of cAMP, another component of guidance modulation, 14-3-3 expression may partly determine the ability of a growth cone to respond to antirepellent cues.

Another mechanism for changing growth cone responsiveness to particular cues is alteration of the surface expression of receptors for those cues. This can be achieved either by enhancing or reducing exocytosis or endocytosis (ligand-induced or otherwise) or by cleavage and proteolysis of the receptor. This can resemble the regulation of robo-mediated repulsion by commissureless, wherein pre-crossing axons have little surface expression of robo because commissureless sequesters it and leads to its degradation (Kidd et al., 1998; Keleman et al., 2002). Guidance cues modulating surface expression of their own receptors can induce shifts in either direction, as with netrin's ability to increase DCC membrane insertion in rat cortical neurons after long-term exposure (Bouchard et al., 2004) or to decrease surface DCC in rat cortical neurons exposed to netrin more briefly (Kim et al., 2005).

Though not directly related to axon guidance, recent findings from Heinisch et al. (2010) suggest another possible mechanism for signal integration. They showed that, in slice preparations from rat brain, the chemokines SDF1 and CX3CL1 could reduce hyperpolarization induced by morphine, while neither SDF1 nor CX3CL1 alone altered the cells' membrane potentials. Axon branching and synaptogenesis are known to be affected by electrical activity (Uesaka et al., 2006; Saneyoshi et al., 2010), and neurotransmitters can act as guidance cues (Lipton et al., 1988; Zheng et al., 1994; Xiang et al., 2002; Berghuis et al., 2007) or as modulators of guidance cues (Kreibich et al., 2004; Bonnin et al., 2007). Thus, alteration of neurons' electrophysiological properties might also affect their targeting.

In the next chapter, I will show that SDF1 antirepellent signaling requires $G\alpha_i$, $G\alpha_q$, and $G\beta\gamma$ in combination, as well as activation of phospholipase C. I will then go on to show that SDF1 antirepellent activity requires the metalloprotease ADAM10, potentially to clear repellent receptors from the growth cone surface. Finally, I will discuss a few important open questions regarding SDF1 and G proteins in modulation of axon guidance.

# *SDF1-INDUCED ANTAGONISM OF AXONAL REPULSION REQUIRES MULTIPLE G-PROTEIN COUPLED SIGNALING COMPONENTS THAT WORK IN PARALLEL.*

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**Key words:** Stromal-cell derived factor 1, SDF1, CXCR4, G protein, GPCR, axon guidance, modulation, semaphorin, sema3A, cAMP

**Abstract**.

SDF1 reduces the responsiveness of axonal growth cones to repellent guidance cues in a pertussis-toxin-sensitive, cAMP-dependent manner. Here, we show that SDF1's antirepellent effect can be blocked in embryonic chick dorsal root ganglia (DRGs) by expression of peptides or proteins inhibiting either $G\alpha_i$, $G\alpha_q$, or $G\beta\gamma$. SDF1 antirepellent activity is also blocked by pharmacological inhibition of PLC, a common effector protein for $G\alpha_q$. We also show that SDF1 antirepellent activity can be mimicked by overexpression of constitutively active $G\alpha_i$, $G\alpha_q$, or $G\alpha_s$. These results suggest a model in which multiple G protein components cooperate to produce the cAMP levels required for SDF1 antirepellent activity.

**Introduction.**

The development of the nervous system requires the formation of numerous precise connections between neurons and their targets. Growth cones navigate through complex environments in which they are simultaneously exposed to many different guidance cues. Understanding how a growth cone integrates competing cues into a unitary guidance decision is a major challenge. One region of the developing nervous system in which axons are faced with competing guidance information is the developing optic nerve. For example, as axons leave the eye, they are simultaneously exposed to the potent repellent slit2 and to the chemokine SDF1, both of which are expressed along the optic stalk (Niclou et al., 2000; Erskine et al., 2000; Chalasani et al., 2003b; Chalasani et al., 2007; Li et al., 2005). The presence of slit2 might be expected to preclude retinal extension, but SDF1 can mitigate its repellent effects. SDF1, acting through its G-protein coupled receptor CXCR4, has been shown to reduce the sensitivity of growth cones to a variety of repellents *in vitro* including slit2 (Chalasani et al., 2003a).

The signaling pathway through which SDF1 reduces growth cone responses to repellents has been studied using wholly pharmacological approaches (Chalasani et al., 2003a; Kreibich et al., 2004). SDF1's anti-repellent activity in primary neurons is blocked by pertussis toxin, which inhibits $G\alpha_i$ or $G\alpha_o$, and calmidazolium chloride, which inhibits calmodulin. SDF1 activity is also blocked by the PKA inhibitors PKI and Rp-cAMPs, and mimicked by the cAMP analogue Sp-cAMPs. Further, SDF1 activity is blocked by knockdown of the calcium/calmodulin-stimulated adenylate cyclase ADCY8 (Xu et al., 2010). These findings suggest that increased cAMP levels are a component of the SDF1 antirepellent pathway, despite the apparent requirement for G proteins that canonically induce decreased cAMP levels. Although these studies provide an essential outline of the pathway, they leave many questions unanswered. One of these is how a pertussis toxin-sensitive pathway could lead to increased, rather than decreased, cAMP.

To better understand how CXCR4 activation increases cAMP levels, we began by investigating the identities of the G proteins required for antirepellent activity. We transfected primary neuronal cultures with constructs designed to block specific Gα or Gβγ subunits and assayed their effects on antirepellent signaling. Working downstream from these signaling components, we then examined the involvement of phospholipase C (PLC) in SDF1 signaling.

Here, we demonstrate that SDF1's antirepellent activity requires two distinct G alpha subunits, $G\alpha_i$ and $G\alpha_q$. We also show that anti-repellent signaling is abrogated by a Gβγ scavenger, GRK-CT. These results suggest that $G\alpha_i$, $G\alpha_q$, and Gβγ all cooperate to generate SDF1 antirepellent activity. We also show that antirepellent signaling is blocked by PLC inhibitors. Taken together with previous findings, these results are consistent with SDF1/CXCR4 signaling acting through multiple G protein subunits that work together to activate PLC, which in turn ultimately leads to elevated internal calcium levels that stimulate the calcium/calmodulin-dependent adenylate cyclase ADCY8 to produce cAMP.

## Materials and Methods

*Ethics statement.* Chick embryos were maintained according to University of Pennsylvania Institutional Animal Care and Use Committee (IACUC) guidelines, approved as protocol #802243.

*Cell culture and explant-based collapse assays.* Fertile chicken eggs were purchased from B&E Eggs, York Springs, PA. DRGs were dissected from E7 chick embryos and grown on laminin-coated coverslips in F12 supplemented medium as previously described (Niclou et al., 2000). Explants were cultured for 18-20 hours before treatment. SDF1 (50nM, Invitrogen), supernatant from sema3A-transfected 293T cells, and/or pharmacological inhibitors as noted were added to wells at the same time. Cells were returned to the incubator for 30 minutes and then fixed for at least 30 minutes with 4% paraformaldehyde plus 10% sucrose in PBS. Growth cones were examined on an Axiovert 35 (Zeiss) with phase optics and scored as collapsed if they had no lamella and no more than two filopodia as described in Kapfhammer et al. (2007). Numbers of collapsed and uncollapsed growth cones from pairs of treatment conditions were compared with a two-tailed Fisher Exact Test and considered significant if $p < 0.05$. Statistical comparisons were performed with Prism (GraphPad, La Jolla, CA).

*Transfection.* E7 chick DRGs were dissociated by incubation with 0.25% trypsin-EDTA (Invitrogen) for 20 minutes at 37ºC and then resuspended in Amaxa nucleofector solution. Cells from 12 ganglia were electroporated with 4µg total plasmid DNA using the G-013 program for the rat neuron kit and the Amaxa nucleofector (Lonza). Plasmid volume varied from 3-10µL, depending on plasmid concentration. Cells were cotransfected with EYFP or Citrine (2µg) and an experimental plasmid (2µg). Transfected cells were cultured as described above for 24 hours before treatment with sema3A supernatant. Plasmid-expressing cells were identified by expression of EYFP or Citrine and counts of brightly green growth cones were analyzed as above.

*Plasmids and Reagents.* Expression plasmids for constitutively active G proteins, RGS proteins, and dominant-negative $G\alpha_i$ were obtained from the Missouri Science and Technology cDNA Resource Center (Rolla; cdna.org). An expression plasmid containing GRK-CT was provided by P. Alberts (Ghahremani et al., 1999). Expression plasmids encoding G protein interfering peptides were obtained from Cue Biotech (Gilchrist et al., 1999). The PLC inhibitor U73122 (Sigma) was used at 20nM.

*Immunostaining.* Fixed cultures were washed once with PBS and 3 times with PBS + 0.1% Triton-X100, then blocked for half an hour in blocking reagent: PBS + 3% bovine albumin, 1% PVP-10, 1% PVP-40, and 0.1% PVP-360 (Sigma) with 0.2% Triton-X100 added. Goat anti-GFP (Rockland) or mouse anti-HA (Covance) were used at 1:500 and visualized with AlexaFluor secondary antibodies (Invitrogen). Cultured cells were imaged either on a Zeiss Axiovert 35 with a 63x objective or on a (Leica Confocal) with a 63x objective and 3x zoom. Multiple colors were imaged with line-by-line sequential scanning.

**Results.**

***Blocking $G\alpha_i$ or $G\alpha_q$ blocks SDF1 antirepellent activity.***

Semaphorin 3A (sema3A) is a powerful repellent for dorsal root ganglion (DRG) axons (Luo et al., 1993). Bath application of sema3A to DRG growth cones induces them to transition from a spread motile morphology to a collapsed shape without lamellae and few filopodia (Luo et al., 1993). This dramatic change in morphology can be used to measure the strength of repellent cues or to measure the relative susceptibility of growth cones to repellent cues. Using this assay, the repellent responses of DRG growth cones to sema3A, sympathetic growth cones to sema3C, or retinal growth cones to slit2, have all been shown to be greatly reduced in the presence of the chemokine SDF1 (Chalasani et al., 2003a). SDF1 by itself has little discernible effect on these growth cones, but when SDF1 is present, 5 to 8 times more repellent is required to induce half maximal growth cone collapse (Chalasani et al., 2003a).

SDF1 acts through its seven transmembrane receptor, CXCR4, to mitigate the ability of repellents to collapse growth cones (Chalasani et al., 2003a). Paradoxically, although its signaling pathway in primary neurons is blocked by the $G\alpha_{i/o}$ blocker pertussis toxin, SDF1 appears to induce increased cAMP levels. Previous work from our laboratory showed that SDF1's antirepellent effects can be blocked by the cAMP antagonist RpcAMPs or mimicked by the cAMP analogue SpcAMPs (Chalasani et al., 2003a). An SDF1-induced rise in cAMP has been observed in cultured primary chick retinal neurons (Xu et al., 2010). To better define the specific G-protein components through which SDF1 acts, dissociated DRGs were co-transfected with expression constructs for EYFP along with plasmids encoding short peptides that selectively block signaling through specific Gα containing G-proteins. These peptides are derived from the C termini of the Gα proteins they target and they selectively compete with the targeted Gα proteins for receptor binding (Gilchrist et al., 1999). Their selectivity and effectiveness has been demonstrated in several other systems, including zebrafish (Lin et al., 2005) and fly (Yao and Carlson, 2010).

DRG neurons transfected with EYFP alone collapse in response to sema3A (Fig. 1A; compare the first and second grey bars in Fig. 1C, D). The presence of SDF1 makes DRG growth cones resistant to sema3A (Fig. 1A; compare second and third grey bars in Fig.1C, D). For these experiments, transfected DRG cultures were stained for EYFP and only those growth cones that were brightly fluorescent were counted. In EYFP-only conditions, cultures show low background collapse. The percentage of collapsed growth cones increases in the presence of sema3A but increases significantly less when SDF1 is added along with sema3A. Co-transfection of expression plasmids encoding EYFP along with peptides targeting $G\alpha_{q/11}$ (Fig. 1C, first panel) or $G\alpha_{i1/2}$ (Fig. 1C, second panel) have no effect upon DRG growth cone collapse in the presence of sema3A alone (compare the middle grey bars to the middle black bars). However, the $G\alpha_{q/11}$ or $G\alpha_{i1/2}$ peptides do block SDF1's ability to reduce collapse in response to sema3A (compare the third grey bars to the third black bars). This suggests that both $G\alpha_q$ and $G\alpha_i$ mediated G-protein coupled signaling are each required for SDF1's antirepellent effect. A full-length dominant negative $G\alpha_i$ that has been shown to be effective in transfected CHO cells (Winitz et al., 1994) was tested for its ability to block SDF1-mediated signaling. This construct also blocked the SDF1 antirepellent effect, corroborating the finding with the $G\alpha_i$ based peptide (Fig. 1C, third panel). Co-transfection of EYFP with peptides targeting $G\alpha_s$ or $G\alpha_{o1}$ had no effect on DRG responses to sema3A or to SDF1 (Fig. 1D). Because the effectiveness of the $G\alpha_s$ and $G\alpha_{o1}$ peptides has been tested in other systems (Rasenick et al., 1994; Vanhauwe et al., 2002), the $G\alpha_{i1/2}$ and $G\alpha_q$ peptides were effective, and all of the interfering peptides were expressed from identical expression plasmids, we conclude that $G\alpha_s$ and $G\alpha_o$ are unlikely to be required for antirepellent activity.

***Both Gβγ and Gα are necessary for SDF1 antirepellent activity.***

Because the short inhibitory peptides we used block the initial receptor mediated

dissociation and activation of G proteins, they cannot determine whether SDF1 signaling depends upon alpha or beta-gamma subunits to activate downstream targets. We used the C-terminal portion of GRK2, or GRK-CT, as a Gβγ scavenger that should prevent the complex from stimulating downstream targets (Fig. 2A). Ghahremani et al. (1999) showed that this protein fragment could block Gβγ-specific calcium release in LD2S cells and this construct has since been widely used. Coexpression of GRK-CT with EYFP does not increase background collapse or interfere with growth cones' responses to sema3A (Fig. 2B). GRK-CT does, however, block SDF1-induced reduction in sema3A-mediated growth cone collapse, suggesting that SDF1 antirepellent activity requires Gβγ-induced activation of downstream targets.

We next set out to determine whether specific Gα subunits activate downstream targets in SDF1 mediated antirepellent signaling. RGS proteins act as GAPs for Gα subunits (Fig 2C). RGS2 specifically binds and inactivates $G\alpha_q$, and RGS4 primarily binds $G\alpha_i$ but also binds $G\alpha_q$ to a lesser extent (Heximer, 2004; Huang et al., 1997). Coexpression of either RGS2 or RGS4 with EYFP does not affect background levels of collapse, nor does it interfere with sema3A induced collapse (Fig. 2D). Expression of either RGS2 or RGS4 does however, interfere with SDF1's ability to reduce collapse in response to sema3A (Fig. 2D). These results suggest that $G\alpha_q$, and possibly $G\alpha_i$, activate downstream targets in the SDF1 mediated antirepellent pathway.

***Constitutively active Gα subunits.***

We next asked whether overexpression of specific constitutively active Gα subunits can mimic SDF1 induced antirepellent activity. Gα subunits with a Q to L mutation in the nucleotide-binding region are unable to cleave GTP and are thereby made constitutively active (Graziano and Gilman, 1989; Hermouet et al., 1991; Kroll et al., 1992; Kalinec et al., 1992). Coexpression of QL $G\alpha_s$ with EYFP made DRG growth cones insensitive to sema3A in a manner similar to SDF1, and what is more, SDF1 induced little additional antirepellent effect (Fig. 3A). Similar results were obtained with constitutively active $G\alpha_i$

(Fig. 3C) or QL $G\alpha_q$ (Fig. 3D). QL $G\alpha_o$ had no effect on growth cone responses to either sema3A or SDF1 (Fig. 3B). These results suggest that $G\alpha_s$, $G\alpha_q$, or $G\alpha_i$ each individually have the capability of initiating signaling events similar to those induced by SDF1, whether or not they participate in SDF1 signaling under normal circumstances.

***Inhibition of phospholipase C blocks SDF1 antirepellent activity.***

Because phospholipase C (PLC) is a classical effector of $G\alpha_{q/11}$-class G proteins (Taylor et al., 1991; Smrcka et al., 1991), and since our results show a requirement for $G\alpha_{q/11}$ activity in SDF1 mediated antirepellent signaling, we hypothesized that PLC is required for SDF1's antirepellent activity. The PLC inhibitor U73122 has no effect on background collapse or on growth cone responsiveness to sema3A (Fig. 4, grey bars). U73122 does, however, block SDF1's ability to reduce growth cone responses to sema3A (Fig. 4, black bars).

***Inhibition of phospholipase C blocks antirepellent effects induced by constitutive $G\alpha_q$ activity.***

We next tested whether $G\alpha_q$ activation can induce an anti-repellent response through the activation of PLC. As already demonstrated, sensory axons expressing a control Citrine construct collapse in response to sema3A and this collapse is largely mitigated in the presence of SDF1 (Figure 5, empty bars). In contrast, growth cones expressing the constitutively active QL $G\alpha_q$ are insensitive to sema3A. (Figure 5, grey bars). Significant sensitivity to sema3A is restored, however, when PLC is blocked. Growth cones expressing QL $G\alpha_s$ are also insensitive to sema3A, but this insensitivity is not reversed by blocking PLC (Figure 5, black bars). These findings are consistent with the idea that SDF1 induced antirepellent activity is mediated by $G\alpha_q$ activation of PLC, while constitutively active $G\alpha_s$ mediated antirepellent activity is not. As discussed in more detail below, one attractive explanation for these observations is that SDF1 induced activation of PLC indirectly induces elevated cAMP levels through a separate mechanism from the more traditional direct activation of adenylate cyclases by $G\alpha_s$.

**Discussion.**

Although G protein coupled receptors (GPCRs) are often pictured as acting through specific, dedicated G proteins, it is now known that a single GPCR can bind and activate G proteins from more than one G alpha class (see Hermans, 2003). PAR1, a thrombin receptor, can bind to $G\alpha_{i/o}$, $G\alpha_q$, or to $G\alpha_{12/13}$ (Gilchrist et al., 2001). β2-adrenergic receptors, when phosphorylated by PKA, switch affinities from $G\alpha_s$ to $G\alpha_i$ (Daaka et al., 1997). The class I metabotropic glutamate receptor mGluR1 has been shown to bind $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_s$, at least in certain cell types (Selkirk et al., 2001). Wang et al. (2010) found that parathyroid hormone receptor 1 regulates different genes with different G proteins or combinations of G proteins, suggesting that individual G proteins might be required for some cell behaviors but not for others. These are just a few of the many examples of GPCRs coupling to multiple G proteins.

Chemokine receptors as a class are generally thought to signal through $G\alpha_{i/o}$-type G proteins to decrease cAMP, activate PI3K, and activate both p38 and ERK1/2 MAP Kinases (see Rubin, 2009; Teicher and Fricker, 2010, for reviews). PI3K activation leads to activation of a number of other kinases, including Akt. SDF1 signaling through the chemokine receptor CXCR4 is also associated with changes in transcription, usually mediated through MAPK or Akt, that contribute to cell survival (Chalasani et al., 2003b; Suzuki et al., 2001; Vlahakis et al., 2002; Zheng et al., 2008). However, several groups have found that CXCR4 signals through other classes of G proteins. Maghazachi (1997) reported that antibodies targeting $G\alpha_o$ or $G\alpha_q$, but not $G\alpha_i$, $G\alpha_s$, or $G\alpha_z$, could block SDF1-induced chemotaxis in natural killer cells. Soede et al. (2000) found that CXCR4-dependent migration of myeloid leukemia cells require either the combination of Gαi and $G\alpha_q$ or $G\alpha_q$ alone, depending on the destination tissue. Tan et al. (2006) showed that SDF1/CXCR4-induced migration of Jurkat T cells required both $G\alpha_{13}$, which activated Rho, and $G\alpha_i$. These and other studies raised the possibility that SDF1/CXCR4 signaling in axon guidance might be more complex than that of the classic chemokine signaling

pathway. Previous work from our laboratory (Chalasani et al., 2003a) identified several components of SDF1/CXCR4 signaling in the antirepellent pathway, including a pertussis toxin-sensitive G protein, increased cAMP, and activation of PKA. In addition to the surprising apparent increase in cAMP levels observed in these previous studies, the effects of SDF1 on axonal responses to repellents were found to be independent of PI3K/Akt signaling and of MAPK.

The findings in this study show that SDF1's antirepellent activity can be blocked separately by $G\alpha_i$, $G\alpha_{q/11}$, or $G\beta\gamma$specific competitive inhibitors. These data suggest that each is required for the normal function of the antirepellent pathway. However, we also found that overexpression of constitutively active forms of $G\alpha_i$ or of $G\alpha_q$ can mimic application of SDF1. This suggests that either one of these signaling components is capable of stimulating a common downstream element that is sufficient for activation of the pathway. These findings are consistent with the idea that SDF1 stimulates multiple G protein coupled pathways to a degree that is insufficient for any one of them alone to induce a physiological response, but in combination, their actions sum to a level above a threshold for activation to produce an anti-repellent response.

We also found that overexpression of a constitutively active $G\alpha_s$ can mimic SDF1 even though a competitive inhibitor of $G\alpha_s$ does not block SDF1 mediated signaling. As $G\alpha_s$ is a canonical stimulator of adenylate cyclase activity and would be expected to elevate cAMP levels, this finding is consistent with the idea that the common element upon which $G\alpha_i$, $G\alpha_q$, and $G\beta\gamma$ all converge downstream from SDF1 activation of CXCR4 is elevated cAMP levels. Thus, our proposed model of the signaling pathway is that $G\alpha_i$, $G\alpha_q$, and their associated $\beta\gamma$ subunits all cooperate to increase the local concentration of cAMP, leading to suppression of axonal repulsion (Fig. 6). The ability of $G\alpha_s$ to accomplish the same thing through a different route raises the possibility that a very wide range of GPCRs could influence axonal responses to repellents and axonal pathfinding.

Previous work has shown that SDF1's antirepellent activity requires calmodulin and the calcium/calmodulin-stimulated cyclase ADCY8 (Chalasani et al., 2003a; Xu et al., 2010). Xu (2010) also showed by Förster resonance energy transfer (FRET) that SDF1 stimulates increased cAMP levels, and that this can be blocked by inhibition of calmodulin. $G\alpha_i$ and $G\alpha_q$ are not ordinarily associated with increases in cAMP, yet our results show that they are required components in the antirepellent signaling pathway. $G\alpha_q$ and $G\beta\gamma$ activity, through the activation of PLC, can produce diacylglycerol and inositol trisphosphate and thereby increase intracellular calcium (Guttridge et al., 1995). Thus, our present finding that both $G\alpha_{q/11}$ and PLC are required for SDF1 antirepellent activity provides a connection between the G proteins activated by SDF1 and the calmodulin and calcium/calmodulin-stimulated cyclase that has been shown to increase cAMP downstream of SDF1. Our results are consistent with a signaling pathway (Fig. 6) in which multiple G protein components stimulate PLC activity that induces an increase in intracellular calcium levels and leads to the activation of calmodulin. Calmodulin, in turn, activates calcium/calmodulin-stimulated adenylate cyclases, such as ADCY8, and thereby increases cAMP.

Some of the important questions that remain include how elevated cAMP levels decrease growth cone responses to repellents and the degree to which this modulation of repellent effectiveness is important in axonal pathfinding *in vivo*. Both SDF1/CXCR4 activity and activity of the calmodulin-activated adenylate cyclases have a strong influence on axonal responses to the repellent slit *in vivo* (Xu et al., 2010). Our findings in this study suggest that activation of a wide range of GPCRs that signal through $G\alpha_i$, $G\alpha_q$, or $G\alpha_s$ could potentially participate in axon guidance decisions.

**Figure 1. Competitive inhibitors of $G\alpha_i$ or $G\alpha_{q/11}$, but not $G\alpha_s$ or $G\alpha_o$, block SDF1-mediated antirepellent activity.** **(A)** Growth cones of dissociated DRGs transfected with EYFP or EYFP + $G\alpha_{q/11}$ inhibitory peptide have motile lamellae and filopodia. **(B)** Specific inhibitory Gα peptides (medium grey) bind selected GPCRs and prevent their association with functional G proteins containing the same Gα peptide sequence. **(C,D)** Dissociated DRGs were transfected with EYFP-only (grey bars) or with EYFP and an experimental construct (black bars). After 24h in culture, cells were treated for 30' with sema3A or with sema3A + SDF1. **(C)** The SDF1 antirepellent response is blocked by a by peptides targeting $G\alpha_i$ or $G\alpha_{q/11}$, and also by a full-length dominant-negative $G\alpha_i$. **(D)** The SDF1 antirepellent response is not affected by peptides targeting $G\alpha_s$ or $G\alpha_o$. *, $p < 0.001$; **, $p < 0.0001$.

**Figure 2. Scavengers of Gβγ, $G\alpha_i$, or $G\alpha_q$ subunits block SDF1 antirepellent activity.** **(A)** GRK-CT sequesters βγ subunits while leaving α free to activate downstream effectors. **(B)** Transfection of dissociated DRGs with GRK-CT blocks the SDF1 antirepellent effect but does not alter background collapse or response to sema3A. **(C)** RGS proteins sequester specific α subunits and hasten their inactivation while leaving βγ subunits free to activate downstream effectors. **(D)** Transfection of dissociated DRGs with either RGS2, a $\alpha_q$ specific GAP, or RGS4, an $\alpha_i$ and to a lesser extent $\alpha_q$ specific GAP, block SDF1 antirepellent activity without affecting background collapse or response to sema3A. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.001$.

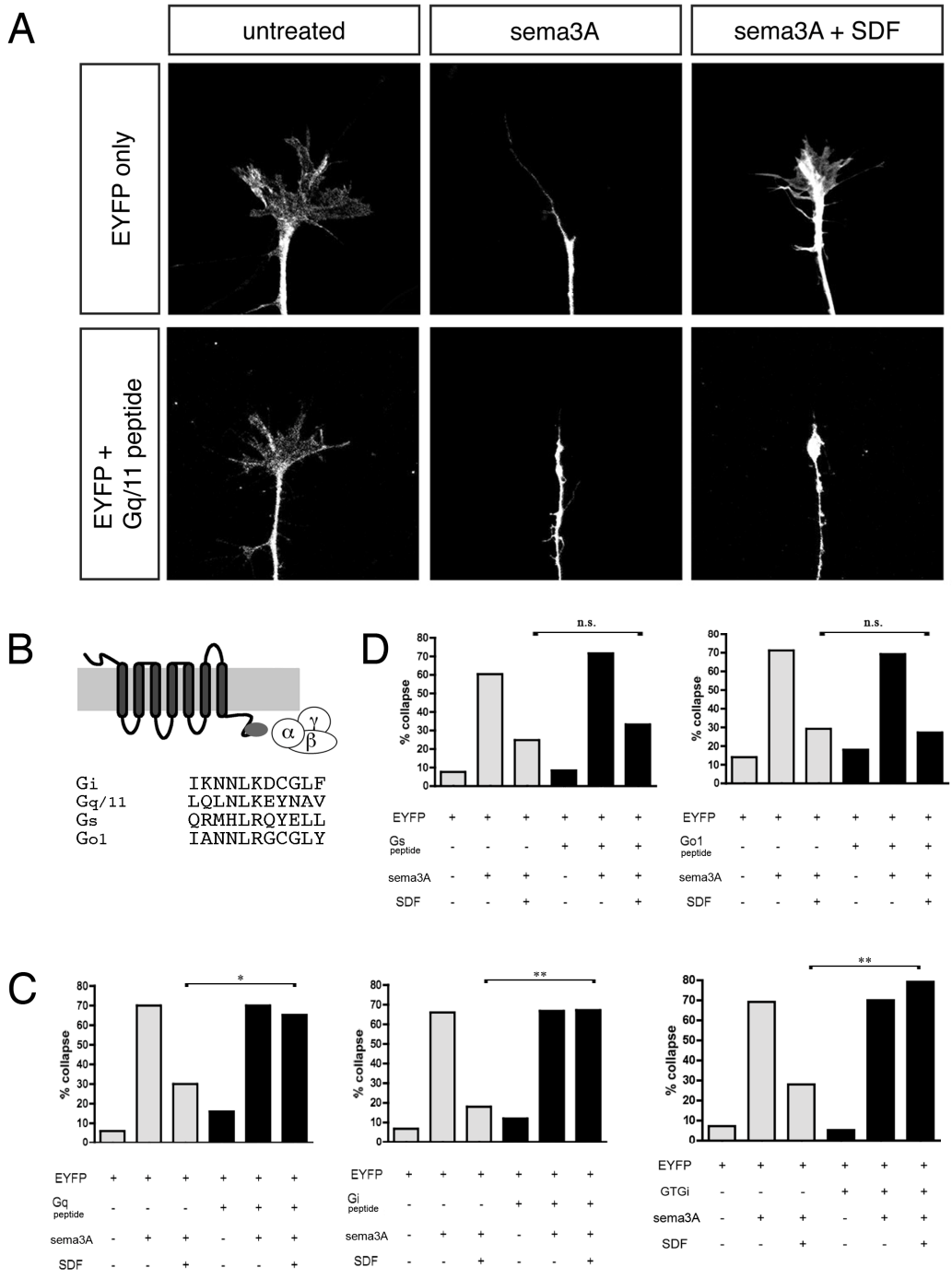
**Figure 3. Constitutively active $G\alpha_q$, $G\alpha_i$, or $G\alpha_s$ mimics SDF1's antirepellent effect.** **(A)** Transfection of QL $G\alpha_s$ into DRGs makes them unresponsive to sema3A. **(B)** Transfection of QL $G\alpha_o$ into DRGs has no effect on their responses to sema3A or SDF1. **(C)** Transfection of DRGs with QL $G\alpha_i$ or with **(D)** QL $G\alpha_q$ makes DRGs unresponsive to sema3A. *, $p < 0.001$, **, $p < 0.0001$.

**Figure 4. Inhibiting PLC blocks SDF1 antirepellent activity.** DRG explants were treated with 20nM PLC inhibitor U73122 (black bars). U73122 does not alter background collapse or DRG responsiveness to sema3A, but does block the antirepellent effect of SDF1. **, $p < 0.0001$.

**Figure 5. Inhibiting PLC blocks antirepellent activity induced by expression of a constitutively active $G\alpha_q$.** DRGs were transfected with expression plasmids for Citrine (control, empty bars), Citrine and QL $G\alpha_q$ (grey bars), or Citrine and QL $G\alpha_s$ (black bars). Expression of QL $G\alpha_q$ makes growth cones insensitive to sema3A unless the PLC blocker U73122 (20nM) is also present. Growth cones expressing QL $G\alpha_s$ are insensitive to sema3A in both the absence and the presence of U73122. *, $p<0.002$.

**Figure 6. A model for antirepellent signaling.** We identify roles for $G\alpha_i$, $G\alpha_q$, and $G\beta\gamma$, as well as PLC, in the antirepellent response to SDF1. Previous work has shown requirements for calmodulin, ADCY8, cAMP, and PKA, along with inhibition of Rho and ROCK. In our model, CXCR4 activates $G\alpha_i$ and $G\alpha_q$, and they and their associated $G\beta\gamma$ subunits cooperate to activate PLC. PLC, through generation of diacylglycerol and inositol trisphosphate (Taylor et al., 1991), increases calcium levels. Increased calcium activates calmodulin, which in turn activates ADCY8 and thereby increases cAMP. Increased cAMP activates PKA, which phosphorylates MAPK1/2, leading to its activation and function in cell survival. PKA also phosphorylates Rho, which is thereby inactivated. This inactivation and the subsequent inactivation of ROCK are required for the antirepellent response to SDF1.

Figure 1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sema3A + SDF
EYFP only
EYFP + Gq/11 peptide
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α
γ
β
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Gq/11 LQLNLKEYNAV
Gs QRMHLRQYELL
Go1 IANNLRGCGLY
D
n.s.
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Go1 peptide
sema3A
SDF
C
*
**
**
Gq peptide
Gi peptide
GTGi

Figure 2

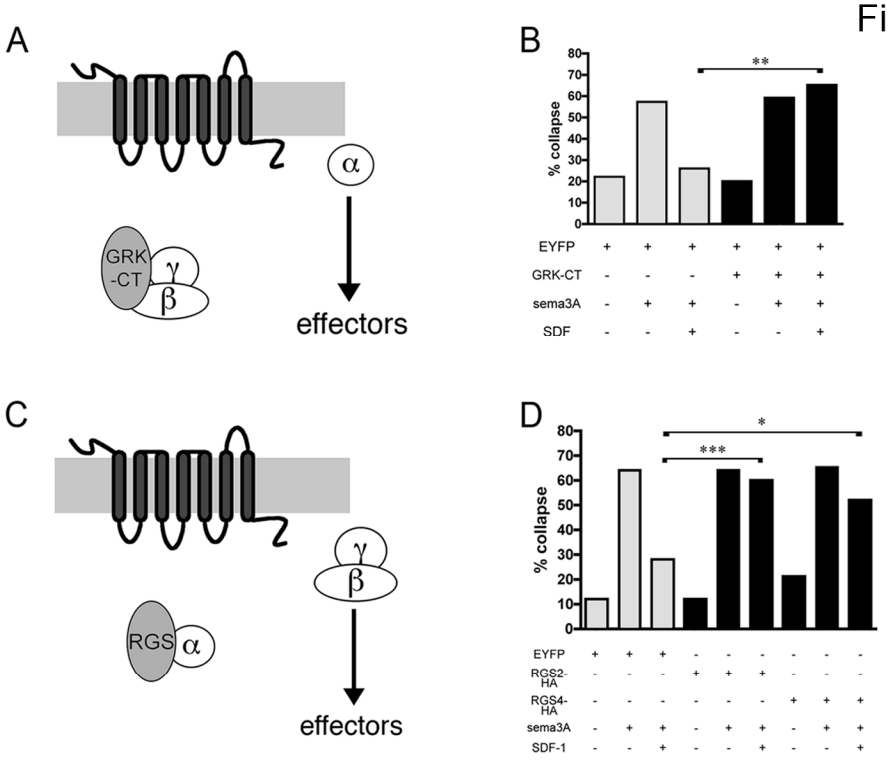



Figure 3

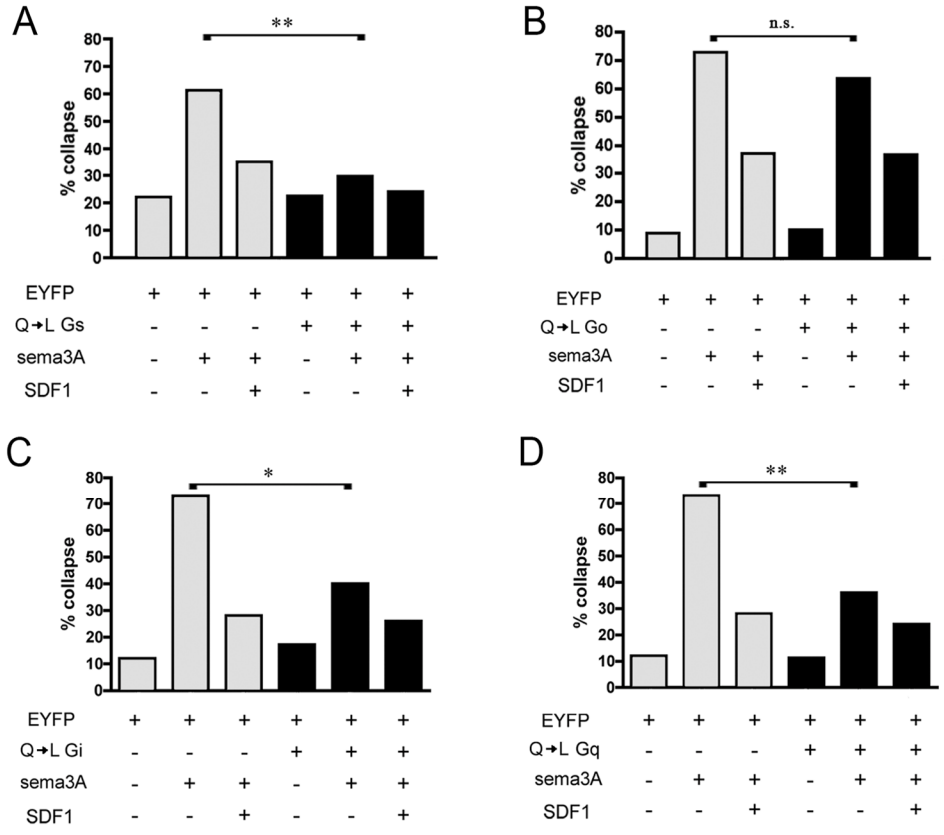


Figure 4

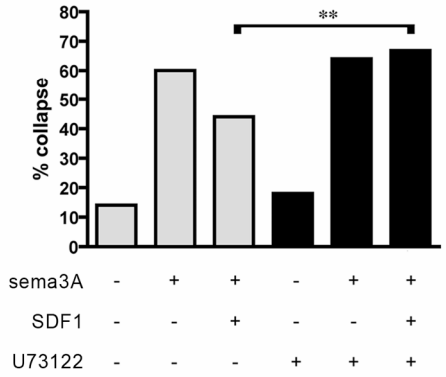



Figure 5

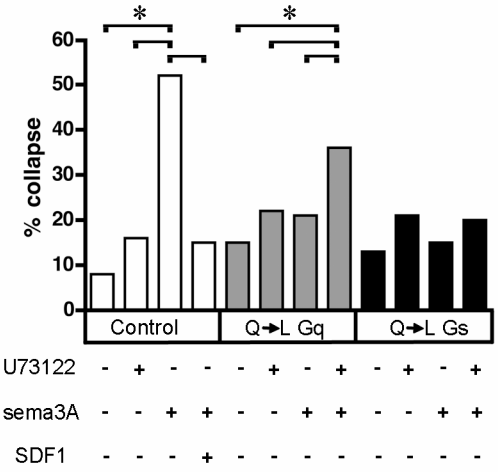
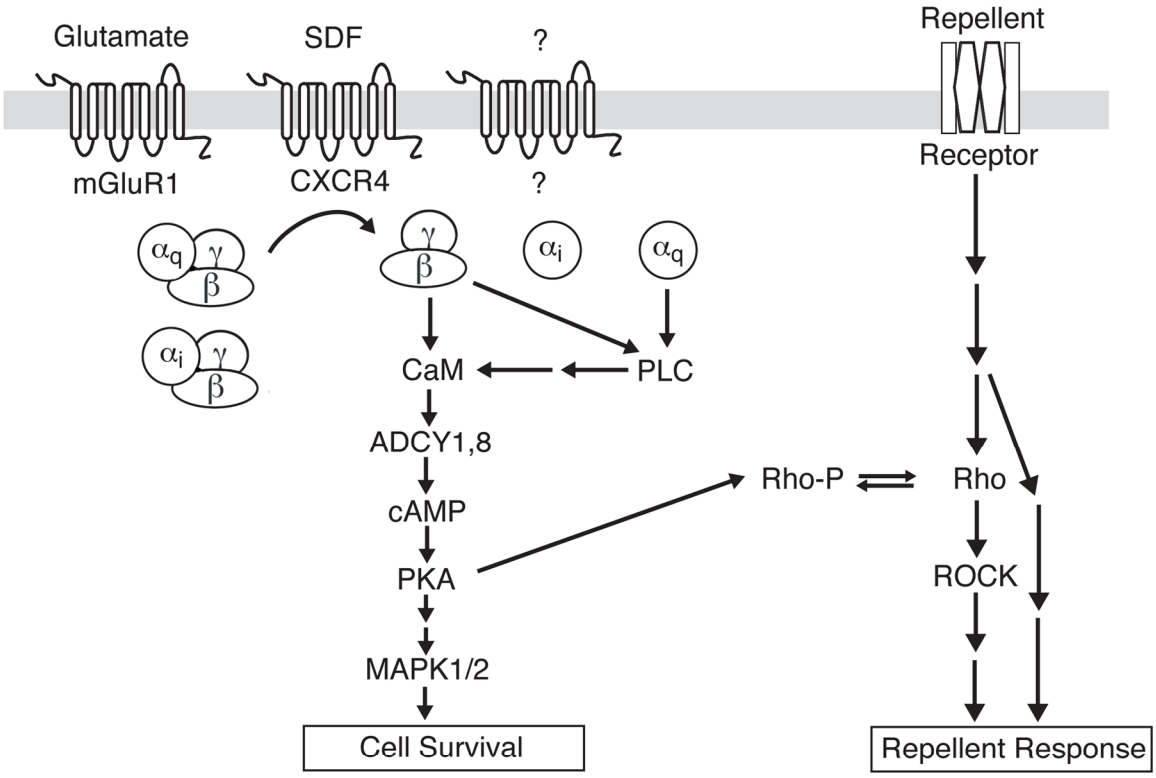


Figure 6


Glutamate
SDF
?
Repellent
mGluR1
CXCR4
?
Receptor
αq
γ
β
αi
γ
β
αi
αq
γ
β
CaM
PLC
ADCY1,8
cAMP
PKA
Rho-P
Rho
ROCK
MAPK1/2
Cell Survival
Repellent Response

## TABLE 1: RAW DATA, FIGS. 1-4

| condition | collapsed | not collapsed | condition | collapsed | not collapsed |
|---|---|---|---|---|---|
| **GTGi** | | | **Gq/11 mini** | | |
| EYFP ctrl | 5 | 31 | EYFP ctrl | 5 | 31 |
| sema | 80 | 32 | sema | 80 | 32 |
| sema + SDF1 | 17 | 41 | sema + SDF1 | 17 | 41 |
| GTGi ctrl | 12 | 41 | Gq/11 mini ctrl | 4 | 23 |
| sema | 75 | 49 | sema | 38 | 19 |
| sema + SDF1 | 56 | 41 | sema + SDF1 | 21 | 13 |
| EYFP ctrl | 2 | 28 | EYFP ctrl | 3 | 46 |
| sema | 11 | 5 | sema | 24 | 10 |
| sema + SDF1 | 5 | 13 | sema + SDF1 | 14 | 32 |
| GTGi ctrl | 1 | 19 | Gq/11 mini ctrl | 8 | 41 |
| sema | 19 | 8 | sema | 38 | 16 |
| sema + SDF1 | 31 | 8 | sema + SDF1 | 32 | 17 |
| EYFP ctrl | 2 | 28 | citrine ctrl | 138 | 322 |
| sema | 11 | 5 | sema | 230 | 71 |
| sema + SDF1 | 5 | 13 | sema + SDF1 | 66 | 108 |
| GTGi ctrl | 1 | 19 | Gq/11 mini ctrl | 142 | 246 |
| sema | 19 | 8 | sema | 180 | 83 |
| sema + SDF1 | 31 | 8 | sema + SDF1 | 236 | 80 |
| **Go1 mini** | | | **Gi mini** | | |
| EYFP ctrl | 5 | 31 | EYFP ctrl | 5 | 72 |
| sema | 80 | 32 | sema | 27 | 34 |
| sema + SDF1 | 17 | 41 | sema + SDF1 | 5 | 30 |
| Go1 mini ctrl | 11 | 49 | Gi mini ctrl | 11 | 66 |
| sema | 20 | 9 | sema | 45 | 67 |
| sema + SDF1 | 9 | 24 | sema + SDF1 | 34 | 51 |
| EYFP ctrl | 5 | 18 | EYFP ctrl | 4 | 61 |
| sema | 17 | 13 | sema | 49 | 29 |
| sema + SDF1 | 10 | 28 | sema + SDF1 | 7 | 30 |
| Go1 mini ctrl | 5 | 22 | Gi mini ctrl | 4 | 27 |
| sema | 23 | 15 | sema | 16 | 8 |
| sema + SDF1 | 5 | 15 | sema + SDF1 | 36 | 15 |
| citrine ctrl | 3 | 33 | EYFP ctrl | 21 | 61 |
| sema | 22 | 20 | sema | 71 | 27 |
| sema + SDF1 | 5 | 28 | sema + SDF1 | 39 | 74 |
| Go1 mini ctrl | 5 | 42 | Gi mini ctrl | 31 | 52 |
| sema | 70 | 66 | sema | 48 | 24 |
| sema + SDF1 | 9 | 37 | sema + SDF1 | 60 | 26 |
| **Gs mini** | | | **GRK-CT** | | |
| EYFP ctrl | 5 | 72 | EYFP ctrl | 2 | 28 |
| sema | 27 | 34 | sema | 11 | 5 |

| | | | | | |
|---|---|---|---|---|---|
| sema + SDF1 | 5 | 30 | sema + SDF1 | 5 | 13 |
| Gs mini ctrl | 8 | 59 | GRK-CT ctrl | 5 | 36 |
| sema | 32 | 36 | sema | 22 | 14 |
| sema + SDF1 | 6 | 36 | sema + SDF1 | 11 | 8 |
| | | | | | |
| EYFP ctrl | 4 | 54 | EYFP ctrl | 5 | 18 |
| sema | 24 | 18 | sema | 17 | 13 |
| sema + SDF1 | 11 | 38 | sema + SDF1 | 10 | 28 |
| Gs mini ctrl | 3 | 22 | GRK-CT ctrl | 7 | 27 |
| sema | 37 | 14 | sema | 26 | 18 |
| sema + SDF1 | 5 | 10 | sema + SDF1 | 13 | 7 |
| | | | | | |
| citrine ctrl | 138 | 322 | citrine ctrl | 60 | 179 |
| sema | 230 | 71 | sema | 127 | 68 |
| sema + SDF1 | 66 | 108 | sema + SDF1 | 69 | 110 |
| Gs mini ctrl | 161 | 230 | GRK-CT ctrl | 121 | 184 |
| sema | 226 | 78 | sema | 114 | 45 |
| sema + SDF1 | 123 | 128 | sema + SDF1 | 174 | 74 |

| **condition** | **collapsed** | **not collapsed** | **condition** | **collapsed** | **not collapsed** |
|---|---|---|---|---|---|
| **RGS2** | | | **QLO** | | |
| EYFP ctrl | 6 | 52 | citrine ctrl | 40 | 230 |
| sema | 48 | 16 | sema | 213 | 78 |
| sema + SDF1 | 16 | 36 | sema + SDF1 | 106 | 110 |
| RGS2 ctrl | 3 | 32 | QLO ctrl | 35 | 192 |
| sema | 30 | 6 | sema | 152 | 60 |
| sema + SDF1 | 36 | 23 | sema + SDF1 | 89 | 81 |
| | | | | | |
| EYFP ctrl | 6 | 45 | citrine ctrl | 18 | 192 |
| sema | 32 | 11 | sema | 178 | 67 |
| sema + SDF1 | 24 | 47 | sema + SDF1 | 64 | 108 |
| RGS2 ctrl | 20 | 105 | QLO ctrl | 13 | 120 |
| sema | 81 | 44 | sema | 106 | 61 |
| sema + SDF1 | 81 | 66 | sema + SDF1 | 49 | 75 |
| | | | | | |
| EYFP ctrl | 4 | 28 | EYFP ctrl | 10 | 35 |
| sema | 36 | 20 | sema | 43 | 27 |
| sema + SDF1 | 18 | 45 | sema + SDF1 | 14 | 26 |
| RGS2 ctrl | 19 | 135 | QLO ctrl | 15 | 50 |
| sema | 61 | 35 | sema | 18 | 28 |
| sema + SDF1 | 154 | 104 | sema + SDF1 | 15 | 23 |
| | | | | | |
| **RGS4** | | | **QLQ** | | |
| EYFP ctrl | 4 | 28 | EYFP ctrl | 3 | 21 |
| sema | 36 | 20 | sema | 30 | 11 |
| sema + SDF1 | 18 | 45 | sema + SDF1 | 12 | 30 |
| RGS4 ctrl | 25 | 96 | QLQ ctrl | 6 | 49 |
| sema | 105 | 56 | sema | 22 | 39 |
| sema + SDF1 | 85 | 80 | sema + SDF1 | 6 | 19 |
| | | | | | |
| EYFP ctrl | 6 | 52 | EYFP ctrl | 10 | 35 |
| sema | 48 | 16 | sema | 43 | 27 |

| | | | | | |
|---|---|---|---|---|---|
| sema + SDF1 | 16 | 36 | sema + SDF1 | 14 | 26 |
| RGS4 ctrl | 20 | 108 | QLQ ctrl | 10 | 26 |
| sema | 70 | 24 | sema | 28 | 38 |
| sema + SDF1 | 92 | 44 | sema + SDF1 | 16 | 51 |
| | | | | | |
| EYFP ctrl | 8 | 68 | EYFP ctrl | 5 | 22 |
| sema | 45 | 15 | sema | 22 | 30 |
| sema + SDF1 | 10 | 18 | sema + SDF1 | 11 | 34 |
| RGS4 ctrl | 15 | 89 | QLQ ctrl | 9 | 48 |
| sema | 63 | 36 | sema | 17 | 26 |
| sema + SDF1 | 61 | 71 | sema + SDF1 | 7 | 19 |
| | | | | | |
| **QLS** | | | **QLI** | | |
| EYFP ctrl | 14 | 70 | EYFP ctrl | 4 | 28 |
| sema | 41 | 18 | sema | 36 | 20 |
| sema + SDF1 | 24 | 47 | sema + SDF1 | 18 | 45 |
| QLS ctrl | 6 | 25 | QLI ctrl | 9 | 41 |
| sema | 23 | 67 | sema | 13 | 23 |
| sema + SDF1 | 17 | 51 | sema + SDF1 | 5 | 20 |
| | | | | | |
| EYFP ctrl | 10 | 35 | EYFP ctrl | 4 | 58 |
| sema | 43 | 27 | sema | 26 | 19 |
| sema + SDF1 | 14 | 26 | sema + SDF1 | 7 | 21 |
| QLS ctrl | 24 | 82 | QLI ctrl | 2 | 35 |
| sema | 28 | 67 | sema | 15 | 13 |
| sema + SDF1 | 16 | 51 | sema + SDF1 | 11 | 36 |
| | | | | | |
| citrine ctrl | 3 | 33 | EYFP ctrl | 3 | 21 |
| sema | 22 | 20 | sema | 30 | 11 |
| sema + SDF1 | 5 | 28 | sema + SDF1 | 12 | 30 |
| QLS ctrl | 8 | 55 | QLI ctrl | 6 | 30 |
| sema | 20 | 116 | sema | 23 | 32 |
| sema + SDF1 | - | - | sema + SDF1 | 11 | 31 |

## Chapter 3: Involvement of an ADAM metalloprotease in SDF1 antirepellent activity

Our standing model for antirepellent signaling (see Chapter 2, Fig. 5) has been that CXCR4 or other modulatory receptors, through the phosphorylation of Rho, block repellent-induced alterations in the growth cone cytoskeleton. We have identified several steps in the antirepellent pathway upstream of PKA and the inactivation of Rho (chapter 2; Chalasani et al., 2003). However, this is not the only way that antirepellent cues might block repellent activity. For example, work from the Tessier-Lavigne lab (Keino-Masu et al., 1996) showed that a metalloprotease inhibitor could increase both axon outgrowth in response to netrin and expression of the attractive netrin receptor DCC, suggesting that guidance receptors can be regulated by inhibitory shedding. Work in other systems has shown that for ADAM (A Disintegrin And Metalloprotease) proteases are required for normal axon guidance (Schimmelpfeng et al., 2001; Chen et al., 2007). Epithelial growth factor receptor (EGFR) can be activated by ligands other than EGF, such as HB-EGF, which is activated as a ligand by cleavage from the same cell membrane as the receptor. GPCRs can activate ADAMs to produce these EGFR ligands through a shedding mechanism (Asakura et al., 2002; Schafer et al., 2004; Tanida et al., 2004; Mifune et al., 2005). We were therefore interested in the possibility that the inhibitory shedding of repellent receptors might be another mechanism for antirepulsion. We focused on ADAM10 and ADAM17 because they are required for several aspects of neural development, including development of the optic cup and otic pit (Hartmann et al., 2002), formation of the neocortex (Jorissen et al., 2010), and synaptic plasticity (Malinverno et al., 2010), in addition to the guidance roles referenced above.

The ADAM proteases are named for their unique pair of extracellular protein-interaction domains. ADAMs include a pro domain, which is cleaved off during posttranslational processing, the metalloprotease and disintegrin domains which give their names, a cysteine-rich domain on their extracellular sides, a transmembrane domain, and a cytoplasmic tail (Fig. 1; see Edwards et al. 2008).

ADAM17 was originally identified as TACE, TNFα converting enzyme, but it has many other substrates as well. These other substrates include Notch, APP, L1-CAM, N-CAM and neuregulin (see Edwards et al., 2008). ADAM10 is also called α-secretase for its role in processing APP and is known in *Drosophila* as Kuzbanian (kuz). Aside from APP, ADAM10 has been reported to cleave L1-CAM, EGFR ligands, Notch, and several cadherins, among others (see Edwards et al., 2008). ADAM10/Kuzbanian was initially identified for its involvement in neural fate specification (Rooke et al., 1996) and for facilitating axon extension (Fambrough et al., 1996; Pan and Rubin, 1997). Soon thereafter, ADAM10/Kuzbanian was linked with slit/robo signaling by the finding that *kuz*, *slit*, and *robo* mutants genetically interact in the fly embryo midline, with combinations of mutations yielding more severe phenotypes (Schimmelpfeng et al., 2001).

A requirement for proteolytic function in axon guidance is also well-established in the ephrin/Eph system. Ephrins are most commonly considered as contact repellents. Both they and their receptors, the Ephs, are attached to the cell membrane, so at least one

signaling partner must be cleaved for the growth cone to withdraw in response to repulsive signaling. Hattori et al. (2000) first demonstrated this, showing that ADAM10 cleaves ephrinA2 when clustered by EphA3-Fc and that an uncleavable ephrinA2 slowed axon retraction. Litterst et al. (2007) showed that Eph could be cleaved in two ways. Ligand binding induced a presumably activating cleavage, yielding increased internalization of C-terminal fragments, and this was not sensitive to metalloprotease inhibitors. Calcium and NMDA activation caused a presumably inhibitory cleavage, as shown by shedding of Eph N-termini. This inhibitory cleavage was sensitive to metalloprotease inhibitors but not to the gamma-secretase inhibitor that blocked the activating cleavage. This demonstrates that proteolysis of an individual repellent receptor can either increase or decrease repellent signaling, depending on the protease and cleavage site.

Here, we show that SDF1 antirepellent activity in primary chick neurons can be blocked by pharmacological inhibition of metalloproteases or by a dominant negative ADAM10, but not by a dominant negative ADAM17. We also show that shedding of neuropilin-1 overexpressed in 293 cells is enhanced by overexpression of ADAM10 and reduced by expression of a dominant negative ADAM10. Pharmacological inhibition of metalloproteases does not block the SDF1-mimicking effect of a cAMP analogue, suggesting that ADAM10 activation is not downstream of increased cAMP.

**Results:**

We wanted to determine whether SDF1 antirepellent signaling includes an inhibitory cleavage of repellent receptors. To address this question, we began by applying the metalloprotease inhibitor TAPI-2 to explant collapse assays. We saw no effect on background collapse or response to sema3A (Fig. 2, compare first green bar to first black bar and second green bar to second black bar), but TAPI-2 does block the reduction in collapse produced by SDF1 (Fig. 2, compare third green bar to third black bar; **, $p < 0.005$).

TAPI-2, though not a universal metalloprotease inhibitor, blocks several proteases, including ADAM10, ADAM17, and a small number of matrix metalloproteases. Therefore, we wanted to identify which of these proteases might underlie TAPI-2's SDF1-blocking function. We took advantage of previously published dominant-negative constructs, composed of most of ADAM10 or ADAM17 that are missing their proteolytic domains and/or pro domain (Fig. 1). The effectiveness of dnADAM10 has been demonstrated in the Eph/ephrin system in NIH 3T3 cells, where it blocked the cleavage of ephrinA2. In the same set of experiments, cleavage of ephrinA2 was enhanced by overexpression of full-length ADAM10 (Hattori et al., 2000). The dnADAM17 was originally tested in COS-7 cells and HEK293 cells, where it blocked the shedding of TNF-α and FasL (Itai et al., 2001). In collapse assays on dissociated DRGs, neither dn-ADAM construct altered background collapse or response to sema3A (Fig. 3A,B, compare first green bar to first black bar and second green bar to second black bar).

dnADAM10 blocked the influence of SDF1 (Fig. 3A, compare third green bar to third black bar; **, $p < 0.001$), but dnADAM17 did not (Fig. 3B, compare third green bar to third black bar). Expression of the dn-ADAM constructs was confirmed by staining for the HA tags on the constructs.

Recent work in the *Drosophila* slit-robo system (Coleman et al., 2010) found a pro-repulsion role for ADAM10. Since this differed from our finding that blocking ADAM10 had no effect on the repellent response to sema3A, we investigated the effects of dnADAM10 on the responses of dissociated chick retina to slit2 and SDF1. Consistent with our DRG results, we found that dnADAM10 did not disrupt repellent signaling but did block the antirepellent response to SDF1 (Fig. 3C, compare second green bar to second black bar and third green bar to third black bar; ***, $p < 0.0001$).

The ADAM proteases are found at the cell surface, are known to cleave receptors in other systems (EGF, Eph/ephrin), and may have a role in antirepellent signaling. We therefore asked whether ADAM10 could cleave neuropilin-1 (NP1), the ligand-binding component of the sema3A receptor complex. HEK293 cells were transfected with NP1 and ADAM10, dnADAM10, or β-galactosidase (as a transfection control). I collected supernatants and lysates from these cells and ran matched Western blots, stained for HA-tag (ADAM10 or dnADAM10) or myc (N-terminus of NP1), or for tubulin as a cell-density control. Some NP1 N-terminus appeared in the supernatant even in the control condition, in which cells were transfected with NP1 and β-gal (Fig. 4, lane 1), suggesting the existence of endogenous protease activity in the HEK293 cells. Cotransfection of

NP1 with full-length ADAM10, however, produced a marked increase in cleaved NP1 (Fig. 4, lane 2). Cotransfection of dnADAM10 with NP1 blocked the release of cleaved NP1 into the medium (Fig. 4, lane 3). These findings show that ADAM10 can cleave NP1 and that thednADAM10 blocks that cleavage. If ADAM10 functions in the SDF1/sema3A interaction the way it works in ephrin/Eph signaling (Litterst et al., 2007), then SDF1 may activate ADAM10 to cause a sema3A-inhibitory shedding event.

After having found a potential mechanism for ADAM10 function in SDF1 antirepellent activity, we wanted to know where in the SDF1 pathway ADAM10 activation might fall. To that end, we performed explant collapse assays with TAPI-2 and SpcAMPS, a cAMP analogue that mimics SDF1's antirepellent effect (Chalasani et al., 2003). We reasoned that, if ADAM10 activation is required downstream of increased cAMP, TAPI-2 should block the antirepellent effect of SpcAMPS. SpcAMPS reduced collapse with or without TAPI-2 (Fig. 5A, compare third green bar and third black bar). We therefore conclude that ADAM10 activity is not downstream of the SDF1-induced increase in cAMP. Consistent with this result, preliminary experiments (Fig. 5B, compare third green bar and third black bar) with the ROCK inhibitor Y-27632, which was previously shown to mimic SDF1's antirepellent effect (Chalasani et al. 2003), showed that TAPI-2 could not block this even further-downstream antirepellent signal.

Because ADAM10 activity is not downstream of cAMP, it must either be upstream of cAMP or in a parallel pathway. Since other work has shown that ADAM10 can be activated by increased intracellular calcium (Litterst et al., 2007), we hypothesize (Fig. 6)

that the calcium produced by SDF1-induced PLC activation not only activates calmodulin and ADCY8 but also activates ADAM10.

**Discussion.**

There are several possible mechanisms by which antirepellent and repellent signaling pathways might interact. These include opposing effects on the inactivation and activation of small G proteins such as Rho, opposing effects on the growth cone cytoskeleton, or interference with the repellent receptor or receptor complex. The latter possibility can be divided further, into alteration of expression, membrane insertion, membrane localization, endocytosis, and shedding of repellent receptors. Shedding of the receptor may be a more elegant system, or at least an elegant supporting mechanism, than several of the other possibilities, since it would prevent the activation of second messengers and thus require alteration of many fewer proteins.

Here, we show that protease activity is required not for the repellent response to sema3A or slit2 but for the reduction in that response by co-application of SDF1. We further demonstrate that the protease requred for SDF1 antirepellent activity is ADAM10 and that ADAM10 can cleave NP1, the component of the sema3A receptor complex which confers ligand specificity. Since ADAM10 has previously been shown to cleave L1 (Maretzky et al., 2005), another component of the sema3A receptor complex, it seems

likely that ADAM10's role in SDF1 activity could be to cleave repellent receptors, thereby preventing their activation.

Though Coleman et al. (2010) found that Kuz/ADAM10 is required for Robo signaling in the fly, we found that a dominant-negative ADAM10 had no effect on chick RGCs' responsiveness to hSlit2. Reasons for this difference may include divergences between fly and human Slit or fly and chick Robo, or different signaling machinery in fly embryo midline and chick RGCs.

Though previous work had shown requirements for ADAM10 in axon guidance, this is the first association of ADAM10 with a modulatory guidance cue. ADAM10 activation and repellent receptor shedding also comprise a novel mechanism for antirepellent signaling. As the study of signaling in guidance modulation shifts toward *in vivo* work, the existence of this additional pathway may provide useful insights.

**Materials and Methods.**

*Plasmids and reagents*. TAPI-2 (Peptides International) was used at 100nM. Full-length ADAM10-HA and DNADAM10-HA were provided by J. Flanagan (Harvard Medical School). DNADAM17-HA was provided by S. Nagata (Osaka University). SpcAMPS (Sigma A166) was used at 20µM. Y-27632 (Sigma Y0503) was used at 10µM. Anti-myc (9E10, Cell Center), anti-HA-tag (MMS-101R, Covance), and anti-tubulin (YL 1/2)

were each used at 1:1000. Appropriate HRP-tagged secondary antibodies (Jackson Immuno) were used at 1:2000.

*Cell culture and collapse assays*. TAPI-2 experiments were performed on explants. Dominant-negative ADAMs were transfected as described in Chapter 2. Cell culture and assay protocols were as described in Chapter 2.

*Shedding assay*. HEK293T cells plated on polylysine were transfected with the listed plasmids using Lipofectamine 2000. Total DNA per transfection was 8µg, per manufacturer's guidelines. Samples of medium (supernatant) or total cell lysate were run on matched 10% SDS-PAGE gels. Blots were stained as noted, developed with ECL-Plus reagents (GE Life Sciences) and exposed to film before being scanned and then analyzed with ImageJ (NIH).

**Figure 1. Domain structures of ADAM10, ADAM17, DN-ADAM10, and DN-ADAM17.** ADAM10 and ADAM17 are closely related members of the ADAM family and share the same domain structure. Each contains a pro domain which is cleaved off during posttranslational processing, a protease domain, a cysteine-rich domain, a disintegrin domain, and a transmembrane domain and cytoplasmic tail. DN-ADAM10 lacks the pro domain and protease domain; DN-ADAM17 lacks the protease domain.

**Figure 2. The metalloprotease inhibitor TAPI-2 blocks SDF1 antirepellent activity.** Explants from E7 chick DRGs were treated for 30' with sema3A, SDF1, and TAPI-2, as noted. TAPI-2 (black bars) blocks the SDF1-induced reduction in collapse. **, $p < 0.005$

**Figure 3. SDF1 antirepellent activity is not blocked by DN-ADAM17 but is blocked by DN-ADAM10 in either DRG or RGC.** (**A,B**) Dissociated E7 chick DRGs were transfected with EYFP, DN-ADAM10, and DN-ADAM17 as noted and then treated with sema3A, SDF1, or sema3A + SDF1. **(A)** DN-ADAM10 does not block sema3A-induced collapse but does block SDF1 antirepellent activity. **, $p < 0.001$. **(B)** DN-ADAM17 blocks neither sema3A-induced collapse nor SDF1's antirepellent effect. (**C**) Dissociated E6 chick RGCs were transfected with EYFP or EYFP + DN-ADAM10 as noted and then treated with slit2, SDF1, or slit2 + SDF1. DN-ADAM10 does not block slit2-induced collapse but does block SDF1 antirepellent activity. ***, $p < 0.0001$.

**Figure 4. ADAM10 can cleave NP1.** HEK293 cells were transfected with NP1 and β-galactosidase, ADAM10, or DN-ADAM10. Samples from total cell lysate or conditioned medium were run on matched SDS-PAGE gels and blotted for myc (NP1), HA-tag (ADAM10, DN-ADAM10), or tubulin. Overexpression of ADAM10 increases shedding of NP1, whereas DN-ADAM10 reduces shedding of NP1 to below baseline.

**Figure 5. The metalloprotease inhibitor TAPI-2 does not block the SDF1 mimicking effects of a cAMP analogue or a ROCK inhibitor.** Explants from E7 chick DRGs were treated for 30' with sema3A, TAPI-2, and Sp-cAMPs or the ROCK inhibitor Y-27632, as noted. **(A)** Sp-cAMPs (third green bar and third black bar) reduces the effect of sema3A in both the absence and presence of TAPI-2. **(B)** Y-27632 (third green bar and third black bar) reduces the effect of sema3A in both the absence and presence of TAPI-2.

**Figure 6. A model for ADAM10 involvement in antirepellent signaling.** In our model, CXCR4 activates $G\alpha_i$ and $G\alpha_q$, and they and their associated $G\beta\gamma$ subunits cooperate to activate PLC. PLC, through the production of diacylglycerol and inositol trisphosphate, increases calcium levels. Increased calcium activates ADAM10, which blocks repulsion through shedding of repellent receptors, and also activates calmodulin. Calmodulin, in turn, activates ADCY8 and thereby increases cAMP. Increased cAMP activates PKA, which phosphorylates MAPK1/2, leading to cell survival. PKA also phosphorylates Rho, which is thereby inactivated. This inactivation and the subsequent inactivation of ROCK are required for the antirepellent response to SDF1.

Figure 1

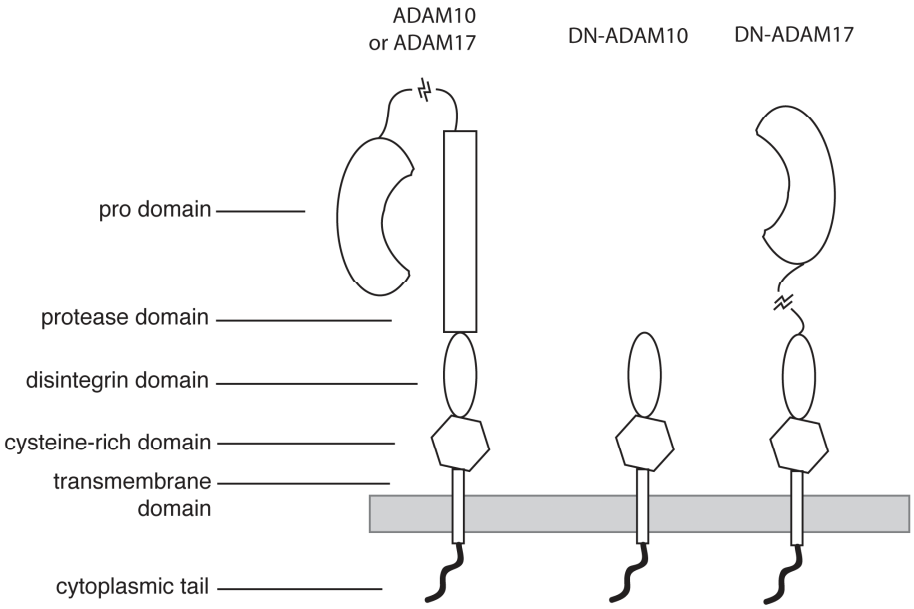



Figure 2

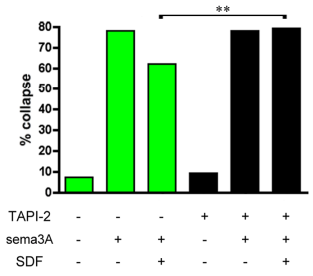


Figure 3

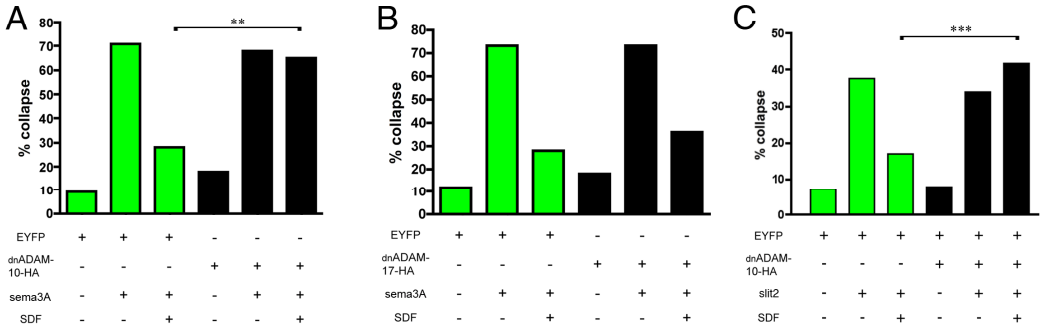



Figure 4

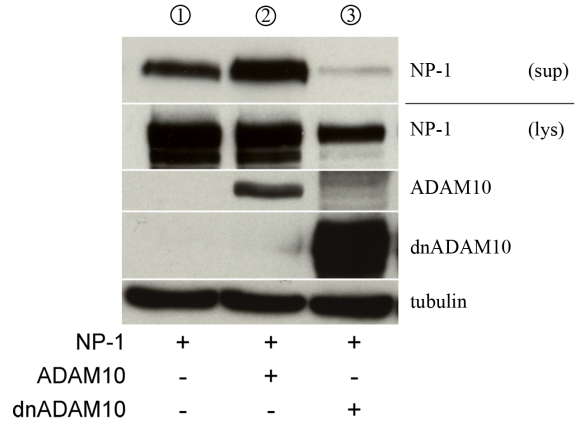


Figure 5

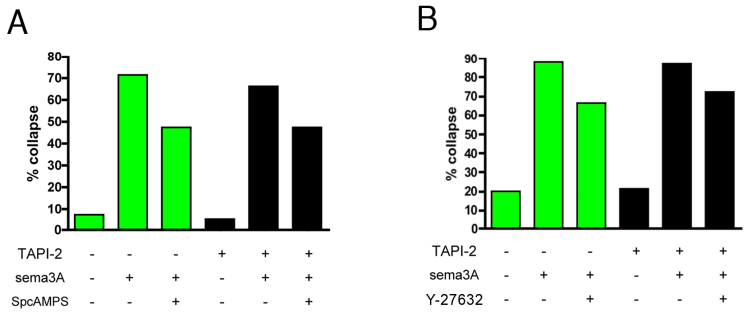
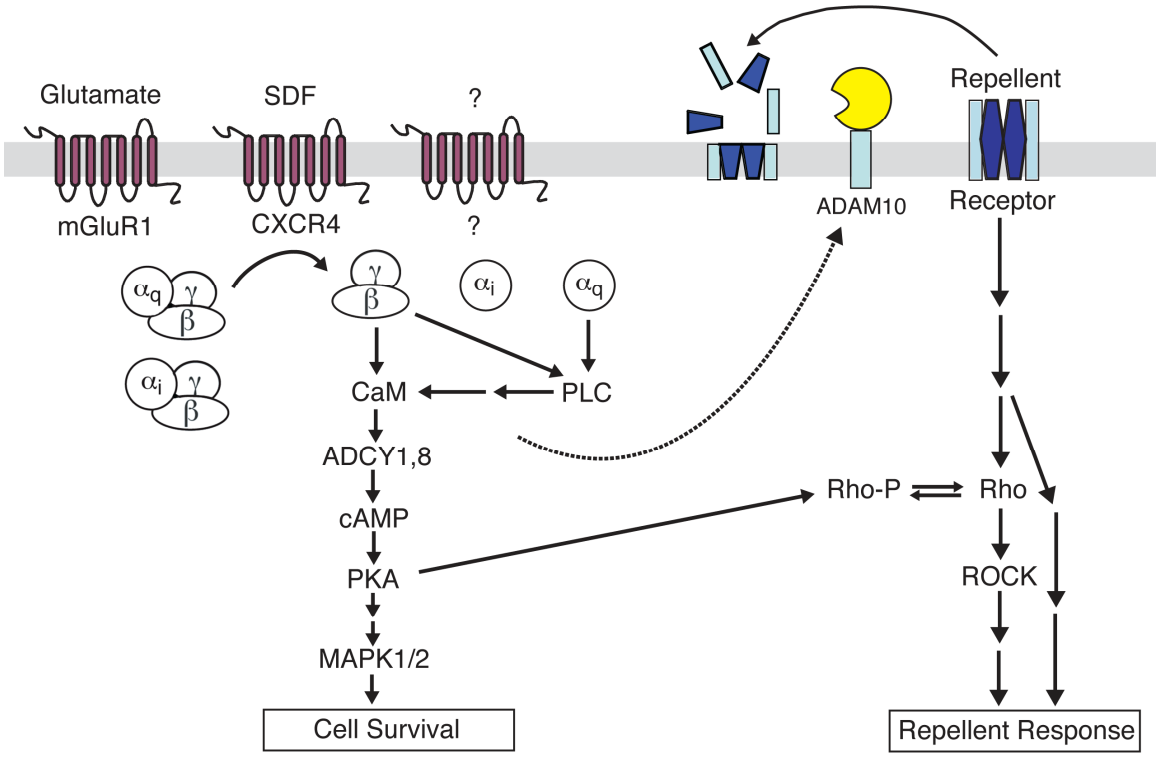



Figure 6



## Chapter 4: Future Directions

At the conclusion of these studies, there are many avenues of inquiry that remain open. In this chapter, I will first discuss a few gaps remaining from my work and ideas about how to fill them, after which I will go on to discuss larger outstanding questions in the roles of SDF1 and modulatory signaling in axon guidance.

### Part 1: Short-term questions

I will begin with a question from our model of antirepellent signaling, which is whether $G\alpha_i$, named for its ability to inhibit adenylate cyclase, does indeed increase cAMP levels in SDF1 signaling. Though separate manipulations of cAMP (Chalasani et al. 2003) and G protein alpha subunits (Chalasani et al. 2003; chapter 2) place both $G\alpha_i$ and cAMP in the SDF1 pathway, we lack direct evidence that $G\alpha_i$ activation is required for increased cAMP.

Recent work in the lab (Xu et al., 2010) showed that SDF1-induced increases in cAMP can be detected by FRET in chick RGCs. A relatively straightforward next step, therefore, would be to apply the $G\alpha_{i/o}$ inhibitor pertussis toxin (PTX) to this FRET assay. Our model would predict that PTX would block the SDF1-induced increase in cAMP but not the cAMP increase induced by forskolin. If PTX does not block the SDF1-induced increase in cAMP, the next likely model is that $G\alpha_i$ contributes to the antirepellent effect

via a different mechanism and that it is either $G\beta\gamma$ or $G\alpha_q$ that produces the increase in cAMP. Especially in that case, it would be interesting to test the $G\alpha_{q/11}$ inhibitor YM-254890 (Taniguchi et al., 2003). Though it is much less widely used than PTX, applying YM-254890 to this FRET paradigm would complement the PTX experiment. Genetic manipulations, though more elegant in some ways, would be more complicated in others. The cotransfection system I used in chapters 2 and 3 has a high but incomplete coexpression. It worked well in the collapse assay but would be problematic in a FRET assay. Cells appropriate for FRET imaging are few enough that one would want to be certain that all imaged cells expressed both the FRET construct and the experimental construct to be tested. This could only be confirmed if the non-FRET construct also fluoresces. A red or, preferably, far-red fluorescent tag might make the minigenes or GRK-CT usable in this context, but the pharmacological inhibitors are more immediately usable and may prove quite useful.

cAMP-sensing FRET may also prove useful in more precisely placing ADAM10 activity in the SDF1 pathway. It seems likely that ADAM10, activated by calcium, is in a separate downstream signaling pathway, parallel to that of cAMP. Using cAMP FRET to determine whether TAPI-2 blocks the increase in cAMP would confirm that ADAM10 is not upstream of the SDF1-induced increase in cAMP. Since we would predict a negative result, parallel dishes of transfected cells subjected to the same changes of media could be used in a collapse assay, as a positive control for TAPI-2 function.

The larger question remaining from chapter 3, however, is whether SDF1 causes shedding of repellent receptors by activation of ADAM10. Since cell lines proved useful in ascertaining ADAM10's ability to cleave NP1, perhaps a cell-line-based approach would be useful in this case, as well. COS cells expressing Plexin-A1 (PlexA1) and NP1 contract in a collapse-like manner upon treatment with sema3A (Takahashi et al., 1999). COS cells might, therefore, be a useful system for studying receptor surface localization and shedding. In such a system, it would be interesting first to determine whether cotransfection of CXCR4 and SDF1 treatment could block the contractile response. If sufficient signaling machinery is present for SDF1 treatment to block the sema3A-induced collapse-like contraction in cells expressing PlexA1, NP1, and CXCR4, these cells could be used to determine whether SDF1 could increase shedding of NP1 or PlexA1. It is difficult to use untransfected primary neurons because enough high quality antibodies to the receptors are unavailable and because primary neurons are particularly susceptible to permeabilization by even detergent-free fixation (not shown). Alternatively, a similar set of experiments with surface biotinylation of dissociated chick DRG would be a step toward confirming physiological relevance. For that approach, dissociated neurons could be treated with SDF1, TAPI-2, or SDF1 + TAPI-2, and the cell-surface proteins in these cultures could be isolated through biotinylation and recovery with streptavidin beads. Those surface-protein samples could then be probed for NP1, PlexA1, or CXCR4. If SDF1 does induce ADAM10-mediated shedding of NP1 or PlexA1, less of the repellent receptor should be present at the cell surface in the SDF1-treated condition.

**Part 2: Long-term questions.**

The most fundamental question here is how SDF1 signaling changes growth cones' responsiveness to repellents. It has been shown that sema3A reduces actin polymerization, especially in the periphery of the growth cone, within about five minutes (Fan et al., 1993). Though SDF1 by itself does not cause dramatic changes in F-actin density or localization (not shown), it may change the rate at which sema3A does so. This could potentially be assayed with fluorescent speckle microscopy in growth cones expressing low levels of fluorescent actin. A simpler but still interesting first step might be to compare the rates of lamellar and filopodial retraction in sema3A-treated versus sema3A- and SDF1-treated growth cones. SDF1 certainly reduces the percentage of collapsed growth cones after 30' treatment, but whether it slows the collapse rate for those growth cones that still collapse or whether it allows some collapsed growth cones to recover might provide insights into its mode of action.

In addition to altering the ability of repellents to alter the growth cone cytoskeleton, it is possible that SDF1 reduces the ability of repellent receptors to begin signaling. I have discussed this possibility in terms of shedding of repellent receptors, but localization within the membrane is also worth considering. Since many receptors are thought to function primarily when they are present in lipid rafts (see Simons and Gerl, 2010), SDF1 may function in part by increasing the ratio of non-raft:raft localization of repellent receptors. This could be tested by immunofluorescence for repellent receptors along with

fluorescent cholera toxin B, which is a stain for lipid rafts (Wolf et al., 1998). Alternatively, if L1 is required for sema3A receptor complex endocytosis and signaling (Castellani et al., 2004), perhaps SDF1 induces a signaling pathway that blocks the association of L1 with PlexA1 and NP1, the rest of the receptor complex. This, again, could be tested by immunofluorescence for the receptors in question. With sufficient quantities of good antibodies against L1, PlexA1, and NP1, immunoprecipitation would be another useful approach. If SDF1 blocks raft localization of any of those receptors, their coprecipitation would be predicted to decrease upon SDF1 treatment.

The other major question about SDF1 antirepellent signaling is how and where it is important *in vivo*. Chalasani et al. (2007) showed that knockdown of SDF1 or CXCR4 could rescue some of the optic tract pathfinding defects of a Robo2 hypomorphic zebrafish (*astray*).

Here, the G protein-targeting reagents I used in chapters 2 and 3 are already proving useful in examining the potential roles of G protein signaling in axon guidance *in vivo*. Since I have confirmed their effectiveness in chick neurons *in vitro*, we can express the G protein inhibitory peptides, GRK-CT, dominant-negative ADAMs, or other reagents *in vivo*, with greater confidence in their function and specificity. Because we do not want to affect earlier developmental processes, we are using the Gal4/UAS system to express these constructs in specific populations of neurons. Gal4 is a transcriptional activator derived from yeast. We can express it under the control of cell-type-specific promoters such as atonal-5 (Ath5), which is expressed in retinal ganglion cells and a few other cell

types in the zebrafish. UAS, Gal4's recognition sequence, then drives expression of our construct of choice in those cells expressing Gal4. By including fluorescent tags in our constructs, either as fusion proteins or under a second UAS, we can visualize the cells expressing those constructs. We have used a CMV-Gal4 plasmid to test the UAS-controlled versions of GRK-CT and the $G\alpha_i$, $G\alpha_q$, and $G\alpha_s$ inhibitory peptides in chick DRG, with the same results as directly CMV-controlled expression of the dominant negatives (Twery, Dell, and Raper, not shown).

Ongoing work in the lab (Dell and Raper, unpublished) has found that blocking $G\alpha_i$ or $G\alpha_q$ has only small effects on the zebrafish optic projection and that blocking $G\beta\gamma$ has similar but statistically significant effects. In contrast, blocking $G\alpha_s$ induces ectopic ipsilateral projections from approximately 10-30% of eyes, depending on UAS dosage. Since $G\alpha_s$ typically activates adenylate cyclase, this is consistent with findings from our lab and the Poo lab (Chalasani et al., 2003; Nishiyama et al., 2003) that cyclic nucleotide levels alter growth cone responses to various guidance cues. In the *in vivo* assays, the cells expressing these reagents are facing many cues, including but certainly not limited to SDF1. Thus, a more obvious, perhaps genuinely larger, role for $G\alpha_s$ than for $G\alpha_i$ or $G\alpha_q$ is not inconsistent with my in vitro work with SDF1 and sema3A as the only guidance cues presented. This result also supports a model in which G protein signaling is required for proper axon guidance, whatever receptors may be activating those G proteins.

The last outstanding questions I wish to discuss pertain to ADAM10 activity in axon pathfinding *in vivo*. Work in frog (Chen et al., 2007) has shown that ADAM10 is required for proper retinotectal guidance at two choice points, one at the mid-diencephalon and one at the entrance to the tectum. Inhibition of ADAM10 with the pharmacological inhibitor GI254023X caused inappropriate turns away from the tectum at low levels and defasciculation and failure to turn in the diencephalon at higher levels. Work in fly, discussed in chapter 3, showed a requirement for ADAM10 in axon extension (Rooke et al., 1996; Fambrough et al., 1996) and in slit/robo-mediated repulsion (Coleman et al., 2010). This suggests that ADAM10 functions both positively and negatively in axon guidance.

Since ADAM10 is thought to facilitate slit/robo signaling in fly and slit/robo signaling is a major contributing factor in zebrafish retinotectal guidance, the fish optic projection might be a good system in which to study requirements for ADAM10 in axon guidance *in vivo*. We have made in the lab a transgenic line expressing DN-ADAM10 under a UAS promoter. Fish expressing the DN-ADAM10 in RGCs do not have ectopic ipsilateral projections at 5 days post fertilization, but they have not been thoroughly analyzed (Dell and Raper, unpublished). Crossing the robo hypomorph mentioned above with fish expressing dn-ADAM10 would provide a more sensitive assay for ADAM10 function in axon guidance, since either enhancer or suppressor phenotypes could be identified. If ADAM10 is required for slit/robo signaling, the *astray* phenotype should be intensified; if ADAM10 is more involved in SDF1/CXCR4 signaling in the zebrafish optic projection, the *astray* phenotype should instead be mitigated. An alternate approach

would be to use the pro domain of ADAM10, which inhibits the function of the mature protein (Moss et al., 2007). Expression of the pro domain at the midline, either under a promoter specific to midline glia or by electroporation, might produce a stronger or more localized effect than the expression of dn-ADAM10 in RGCs.

In conclusion, I have shown that SDF1 antirepellent activity requires $G\alpha_i$, $G\alpha_q$, and $G\beta\gamma$. I have also shown that SDF1 antirepellent activity can be mimicked by overexpression of constitutively active forms of $G\alpha_q$, $G\alpha_i$, or $G\alpha_s$. These findings suggest that SDF1 antirepellent activity requires multiple G protein components working in parallel. I have provided additional support for the contribution of $G\alpha_q$ by showing that inhibition of phospholipase C blocks SDF1 antirepellent activity or antirepellent activity induced by a constitutively active $G\alpha_q$. Further, I have shown that SDF1 antirepellent activity is blocked by either a metalloprotease inhibitor or a dominant-negative ADAM10. Since ADAM10 can cleave the sema3A receptor component NP1, ADAM10 activation and repellent receptor clearing may be another important point of interaction between antirepellent and repellent signaling.

I see the open questions in the role of SDF1/CXCR4 and modulatory signaling in axon guidance as threefold: What are the remaining details of the SDF1 pathway? How does SDF1 signaling reduce growth cone responsiveness to repellents? How important is modulatory signaling in vivo? Additionally, ADAM10 function in axon guidance, both in antirepellent signaling and in other contexts, is worthy of further investigation.

**References**

**Chapter 1**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