## Mesendoderm Patterning by the Nodal/Lefty Activator/Inhibitor System

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Mesendoderm patterning by the Nodal/Lefty activator/inhibitor system

A dissertation presented
by
Katherine Wallace Rogers
to
The Department of Molecular and Cellular Biology
in partial fulfillment of the requirements
for the degree of
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Mesendoderm patterning by the Nodal/Lefty activator/inhibitor system

Abstract

During zebrafish embryogenesis, a gradient of the secreted TGFβ ligand Nodal induces expression of mesendodermal genes in an exposure-dependent manner, and also induces expression of the secreted Nodal feedback inhibitor Lefty. The long-range Lefty gradient dampens signaling by the shorter-range Nodal gradient, and together they are required for proper patterning of the germ layers. My research has addressed two fundamental questions about this patterning system: 1) How are the distinct ranges of Nodal and Lefty achieved, and 2) What is the role of Lefty-mediated feedback inhibition in this patterning system?

To understand why Nodal and Lefty have different activity ranges, we developed fluorescent fusion proteins and microscopy techniques to measure the distributions, degradation rates, and diffusion coefficients of Nodal and Lefty in living zebrafish embryos. We found that Nodal-GFP forms a shorter-range gradient than Lefty-GFP in vivo, consistent with the shorter activity range of Nodal. The extracellular half-lives of Nodal-Dendra2 and Lefty-Dendra2 are similar, but the diffusion coefficient of Nodal-GFP is much lower than that of Lefty-GFP. Differential diffusivity therefore explains the shorter range of Nodal compared to Lefty.

To better understand the function of Lefty during vertebrate embryogenesis, we generated and analyzed zebrafish lefty mutants. Although patterning is highly robust to lefty gene dosage, complete loss of lefty causes upregulation of mesendodermal genes
during gastrulation, aberrant cell internalization, and severe patterning defects, consistent with excess Nodal signaling. Mutations in *nodal* partially suppress patterning defects caused by *lefty* loss, and *lefty* mutants can be fully rescued by ectopic expression of *lefty-gfp* or by treatment with low levels of a Nodal inhibitor drug. Further, *lefty* expression is highly responsive to Nodal signaling. These results demonstrate that Lefty acts as a fast-responding, global dampener of Nodal signaling that restricts mesendoderm specification.
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CHAPTER 1
Morphogen gradients: From generation to interpretation

PREFACE

This chapter was previously published in the Annual Review of Cellular and Developmental Biology on July 29th, 2011. Alexander F. Schier and I wrote the review together. Sections have been updated as appropriate, and a final section reviewing the Nodal/Lefty patterning system has been added.

ABSTRACT

Morphogens are long-range signaling molecules that pattern developing tissues in a concentration-dependent manner. The graded activity of morphogens within tissues exposes cells to different signal levels and leads to region-specific transcriptional responses and cell fates. In its simplest incarnation, a morphogen signal forms a gradient by diffusion from a local source and clearance in surrounding tissues. Responding cells often transduce morphogen levels in a linear fashion, which results in the graded activation of transcriptional effectors. The concentration-dependent expression of morphogen target genes is achieved by their different binding affinities for transcriptional effectors as well as inputs from other transcriptional regulators. Morphogen distribution and interpretation are the result of complex interactions between the morphogen and responding tissues. The response to a morphogen is dependent not simply on morphogen concentration but also on the duration of morphogen exposure and the state of the target cells. In this review, we describe the morphogen concept and discuss the mechanisms that underlie the generation, modulation, and interpretation of morphogen gradients.
INTRODUCTION

Multicellular organisms come in many forms and shapes, but all of them face the same fundamental challenge during development: generation of distinct cell types and organs from a single cell. One way that this patterning process can be achieved is through morphogen gradients (Briscoe et al. 2010). Morphogens are long-range signaling molecules that act over a few to several dozen cell diameters to induce concentration-dependent cellular responses. The graded distribution of a morphogen within a tissue exposes cells to different morphogen concentrations. Cells exposed to high levels of morphogen signaling activate different transcriptional programs and adopt different fates than cells exposed to lower levels. Graded morphogen distribution thereby subdivides tissues into distinct cell types that are arranged as a function of their distance from the source (Briscoe et al. 2010).

This review discusses the mechanisms underlying morphogen-mediated fate specification. We refer the reader to recent reviews and studies that discuss other aspects of morphogen signaling, such as axon guidance, cell and tissue polarity, growth control, regeneration, evolution, and the application of morphogens in regenerative medicine (Brockes & Kumar 2008, Crickmore & Mann 2008, Lynch & Roth 2011, Meinhardt 2009, Schwank et al. 2011b, Strutt 2009, Swaney et al. 2010, Umulis et al. 2009, Wichterle et al. 2002). We begin by describing the history of the morphogen concept and the molecular characterization of morphogens. Next, we address how morphogen gradients form and how morphogens move through tissues. We describe how interactions between morphogens and target tissues modify the shape of morphogen gradients. We then discuss how morphogen signals are transduced and how cells interpret different

HISTORY OF THE MORPHGEN CONCEPT

Embryological and Theoretical Studies

The current concept of morphogen-regulated development is a synthesis of several ideas—induction, gradients, thresholds, and diffusion—that emerged from embryological and theoretical studies starting in the early twentieth century. The idea of induction, whereby a cell or tissue instructs neighboring cells to adopt a particular fate, was suggested as a mechanism underlying embryonic patterning. Other studies proposed that concentration gradients of molecules could provide cells with information about their position within a tissue, and concentrations above or below a certain threshold might elicit different responses. Finally, the idea of diffusion was suggested as a mechanism that could generate gradients by the spreading of molecules throughout target fields.

The first examples of induction came from experiments in amphibians by Warren H. Lewis, Hans Spemann, and Hilde Mangold. Lewis found that transplantation of the optic cup into the epidermis caused ectopic lens formation (Lewis 1904, Wolpert 1986).
Spemann and Mangold found that transplanting the dorsal pole of a gastrula embryo to the ventral side of a host embryo could induce a second embryonic axis (Spemann & Mangold 1924, Wolpert 1986). These studies revealed the inductive abilities of certain tissues but raised the question of how a single tissue could induce multiple cell types.

In the early twentieth century, Thomas Hunt Morgan and others proposed the idea that gradients might coordinate development (Boveri 1901, Morgan 1901). Morgan suggested that a gradually decreasing distribution of “material” from the animal to the vegetal pole of the developing sea urchin embryo could control gastrulation and generate patterns within developing tissues. This idea did not gain immediate acceptance, however, because it was unclear how the continuously graded distribution of a material could generate discrete regions of specification. Albert Dalcq and Jean Pasteels introduced the idea of thresholds in morphogenesis (Dalcq 1938), and Klaus Sander provided experimental evidence supporting the idea that exposure to a range of concentrations might be sufficient to induce a particular fate, as long as signaling levels were within certain boundaries (Sander 1960). Signaling levels below or above these boundaries would result in adoption of different fates. Leopold von Ubisch connected the concept of morphogen gradients to differential gene activation and thus was able to provide a molecular framework for morphogen gradient interpretation (von Ubisch 1953).

Hildegard Stumpf made the link between gradients and induction in the 1960s. On the basis of transplantation studies in moth pupae, she suggested that the graded distribution of inductive molecules within tissues could account for developmental patterning (Stumpf 1966). Stumpf proposed that an inductive signal was released from a
localized source—i.e., an inductive tissue—and that it instructed the concentration-dependent generation of several distinct cell types in surrounding tissues. In the related positional information theory, Lewis Wolpert and others proposed that each cell within a field is assigned a “positional value” that provides information about the cell’s position with respect to other cells (Lawrence 1966, Stumpf 1966, Wolpert 1969). Cells with different positional values could adopt different fates, and positional values could be assigned on the basis of morphogen exposure. Importantly, a signal providing positional information would not give specific instructions about the particular fate a cell should adopt. Instead, a combination of genotype, prior exposure to developmental signals, and positional information would control cell fate decisions. Thus, the same morphogen could be used in multiple tissue types or animals to provide positional information without dictating a particular cell fate (Wolpert 1969). The influential “French flag” model synthesized the concepts of induction, thresholds, and positional information and illustrated how gradients of inductive molecules could subdivide developing tissues into discrete regions of differentiation (Fig. 1.1).

The French flag model provided one explanation for how gradients could pattern developing tissues, but how would these gradients be formed? As early as 1952, Alan Turing, who coined the term morphogen (“form producer”), and later Alfred Gierer and Hans Meinhardt, developed mathematical models describing how gradients could form in tissues (Gierer & Meinhardt 1972, Turing 1952). Their models described how interactions between diffusing molecules could lead to the formation of gradients (and other patterns) across a field of cells. In such models, a locally acting activator induces both its own synthesis and the synthesis of a long-range inhibitor. This can result in self-
organization that generates patterns in an initially homogenous field of cells (Kondo & Miura 2010).

**Figure 1.1. The French flag model.** Morphogen is secreted from a source cell (green) and forms a concentration gradient within the tissue. Cells exposed to morphogen concentrations above threshold 1 exhibit a distinct response (blue). Cells exposed to intermediate morphogen concentrations (between thresholds 1 and 2) exhibit the “white” response, whereas cells exposed to levels below threshold 2 exhibit the “red” response. In this way, a concentration gradient of a single, diffusing substance could give rise to multiple cell fates and assign positional values to cells. Figure based on Kicheva & González-Gaitán (2008).

In 1970, Francis Crick proposed a simple “source-sink” model for the generation of morphogen gradients (Crick 1970). He suggested that localized cells produce a morphogen and secrete it into surrounding tissue. Morphogen molecules then diffuse and are destroyed by “sink” cells that are located at the opposite end of the tissue. These processes of diffusion and destruction together would result in a stable concentration gradient of morphogen, with the highest concentration located near the source cells and the lowest near the sink.
By the beginning of the 1970s, theoretical models and further embryological studies (Summerbell et al. 1973, Tickle et al. 1975, Wolpert et al. 1971) provided possible explanations of how morphogen gradient–mediated development could occur, but the discovery and characterization of morphogens had to await the advent of developmental genetics and molecular biology.

*Molecular Studies*

Despite the wealth of theories on gradient-mediated biological patterning, direct evidence that gradients control pattern formation *in vivo* was lacking until the late twentieth century. For example, molecular studies in the 1980s identified molecules that were distributed in gradients within developing tissues but did not function as morphogens. The small molecule retinoic acid (RA), for example, was found to form a shallow gradient within developing limb buds, but an essential patterning role for this gradient has not been demonstrated (Thaller & Eichele 1987). Graded distribution of the homeodomain protein Caudal was found along the anterior-posterior axis in *Drosophila* embryos (Macdonald & Struhl 1986, Mlodzik & Gehring 1987, Mlodzik et al. 1985), but the patterning defects in caudal mutants could be rescued by non-graded expression of Caudal protein, which argues against a requirement for the gradient (Macdonald & Struhl 1986).

The graded distribution and function of the Bicoid protein in Drosophila embryos provided the first clear connection between a molecular gradient and pattern formation. The Bicoid transcriptional regulator forms an anterior-to-posterior gradient in the syncytial blastoderm (Driever & Nüsslein-Volhard 1988a, Struhl et al. 1989)
High levels of Bicoid are required for expression of anterior marker genes, whereas genes with more posterior expression domain boundaries are expressed at lower levels of Bicoid (Driever & Nüsslein-Volhard 1988b, Struhl et al. 1989). Dampening of the Bicoid gradient resulted in the loss of anterior markers and the anterior shift of posterior markers. Although Bicoid was the first morphogen to be discovered, its molecular nature (transcriptional regulator) and environment (syncytium) make it unusual compared with most other morphogens, which are extracellular ligands that act in cellularized tissues. However, Bicoid does provide a valuable system for the study of how graded transcriptional effector activity, the ultimate outcome of morphogen gradient activity, leads to differential gene expression. Shortly after the discovery of the anterior-posterior Bicoid gradient, a ventral-to-dorsal nuclear gradient of the transcriptional regulator Dorsal was discovered and found to be required for dorsal-ventral patterning in Drosophila (Roth et al. 1989, Rushlow et al. 1989, Steward 1989). Thus, Bicoid and Dorsal were the first examples of transcriptional regulator gradients that control embryonic patterning.

Members of the transforming growth factor β (TGFβ) family were the first extracellular morphogens to be identified. Graded and long-range activity of Drosophila Decapentaplegic (Dpp) was found to pattern multiple tissues, including the dorsal-ventral embryonic axis and the wing imaginal disc (Affolter & Basler 2007, Ferguson & Anderson 1992, Lecuit et al. 1996, Nellen et al. 1996, Umulis et al. 2009). Exposure of Xenopus cells to different concentrations of the TGFβ signal Activin induced different mesodermal and endodermal cell types (Green & Smith 1990, Green et al. 1992, Gurdon
et al. 1994). In this assay, Activin mimics the effects of TGFβ signals of the Nodal family, which are the endogenous morphogens that pattern the germ layers (Chen & Schier 2001, Schier 2009, Shen 2007). The 1990s also saw the identification of several additional extracellular morphogen ligands ranging from Wingless (Wg)/Wnt to Hedgehog (Hh) (Briscoe et al. 2001, Heemskerk & DiNardo 1994, Katz et al. 1995, Kiecker & Niehrs 2001, Morisato & Anderson 1994, Neumann & Cohen 1997, Tabata & Kornberg 1994, Zecca et al. 1996). Following the discovery of bona fide morphogens, research began to address how morphogen gradients form, how morphogen signals are transduced, and how responding cells interpret graded signals and modulate the formation and interpretation of morphogen gradients. We discuss these aspects of morphogen biology in the following sections.

**GENERATION OF MORPHOGEN GRADIENTS**

How do morphogens move through target fields? Do they diffuse passively, or are they actively transported? Do morphogens move as individual molecules or as higher-order aggregates? Do they travel through extracellular spaces, or do they move through cells? Here we discuss our current knowledge of the mechanisms by which morphogen gradients are formed.

*The Synthesis-Diffusion-Clearance Model of Gradient Formation*

The prevailing model of morphogen gradient formation is the synthesis, diffusion, and clearance (SDC) model [also called the synthesis-diffusion-degradation (SDD) model]. According to this model, morphogen is produced from a localized source,
diffuses through tissues, and is cleared (Crick 1970, Wartlick et al. 2009). In the simplest scenario, a group of secreting cells creates a morphogen flux into an initially homogeneous field of cells. Morphogen molecules then move through the target field by diffusion. Clearance results in the removal of morphogen from the diffusible pool, for example, by immobilization, degradation, or endocytosis (Lander et al. 2009). The combination of constant flux from a localized source, diffusion, and uniform clearance results in a decaying concentration gradient. The distance over which the gradient decays depends on the diffusivity and clearance rates: the higher the diffusivity and lower the clearance rate are, the longer the morphogen’s range (Supplemental Figure 2) (for a detailed mathematical description, see Wartlick et al. 2009).

Recent studies have begun to test these theoretical predictions by measuring morphogen gradient profiles using fluorescent protein fusions or immunohistochemistry (e.g., Callejo 2006, Chamberlain et al. 2008, Driever & Nüsslein-Volhard 1988a, Gregor et al. 2007a,b; Gritli-Linde et al. 2001, Kicheva et al. 2007, Roth et al. 1989, Rushlow et al. 1989, Steward 1989, Strigini & Cohen 2000, Teleman & Cohen 2000, Yu et al. 2009). These studies show that most morphogen gradients approximate an exponentially decaying curve within developing tissues, which supports the SDC model. The exponential profiles of morphogen gradients suggest that clearance of most morphogens occurs throughout tissues rather than at a localized sink far from the source, as proposed by Crick (1970).
Recent biophysical measurements further support the SDC model and suggest that morphogen gradients are formed via diffusion (Abu-Arish et al. 2010, Kicheva et al. 2007, Lander 2007, Yu et al. 2009). For example, the spatial profile and biophysical properties of fluorescently labeled FGF8 (fibroblast growth factor 8) in zebrafish embryos and Dpp in imaginal discs are consistent with the idea that these morphogens spread nondirectionally via a diffusive process (Kicheva et al. 2007, Yu et al. 2009). Surprisingly, measured diffusion coefficients vary dramatically depending on the morphogen, tissue context, and experimental approach. The effective diffusion coefficients of Wg-GFP (green fluorescent protein) and Dpp-GFP in the Drosophila wing disc were found to be 0.05 µm$^2$ s$^{-1}$ and 0.1 µm$^2$ s$^{-1}$, respectively (Kicheva et al. 2007). In the imaginal disc that gives rise to the small wing-like organ called the haltere, the effective diffusion coefficient of Dpp-GFP is much lower, less than 0.003 µm$^2$ s$^{-1}$ (Wartlick et al. 2011). These effective diffusion coefficients are more than 1,000 times smaller than the diffusion coefficient of GFP in solution or the diffusion coefficient of FGF8-GFP in extracellular spaces within zebrafish embryos [50 µm$^2$ s$^{-1}$ (Yu et al. 2009)]. Analysis of Bicoid-GFP in the cortical cytoplasm has yielded diffusion coefficients that range from 0.3 µm$^2$ s$^{-1}$ (Gregor et al. 2007b) to ∼7 µm$^2$ s$^{-1}$ (Abu-Arish et al. 2010, Porcher et al. 2010) at mitotic cycle 14. In the nucleus, two diffusion processes of Bicoid-GFP have been detected with diffusion coefficients of ∼0.2 µm$^2$ s$^{-1}$ and ∼8 µm$^2$ s$^{-1}$, respectively (Porcher et al. 2010).

Why do the measured diffusion coefficients of morphogens differ by four orders of magnitude? The movement of some but not other morphogens may be hindered by
obstructions in the cellular environment and by interactions with other molecules (Rusakov & Kullmann 1998, Thorne et al. 2008). However, there is a striking correlation between diffusion coefficients and experimental approaches. The high diffusion coefficients were deduced from fluorescence correlation spectroscopy (FCS) measurements, whereas the lower diffusion coefficients were determined by fluorescence recovery after photobleaching (FRAP). For example, more recent FCS measurements of Dpp-Dendra2 in the wing disc revealed a diffusion coefficient of ~20 \( \mu m^2 s^{-1} \) for the majority of DppDendra2 molecules (Zhou et al. 2012), whereas previous FRAP experiments produced a diffusion coefficient of 0.1 \( \mu m^2 s^{-1} \) for Dpp-GFP(Kicheva et al. 2007). These two techniques measure diffusion within different environmental contexts, time windows, and length scales (Supplemental Figure 3) (see (Grimm et al. 2010) for a detailed discussion). The FCS experiments observed diffusion in small volumes (<0.5 \( \mu m^3 \)) and over short timescales (<100 s). In contrast, the FRAP experiments observed diffusion over multiple cells or nuclei (>1,000 \( \mu m^3 \)) and long time windows (~1 h).

Considering that morphogen gradients range from 10–200 \( \mu m \) and are formed within 30–300 min, Grimm et al. (2010) have argued that FRAP is a better measure of overall morphogen movement than FCS. For example, FCS might detect mainly the short-term diffusion of mobile molecules in a sub-environment within a tissue. In contrast, FRAP examines a cohort of molecules in different environments. The FRAP-based measurements thus provide effective diffusion coefficients that potentially reflect long-term movement through multiple environments (e.g., apical and basolateral, extracellular and intracellular, matrix and membrane) or that might be dominated by processes that trap molecules and cause them to accumulate locally (e.g., binding to extracellular...
matrix, endocytosis) (Bergmann et al. 2007). In contrast, the high diffusion coefficient of FGF8 measured by FCS (∼50 μm$^2$ s$^{-1}$) might correspond to free diffusion in extracellular spaces located at a distance from cell membranes. Indeed, the observation that a fraction of FGF8 signals has a diffusion coefficient of ∼5 μm$^2$ s$^{-1}$ (Yu et al. 2009) suggests that the overall effective diffusion of morphogens is a composite of interactions with multiple environments. Additional studies are needed to determine whether FRAP is the more accurate measure of long-range diffusivity, whereas FCS provides a potential means to dissect movements and interactions at smaller scales.

Clearance rate coefficients provide an additional test of SDC models but have been determined directly only for recombinant, bacterially produced FGF8 in zebrafish. Consistent with an SDC mechanism, FGF8 has a half-life between 9 and 18 min (Yu et al. 2009). On the basis of the shape of the gradient and the diffusion coefficients, the half-lives of Dpp, Wg, and Bicoid have been estimated as 45, 8, and 40 min, respectively (Abu-Arish et al. 2010, Kicheva et al. 2007). The short half-life of Wg may contribute to the comparatively short range of the Wg gradient. Proteasome-mediated degradation of Bicoid is crucial for normal gradient formation (Liu & Ma 2010). Interestingly, Wartlick et al. (2011) suggested that clearance rates for Dpp decrease as the wing disc grows, which allows the formation of a longer-range gradient in larger tissues.

For clarity, we have thus far made some simplifying assumptions when discussing the SDC model (e.g., the tissue being patterned is flat and completely uniform in composition, and clearance rates are equal throughout the tissue). In reality, gradient formation takes place in a dynamic 3D tissue. Therefore, although the basic concept of synthesis, diffusion, and clearance remains valid, several additional factors must be
considered when modeling gradient formation \textit{in vivo}. For example, target tissues are often heterogeneous, and feedback mechanisms can modify diffusion and clearance (Bollenbach et al. 2008, Cadigan et al. 1998, Chen & Struhl 1996, Dessaud et al. 2007, Lecuit & Cohen 1998). In addition, the sources of some morphogens are dynamic and not tightly localized. For example, Bicoid mRNA is not localized solely to the anterior-most pole; an extended SDC model is required to account for Bicoid protein distribution (Little et al. 2011, Spirov et al. 2009, St Johnston et al. 1989).

Surprisingly, recent experiments have demonstrated that a membrane-tethered form of Wingless expressed in the endogenous Wg domain supports relatively normal patterning, demonstrating that spreading of Wg is not strictly necessary for normal patterning (Alexandre et al. 2013). It has been suggested that early \textit{wg} expression in the wing primordium is sufficient for expression of Wg target genes, and that subsequent formation of the Wg gradient enhances proliferation. Thus, although the Wg gradient may normally form by SDC, this mechanism is apparently dispensable for normal patterning under optimal environmental conditions.

Some gradients form by mechanisms other than SDC. For example, a gradient of FGF8 mRNA in the embryonic mouse tail bud results from localized transcription followed by cell division and transcript degradation (Dubrulle & Pourquié 2004). Thus, the FGF8 mRNA and protein gradients in the tail bud form by a cell lineage transport mechanism rather than by diffusion from a localized source. Other variations in gradient formation are exemplified by RA and bone morphogenetic protein (BMP). The RA gradient in the zebrafish hindbrain may be shaped mainly by spatially regulated expression of enzymes that degrade RA, such that a localized source of RA is not strictly

*Morphogen Vehicles*

In the simplest case, morphogens diffuse through tissues as individual monomers or dimers. Beads soaked with RA, Activin, or FGF8 can serve as ectopic signal sources and induce long-range signaling (Gurdon et al. 1994, Nowak et al. 2011, White et al. 2007). In these cases, there is no apparent requirement for modification of signaling molecules before release. It is becoming clear, however, that most morphogens oligomerize, interact with other diffusible proteins, or are lipid modified (Supplemental Figure 4). For example, formation of the Dpp/BMP gradients in Drosophila, Tribolium, and Xenopus embryos involves the association of these molecules with secreted antagonists (Ben-Zvi et al. 2008, Eldar et al. 2002, Holley et al. 1996, Lewis 2008, Marqués et al. 1997, Reversade & De Robertis 2005, Shimmi 2003, Shimmi et al. 2005, van der Zee et al. 2006, Wang & Ferguson 2005, Zakin & De Robertis 2010). BMPs are initially found both ventrally and dorsally in Xenopus blastulae. They are mobilized when complexed with Chordin, a BMP antagonist that is secreted on the dorsal side. As Chordin diffuses away from its dorsal source, it binds to, inhibits, and mobilizes BMPs until the extracellular protease Tolloid degrades the Chordin portion of this complex (Fig. 1.2; Lewis 2008, Zakin & De Robertis 2010). This shuttling results in the deposition of BMPs near the site of Chordin degradation and reshapes the distribution and activity of
BMPs into a gradient that peaks in the ventral region. Several additional extracellular molecules act as anti- or pro-BMPs and regulate the movement of BMPs within the embryo (Zakin & De Robertis 2010). A homologous mechanism is used during dorsal-ventral patterning in Drosophila (Umulis et al. 2009). More recently, a similar shuttling-based mechanism has been proposed to generate a sharp gradient of the ligand Spätzle during dorsoventral patterning in *Drosophila* (Haskel-Ittah et al. 2012).

**Figure 1.2. The bone morphogenetic protein (BMP) shuttling mechanism.** In *Xenopus* embryos, Chordin (red) is secreted from the dorsal region, whereas BMP (green) is initially uniformly expressed. 1) Chordin, upon secretion from the dorsal region, forms a complex with and antagonizes BMP. 2) This interaction mobilizes BMP as complexes diffuse in the extracellular space. 3) Chordin is cleaved by an extracellular protease, which causes it to release and deposit BMP at the site of cleavage. This shuttling generates a ventral-to-dorsal gradient. Figure based on Lewis (2008).
Lipidation modulates the movement of some morphogens (Eaton 2008, Steinhauer & Treisman 2009). For example, the mature, secreted Hh peptide is covalently modified by both cholesterol and palmitic acid (Gallet 2011). Modified Hh remains associated with the outer leaflet of the plasma membrane, possibly in lipid rafts, until its release, which is dependent on the transmembrane protein Dispatched (Gallet 2011). The release of Hh from the plasma membrane allows long-range spread and signaling.

Some morphogens appear to form higher-order structures (Eaton 2008, Gallet 2011). At the smallest scale, electrostatic interactions mediate the formation of Hh oligomers that cluster at the cell surface (Vyas et al. 2008). Mutant Hh proteins that retain signaling competence but lack the ability to aggregate lose long-range signaling capabilities. Other studies suggest that Hh and Wg may be packaged through their lipid modifications into larger-scale lipoprotein particles (Supplemental Figure 4) (Callejo et al. 2008, Eaton 2008, Eugster et al. 2007, Gallet 2011, Panáková et al. 2005). As discussed below, lipidation might enhance the interactions with extracellular proteoglycans and act to concentrate morphogens at cell surfaces. Moreover, packaging of Hh and Wg into lipoprotein particles might account for the observations that lipoprotein receptor-related proteins can act as coreceptors for Hh and Wg (Fisher & Howie 2006, He 2004) and that the Hh receptor Patched is itself a lipoprotein receptor (Callejo et al. 2008). Although these studies suggest important roles for Hh-containing particles, the composition, arrangement, and role of higher-order morphogen assemblies are still poorly understood.
Extra- and Intracellular Morphogen Routes

Low-affinity interactions with extracellular matrix components such as heparan sulfate proteoglycans (HSPGs) are thought to modulate several aspects of morphogen biology. HSPGs are composed of protein cores to which sulfated glycosaminoglycan chains are attached. They confine the movement of secreted morphogens to cell surfaces, thereby effectively increasing the concentration of molecules at the surface, promoting productive morphogen-receptor interactions, and preventing the release of morphogens into the lumen that overlies epithelia (Supplemental Figure 5) (Baeg et al. 2001, Belenkaya et al. 2004, Callejo 2006, Gallet 2006, Han 2005, Takei 2004, Vincent & Dubois 2002, Yan & Lin 2009, Yan et al. 2009). For example, extracellular Dpp is lost from the surface of cells lacking functional HSPGs, and HSPG mutant cells exhibit attenuated Dpp responses compared with their HSPG-expressing neighbors (Belenkaya et al. 2004, Takei 2004). Thus, extracellular HSPG-mediated tethering of Dpp is required for morphogen signaling and spreading along the cell surface. Interactions with HSPGs can have multiple additional effects. For example, HSPGs may act as coreceptors for morphogens (Fujise 2003, Strigini & Cohen 2000, Tsuda et al. 1999). Morphogen binding to HSPGs can restrict movement not only to the surface of a cell but also to areas near the source, which shortens the signal’s range. For example, cleavage of heparin sulfate chains from HSPGs in zebrafish embryos causes an expansion of the FGF8 activity range, presumably by allowing FGF8 to move farther from its source (Yu et al. 2009). In contrast, HSPGs are required for the long-range spreading of some morphogens (Baeg et al. 2001, Belenkaya et al. 2004, Marjoram & Wright 2011, Oki et al. 2007, Takei 2004, The et al. 1999). For example, Vyas et al. (2008) proposed that the
aggregation of Hh molecules discussed above allows Hh to interact with HSPGs, thereby facilitating its ability to interact with receptors as it moves through the wing disc. Lipoprotein particles can also interact with HSPGs and contribute to morphogen spreading (Eugster et al. 2007). HSPGs might even be involved in the release and packaging of morphogens at the source. For example, HSPGs might facilitate the assembly of lipidated Hh into Hh oligomers or lipoprotein particles (Eaton 2008, Eugster et al. 2007, Gallet 2011, Panáková et al. 2005). Finally, HSPGs might promote the stabilization and spreading of morphogens by preventing internalization and clearance (Akiyama et al. 2008, Takei 2004). For example, Dpp mutant proteins that are unable to interact with the HSPG Dally are less stable in the extracellular space than wild-type Dpp and form shallower extracellular gradients of lower amplitude and decreased range (Akiyama et al. 2008).

The complex roles of HSPGs are also exemplified by their functions in Wg morphogen signaling. Strikingly, low levels of Dally-like protein (Dlp), a protein core of *Drosophila* HSPG, enhance Wg signaling, whereas higher levels inhibit signaling (Gallet et al. 2008, Yan et al. 2009). One model explaining this biphasic behavior of Dlp suggests that low (agonistic) levels of Dlp enhance signaling by concentrating Wg on the cell surface, which prevents its loss into the extracellular space and makes it available to its receptor Frizzled2 (Fz2) (Fig. 1.3). Conversely, high (antagonistic) levels of Dlp compete with Fz2 for Wg binding (Yan et al. 2009). Other biphasic modulators of morphogen signaling include *Drosophila* Crossveinless2 as well as *Xenopus* Syndecan-1 (Olivares et al. 2009, Serpe et al. 2008) and Ont1 (Inomata et al. 2008), which modulate BMP signaling, and *Drosophila* Ihog, which modulates Hh signaling (Dessaud et al.)
Biphase modulation of receptor-morphogen interaction may help generate sharp borders of gene expression by creating bistable states in which morphogen binds either many or few receptors (Olivares et al. 2009, Serpe et al. 2008).

Figure 1.3. Biphasic activity of heparan sulfate proteoglycans (HSPGs). (a) In the absence of HSPGs (blue), morphogen molecules (green) are not concentrated at the cell surface; therefore, receptor-morphogen interactions are reduced and target genes are not induced. (b) When HSPGs are present in low abundance, they bind morphogen molecules, concentrating them at the cell surface and promoting receptor-morphogen interactions, resulting in target gene expression. (c) When HSPGs are present in high abundance, morphogen molecules are still retained at cell surfaces. However, because HSPG molecules bind morphogen, they outcompete receptors for morphogen binding. Thus, environments with high levels of HSPGs contain many additional binding sites that compete with receptors for morphogen binding, whereas environments with low levels of HSPGs contain fewer competing binding sites but still concentrate morphogen molecules at cell surfaces.
The multiple potential roles of HSPGs have made it difficult to interpret the results of mutant analyses. For example, although the loss of an HSPG might lead to reduced signaling, it is unclear if this is due to changes in diffusion, increased clearance, release from the cell surface, reduced presentation to receptors, abnormal packaging at the source, or other aspects of morphogen signaling. Quantitative imaging and biophysical studies will be needed to address the exact roles of HSPGs and their modulators in different systems (Kleinschmit et al. 2010, Szuperak et al. 2011, Vuilleumier et al. 2010).

Importantly, extracellular environments vary widely between different tissues and even within a tissue. For example, Dpp is much more mobile in early Drosophila embryos than in the wing discs. This increased mobility is thought to be caused, at least in part, by the absence of HSPGs in early embryos (Bornemann et al. 2008). Even within the same tissue, a single morphogen can have different ranges. For example, Hh is secreted both apically and basolaterally from the wing disc epithelium (Supplemental Figure 6). Strikingly, basolateral Hh acts only at a short range, whereas apically released Hh acts at a long range (Ayers et al. 2010). Apical Hh is dispersed more widely due to its interaction with the HSPG Dally. Dally binds Hh and is released from the cell surface by the hydrolase Notum, which increases the mobility and range of Hh. In contrast, basolateral Hh signals move through cell-cell contact or through limited diffusion. This example highlights that overall morphogen distribution is a composite of different gradients that form by distinct mechanisms. Finally, even highly related morphogens can have different ranges within the same tissue. For example, the Nodal signal Squint has a
longer range in zebrafish embryos than the related Cyclops signal (Chen & Schier 2001, Schier 2009).

In addition to the composition of the extracellular matrix, endocytosis has been implicated in modulating morphogen gradient formation. Blockage of endocytosis can prevent morphogen movement (Bejsovec & Wieschaus 1995, Entchev et al. 2000, Gallet et al. 2008, Kicheva et al. 2007, Moline et al. 1999). One interpretation of this result is that morphogens may be transported intracellularly by a process called transcytosis, in which cellular uptake and subsequent release mediate the spread of morphogen molecules through a tissue (Supplemental Figure 6; (Entchev et al. 2000, González et al. 1991, Kruse 2004)). An alternative interpretation is that preventing endocytosis may lead to a buildup of extracellular molecules (e.g., receptors), thereby sequestering and inhibiting the spreading of morphogens (Belenkaya et al. 2004, Lander et al. 2002). Interestingly, however, transient blockage of endocytosis using temperature-sensitive mutants almost immediately precludes movement or accumulation of Dpp, which would be inconsistent with the latter interpretation, because receptor buildup should take time (Kicheva et al. 2007). In some cases, blocking endocytosis does not preclude gradient formation and can even increase gradient range, potentially by decreasing ligand clearance rates or altering trafficking (Dubois et al. 2001, Nowak et al. 2011, Scholpp & Brand 2004, Strigini & Cohen 2000, Yu et al. 2009).

Although extracellular diffusion appears to be a major mode of morphogen movement, long filopodia-like extensions also have been implicated in morphogen signaling (Bischoff et al. 2013, Briscoe & Vincent 2013, Gradilla et al. 2014, Hsiung et al. 2005, Kornberg & Roy 2014, Ramírez-Weber & Kornberg 1999, Roy et al. 2011,
Although such structures are used during cell communication in processes such as synapse formation and Notch signaling (Cohen et al. 2010, de Joussineau et al. 2003), their importance in morphogen signaling has not been established. However, more recently, experiments have demonstrated that modulating the length of Hh-containing cytonemes leads to corresponding alterations in the range of the Hh gradient and Hh target gene expression (Bischoff et al. 2013, Briscoe & Vincent 2013). Similarly, mutations that perturb Dpp-containing cytonemes lead to reduced Dpp signaling (Roy et al. 2014). Further research will be required to understand the role of these thin filopodia during patterning.

Modulation of Morphogen Distribution by Target Tissue Feedback

Although the target tissues of morphogens have classically been portrayed as simple responders or readers of gradients, it is now clear that the dialogue between morphogens and target cells contributes to gradient shape and interpretation. The feedback regulation of morphogen receptor expression exemplifies this (Cadigan et al. 1998, Chen & Struhl 1996, Dessaud et al. 2007, Lecuit & Cohen 1998). Expression of the Hh receptor Patched (Ptc), for example, is upregulated by Hh signaling (Chen & Struhl 1996). Ptc is an unusual receptor in that it actively represses activity of the Hh signaling pathway in the absence of Hh. Binding of Sonic hedgehog (Shh) to Ptc sequesters Shh and prevents it from spreading farther from its source. Moreover, Shh-induced Ptc expression promotes the endocytosis and degradation of Shh (Incardona et al. 2000). The extracellular vertebrate protein Hip1 also binds and sequesters Shh, antagonizing Shh signaling (Dessaud et al. 2008). In contrast, members of the Ihog/Boi/Cdo/Boc family
and Gas1 (in vertebrates) bind Shh to promote pathway activation (Dessaud et al. 2008). Before Shh exposure, target tissues such as the developing spinal cord express low levels of Ptc and higher levels of Gas1, Cdo, and Boc (Fig. 1.4a,b). Therefore, upon secretion Shh moves into a target field that is highly responsive to Shh signaling. Binding of Shh to Ptc activates the Shh signaling pathway, induces Ptc and Hip1 expression, and represses Gas1, Cdo, and Boc expression. As a result, the target field becomes less responsive and sequesters Hh, preventing its spread to regions more distant from the source. Moreover, Shh-induced Ptc expression promotes the endocytosis and degradation of Shh (Incardona et al. 2000). Thus, negative feedback loops can modulate the activity and spread of morphogen signals and are thought to contribute to the robustness of morphogen signaling (Barkai & Shilo 2009, Eldar et al. 2003, Irons et al. 2010, Lander et al. 2009).

In another example of morphogen-tissue dialogue, high levels of Dpp signaling in the wing disc repress the expression of the Dpp receptor Thickveins (Tkv), leading to an increase in the range of Dpp (Lecuit & Cohen 1998). This increase in range could be the result of an effective increase in Dpp diffusivity because fewer receptors are present to hinder Dpp mobility. In addition, a decrease in receptor expression could reduce the frequency of receptor-mediated endocytosis, thus decreasing clearance rates and increasing range. Strikingly, the repression of Tkv expression by Dpp is blocked in the developing haltere disc by the Hox transcriptional regulator Ultrabithorax (Ubx) (Crickmore 2006). Moreover, Ubx represses Dally (de Navas et al. 2006). Tkv expression and Dally repression restrict the range of the Dpp gradient in the haltere disk (Crickmore 2006, de Navas et al. 2006). Indeed, the Dpp diffusion coefficient in the haltere disc is smaller than in the wing disc (Wartlick et al. 2011). Thus, the limited diffusion and range
Figure 1.4. **Target tissue feedback inhibition.** (a,b) Model for tissue feedback during Sonic hedgehog (Shh) signaling in the developing spinal cord. (a) Before Shh secretion, levels of Gas1, Cdo, and Boc (teal) are high. Gas1, Cdo, and Boc are coreceptors for Hedgehog (Hh) and enhancers of Hh signaling. Patched (Ptc, pink) is expressed at low levels. Ptc represses Hh signaling in the absence of Hh but is inactive upon binding to Hh and sequesters Hh. Hip1 (orange), which binds and sequesters Hh, is not expressed in the absence of Shh. (b) Shh (green) is secreted into the ventral neural tube from the notochord (not shown) and floor plate (the ventral-most region in the neural tube). Upon Shh secretion, Shh signaling downregulates expression of Gas1, Cdo, and Boc and upregulates expression of Ptc and Hip1 near the source, which results in the sequestration of Shh and the dampening of pathway activation. (c) Model for feedback inhibition of Nodal signaling by Lefty. Nodal activity induces expression of the secreted Nodal inhibitor Lefty (orange), which is co-expressed with Nodal but has a longer activity range than Nodal (green). Model based on Chen & Schier (2002) and Shen (2007).

In addition to morphogen receptors, secreted feedback regulators also modify the formation and interpretation of morphogen gradients (Piddini & Vincent 2009, Schier 2009, Szuperak et al. 2011, Vuilleumier et al. 2010). For example, Nodal ligands induce expression of Lefty proteins, which are secreted Nodal signaling inhibitors (Schier 2009).
Lefty expression dampens Nodal activity and ensures that it does not extend beyond the appropriate range (Fig. 1.4c). Interestingly, the Nodal morphogen system shares several similarities with classical reaction-diffusion systems (Gierer & Meinhardt 1972, Kondo & Miura 2010, Meinhardt 2009) (see above section on Embryological and Theoretical Studies). In particular, Nodal and Lefty share the activator/inhibitor and self-enhancement features of this system. For example, in the zebrafish blastula, Nodal activates Nodal and Lefty transcription, and Lefty is required to restrict the range of Nodal signaling by blocking both the generation of Nodal locally and the response to Nodal at a distance (Chen & Schier 2002). The balance of Nodal and Lefty levels is further regulated by the microRNA miR-430 (Choi et al. 2007). The Nodal/Lefty activator/inhibitor pair also plays a role during left-right specification (Nakamura et al. 2006). In this system, Nodal/Lefty interactions appear to amplify small differences between the left and right sides. Analogous activator-feedback inhibitor pairs also have been implicated in head regeneration in Hydra (Meinhardt 2009), pigment stripe formation in zebrafish (Kondo & Miura 2010), and hair follicle spacing in mouse (Sick et al. 2006). However, in none of these systems have the tenets of reaction-diffusion models been tested by quantitative analyses.

TRANSDUCTION OF MORPHOGEN SIGNALING

As morphogens move through tissues, they bind receptors displayed on cell surfaces and initiate intracellular signaling cascades that result in the activation of transcriptional effectors. Different levels of effector activate and repress different sets of genes, which results in the execution of distinct developmental programs. In the
following section, we discuss how binding of morphogens to receptors is translated into differential gene expression.

Reading Different Morphogen Concentrations

To detect differences in morphogen concentration, cells could measure either the absolute number of occupied receptors or the ratio of bound to unbound receptors. With the possible exception of Hh signaling (Casali & Struhl 2004), the absolute number of activated, ligand-bound receptors is thought to determine morphogen signal transduction. For example, regardless of the total number of Activin receptors on a cell’s surface, expression of a low-threshold target gene requires at least 100 occupied Activin receptors per cell, whereas expression of a high-threshold target gene requires at least 300 occupied receptors (Dyson & Gurdon 1998). This argues against models in which Activin signaling levels are measured by the ratio of bound to unbound receptors. Instead, Activin concentrations are transmitted by absolute receptor occupancy. Importantly, at the low morphogen concentrations found in developing tissues (i.e., concentrations lower than the dissociation constant of ligand-receptor complexes), receptor activation increases almost linearly with ligand level. Thus, total receptor activity is roughly proportional to morphogen concentration.

Linear Signal Transduction

Some biological signaling pathways can generate “all-or-none” or “switch-like” behavior of signal effectors or transducers. For example, exposure to increasing levels of progesterone, a signal that does not act as a morphogen, induces a switch-like activation
of mitogen-activated protein kinase (MAPK) in immature *Xenopus* oocytes that leads to oocyte maturation (Ferrell & Machleder 1998). This all-or-none behavior generates a single response (oocyte maturation) from a graded input (different progesterone concentrations). Morphogen signaling, in contrast, generates multiple responses from a graded input (Supplemental Figure 7). In many cases, graded morphogen signaling is translated into graded transcriptional regulator activity, which leads to the expression of different sets of genes (Ashe 2006, Harvey & Smith 2009, Moussian & Roth 2005, Reeves & Stathopoulos 2009, Shimizu & Gurdon 1999, Stamataki 2005, Wilson et al. 1997). For example, a 3× higher Activin receptor occupancy is transduced into a 3× higher nuclear concentration of the transcriptional regulator Smad2 (Dyson & Gurdon 1998, Shimizu & Gurdon 1999). Accordingly, the gradient of nuclear Smad2 along the vegetal-animal axis of the zebrafish blastula is thought to reflect the concentration gradient of both Nodal and Nodal-receptor complexes (Harvey & Smith 2009). Thus, extracellular morphogen concentration gradients can be maintained intracellularly as concentration gradients of activated transcriptional regulators. In this model, regulators have either an active or inactive form, and the concentration of active transcriptional regulators determines target gene expression.

Some morphogens may also utilize a mechanism in which individual signal transduction molecules have graded activities. For example, transduction of Hh signaling is mediated by the phosphorylation of the Smoothened (Smo) C terminus. Phosphorylation at individual sites leads to incremental changes in Smo activity (Jia et al. 2004, Zhao et al. 2007). This suggests a mechanism in which graded Hh signaling leads to graded Smo activity by inducing progressive phosphorylation of Smo. Increasing
levels of Hh lead to a more extensive phosphorylation of individual Smo molecules as well as to a higher number of phosphorylated Smo molecules. Graded Smo activity results in the graded activation of Ci/Gli transcriptional regulators. In the absence of Hh, Ci is processed into a repressor form. Increasing levels of Hh block processing and convert Ci into an activator form. Thus, the extracellular gradient of Hh morphogen is translated into opposing nuclear gradients of Ci activator and Ci repressor.

INTERPRETATION OF MORPHOGEN SIGNALING

Graded morphogen distribution results in distinct domains of target gene expression within a tissue. In the following section, we discuss how DNA-binding affinity, combinatorial interactions, and duration of signaling contribute to differential target gene induction.

Differential Affinities of Transcriptional Regulators for DNA Elements in Target Genes

A relatively simple mechanism by which graded activities of transcriptional regulators induce differential gene expression involves the DNA-binding affinity for cis-regulatory elements (Ashe 2006, Driever et al. 1989). In this model, regulatory elements with high affinity for the transcriptional effector control genes activated at low levels of morphogen signaling, whereas elements with low affinity for the transcriptional regulator control genes induced only at high levels of morphogen signaling (Fig. 1.5). In support of this model, the expression domains of morphogen-responsive reporter genes can be broadened when enhancer affinity for transcriptional regulators is increased (Ashe 2006, Driever et al. 1989, Jiang & Levine 1993, Struhl et al. 1989, Wharton et al. 2004).
Figure 1.5. Gradient interpretation. (a) Interpretation by DNA-binding sites with varying affinity for transcriptional regulator (gold). The promoter of the top gene contains three low-affinity binding sites (blue; high-threshold gene); the promoter of the bottom gene contains three high-affinity binding sites (red; low-threshold gene). At high regulator concentrations, all sites in both promoters are bound, and both genes are expressed. At low concentrations, only the high-affinity sites are occupied, and only the gene with high-affinity sites is expressed. Based on Ashe & Briscoe (2006). (b) The ventral-to-dorsal nuclear Dorsal gradient (green) in Drosophila embryos is illustrated in a cross section. The expression domains of the Dorsal target genes Snail (blue), Twist (red), and Vnd (orange) are indicated. Based on Reeves & Stathopoulos (2009). (c) A coherent feed-forward loop initiated by Dorsal. (d) An incoherent feed-forward loop initiated by Dorsal. This loop restricts the expression of Vnd to the lateral regions of the embryo.

Morphogen Interpretation by Combinatorial Interactions

Despite the importance of distinct DNA-binding affinities in responses to morphogen signaling, this parameter alone cannot predict the expression boundaries of most morphogen targets. For example, some genes controlled by high-affinity Bicoid binding sites have more restricted expression domains than do genes controlled by lower-
affinity sites (Burz et al. 1998, Ochoa-Espinosa et al. 2005, Segal et al. 2008). In addition, when the Bicoid gradient is experimentally flattened, the expression boundaries of some target genes remain correctly positioned with respect to each other (Löhr et al. 2009, Ochoa-Espinosa et al. 2009). This suggests that additional inputs influence the positioning of target gene expression boundaries. One of these inputs is the transcriptional repressor Capicua (Löhr et al. 2009), which is repressed at the poles of the *Drosophila* embryo and binds to DNA regulatory elements that control expression of some Bicoid target genes. The balance between activation by Bicoid and repression by Capicua determines the expression boundaries of some Bicoid target genes (Fig. 1.6). The modulation of Bicoid activity by the presence of Capicua is an example of pre-patterning, in which preexisting factors modify the response to morphogen signaling. Another example of pre-patterning is the cooperation of TGFβ signaling with asymmetrically localized, maternally deposited factors (Schier 2009). For example, the vegetally localized transcriptional regulator Eomesodermin cooperates with Nodal morphogen signaling to induce endoderm in zebrafish, whereas animally localized Ectodermin restricts Nodal signaling and promotes ectoderm formation in *Xenopus* (Bjornson et al. 2005, Dupont et al. 2005). In addition, a recent study suggests that distinct BMP/Nodal ratios organize the embryonic axis (Xu et al. 2014). Zebrafish explants engineered to secrete Nodal and BMP at opposing ends developed clear anterior-posterior and dorsal-ventral axes and contained morphologically recognizable structures such as notochord. This demonstrates that a surprising amount of information can be contained within two overlapping morphogen gradients.
Figure 1.6. Capicua helps define the expression domains of Bicoid target genes.
Capicua represses the expression of a subset of Bicoid target genes. (a) In wild-type embryos, Bicoid is distributed in an anterior-to-posterior gradient (green), whereas Capicua activity is repressed at the poles (orange). The expression domain of the Bicoid target gene *otd* is depicted in purple below. (b) In the absence of Capicua, the expression domain of *otd* is expanded posteriorly. (c) If the Bicoid gradient is experimentally flattened, mirror duplications of target gene expression are observed at the poles. For details see Löhr et al. (2009) and Ochoa-Espinosa et al. (2009).

Modulation of morphogen interpretation by multiple inputs is also highlighted by the combinatorial regulation of Dorsal target genes (Reeves & Stathopoulos 2009). The transcriptional regulator Dorsal forms a ventral-to-dorsal nuclear gradient in *Drosophila* embryos and is required for patterning of mesoderm and ectoderm (Fig. 1.5). Dorsal regulates the expression of at least 50 target genes and determines three activation thresholds. Although different Dorsal DNA-binding affinities contribute to target gene induction, the expression of Dorsal target genes is also controlled by additional transcriptional regulators. For example, Dorsal induces expression of the transcriptional regulator Twist, which, together with Dorsal, promotes expression of several Dorsal target genes (Ip et al. 1992a, Kosman et al. 1991; Fig. 1.5). Expression of reporter genes containing only Dorsal binding sites is patchy and stochastic, whereas reporters containing only Twist sites are not expressed. In contrast, reporters containing both
Dorsal and Twist binding sites are expressed uniformly in domains with sharp borders (Szymanski & Levine 1995). Thus, the Dorsal-Twist coherent feed-forward loop leads to synergistic and robust activation of downstream genes. Robust gene expression may also be achieved by the preloading of RNA polymerase on the promoters of Dorsal target genes as well as by the presence of two or more enhancers that are both responsive to Dorsal (Boettiger & Levine 2009, Hong et al. 2008).

Dorsal-mediated patterning also employs incoherent feed-forward loops (Fig. 1.5). Dorsal and Twist activate Snail expression, which in turn represses the expression of the Dorsal target gene Vnd (Ip et al. 1992b, Reeves & Stathopoulos 2009, Kosman et al. 1991). Thus, some Dorsal targets are repressed at high levels of Dorsal, at which Snail is induced, but activated at lower levels of Dorsal, at which Snail is not expressed. In this way, interactions between target genes can play an important role in defining target gene expression domains.

Although most current studies focus on DNA-binding affinity and transcriptional regulator networks in morphogen interpretation, additional factors such as nucleosome positioning (Kim & O'Shea 2008) or chromatin modifications might also affect target gene induction (Dahle et al. 2010, Vastenhouw et al. 2010). For example, studies in yeast suggest that changes in nucleosome positioning in promoter regions can modulate gene expression in response to graded signals (Kim & O'Shea 2008, Lam et al. 2008).

**Temporal Effects of Morphogen Signaling**

Formation of a morphogen gradient is dynamic and occurs over time. Therefore, not only are cells exposed to different concentrations of morphogen, they are also
exposed to morphogen for different durations (Supplemental Figure 8). In some fast-developing early embryos, morphogen-mediated patterning is so rapid (2–5 h) that it is often assumed that increases in morphogen concentration are directly translated into expression of target genes (Bourillot et al. 2002, Harvey & Smith 2009, Schier 2009). However, even in these cases, the duration of morphogen signaling is important for proper cell fate specification. For example, premature inactivation of Nodal signaling blocks the induction of genes whose activation depends on high levels of Nodal (Gritsman et al. 2000, Hagos & Dougan 2007).

In more slowly developing tissues, the duration of exposure to morphogen can have dramatic effects on the response of cells (Ahn & Joyner 2004, Dessaud et al. 2007, 2010; Harfe et al. 2004, Kutejova et al. 2009, Nahmad & Stathopoulos 2009, Ribes & Briscoe 2009, Scherz et al. 2007, Yang et al. 1997). The dynamic nature of morphogen gradient interpretation is illustrated in the patterning of the spinal cord by Shh (Ribes & Briscoe 2009). A ventrally localized Shh source generates a ventral-to-dorsal gradient of Shh protein that patterns the nervous system. Shh is first detected close to its ventral source and then extends dorsally over several hours (Chamberlain et al. 2008). As the gradient emerges, target genes are activated, but importantly, their expression domains are induced and refined progressively. For example, Olig2 is first expressed close to the Shh source and then expands dorsally, whereas Nkx2.2 is expressed ventrally only later, when higher levels of Shh have accumulated (Chamberlain et al. 2008, Stamataki 2005). Nkx2.2 expression then extends dorsally, repressing Olig2 in its wake. Thus, Shh, Olig2, and Nkx2.2 are engaged in an incoherent feed-forward loop (similar to that generated by Dorsal activity, as discussed above) that generates distinct gene expression domains in
the developing spinal cord. In this scenario, the role of time could be to establish the Shh gradient and allow regulatory interactions to occur between target genes.

Consistent with a requirement for Shh accumulation over time, higher levels of Shh are required to induce Nkx2.2 than Olig2 in neural plate explants. However, exposing neural tube explants to identical Shh concentrations for progressively longer times initially activates Olig2 and subsequently induces Nkx2.2 expression (Fig. 1.7; (Dessaud et al. 2007, 2010)). Although it remains to be determined why Nkx2.2 induction requires longer durations of Shh exposure than Olig2, differential timing alone is sufficient to induce different target genes in this situation. These findings suggest that the adoption of distinct cell fates depends not only on the correct Shh concentration but also on the proper duration of Shh exposure.

The duration of Shh exposure alters cellular responses in several ways. First, extended exposure to Shh leads to an upregulation of Ptc expression (Dessaud et al. 2008). This negative feedback mechanism leads to the progressive desensitization of cells to Shh (Chen & Struhl 1996, Dessaud et al. 2007, Ribes & Briscoe 2009, Ribes et al. 2010). Cells that continue to be exposed to high levels of Shh maintain transduction of Shh signaling and can induce Nkx2.2 expression. In contrast, cells that are exposed to lower Shh concentrations and hence maintain transduction of signaling for shorter durations are unable to activate Nkx2.2 expression (Fig. 1.7). Second, the transcriptional regulators that are activated or repressed by Shh signaling can modulate transduction of Shh signaling (Lek et al. 2010). For example, Nkx2.2 alters the transduction of Shh signaling, at least in part, by inhibiting the generation of repressive forms of Gli transcriptional regulators. This positive feedback loop could amplify Shh signaling and
augment Nkx2.2 expression. These temporal adaptation mechanisms change the response of target cells even if extracellular Shh levels remain constant. Third, withdrawal of Shh can reverse patterning and result in the acquisition of a more dorsal fate. For example, cells that express Nkx2.2 can re-express Olig2 after Shh withdrawal (Dessaud et al. 2010). Thus, Shh-mediated patterning of the spinal cord depends on at least four factors: (a) the concentration of Shh, (b) the length of exposure to Shh, (c) feedback loops between the pathway and target genes, and (d) complex cross-regulatory interactions between target genes (Chamberlain et al. 2008, Dessaud et al. 2008, 2007, 2010; Lek et al. 2010, Ribes & Briscoe 2009).

Figure 1.7. Temporal integration of Sonic hedgehog (Shh) signaling. (a) Hypothetical phase diagram describing the relationships between Gli activity, time, and gene expression. Nkx2.2 expression (blue) requires higher concentrations of Shh signaling for longer periods of time than Olig2 expression (orange). Nkx2.2 represses Olig2 expression. The thick line represents the temporal Gli activity in cells exposed to a constant, high concentration of Shh. The thin line represents Gli activity in cells exposed to a constant, low concentration of Shh. (b) Gene expression in cells exposed to a constant, high Shh concentration. Cells first express Olig2. Olig2 expression decays as Nkx2.2 expression initiates and represses Olig2. (c) Gene expression in cells exposed to a constant, low Shh concentration. Cells express Olig2 but never reach the activity level required for Nkx2.2 expression. Figure based on data from Dessaud et al. (2010).
Time appears to play important roles in the interpretation of other morphogens as well. For example, the ability of embryonic zebrafish cells to respond to BMP signaling changes over time, resulting in temporal patterning of the anterior-posterior axis (Tucker et al. 2008). In this case, it is thought that target tissue sensitivity changes so that BMP signaling has different effects during different periods. In addition, the interpretation of Wnt signaling may depend on the fold change in the level of the Wnt effector β-catenin in the nucleus over time (Goentoro & Kirschner 2009). Surprisingly, target genes appear to respond not to absolute levels of β-catenin but to the ratio of β-catenin before and after an increase in Wnt signaling. An analogous mechanism might be at play in the wing imaginal disc, where the timing of cell division correlates with 50% increases in Dpp signaling (Wartlick et al. 2011). Thus, temporal changes in morphogen signaling levels dictate target responses in several systems, but in none of these cases are the molecular mechanisms that mediate temporal integration understood. Interestingly, several other systems use temporal gradients to control the timing of target gene expression. For example, increasing concentrations of the FoxA transcriptional regulator Pha-4 contribute to the activation of different gene batteries at different times during C. elegans pharynx development (Gaudet 2002). Analogously, “just-in-time” transcription programs ensure the ordered activation of genes encoding proteins involved in the SOS response (Ronen et al. 2002) and flagellum assembly (Kalir 2001). The different temporal onsets of target gene expression may be caused by differences in the affinity of a common transcriptional regulator for the promoters of the various genes in the pathway. It is conceivable that a similar mechanism underlies the interpretation of morphogen duration.
Although the duration of morphogen signaling may strongly affect responses in some contexts, it has relatively negligible effects in others. For example, cells exposed to Activin for as little as 10 min continue to express target genes for several hours after culture in Activin-free medium (Gurdon et al. 1995, Jullien 2005). The target genes that continue to be expressed are appropriate for the concentration of Activin previously experienced. When these cells are subsequently exposed to higher concentrations of Activin, they respond by expressing genes appropriate for the higher concentrations. Thus, a cell can increase its response, but it does not revert to a response appropriate for lower concentrations (Supplemental Figure 9). This “ratchet” mechanism affords a memory of the original positional information communicated to a cell in dynamic environments, such as when cells are changing their positions during gastrulation. The molecular basis of the Activin ratchet mechanism may involve long-lived Activin/receptor complexes that are endocytosed and elicit persistent cellular responses by maintaining a constant flow of phosphorylated Smad2 into the nucleus (Bourillot et al. 2002, Dyson & Gurdon 1998, Jullien 2005). However, the ratchet mechanism is not utilized by all morphogens. For example, as discussed previously, premature removal of Shh results in a loss of target gene expression, arguing against a long-term memory of previous exposure (Dessaud et al. 2010).

PRECISION, ROBUSTNESS, AND SCALING OF MORPHOGEN-MEDIATED PATTERNING

Despite its complexity, development is a reproducible, robust process. Embryos develop faithfully even in the face of environmental perturbations (such as low temperatures or oxygen availability), genetic perturbations (such as heterozygosity for
developmental control genes), and noise introduced by the stochasticity of gene expression and biochemical reactions. Even differently sized embryos of the same species give rise to mature organisms with proportionally patterned bodies. Here we discuss three important aspects of morphogen-mediated development that may influence reproducibility: precision, robustness, and scaling.

**Precision**

How do gradients give rise to precise gene expression domains that are at almost identical positions in different individuals? For example, the Bicoid-dependent posterior boundary of hunchback (hb) expression in the early *Drosophila* embryo varies by about 2% of embryo length in different individuals (Gregor et al. 2007a, He et al. 2008, Houchmandzadeh et al. 2002, Manu et al. 2009, Porcher et al. 2010, Porcher & Dostatni 2010). Strikingly, Bicoid levels in adjacent nuclei at this boundary differ only by 10%. Several studies suggest that the Bicoid gradient is very reproducible from embryo to embryo (~2–10% variability of embryo length) (Gregor et al. 2007a, He et al. 2008, Manu et al. 2009), and artificial cis-regulatory elements that contain only Bicoid binding sites drive expression in remarkably sharp and reproducible domains (Crauk & Dostatni 2005). How small differences in Bicoid levels are translated into sharp target gene thresholds is unclear (Porcher & Dostatni 2010). One model proposes that cooperative binding of Bicoid to regulatory elements can generate sharp on/off responses (Burz et al. 1998, Lebrecht et al. 2005, Ma et al. 1996, Struhl et al. 1989).

Although the Bicoid gradient appears to be remarkably precise, whether it provides positional information that is sufficiently precise to generate the observed low
variability in the hb gene expression domain is controversial (Gregor et al. 2007a, He et al. 2008, Houchmandzadeh et al. 2002, Manu et al. 2009, Porcher & Dostatni 2010). The expression of many Bicoid target genes is controlled by multiple regulatory elements that bind transcriptional regulators other than Bicoid. It has therefore been proposed that hb expression is refined by the input of these transcriptional regulators [e.g., maternal Hb protein and Capicua (Fig. 1.6)] and by cross-repressive interactions among transcriptional regulators that are coexpressed with hb (e.g., Kruppel and Knirps) (Clyde et al. 2003, Jaeger et al. 2004, Löhr et al. 2009, Manu et al. 2009, Ochoa-Espinosa et al. 2005, 2009). In this view, Bicoid is an essential component of a gene regulatory network that positions gene expression domains. Moreover, hb expression is dynamic during early development. Initially, nascent hb transcripts are found in a gradient that reflects the Bicoid gradient (Porcher et al. 2010). A sharp posterior boundary becomes apparent only at later stages. The molecular basis of this transition is not understood, further highlighting the complexities in deciphering when, where, and how morphogen gradients regulate precise expression boundaries (Bergmann et al. 2007, Bollenbach et al. 2008, de Lachapelle & Bergmann 2010, Gregor et al. 2007a, He et al. 2008, Houchmandzadeh et al. 2002, Manu et al. 2009, Porcher & Dostatni 2010).

Robustness

Modeling has demonstrated that feedback mechanisms can enhance the robustness (i.e., the resistance to perturbations) of morphogen systems (Barkai & Shilo 2009, Dassow et al. 2000, Eldar et al. 2002, 2003; Irons et al. 2010, Lander et al. 2009, Meinhardt 2009). For example, as discussed above, Dpp and Hh signaling controls the
expression of numerous genes that affect morphogen movement or interpretation. In particular, self-enhanced clearance is thought to help buffer changes in morphogen production rates (Barkai & Shilo 2009, Eldar et al. 2003, Lander et al. 2009). In this model, the higher the morphogen concentration, the more likely it is that a morphogen molecule will be cleared, thereby reducing transient increases in morphogen concentration. In the absence of feedback mechanisms, decoding the presteady state of morphogen profiles can also enhance robustness (Bergmann et al. 2007, Saunders & Howard 2009). Similar to self-enhanced clearance, this strategy can reduce patterning errors caused by fluctuations in morphogen production rates.

Scaling

Although organisms can vary dramatically in size, the variations in proportion are often much less substantial, similar to a flag whose pattern is size invariant (Wolpert 2011). For example, the length of different dipteran embryos can vary by up to five-fold, but the Bicoid gradient and the expression of segmentation genes scale with embryo length (Gregor et al. 2005, Lott et al. 2007, Sommer & Tautz 1991). Thus, on average, Bicoid molecules must travel significantly farther in the larger embryos of some species. Even in differently sized Drosophila embryos, Bicoid gradients have congruent shapes when distribution is analyzed with respect to percent of egg length (He et al. 2008). The molecular basis for this scaling is unknown (Gregor et al. 2008), but longer Drosophila embryos express slightly higher levels of Bicoid than do shorter embryos (He et al. 2008). This suggests that egg length might be coupled to Bicoid RNA production during oogenesis or to Bicoid translation or stability in the embryo (Cheung et al. 2011).
Morphogen gradient scaling is also observed during tissue growth. For example, the decay length of the Dpp gradient increases over time but remains proportional to the size of the wing imaginal disc even as it grows more than five-fold (Wartlick et al. 2011). The diffusion coefficient of Dpp appears to stay constant during tissue growth, which suggests that other parameters of gradient formation change to increase the range of signaling. For example, the Dpp source widens during growth, and the clearance of Dpp might decrease, resulting in an increase in gradient amplitude and range. Because growth is uniform during gradient scaling, all cells experience the same relative, but not absolute, change in Dpp signaling over time. Cell division exhibits a striking correlation with a relative Dpp signaling increase of ~50%. Thus, cells are thought to divide every time they experience a 50% increase in Dpp signaling, but how cells measure such signaling changes over time is unclear.

In addition, it has been proposed that an extracellular “expander” molecule—possibly Pentagone (Pent) (Vuilleumier et al. 2010)—acts as a size sensor during patterning of the wing disc (Ben-Zvi et al. 2011). In this model, Pent is mobile and expands the Dpp gradient, either by stabilizing Dpp or enhancing its diffusivity. Dpp represses the expression of Pent. As Dpp spreads and accumulates, Pent is progressively inhibited, ensuring that the gradient continues to expand until Pent is fully repressed by Dpp (Ben-Zvi et al. 2011).

Another striking example of scaling is observed during the earliest stages of embryogenesis, when many embryos can be split in two and one or both halves develop into smaller but normal animals (Cooke 1981). Studies in *Xenopus* suggest that multiple feedback mechanisms contribute to the scaling of BMP signaling in the smaller, dorsal
half of split embryos (Barkai & Shilo 2009, Ben-Zvi et al. 2008, Eldar et al. 2002, Reversade & De Robertis 2005). Several theoretical models have been proposed to account for scaling, but which of these models best reflects the in vivo response of organisms to size differences is unclear (Ben-Zvi & Barkai 2010, McHale et al. 2006, Othmer & Pate 1980, Umulis et al. 2010).

Not all embryos are capable of developing into normal adults after being split, and ligation of Drosophila eggs does not result in scaling of the Bicoid gradient (Boring et al. 1993). Moreover, scaling of morphogen gradients is only one potential mechanism to deal with size differences. For example, embryos that contain higher levels of Bicoid initially display a posterior shift of target gene expression (Supplemental Figure 1) but develop into normal flies (Berleth et al. 1988). Cell death in the expanded regions appears to correct for abnormal patterning, which reveals that initial variations in patterning can be corrected at later stages (Namba et al. 1997). In addition, scaling could in theory be achieved by a change of competence in the cells themselves such that tissues are patterned correctly even if the morphogen gradient itself does not scale.

CONCLUSIONS AND PROSPECTS

The study of morphogens has seen remarkable progress in the past 25 years. Morphogens are no longer a theoretical concept but have been identified in many developmental contexts. A plethora of regulatory interactions have been demonstrated to regulate morphogen gradient formation and interpretation. Although there are many variations on the theme, the general picture that emerges for morphogen gradient formation and interpretation is as follows. Morphogen molecules are released from a
local but dynamic source, assemble with themselves and/or other molecules, and move via restricted diffusion through the extracellular milieu. Gradient shape is determined by the flux from the source, the diffusivity of the morphogen, and its clearance kinetics in target tissues. These properties are modulated by interactions with HSPGs and other extracellular proteins that tether morphogens to the cell surface, prevent loss of morphogen to receptor-free domains, and affect morphogen trafficking and reception. Morphogen concentration can be transmitted linearly to intracellular transduction molecules, which results in the graded activity of transcriptional effectors. These transcriptional regulators are part of complex regulatory networks that modulate the target gene response to morphogen concentration and duration. Thus, target genes are regulated not only by the morphogen signaling pathway but also by preexisting factors, cross-regulatory interactions, and feed-forward loops. Feedback mechanisms buffer fluctuations in morphogen production, affect signaling interpretation, and confer scalability and robustness to morphogen-mediated patterning.

Despite this general understanding of morphogen signaling, many questions remain to be addressed. In particular, sophisticated mathematical models have been proposed to explain how morphogen gradients form and how this information is translated into target gene induction. Strikingly, however, few of the parameter values in these models have been determined experimentally. Thus, how well theoretical considerations of robustness, scaling, and precision hold up to experimental scrutiny is unclear. For example, for most morphogen systems we lack measurements of diffusion coefficients, clearance kinetics, binding constants, on and off rates, and temporal dynamics. The current controversies surrounding the simple Bicoid gradient in the
extensively studied Drosophila embryo indicate that gaining a full quantitative understanding will not be a trivial undertaking. At the molecular and subcellular levels, it is largely unclear how morphogens assemble to move through the extracellular milieu, what routes they take, and how they interact dynamically with other molecules. Finally, it remains largely unclear how morphogen function is modified to contribute to the striking variation of forms and shapes found in nature.

THE NODAL/LEFTY PATTERNING SYSTEM

Nodal is a highly conserved member of the TGFβ superfamily with roles in germ layer patterning and the establishment of left-right asymmetry in multiple animal phyla (Schier 2009, 2003). nodal gene numbers vary between species: humans and mice have a single nodal, zebrafish have three, and Xenopus have five (Erter et al. 1998, Feldman et al. 1998, Jones et al. 1995, Joseph & Melton 1997, Long 2003-b; Sampath et al. 1998, Takahashi et al. 2000, Zhou et al. 1993). Nodal signaling during early vertebrate embryogenesis induces expression of genes specifying mesoderm and endoderm (collectively referred to as mesendoderm), and dysregulation of Nodal signaling is implicated in multiple human pathologies, including cancer, congenital heart defects, and situs inversus (mis-positioning of bilaterally asymmetric organs) (see Appendix) (Deng et al. 2014, Joziassse et al. 2008, Lowe et al. 1996, Quail et al. 2013, Su et al. 2013).

Nodal signals through a receptor complex comprised of type I and type II Activin receptors and a membrane-anchored EGF-CFC protein that appears to act as a Nodal coreceptor (Reissmann et al. 2001, Yan et al. 2002, Yeo & Whitman 2001, Zhang et al. 1998). Nodal signaling via this receptor complex results in the phosphorylation of the
signal transducers Smad2 and Smad3 and their subsequent nuclear accumulation (reviewed in Shi & Massagué 2003). Together with Smad4 and other transcription factors (including FoxH1 and Mixer), phosphorylated Smad2/3 activates expression of Nodal target genes. In zebrafish, 47 direct Nodal target genes—including nodal itself—have been identified (Dubrulle et al., submitted, Bennett et al. 2007).

One of the target genes induced by Nodal signaling is the secreted Nodal feedback inhibitor, lefty. Lefty is a divergent member of the TGFβ superfamily, but lacks an alpha helix and cysteine residue that are thought to be involved in the dimerization of TGFβ ligands (Thisse & Thisse 1999). Similar to nodal, the number of lefty genes varies from species to species: humans, mice, and zebrafish all have two lefty genes that appear to have resulted from distinct duplication events in mammals and ray-finned fish, respectively, whereas flounder and fugu have a single lefty (Hashimoto et al. 2007). Lefty has been shown to inhibit Nodal signaling by competitively binding to the EGF-CFC coreceptor, preventing Nodal from signaling through the receptor complex (Chen & Shen 2004, Cheng et al. 2004). There are also indications that Nodal and Lefty may interact directly (Chen & Shen 2004).

Experiments in which zebrafish Nodals (Squint and Cyclops) and Leftys (Lefty1 and Lefty2) were ectopically expressed from clones in zebrafish embryos demonstrated that Nodals and Leftys have distinct activity ranges (Chen & Schier 2001, 2002). Nodal target gene expression was induced farther from Squint-expressing clones than from Cyclops-expressing clones. Ectopic expression of Lefty1 or Lefty2 at the animal pole was able to repress Nodal signaling at the margin, far from the ectopic source of Lefty. This demonstrated that Leftys are long-range Nodal repressors, whereas Squint and Cyclops
are medium- and short-range activators, respectively. The properties of the Nodal/Lefty patterning system thus appear to be consistent with proposed reaction/diffusion patterning systems in which a short-range activator (Nodal) activates a long-range inhibitor (Lefty) (Kondo & Miura 2010, Meinhardt 2009, Schier 2009, Shen 2007, Shiratori 2006).

The medium-range Nodal Squint is thought to act as a morphogen during germ layer patterning in zebrafish embryos (Chen & Schier 2001). Squint and the short-range Nodal Cyclops are expressed in a localized region of zebrafish embryos (the margin) just prior to and during gastrulation, and are thought to form a protein gradient along the animal-vegetal axis (Schier 2009). Although the endogenous Nodal gradient has never been visualized, at least two key observations support the existence of a Nodal protein gradient: 1) a gradient of nuclear SMAD2 is clearly visible during the stages that *nodal* is expressed, and nuclear accumulation is highest at the margin where Nodal expression is strongest (Dubrulle et al., submitted, Harvey & Smith 2009), and 2) expression of Nodal target genes with distinct ranges emanates from the margin (Dubrulle et al., submitted, Bennett et al. 2007). Cells exposed to high levels of Nodal signaling (nearest the margin) become endoderm, cells exposed to moderate Nodal levels become mesoderm, and cells exposed to low levels of Nodal or not exposed to Nodal give rise to ectoderm.

*squint<sup>−/−</sup>;cyclops<sup>−/−</sup> zebrafish mutants and embryos lacking the EGF-CFC coreceptor do not produce head and trunk mesendoderm, cells fail to internalize during gastrulation, and the resulting embryos are non-viable (Carmany-Rampey & Schier 2001, Feldman et al. 1998, Gritsman et al. 1999). Interestingly, whereas *cyclops<sup>−/−</sup>* mutants exhibit cyclopia and axis patterning defects and are non-viable, *squint<sup>−/−</sup>*, *cyclops<sup>−/−</sup>* and *squint<sup>+/−</sup>;cyclops<sup>−/−</sup>* mutants are often relatively normally patterned and viable (Dougan
2003, Feldman et al. 1998, Pei et al. 2007). This demonstrates that patterning is robust to

*nodal* gene dosage, and that the activity of the short-range ligand Cyclops is sufficient to

pattern the mesendoderm.

*lefty* mutants have never been generated in zebrafish, but experiments with

morpholinos suggest that in the absence of *lefty*, excess Nodal signaling generates extra

mesendoderm, resulting in severe patterning defects (Agathon et al. 2001, Chen & Schier

2002, Feldman et al. 2002). Overexpression of *lefty* mRNA phenocopies

squint<sup>−/−</sup>;cyclops<sup>−/−</sup> mutants and embryos lacking the EGF-CFC coreceptor, indicating that

Lefty is a Nodal inhibitor (Bisgrove et al. 1999, Meno et al. 1999, Thisse & Thisse 1999).

In Chapters 2-4, I discuss our efforts to examine the biophysical properties of

Nodal and Lefty and determine how their different activity ranges are established. In

Chapter 5, I describe the generation and analysis of zebrafish *lefty* mutants, and discuss

evidence indicating that the feedback inhibitor Lefty acts as a rapidly-responding global

Nodal dampener. In Chapter 6, I propose future experiments to follow up on my PhD

work, and speculate about possible benefits of the design of the Nodal/Lefty

mesendoderm patterning system.
CHAPTER 2
Differential diffusivity of Nodal and Lefty underlies a reaction-diffusion patterning system

PREFACE

This chapter was previously published in Science on May 11th, 2012 (Müller et al. 2012). Patrick Müller developed the Fluorescence Recovery After Photoconversion (FRAP) assay and performed the Cyclops-GFP, Squint-GFP, and Lefty2-GFP FRAP experiments, and designed and performed the gradient measurement experiments. I generated and tested the activity of the fusion proteins, developed the Fluorescence Decay After Photoconversion (FDAP) assay and performed the FDAP experiments, and carried out the published Lefty1-GFP Fluorescence Recovery After Photoconversion (FRAP) experiments. Ben M. Jordan and Patrick Müller developed software and techniques to analyze FRAP and FDAP data. Joon S. Lee established the FRAP assay in zebrafish and generated initial versions of the fusion proteins. Drew Robson and Sharad Ramanathan aided with mathematical modeling. Alexander F. Schier helped conceive, design, and support the project.

ABSTRACT

Biological systems involving short-range activators and long-range inhibitors can generate complex patterns. Reaction-diffusion models postulate that differences in signaling range are caused by differential diffusivity of inhibitor and activator. Other models suggest that differential clearance underlies different signaling ranges. To test these models, we measured the biophysical properties of the Nodal/Lefty activator/inhibitor system during zebrafish embryogenesis. Analysis of Nodal and Lefty
gradients revealed that Nodals have a shorter range than Lefty proteins. Pulse-labeling analysis indicated that Nodals and Leftys have similar clearance kinetics, whereas fluorescence recovery assays revealed that Leftys have a higher effective diffusion coefficient than Nodals. These results indicate that differential diffusivity is the major determinant of the differences in Nodal/Lefty range and provide biophysical support for reaction-diffusion models of activator/inhibitor-mediated patterning.

MAIN TEXT

In 1952, Alan Turing put forward the reaction-diffusion model, in which two interacting and diffusing species of molecules can generate complex patterns (Turing 1952). Gierer and Meinhardt postulated that pattern formation in reaction-diffusion models requires a short-range activator that enhances both its own production and that of a long-range inhibitor (Gierer & Meinhardt 1972) (Fig. 2.1A). Despite the prominence of reaction-diffusion models and the widespread occurrence of short-range activators and long-range inhibitors in development (Ben-Zvi et al. 2008, Chen & Schier 2002, Economou et al. 2012, Klein et al. 2004, Kondo & Miura 2010, Meinhardt 2009, Shiratori 2006, Sick et al. 2006), it is unclear how differences in activator and inhibitor ranges arise in vivo. The classic reaction-diffusion models postulate that the inhibitor is more diffusive than the activator (see Supplementary Materials in the Appendix, or www.sciencemag.org/cgi/content/full/science.1221920/DC1), but more recent studies suggest that differential signal clearance might be a major determinant of differences in signaling range (Kicheva et al. 2007, Le Good et al. 2005, Müller & Schier 2011, Nakamura et al. 2006, Rogers & Schier 2011, Wartlick et al. 2011, Yu et al. 2009, Zhou
et al. 2012) (Fig. 2.1B). This question has not been resolved because the biophysical properties of diffusion and clearance have not been measured for any activator/inhibitor pair.

Figure 2.1. Model of the Nodal/Lefty activator/inhibitor reaction-diffusion system and regulation of range. (A) In the source, Nodal signals (blue) activate their own expression as well as the expression of Lefty (red), which inhibits Nodal production. Nodal signaling in the responsive field is inhibited by the long-range inhibitor Lefty. (B) Distribution is controlled by both diffusivity, D, and clearance, k_i. Highly mobile molecules that are rapidly cleared from the extracellular space (black circles) can form gradients similar to those formed by poorly diffusive molecules that are slowly cleared (blue). Decreasing the clearance of the more diffusive species results in a long-range gradient (red). Simulations were performed as described in the Supplementary Materials (see Appendix).

own expression, and (ii) Leftys are long-range inhibitors that are activated by Nodals (Kondo & Miura 2010, Meinhardt 2009, Schier 2009, Shen 2007, Shiratori 2006). Genetic and embryological studies in zebrafish have shown that during mesendoderm induction, the two Nodal signals Cyclops and Squint and the two Nodal signaling inhibitors Lefty1 and Lefty2 have different activity ranges: Cyclops is a short-range activator of mesendodermal gene expression, Squint acts at a medium range, and Lefty1 and Lefty2 are long-range inhibitors (Chen & Schier 2001, 2002). Moreover, these Nodal signals induce their own expression as well as the expression of Leftys (Schier 2009, Shen 2007, Shiratori 2006) (Fig. 2.1A). However, the biophysical properties that control the different activity ranges of Nodals and Leftys are unknown. It therefore remains unclear whether one of the central tenets of reaction-diffusion models—differential diffusivity of activator and inhibitor—is fulfilled \textit{in vivo} (Chen & Schier 2002, Le Good et al. 2005, Marjoram & Wright 2011, Meinhardt 2009, Nakamura et al. 2006, Schier 2009, Shen 2007, Shiratori 2006). To address this question, we performed measurements of Nodal and Lefty distribution, clearance, and diffusivity during zebrafish embryogenesis.

To visualize Nodal and Lefty proteins \textit{in vivo}, we generated active fusions of green fluorescent protein (GFP) with Cyclops, Squint, Lefty1, and Lefty2 (see Figs. A.4 to A.13 for analyses of fusion protein activity, processing, localization, and distribution). When expressed from a localized source in blastula embryos, the fusion proteins had activity ranges similar to those of their untagged counterparts (Figs. A.7 and A.12). \textit{In vivo} imaging revealed that the distribution profiles of the fusion proteins reflected their activity ranges: The gradient formed by Cyclops-GFP exhibited a punctate distribution
and dropped steeply as the distance from the source increased, whereas the gradient
formed by Squint-GFP was more diffuse and reached farther (Fig. 2.2, C and 2.2D). The
distributions of Lefty1-GFP and Lefty2-GFP were more shallow and were long-range and
super-long-range, respectively (Fig. 2.2, E and F).

**Figure. 2.2.**
**Measurement of Nodal-GFP and Lefty-GFP distributions.** (A) At late blastula stages,
about 40 cells secreting Nodal-GFP or Lefty-GFP proteins were transplanted from
donor embryos into wild-type hosts (see Supplementary Materials in the Appendix). The
gradient profile was determined using a maximum-intensity projection of five
confocal slices encompassing a depth of 20 mm (about one cell). (B) A representative
projection is shown for Squint-GFP. (C to F) Construct schematic, representative
maximum-intensity projection, and distribution profiles 30, 60, and 120 min after
transplantation for Cyclops-GFP (C), Squint-GFP (D), Lefty1-GFP (E), and Lefty2-GFP
(F). Embryos that did not undergo transplantation were used for background subtraction,
and all intensities were normalized to the value most proximal to the source. Error bars
indicate SE. Numbers of embryos analyzed at 30, 60, and 120 min after transplantation,
respectively: for Cyclops-GFP, n30 = 7, n60 = 7, n120 = 7; for Squint-GFP, n30 = 12,
n60 = 17, n120 = 20; for Lefty1- GFP, n30 = 8, n60 = 8, n120 = 13; for Lefty2-GFP, n30
= 12, n60 = 10, n120 = 12.
To determine whether differential clearance underlies the different ranges of Nodal and Lefty signals, we developed a pulse-labeling assay to measure extracellular clearance rate constants. We fused the monomeric green-to-red photoconvertible protein Dendra2 (Zhang et al. 2007) to Nodals and Leftys and uniformly expressed these active fusions in blastula embryos (Figs. A.4 to A.12). We then switched the fluorescent state of Dendra2 from green to red throughout the embryo and monitored the decrease in intensity of the extracellular red signal for 300 min (Fig. 2.3). By fitting the data with an exponential decay model, we obtained clearance rate constants ($k_i$) and calculated the inversely related extracellular half-lives, $\tau = \ln(2)/k_i$ (see Figs. A.14 to A.18 for control experiments and detailed measurements). We found half-lives of 95 to 218 min with clearance rate constants of $1.22 (\pm 0.13) \times 10^{-4}/s$ for Cyclops-Dendra2, $1.00 (\pm 0.06) \times 10^{-4}/s$ for Squint-Dendra2, $0.53 (\pm 0.05) \times 10^{-4}/s$ for Lefty1-Dendra2, and $0.69 (\pm 0.07) \times 10^{-4}/s$ for Lefty2-Dendra2 (Fig. 2.3B and Fig. A.14). Thus, protein half-lives increased only slightly as ranges increased, which suggests that differential clearance is only a minor contributor to the differences in Nodal and Lefty range.
Figure 2.3. Measurement of extracellular clearance rate constants. (A) Uniformly expressed Nodal-Dendra2 or Lefty-Dendra2 fusion proteins were photoconverted using an ultraviolet pulse. The average extracellular photoconverted Dendra2 intensity was monitored over time and used to determine the clearance rate constants ($k_1$) and half-lives [$\tau = \ln(2)/k_1$] by fitting exponential functions to data from individual embryos (see Supplementary Materials in the Appendix). The normalized average intensity from Squint-Dendra2 experiments at 10-min intervals (black, n = 11) is shown fitted with an exponential function (red). Error bars indicate SD. See Fig. A.14 for Cyclops-Dendra2, Lefty1-Dendra2, and Lefty2-Dendra2 results. (B) Summary of mean extracellular $k_1$ values. Error bars indicate SE.

To determine whether differential diffusivity underlies the different ranges of Nodals and Leftys, we measured their effective diffusion coefficients using fluorescence recovery after photobleaching (FRAP) (Gregor et al. 2007b, Kicheva et al. 2007, Rogers & Schier 2011, Wartlick et al. 2011). FRAP assays can measure diffusivity over developmentally relevant length and time scales by observing the diffusion-dependent reappearance of fluorescence after photobleaching (see Supplementary Materials in the Appendix). To perform FRAP assays, we ubiquitously expressed Nodal-GFP and Lefty-GFP fusion proteins in blastula embryos and photobleached a cuboidal volume containing several hundred cells. We then monitored the recovery of fluorescence in the
bleached region over a period of 50 min (3000 s) (Fig. 2.4A and Figs. A.20 to A.25). Photobleaching was nearly uniform (Fig. A.24) and had no apparent toxic effects on the embryo, and fluorescence recovery occurred from regions adjacent to the bleached window (Fig. A.25). We determined the effective diffusion coefficients of the fusion proteins by fitting a three-dimensional diffusion model to recovery profiles (Figs. A.21 to A.23). We obtained effective diffusion coefficients of 0.7 +/- 0.2 µm²/s for Cyclops-GFP, 3.2 +/- 0.5 µm²/s for Squint-GFP, 11.1 +/- 0.6 µm²/s for Lefty1-GFP, and 18.9 +/- 3.0 µm²/s for Lefty2-GFP (Fig. 2.4B, Figs. A.20 to A.25). Thus, increased protein diffusivities reflect increased ranges, indicating that differential diffusivity is a major contributor to the differences in Nodal and Lefty range.

**Figure 2.4. Measurement of effective diffusion coefficients.** (A) Uniformly expressed Nodal-GFP or Lefty-GFP fusion proteins were locally photobleached (yellow boxes) at blastula stages. Fluorescence recovery was monitored over time, and the effective diffusion coefficient D was determined by fitting the resulting recovery profile (black) with simulated recovery curves (red) that were numerically generated using a model that includes diffusion, production, and clearance in a three-dimensional embryo-like geometry (see Supplementary Materials in the Appendix). Results for an individual Squint-GFP embryo are shown. See Fig. A.20 for Cyclops-GFP, Lefty1- GFP, and Lefty2-GFP results. (B) Summary of mean diffusion coefficients. Error bars indicate SE.
To test whether the experimentally determined values for diffusivity and clearance accurately predict the measured distribution profiles, we numerically simulated signal secretion from a localized source, diffusion, and clearance (Gregor et al. 2007b, Kicheva et al. 2007, Yu et al. 2009) in a three-dimensional geometry appropriate for blastula embryos (see Supplementary Materials in the Appendix). Using the measured values for diffusivity and clearance, these simulations yielded distribution profiles similar to the experimentally determined protein distributions (Fig. A.28) and thus provided independent support for the validity of the experimental approaches.

Our results have two major implications. First, differential diffusivity underlies differences in activator/inhibitor range. The differences in range (Cyclops < Squint < Lefty1 < Lefty2) are reflected in the differences in effective diffusion coefficients (Cyclops < Squint < Lefty1 < Lefty2). There is a similar trend in half-lives, but the differences in diffusivity are much more pronounced than the differences in clearance. During embryogenesis, the sources of Nodal and Lefty overlap, but Nodal signaling is active near the source and is inhibited by Lefty farther away. Our results suggest that the lower mobility of Nodal allows its accumulation close to the site of secretion, whereas the high mobility of Lefty leads to rapid long-range dispersal and prevents accumulation near the source. Thus, the differential diffusivity of Nodal and Lefty signals serves as the biophysical basis for the spatially restricted induction of cell fates during embryogenesis.

Second, the previously described network topology of the Nodal/Lefty system and the biophysical properties of Nodals and Leftys measured here support the activator/inhibitor reaction-diffusion model of morphogenesis: A less diffusive activator (Nodal) induces both its own production and that of a more diffusive inhibitor (Lefty)...
(Kondo & Miura 2010, Meinhardt 2009). The Nodal/Lefty reaction-diffusion system is further constrained by pre-patterns and rapid cell fate specification; thus, the system results in graded pathway activation during mesendoderm induction and exclusive pathway activation on the left during left-right specification (see Supplementary Materials in the Appendix for detailed discussion). Mathematical models have postulated that the inhibitor in reaction-diffusion systems must have a higher diffusion coefficient than the activator. Several models suggest that clearance-normalized inhibitor and activator diffusion coefficients differ by a factor of at least 6, that is, $R = (D/k_I)_{\text{inhibitor}}/(D/k_I)_{\text{activator}} > 6$ (Granero et al. 1977, Kondo & Asal 1995, Nakamura et al. 2006, Sick et al. 2006, Yamaguchi et al. 2007). The average ratio of the normalized diffusivities of Leftys and Nodals measured here is $R \approx 14$, providing biophysical support for these modeling studies (see Supplementary Materials in the Appendix for comparison of reaction-diffusion systems). The different diffusivities in the Nodal/Lefty biological system have counterparts in chemical reaction-diffusion systems. For example, patterns can be generated in a starch-loaded gel by combining an activator (iodide) with an inhibitor (chlorite) in the presence of malonic acid (Lengyel & Epstein 1991). In this in vitro system, diffusion of the activator is hindered by binding to the starch matrix and is thought to result in a higher (factor of ~15) diffusivity of the inhibitor. These models and our measurements raise the possibility that differential binding interactions and a ratio of at least a factor of 5 to 15 of inhibitor and activator diffusivities might be a general feature of reaction-diffusion–based patterning.
CHAPTER 3
Measuring protein stability in living zebrafish embryos using Fluorescence Decay After Photoconversion (FDAP)

PREFACE

This chapter is currently in press at the Journal of Visualized Experiments. Here I present a detailed explanation of the Fluorescence Decay After Photoconversion technique developed to measure the half-lives of Nodal and Lefty (see Chapter 2). I developed the zebrafish FDAP technique and wrote the protocol with input from Patrick Müller. The PyFDAP software used to fit FDAP data was written by Alexander Bläßle with input from Patrick Müller. The FDAP technique was developed in the lab of Alexander F. Schier.

ABSTRACT

Protein stability influences many aspects of biology, and measuring the clearance kinetics of proteins can provide important insights into biological systems. In FDAP experiments, the clearance of proteins within living organisms can be measured. A protein of interest is tagged with a photoconvertible fluorescent protein, expressed in vivo and photoconverted, and the decrease in the photoconverted signal over time is monitored. The data is then fitted with an appropriate clearance model to determine the protein half-life. Importantly, the clearance kinetics of protein populations in different compartments of the organism can be examined separately by applying compartmental masks. This approach has been used to determine the intra- and extracellular half-lives of secreted signaling proteins during zebrafish development. Here, we describe a protocol for FDAP experiments in zebrafish embryos. It should be possible to use FDAP to
determine the clearance kinetics of any taggable protein in any optically accessible organism.

INTRODUCTION

The levels of proteins in cells and organisms are determined by their rates of production and clearance. Protein half-lives can range from minutes to days (Belle et al. 2006, Boisvert et al. 2012, Eden et al. 2011, Schwanhäusser et al. 2011). In many biological systems, the stabilization or clearance of key proteins has important effects on cellular activity. Modulation of intracellular protein stability is required for cell cycle progression (Holloway et al. 1993, Parry & O'Farrell 2001), developmental signaling (Chen et al. 2011, Dharmasiri & Estelle 2004, MacDonald et al. 2009), apoptosis (Elmore 2007), and normal function and maintenance of neurons (Rubinsztein 2006, Yi & Ehlers 2007). Extracellular protein stability affects the distribution and availability of secreted proteins, such as morphogens (Eldar et al. 2003, Müller & Schier 2011), within a tissue.

Over the last few decades, protein stability has mainly been assessed in cell culture using radioactive pulse-labeling or cycloheximide chase experiments (Zhou 2004). In such pulse-chase experiments, cells are either transiently exposed to a “pulse” of radioactive amino acid precursors that are incorporated into newly synthesized proteins, or they are exposed to cycloheximide, which inhibits protein synthesis. Cultured cells are then collected at different time points, and either immunoprecipitation followed by autoradiography (in radioactive pulse-chase experiments) or western blotting (in cycloheximide experiments) is used to quantify the clearance of protein over time.
Conventional protein stability assays have several shortcomings. First, proteins in these assays are often not expressed in their endogenous environments, but rather in tissue culture and sometimes in cells from different species. For proteins whose stability is context-dependent, this approach is problematic. Second, it is not possible to follow protein clearance in individual cells or organisms over time, and the data from these assays reflects an average of different populations of cells at different time points. Since individual cells may have started with different amounts of protein, may have taken up the radioactive label or cycloheximide at different times, or may have different clearance kinetics, such aggregate data could be misleading. Finally, in the case of cycloheximide chase experiments, addition of the protein synthesis inhibitor may have unintended physiological effects that could artificially alter protein stability (Kenney 1967, Schimke & Doyle 1970, Woodside 1976). These shortcomings can be avoided by using Fluorescence Decay After Photoconversion (FDAP), a technique that utilizes photoconvertible proteins to measure protein clearance dynamically in living organisms (Drocco et al. 2011, Kiuchi et al. 2011, Miyawaki 2011, Müller et al. 2012, Pantazis & Supatto 2014, Plachta et al. 2011, Zhang et al. 2007) (see Discussion for limitations of the FDAP technique).

Photoconvertible proteins are fluorescent proteins whose excitation and emission properties change after exposure to specific wavelengths of light (Lukyanov et al. 2005). One commonly used variant is Dendra2, a “green-to-red” photoconvertible protein that initially has excitation and emission properties similar to green fluorescent proteins, but after exposure to UV light—“photoconversion”—its excitation/emission properties become similar to those of red fluorescent proteins (Gurskaya et al. 2006, Zhang et al.
Importantly, new protein produced after photoconversion will not have the same excitation/emission properties as the photoconverted protein, allowing decoupling of production and clearance upon photoconversion and observation of only a pool of photoconverted protein. Tagging proteins of interest with photoconvertible proteins thus provides a convenient way to pulse-label proteins in intact, optically accessible living organisms.

In FDAP assays (Fig. 3.1A), proteins of interest are tagged with a photoconvertible protein and expressed in living organisms (Fig. 3.1B). The fusion protein is photoconverted, and the decrease in photoconverted signal over time is monitored by fluorescence microscopy (Fig. 3.1C). The data is then fitted with an appropriate model to determine the half-life of the fusion protein (Fig. 3.1D).

The FDAP assay described here was designed to determine the extracellular half-lives of secreted signaling proteins in zebrafish embryos during early embryogenesis (Müller et al. 2012). However, this approach can be adapted to any transparent model organism that tolerates live imaging, and could be used to monitor the clearance of any taggable intracellular or extracellular protein. Variations of the technique described here have been performed in cultured cells (Kiuchi et al. 2011, Zhang et al. 2007) and *Drosophila* (Drocco et al. 2011) and mouse (Plachta et al. 2011) embryos.
Figure 3.1: Fluorescence Decay After Photoconversion (FDAP) overview. A) Workflow of an FDAP experiment. B) Injection of mRNA and a fluorescent dye into a zebrafish embryo at the one-cell stage. Protein is produced from the mRNA as the embryo develops over about five hours prior to imaging. The dye labels cells (green circles). C) The fusion protein is photoconverted using a UV pulse, and the decrease in the intensity of the photoconverted signal over time is monitored. D) The data is fitted with an exponentially decreasing function to obtain clearance rate constants ($k$) and half-lives ($\tau$).

PROTOCOL

1. Generating a photoconvertible fusion construct and injecting dechorionated zebrafish embryos

1.1) Generate a functional construct containing the protein of interest fused to a green-to-red photoconvertible protein (see Discussion), then use in vitro transcription to generate capped mRNA encoding the fusion protein as in Müller et al., 2012.

1.2) Use pronase to remove the chorions from about 30 zebrafish embryos at the one-cell stage. Alternatively, manually dechorionate embryos using forceps (Zou & Wei 2010).
Note: Embryos must be dechorionated for subsequent imaging. If desired, embryos can be injected through the chorion and dechorionated later, just prior to imaging.

1.2.1) Make a 5 mg/ml stock solution of pronase from *Streptomyces griseus* in standard zebrafish embryo medium (Müller et al. 2012). Rock the solution gently at room temperature for 10 min to allow the protease to dissolve. Aliquot 2 ml into microcentrifuge tubes and freeze at -20 °C.

1.2.2) Transfer one-cell stage embryos to a 5 cm diameter glass or agarose-coated plastic petri dish containing ~8 ml embryo medium. Add 2 ml of thawed pronase stock solution to the dish and incubate at room temperature for 5 to 10 min.

1.2.3) Avoid exposing embryos to air or plastic, as contact with either will cause dechorionated embryos to rupture. Fill a 200 ml glass beaker with embryo medium. Transfer the embryos to the beaker by tilting the petri dish while submerging it in the medium.

1.2.4) After the embryos have settled to the bottom of the beaker, pour out most of the embryo medium, then pour fresh embryo medium into the beaker. The mild swirling of the medium pouring into the beaker causes embryos to lose their weakened chorions.

1.2.5) Repeat step 1.2.4.

1.3) Transfer the dechorionated embryos to an agarose-coated injection dish (Yuan & Sun 2009) using a glass Pasteur pipette with a flamed tip. Flaming the pipette tip prevents jagged edges from injuring embryos.

1.4) Co-inject the mRNA and a 3 kDa Alexa488-dextran conjugate (Rosen et al. 2009, Yuan & Sun 2009) (Fig. 3.1B; see Discussion for suggested mRNA and Alexa488-
Inject directly into the center of the cell (not the yolk) to ensure even distribution of mRNA and fluorescent dye once cleavage commences. Note: The Alexa488 signal will be used during data analysis to generate compartmental masks in order to distinguish between intracellular and extracellular fluorescence.

1.5) Transfer injected embryos to a 1 - 2% agarose-coated well of a six-well plastic dish filled with embryo medium. Incubate in the dark at 28 °C until embryos have reached late sphere stage (Kimmel et al. 1995) (approximately five hours post fertilization). Check embryos every one to two hours under a stereomicroscope and remove any debris generated by embryos that have died.

2. Mounting zebrafish embryos for photoconversion and imaging on an inverted confocal microscope

2.1) Use a stereomicroscope to identify one to five healthy embryos, and use a glass Pasteur pipette with a flamed tip to remove them from the dish.

2.2) Gently eject the embryos into a microcentrifuge tube containing ~1 ml of melted 1% low melting point agarose in 1x Danieau’s embryo medium (Fig. 3.2A). Note: Agarose should have a temperature between 40 and 42 °C; higher temperatures could damage the embryos.

2.3) Draw the embryos back into the pipette along with some agarose. Gently eject the agarose and embryos onto the cover glass of a glass-bottom dish (Fig. 3.2B). Ensure that the thickness of the cover glass is compatible with the objective on the confocal microscope.
2.3.1) Re-use the glass pipette if desired. To clean the residual agarose out of the pipette and prevent clogging, quickly pipette embryo medium up and down. Place a 15 ml tube filled with ~5 ml of embryo medium next to the stereomicroscope for this purpose.

2.4) Use a metal probe to position the embryos so that the animal pole (blastoderm) faces the cover glass. Work quickly since the agarose will solidify in 20 - 30 s. Use the stereomicroscope to monitor the embryos’ positions and readjust as necessary until the agarose hardens.

2.5) Repeat steps 2.1 – 2.4 until the desired number of embryos has been mounted. Note: In a typical experiment, four agarose drops containing four or five embryos each will fit easily on the cover glass. About 16 embryos can be imaged during a single ideal experiment (Fig. 3.2C).

2.6) When the agarose has solidified, fill the glass-bottom dish with 1x Danieau’s embryo medium.

Figure 3.2: Mounting zebrafish embryos for FDAP experiments. A) Zebrafish embryos (blastoderm = white, yolk = black) are transferred from embryo medium (blue) into melted agarose (yellow). B) Embryos and agarose are placed onto the cover glass of a glass-bottom dish. Embryos are then manually positioned so that the animal pole faces the cover glass. A cross-section of a glass-bottom dish is shown. C) Schematic overview of a glass-bottom dish with several agarose drops containing four embryos each (view looking down into the dish).
3. **Photoconverting and measuring the decrease of the photoconverted signal**

Note: A 25x or 40x water objective is appropriate for the size and refractive index of zebrafish embryos. It is best to use immersion oil with the same refractive index as water rather than actual water, since water will evaporate during the course of the five-hour experiment. Ensure that the immersion oil is designed to be used with a water (not oil) objective.

3.1) Place a large drop of immersion oil on the objective to ensure that the oil film between the objective and cover glass will not break as the stage moves to different embryo positions during imaging. Securely place the glass-bottom dish onto the stage so that the dish will not shift when the stage moves. If possible, use a heated stage at 28 °C, the optimal temperature for zebrafish development.

3.2) Define each embryo’s position in the confocal microscope’s software package. Adjust the z-depth for each embryo, and attempt to target roughly the same plane in each embryo. Note: About 30 µm from the animal pole is a good depth since at this depth the enveloping layer of the embryo can be avoided, imaging area is maximized, and light scattering is minimal. A single optical slice with a thickness of ~3.3 µm provides sufficient data; there is no need to acquire a z-stack (see Section 5).

3.3) Collect two signals during the experiment: the “green” signal from the Alexa488-dextran conjugate—which will be used during data analysis to isolate extracellular and intracellular fluorescence—and the “red” signal from the fusion protein after it is photoconverted.

3.3.1) Excite Alexa488 using a 488 nm laser, and collect emitted fluorescence between ~500 and 540 nm. Note: After photoconversion, many green-to-red
photoconvertible proteins (e.g., Dendra2) can be excited with a 543 nm laser and emit fluorescence between ~550 and 650 nm. Adjust as necessary based on the photoconvertible protein used.

3.4) Acquire “pre-photoconversion” images, and configure the confocal microscope’s software to image each of the previously defined positions (from step 3.2) with the appropriate imaging conditions every 10 or 20 min for a five-hour time course (see Section 5 and Discussion).

3.5) To photoconvert the fusion protein, switch to a 10x objective and expose groups of embryos to UV light from a mercury arc lamp with a ~300-400 nm excitation filter at 100% output for 2 min. Shift the focus along the z-axis to promote uniform photoconversion (see Section 5). Ensure that the immersion oil does not drip onto the 10x objective during photoconversion. Note: The shifting of focus during photoconversion could be automated in order to avoid variability among experimenters.

3.6) Switch back to the 25x or 40x objective immediately after photoconversion. Ensure that the previously defined positions from step 3.2 are still accurate. If the dish shifted during photoconversion, re-define the positions of the embryos.

   3.6.1) Start the program created in step 3.4 and allow imaging to continue for five hours. Note the time elapsed between photoconversion and the start of imaging for each embryo.

3.7) Occasionally check on the experiment. Monitor the level of Danieau’s medium and add more if necessary. Restart the software if it has stalled.

3.8) In order to determine the background fluorescence values that will be used during data analysis to estimate the asymptote of an exponentially decreasing model, include
some embryos that have been injected with Alexa488-dextran but not mRNA in the experiment. To determine the instrument noise, which will also be used during subsequent data analysis, acquire an image in the absence of a sample.

4. Analyzing the data using PyFDAP

4.1) Visually inspect the time course data sets from each embryo. Discard data sets from embryos that died during imaging, that shifted significantly, that have very low levels of photoconverted signal, or that contain regions of cells that look unusual and have stopped moving and dividing (typical of injured or sick embryos). Note: Occasionally, bubbles in the immersion oil or other artifacts will appear in one or two images in an otherwise usable data set. Note any images that contain artifacts; they will be discarded later, and the remaining time points from such a data set can still be analyzed.

4.2) Use the Python-based software package PyFDAP to analyze the FDAP data. PyFDAP calculates half-lives by determining the average intracellular and extracellular red fluorescence intensity in each image and fitting the data with an exponentially decreasing function (Fig. 3.3).

4.2.1) Download PyFDAP from the following site:

http://people.tuebingen.mpg.de/mueller-lab

4.2.2) Use PyFDAP to separate intracellular and extracellular photoconverted signal (Fig. 3.3A,B). Use the Alexa488 signal, which is strictly intracellular, to create an intracellular mask. Apply this mask to the corresponding red channel image to prevent intracellular pixels from being considered when calculating
average extracellular intensity. To measure average intracellular intensity, invert the mask.

4.2.3) In PyFDAP, display the masked images generated in step 4.2.2. Visually inspect these images and discard data sets in which masks do not accurately distinguish intracellular from extracellular space (this should be rare; note that cell membranes are included in images in which extracellular space has been masked, but they could be removed by altering the thresholding algorithm or by introducing a membrane mask (e.g., using membrane-CFP)). Also discard any single images containing artifacts (e.g., bubbles in the immersion oil) identified in step 4.1.

4.2.4) Use PyFDAP to calculate average extracellular and intracellular fluorescence intensities for each image. PyFDAP calculates these averages by summing the intensities of pixels that fall outside of the mask and dividing by the total number of pixels summed.

4.2.5) Fit the fluorescence data (Fig. 3.3) with the following exponential function:

\[ c(t) = c_0 e^{-kt} + y_0 \]

where \( t \) is time post-photoconversion, \( c(t) \) is intensity at a given value of \( t \), \( c_0 \) is the intensity at \( t = 0 \), \( k \) is the clearance rate constant, and \( y_0 \) is the asymptote that the function approaches as fluorescence decreases (Fig. 3.1D). \( y_0 \) can be constrained based on the measurements in step 3.8 (Müller et al. 2012).

4.2.6) Use PyFDAP to calculate the extracellular and intracellular protein half-lives (\( \tau \)) from the clearance rate constants (\( k \)) using the following relationship:
Half-life $\tau = \frac{\ln(2)}{k}$

Figure 3.3: Data analysis using PyFDAP. A) PyFDAP uses the Otsu thresholding algorithm (Otsu 1979) to generate intra- and extracellular masks from the intracellular Alexa488 signal (green). B) Photoconverted signal (red) from an embryo expressing a secreted Dendra2 fusion protein (Squint-Dendra2 (Müller et al. 2012)). Average extra- and intracellular fluorescence intensities were calculated using the masks shown in (A). The space outside of the embryo was excluded from these calculations by discarding pixels outside of the yellow circle. C) PyFDAP screenshot displaying extracellular intensity data from an FDAP experiment (black circles) fitted with an exponentially decreasing function (red dashed line). The extracellular half-life is indicated by the red arrow.
5. Control experiments to assess photobleaching, inadvertent photoconversion, and photoconversion uniformity

5.1) Assessing photobleaching. Note: Photobleaching could cause an artifactual decrease in fluorescence intensity that reflects the bleaching properties of the fluorescent protein in addition to the clearance of the protein of interest.

5.1.1) To assess possible photobleaching, perform one set of FDAP experiments with 10 min intervals between imaging and a second set with 20 min intervals between imaging (Fig. 3.4). Analyze the data from both sets of experiments using PyFDAP as described in Section 4.

5.1.2) Compare the half-lives from the 10 and 20 min interval experiments. Longer half-lives from 20 min interval experiments indicate significant photobleaching. If the half-lives from both experiments are identical, photobleaching is not a significant concern.

5.1.3) Alternatively, assess photobleaching by acquiring a series of ~30 images immediately after photoconversion. A significant decrease in fluorescence intensity indicates significant photobleaching.

5.1.4) If photobleaching is detected, use lower laser power, decrease imaging time, or consider using a more photostable photoconvertible protein (McKinney et al. 2009).
Figure 3.4: Representative FDAP results. A) A representative embryo expressing secreted Squint-Dendra2 just prior to photoconversion (far left) and 27, 87, and 287 min post-photoconversion. B) To control for photobleaching and inadvertent photoconversion (see Section 5), experiments with 10 or 20 min intervals were performed (data from (Müller et al. 2012)). In PyFDAP, extracellular intensity profiles were generated, fitted with exponentially decreasing functions, and normalized by subtracting the fitted $y_0$ value from each data point and dividing by the fitted $c_0$ value. Data from the 10 min (black, $n = 11$) and 20 min (blue, $n = 12$) interval experiments were then averaged, respectively. Error bars indicate standard deviation.

5.2) Assessing inadvertent photoconversion. Note: Dendra2 can be photoconverted using 488 nm illumination (Gurskaya et al. 2006). When exciting Alexa488 with the 488 nm laser as described in step 3.3.1, inadvertent photoconversion and therefore an artifactual increase in the apparent half-life of the protein of interest is possible. However, we and others (Dempsey et al. 2014) have found that 488 nm illumination is an inefficient method of photoconversion in zebrafish embryos.
5.2.1) Use the control experiment described in step 5.1.1 to detect inadvertent photoconversion. Compare the half-lives from the 10 and 20 min interval experiments. Shorter half-lives from 20 min interval experiments indicate significant inadvertent photoconversion. If the half-lives from both experiments are identical, inadvertent photoconversion is not a significant concern.

5.2.2) If inadvertent photoconversion is detected, use a lower 488 nm laser power and shorter imaging times to avoid inadvertently photoconverting Dendra2.

5.3) Assessing photoconversion uniformity. Note: If photoconversion is biased toward the animal pole of the embryo, the decrease in fluorescence will be influenced by protein diffusion or cell movement into deeper planes (Fig. 3.5A).

5.3.1) To determine whether photoconversion is uniform, express a secreted photoconvertible protein (for experiments with extracellular fusion proteins) or a cytoplasmic photoconvertible protein (for experiments with intracellular fusion proteins). Photoconvert as usual, then acquire a z-stack encompassing most of the blastoderm every 20 min for 80 min.

5.3.2) If photoconversion is biased toward the animal pole, the fluorescence intensity in deeper planes will increase over time due to diffusion or cell movement (Fig. 3.5B). If non-uniform photoconversion is detected, focus deeper into the embryos during photoconversion.
**Figure 3.5: Assessing photoconversion uniformity.** A) Non-uniform photoconversion of an extracellular protein can lead to an erroneously short apparent half-life if photoconverted protein diffuses into deeper planes over time. B) To determine whether photoconversion is uniform, a z-stack covering most of the blastoderm is acquired at several time points post-photoconversion. Fluorescence intensity will increase in deeper planes over time if photoconversion was non-uniform (note that light scattering causes deeper planes to appear dimmer than higher planes).

**REPRESENTATIVE RESULTS**

FDAP has been used to determine the half-lives of extracellular signaling proteins in zebrafish embryos (Müller et al. 2012). One of these proteins, Squint, induces expression of mesendodermal genes during embryogenesis (Schier 2009). Squint-Dendra2 activates expression of mesendodermal genes at levels similar to untagged Squint, as demonstrated by qRT-PCR and *in situ* hybridization assays (Müller et al. 2012). Embryos were co-injected with Alexa488-dextran and mRNA encoding Squint-Dendra2 and subjected to the FDAP assay. A decrease in the extracellular photoconverted signal intensity over time is evident (Fig. 3.4A). Extracellular intensity
profiles from 23 embryos were generated using PyFDAP. The resulting data was fitted in PyFDAP with a first-order clearance kinetics model, and an average clearance rate constant $k$ of $1.00 \times 10^{-4}/s$, corresponding to an average half-life $\tau$ of 116 min, was determined. Similar intensity profiles and clearance rate constants were obtained when the intervals between imaging were 10 or 20 min, suggesting that photobleaching or inadvertent photoconversion did not contribute significantly to intensity changes (Fig. 3.4B).

DISCUSSION

The success of an FDAP experiment relies on the generation of a functional photoconvertible fusion protein. Tagging a protein can affect its biological activity and/or biophysical properties, including its localization, solubility, and stability (Landgraf et al. 2012, Morimoto et al. 2011, Pédelacq et al. 2005, Quattrocchio et al. 2013, Stadler et al. 2013, Swulius & Jensen 2012). Be prepared to test the activity of several different fusion constructs in order to find one that is active. We have found that changing the position of the photoconvertible protein relative to the protein of interest or using longer linkers (e.g., using the amino acid sequence LGDPPVAT (Müller et al. 2012)) can enhance the activity of the fusion protein. In the case of signaling proteins, the activity of the fusion protein can be determined by testing its ability to induce expression of target genes (Müller et al. 2012). qRT-PCR or in situ hybridization provide good readouts of target gene expression (Müller et al. 2012). Note that the protocol described here is designed to determine the stability of proteins in the early zebrafish embryo and would require modification to assess protein stability in other contexts.
The green-to-red photoconvertible protein Dendra2 (Gurskaya et al. 2006) has been used successfully in zebrafish FDAP experiments (Müller et al. 2012) (Fig. 3.4), but other options are available (Lukyanov et al. 2005, Shaner et al. 2005). To avoid potential artifacts due to aggregation of the fusion protein, choose a monomeric photoconvertible protein. Photoactivatable proteins can also be used in FDAP assays (Drocco et al. 2011, Kiuchi et al. 2011, Lukyanov et al. 2005, Plachta et al. 2011).

Before performing an FDAP experiment, several aspects of the protocol need to be optimized. Inject different amounts of mRNA to determine the lowest amount that provides useable signal after photoconversion; ~50 pg mRNA is a good starting amount. In order to generate meaningful compartmental masks (Fig. 3.3), the Alexa488 signal must be bright enough to compete against the signal from the non-photoconverted fusion protein that is constantly produced from the injected mRNA; inject between 0.2 and 4 ng of Alexa488-dextran to find the optimal amount of fluorescent dye. Find the optimal post-conversion imaging conditions and use the same conditions for all experiments with a given construct. Use good quantitative imaging practices (Waters 2009), and choose an appropriate dynamic range to avoid saturated pixels in the red channel. Determine the appropriate imaging interval for each fusion protein. Proteins with very short half-lives may require more frequent imaging over a shorter total time period. Establish the optimal photoconversion technique based on the organism and photoconvertible protein used. We describe one robust photoconversion method using a mercury arc lamp in step 3.5, but Dendra2 can also be photoconverted with a 405 (Dempsey et al. 2014) or 488 nm laser (Gurskaya et al. 2006).
One limitation of this FDAP protocol is that overexpression of the protein of interest is required. Overexpression could affect protein stability, for instance, through abnormal expression of other genes that modify the protein’s clearance kinetics (Eldar et al. 2003, Moll & Petrenko 2003). If the protein of interest is a signaling molecule, consider performing experiments in the presence of a signaling inhibitor to determine whether blocking expression of target genes affects protein stability. In the future, it may be possible to generate transgenic embryos expressing photoconvertible fusions under the control of endogenous expression elements (Auer et al. 2014, Bedell et al. 2014, Hruscha et al. 2013-b; Zu et al. 2013). If transgenic embryos produce sufficient signal, FDAP experiments in a non-overexpression context are conceivable, perhaps using light-sheet microscopy to observe fluorescence decrease in the entire embryo (Keller et al. 2008).

Possible further applications of FDAP include investigating the mechanisms that regulate protein stability by examining the effects of different perturbations (e.g., expression of putative proteases) or protein modifications (e.g., phosphorylation) on stability. For example, the factors controlling the extracellular stability of Squint are currently unknown. Many secreted developmental signals are internalized by cells (Blanchet et al. 2008b, Incardona et al. 2000, Jullien 2005, Scholpp & Brand 2004), which could contribute to the clearance of Squint and other ligands from the extracellular space. FDAP experiments in which internalization is blocked might provide information about mechanisms controlling extracellular protein clearance.

In contrast to conventional assays for measuring protein stability (Zhou 2004), FDAP offers a microscopy-based alternative in which the clearance of proteins can be monitored over time within living organisms. Similar methods have been used to monitor
protein clearance in model systems other than zebrafish embryos (Drocco et al. 2011, Kiuchi et al. 2011, Plachta et al. 2011, Zhang et al. 2007). This FDAP protocol has the potential to be adapted to determine the half-life of any taggable protein in biological systems where live imaging is feasible.
CHAPTER 4
Factors affecting Nodal and Lefty diffusivity and localization

PREFACE

I designed, performed, and interpreted the following experiments with input from Patrick Müller and Alexander F. Schier. The FGF8-GFP/heparin data discussed in this chapter were published in Development on April 15th, 2013 (Müller et al. 2013).

SUMMARY

The distinct activity ranges of Nodal and Lefty are likely caused by their different diffusivities. I used Fluorescence Recovery After Photobleaching (FRAP) together with a candidate-based approach to search for regulators of Nodal and Lefty mobility. I found that interactions with receptor complexes and heparan sulfate proteoglycans (HSPGs) are unlikely to fully account for the much lower diffusivity of Nodal compared to Lefty, although interactions with receptor complexes can affect Nodal localization and diffusivity, and Nodal can alter the distribution of HSPGs at the membrane. Finally, I found that Lefty facilitates the diffusion of Nodal, and provide evidence that Nodal and Lefty interact in the extracellular space. My results implicate receptor complexes and HSPGs in the regulation of Nodal and Lefty localization, but suggest that other factors must account for the low diffusion coefficient of Nodal.

INTRODUCTION

The activity ranges of Nodal and Lefty determine the spatial extent of mesendoderm induction during embryogenesis, and therefore influence germ layer
patterning. We found that differences in diffusivity likely account for the different activity ranges of Nodal and Lefty (see Chapter 2 (Müller et al. 2012)); however, the mechanisms that give rise to their different diffusivities remain unknown. Our results suggest the existence of extracellular factors that affect the diffusivity of Nodal and Lefty. Such “diffusion regulators” might affect mobility by transiently binding to Nodal or Lefty, hindering their movement through the extracellular space of the embryo (Müller et al. 2013).

In other patterning systems, interactions with receptors and heparan sulfate proteoglycans (HSPGs) have been suggested or demonstrated to affect the mobility and stability of ligands (reviewed in Rogers & Schier 2011, see Chapter 1), but it is unclear whether these or other factors affect Nodal or Lefty mobility. I used confocal microscopy and our zebrafish FRAP assay to determine whether manipulating levels of candidate diffusion regulators influences Nodal or Lefty diffusivity, localization, or distribution. Although I was unable to conclusively identify an individual diffusion regulator that explains the lower diffusivity of Nodal compared to Lefty, I uncovered several factors that influence the diffusivity and localization of Nodal and Lefty, and may therefore have roles in regulating their activity ranges or other aspects of their biology. In addition, I found that Nodal and Lefty affect each other’s localization and diffusion, and obtained evidence that Nodal and Lefty may directly bind in the extracellular space.
RESULTS AND DISCUSSION

*Nodal coreceptor*

An obvious diffusion regulator candidate is the Nodal receptor complex. Receptors have been proposed to regulate the spreading of other developmentally important ligands, such as Shh and Dpp. In the case of Shh, upregulation of the Shh receptor Ptc by Shh signaling is thought to sequester Shh and restrict its signaling range to the ventral region of the neural tube (Chen & Struhl 1996). In contrast, downregulation of the Dpp receptor Tkv by Dpp signaling is thought to promote spreading of Dpp to more distant regions of the wing disc (Lecuit & Cohen 1998).

Nodal binds to a receptor complex consisting of type I and II Activin receptors and the EGF-CFC coreceptor One-eyed pinhead (Oep) (reviewed in Schier 2009 and Chapter 1). In the absence of Oep, Nodal does not bind to the receptor complex and Nodal signaling is not transduced (Cheng et al. 2004, Reissmann et al. 2001, Yeo & Whitman 2001). If interactions with receptors regulate Nodal diffusion, then the diffusion coefficient of Nodal-GFP might be expected to increase in the absence of *oep*, and decrease when *oep* is overexpressed.

Consistent with the latter prediction, the diffusivity of Squint-GFP decreases ~5-fold when *oep* is overexpressed, and *oep* overexpression also causes a dramatic change in the localization of Squint-GFP: Squint-GFP is typically found in membrane-associated clusters as well as diffusely throughout the extracellular space, but when *oep* is overexpressed, only membrane-associated Squint-GFP clusters are visible, similar to the punctate localization of Cyclops-GFP (Fig. 4.1, 4.2).
Figure 4.1: Factors affecting Nodal/Lefty-GFP localization. Localization of GFP-tagged Nodals and Leftys (green) in zebrafish embryos at 5-6 hpf. Membrane-bound RFP (red) indicates cell membranes. Note clustered, membrane-associated distribution of Cyclops-GFP (A). oep overexpression induces similar clustering of Squint-GFP (F). Extracellular heparin injection and lefty overexpression increase the diffuse extracellular Cyclops-GFP signal (I,M). cyclops overexpression induces clustering of Squint- (R) and Lefty-GFP (S,T).
Figure 4.2: Coreceptor levels affect Nodal/Lefty-GFP diffusivity. Nodal- and Lefty-GFP diffusion coefficients measured in zebrafish embryos using FRAP as in Müller et al. 2012. MZoep = maternal-zygotic oep mutants. oep gapmer = embryos injected with an antisense oligonucleotide targeting oep transcripts demonstrated to efficiently knock down oep (data not shown). Oep mRNA = wild-type embryos injected with 50 pg oep mRNA. Error bars represent standard error, N is between 2 and 13 embryos for each condition.

Interestingly, oep overexpression does not obviously affect the development of wild-type embryos (Zhang et al. 1998), suggesting that relatively immobile, presumably short-range membrane-associated Squint can still mediate normal patterning. This is consistent with the observation that squint homozygous mutants are often viable (Pei et al. 2007): squint mutants lack medium-range Squint activity and rely on short-range signaling by Cyclops as their only source of Nodal during germ layer patterning. The mobility of Lefty2-GFP also decreases when oep is overexpressed (Fig. 4.2), although its
localization is unaffected (Fig. 4.1). In contrast, the diffusion coefficients of Cyclops-GFP and Lefty1-GFP are unaffected by \textit{oep} overexpression (Fig. 4.2).

If interactions with receptor complexes are responsible for the lower diffusivity of Nodal-GFP compared to Lefty-GFP, then in the absence of \textit{oep}, Nodal-GFP diffusivity should increase. I performed Nodal- and Lefty-GFP FRAP experiments in maternal-zygotic \textit{oep} mutant embryos (MZ\textit{oep}) or embryos injected with \textit{oep} antisense oligonucleotides, which roughly phenocopy MZ\textit{oep}. Squint-GFP was inefficiently secreted in MZ\textit{oep} mutants, but was secreted normally in embryos injected with \textit{oep} antisense oligos. There are indications that the mouse ortholog of Oep, Cripto, associates with Nodal during its secretion (Blanchet et al. 2008a), but it is unclear whether this is related to the inefficient secretion of Squint-GFP in MZ\textit{oep} mutants.

Surprisingly, the diffusivity of Cyclops-GFP did not change in the \textit{oep} knockdown scenarios, and the diffusion coefficients of Squint- and Lefty-GFP decreased or remained the same as in wild-type embryos (Fig. 4.2). The localizations of Nodal- and Lefty-GFP were also similar in wild-type embryos and embryos lacking \textit{oep}, although Cyclops-GFP puncta tended to be more clustered in MZ\textit{oep} mutants (data not shown). Although overexpression of \textit{oep} causes Squint-GFP to cluster similarly to Cyclops-GFP (Fig. 4.1), Oep does not appear to be required for clustering of Cyclops- or Squint-GFP. Thus, although Oep \textit{can} affect the diffusivity of Nodal-GFP, interactions with Oep/receptor complexes are not sufficient to explain the lower diffusion coefficient of Nodal compared to Lefty.

The inability of \textit{oep} loss to increase Nodal diffusivity was surprising, given that Oep is known to mediate binding between Nodal and its receptor complex. Diffusion
regulators are predicted to have three features: first, diffusion regulators should be extracellular, second, they should be membrane-bound or otherwise immobilized, and third, they should interact transiently with Nodal. Consistent with the first and second predictions, Oep has a signal sequence, is membrane-associated (Zhang et al. 1998), and EGF-CFC family members contain sites for GPI anchor attachment (Minchiotti et al. 2000). However, it is possible that interactions between Nodal and receptor complexes are not transient but are effectively irreversible. For example, Nodal could bind to its receptor complex and subsequently be internalized and degraded. In this case, interactions with receptor complexes would lead to Nodal clearance (see Chapter 3) and would affect Nodal’s activity range, but would not affect its effective diffusivity.

\textit{oep} is a direct target of Nodal signaling (Dubrulle et al., submitted). I showed that \textit{oep} overexpression causes clustering of Squint-GFP and reduces its diffusivity about 5-fold (Fig. 4.1, 4.2). An intriguing possibility is that upregulation of \textit{oep} expression by Nodal signaling could serve to both sensitize cells to Nodal signaling and to restrict the activity range of Nodal. This idea could be explored by characterizing in detail MZoep embryos that have been rescued by overexpression of \textit{oep} mRNA (Zhang et al. 1998); in this scenario, \textit{oep} expression and Nodal signaling are decoupled, which may have previously unappreciated developmental consequences.

\textit{Heparan sulfate proteoglycans}

Heparan sulfate proteoglycans (HSPGs) are ubiquitous in the extracellular environment and are composed of sulfated glycosaminoglycan chains attached to a protein core that is often anchored to the cell membrane. Many developmental signals
interact with HSPGs, including Dpp, Wnt, Hh, FGF, and Nodal (reviewed in Rogers & Schier 2011, see Chapter 1). HSPGs provide several important functions in ligand-based patterning systems: they promote signal-receptor interactions by concentrating ligand at the cell surface, prevent ligand loss, act as coreceptors, affect signal stability and internalization, modulate cells’ abilities to sense signaling, and regulate spreading of secreted signals. In fact, crystal structures demonstrate that HSPGs are involved in the formation of the FGF-receptor complex (Schlessinger et al. 2000), and single molecule tracking has shown that FGF2 spends most of its time transiently bound to HSPG-dependent clusters (Duchesne et al. 2012). HSPGs are therefore important regulators of morphogen signaling and spreading, and another obvious candidate Nodal diffusion regulator.

knypek encodes a zebrafish HSPG core protein (Topczewski et al. 2001). Knypek-GFP fusions localize to the cell membrane when expressed in zebrafish embryos as expected (Fig. 4.3). Interestingly, overexpression of cyclops induces the formation of membrane-associated Knypek-GFP clusters, similar to Cyclops-GFP and FGF8-GFP (Yu et al. 2009) clusters. In contrast, overexpression of cyclops did not induce clustering of membrane-bound GFP (Fig. 4.3). Knypek-RFP fusions were not visible when expressed alone, but when co-expressed with Cyclops-GFP, Knypek-RFP formed bright, membrane-associated clusters that co-localized with Cyclops-GFP clusters (Fig. 4.3). Cyclops can therefore induce clustering of an HSPG core protein, suggesting that HSPGs and Cyclops may interact, and that this interaction may be involved in the formation of Cyclops clusters as is the case for FGF clustering (formation of Cyclops-GFP clusters apparently does not require interactions with the receptor complex, see above). Though
Cyclops and many other important ligands form membrane-associated clusters, the biological relevance of clustering and the mechanisms behind clustering are largely unknown (see Chapter 6).

**Figure 4.3: Cyclops induces HSPG clustering.** (A-D) Cyclops overexpression induces clustering of Knypek-GFP but not membrane-bound GFP in zebrafish embryos. Knypek-GFP is localized to cell membranes (A), similar to membrane-bound GFP (B). Knypek-GFP forms membrane-associated clusters when *cyclops* is overexpressed (C); in contrast, *cyclops* overexpression does not affect the localization of membrane-bound GFP (D). (E-L) Knypek-RFP clusters co-localize with Cyclops-GFP clusters. Cyclops-GFP forms clusters visible in the green channel (E) but not the red (I). In the absence of Cyclopsonduced clustering, Knypek-RFP is not visible (F,J). However, overexpression of untagged *cyclops* causes clustering of Knypek-RFP (G,K). Some, but not all, Cyclops-GFP clusters co-localize with Knypek-RFP clusters when these constructs are co-expressed (H,L). Arrowheads indicate co-localized clusters.

If HSPGs act as Nodal diffusion regulators, extracellular injection of heparin should increase the diffusion coefficient of Nodal-GFP. When exogenous heparin is introduced into the extracellular space, it appears to compete with endogenous, membrane-bound HSPGs for ligand binding, causing release of ligand from cell surfaces and enhancing ligand diffusivity. When extracellular exogenous heparin is introduced into zebrafish embryos expressing FGF8-GFP, I found that membrane-associated FGF8-GFP clusters dissolved and the GFP signal became diffuse in the extracellular space; further, the diffusion coefficient of FGF8-GFP increased ~6-fold (Müller et al. 2013).
Others have demonstrated that more FGF8-GFP becomes mobile when HSPG sugar chains are destroyed by extracellular injection of heparinase I (Yu et al. 2009). This indicates that HSPGs are regulators of FGF8 localization and diffusion in vivo (Müller et al. 2013).

To test whether HSPGs act as Nodal diffusion regulators as well, I injected heparin into the extracellular space of zebrafish embryos expressing Nodal-GFP. I observed a modest increase in the amount of diffuse extracellular Cyclops-GFP signal, and fewer Squint-GFP puncta in the presence of exogenous heparin (Fig. 4.1). I then performed FRAP experiments in embryos injected with extracellular heparin. I observed a modest increase (~2-fold) in Cyclops-GFP diffusivity and no significant change in Squint- and Lefty2-GFP diffusivity (Fig. 4.4). In contrast to what I observed for the FGF8-GFP fusion, extracellular heparin injection has a relatively mild effect on Nodal-GFP localization and diffusion. This suggests that the respective affinities of FGF and Nodal for HSPGs probably differ, and that, whereas FGF8-GFP clustering appears to be highly HSPG-dependent, clustering of Cyclops-GFP may be mediated by additional factors.
Finally, I sought to determine the effects of removing all endogenous heparan and chondroitin sulfate on Nodal-GFP diffusivity. If the sugar chains of HSPGs and/or CSPGs bind and inhibit the mobility of Nodal, then in the absence of these chains Nodal-GFP should have a higher diffusion coefficient. The zebrafish jekyll allele encodes a defective UDP-glucose dehydrogenase, required for synthesis of heparan sulfate (HS), chondroitin sulfate (CS), and hyaluronic acid (HA) (Stainier et al. 1996, Walsh 2001). I used germline transplantation (Ciruna et al. 2002) to generate maternal-zygotic jekyll
(MZjekyll) mutant embryos, in which HS, CS, and HA synthesis is defective (Superina et al. 2014). The localization of Nodal- and Lefty-GFP was similar in MZjekyll mutants and wild-type embryos, and the diffusion coefficient of Cyclops-GFP was similar in MZjekyll and wild-type embryos; the diffusion coefficient of Squint-GFP was slightly lower in MZjekyll embryos compared to wild-type embryos (Fig. 4.4). This indicates that interactions with GAG chains do not significantly hinder the diffusion of Nodal in zebrafish embryos.

Together, my results suggest that Nodal and HSPGs may interact in the extracellular environment, but HSPGs do not appear to be key regulators of Nodal or Lefty diffusion in early zebrafish embryos. However, interactions between Nodal and HSPGs may have other important functions during germ layer patterning (e.g., affecting Nodal stability or interactions with receptor complexes) and should be explored further.

**Nodal and Lefty affect each other’s distribution and diffusivity**

It has been suggested that BMP ligands are mobilized upon binding to their antagonists, and that this facilitated diffusion (Müller et al. 2013) is part of a mechanism that allows patterning to adjust to changes in tissue size (Ben-Zvi et al. 2008) (see Chapters 1 and 6). However, a direct effect of an antagonist on the diffusion of an agonist has never been demonstrated. I found that Nodal and Lefty can affect each other’s localization and diffusivity, and that Lefty overexpression can extend the range of Cyclops-GFP gradients.

Overexpression of Lefty1 and Lefty2 together causes an increase in the amount of diffuse extracellular Cyclops-GFP (Fig. 4.1) and increases its diffusivity about 3-fold
Similarly, the diffusion coefficient of Squint-GFP is doubled when Lefty1 and Lefty2 are overexpressed (Fig. 4.5). Expression of Lefty1 or Lefty2 individually also increases the diffusivity of Nodal-GFP. Interestingly, when Cyclops is overexpressed, Lefty1- and Lefty2-GFP form membrane-associated clusters similar to Cyclops-GFP clusters (Fig. 4.1), and Cyclops overexpression also decreases the diffusivities of Squint-, Lefty1-, and Lefty2-GFP (Fig. 4.5). This indicates that Nodal and Lefty may interact in the extracellular space and influence each other’s localization and diffusivity.

If Lefty overexpression increases the diffusivity of Cyclops-GFP without significantly affecting other properties of Cyclops, the range of the Cyclops-GFP gradient should be increased. I therefore performed transplantation experiments (similar to those described in Chapter 2) to measure the Cyclops-GFP gradient in the presence of excess Lefty. I found that the range of the Cyclops-GFP gradient was significantly, but only slightly, expanded when Lefty was overexpressed (Fig. 4.5).

The biological significance of the ability of Nodal and Lefty to influence each other’s localization and diffusivity is currently unclear. Although it has been proposed that facilitated diffusion can generate sharp gradients via redistribution of signaling molecules (Ben-Zvi et al. 2008, Haskel-Ittah et al. 2012), or allow coordination of patterning and tissue size (Ben-Zvi & Barkai 2010, Ben-Zvi et al. 2011), differences in the geometry and other unique properties of the Nodal/Lefty patterning system suggest that Lefty-mediated facilitated diffusion of Nodal may serve a different function (see Chapter 6).
Figure 4.5: **Nodal and Lefty affect each other’s diffusivity and range.** (A) Embryos were injected with either 60 pg *lefty1*, 60 pg *lefty2*, 30 pg of *lefty1* and *lefty2* together, or 30 pg *cyclops* mRNA and subjected to the FRAP assay as in Müller et al. 2012. Lefty overexpression enhances Nodal diffusivity, whereas Cyclops overexpression decreases Lefty diffusivity. (B) Cyclops-GFP-expressing cells were transplanted into uninjected hosts or hosts injected with 72 pg of each *lefty* mRNA. In the latter case, donors were also injected with *lefty* mRNA. Gradients were quantified and normalized as in Müller et al. 2012. Lefty-overexpressing embryos exhibit slightly longer-range Cyclops-GFP gradients than uninjected embryos. N = 18 uninjected hosts, 15 *lefty*-expressing hosts.
Evidence for binding between Nodal and Lefty

The mechanisms by which Lefty inhibits Nodal signaling and facilitates its diffusion are not completely understood. It is clear that Lefty competes with Nodal for binding to the receptor complex: co-immunoprecipitation experiments demonstrated that Lefty binds to the Nodal coreceptor Oep and prevents Nodal from binding to the receptor complex, thereby inhibiting Nodal signaling (Chen & Shen 2004, Cheng et al. 2004). It has also been proposed that Lefty binds to Nodal directly and prevents Nodal from binding to receptor complexes. Structurally, Lefty is similar to TGFβ ligands, but Lefty is missing an alpha helix and cysteine residue thought to be required for homo- or heterodimerization (Thisse & Thisse 1999). Mouse Lefty can pull down Nodal from conditioned medium of human 293T cells, but these experiments may not represent physiologically relevant conditions (Chen & Shen 2004), and Nodal did not appear to pull down Lefty in Xenopus co-IP experiments (Cheng et al. 2004). My experiments showing that Nodal and Lefty can influence each other’s diffusivity and localization (see above) also suggest that Nodal and Lefty could interact in the extracellular space, but do not conclusively demonstrate this.

Fluorescence cross-correlation spectroscopy (FCCS) can be used to determine whether two diffusing fluorescently labeled species interact either directly or through an intermediary (Ries & Schwille 2012). I expressed functional GFP- and mRFP1-tagged proteins in zebrafish embryos (Müller et al. 2012), then monitored fluctuations in GFP and mRFP1 signal in a very small (~0.5 fl) volume within the extracellular space of individual embryos. If the GFP- and mRFP1-labeled species are bound, their
fluorescence fluctuations will be correlated; if not, they will exhibit little or no cross-correlation.

As a control, I first co-expressed secreted GFP and mRFP1 in zebrafish embryos, which should not bind and therefore should not exhibit significant cross-correlation. I then expressed a secreted GFP-mRFP1 fusion, which should exhibit cross-correlation. As expected, secreted GFP-mRFP1 showed high levels of cross-correlation compared to secreted GFP and secreted mRFP1 in vivo (Fig. 4.6). I then co-expressed Nodal-GFP with either secreted mRFP1, Lefty1-mRFP1, or Lefty2-mRFP1. When Nodal-GFP was co-expressed with secreted mRFP1, low levels of cross-correlation were observed. However, when Nodal-GFP was co-expressed with Lefty-mRFP1, I observed a significant increase in cross-correlation (Fig. 4.6), suggesting that Nodal-GFP and Lefty-mRFP1 may bind in the extracellular space of living zebrafish embryos.

It is not yet clear whether Lefty binding to Nodal is another mechanism by which Lefty inhibits Nodal signaling (in addition to competing with Nodal for binding to the Nodal coreceptor Oep), although it is plausible that this interaction could prevent Nodal from interacting with the receptor complex. In addition, binding to Lefty may prevent Nodal from interacting with diffusion regulators, which could explain how Lefty enhances the diffusion of Nodal. It will also be important to determine whether the interaction between Nodal and Lefty is reversible. If Nodal and Lefty bind reversibly, Lefty may act via facilitated diffusion to counter-intuitively expand the range of Nodal; however, if binding is irreversible, the faster-diffusing complexes may simply represent biologically inert molecules that reflect cleared/degraded Nodal.
binding kinetics of Nodal and Lefty could provide valuable information about how the spatial restriction of mesendoderm specification is regulated.

Figure 4.6: Evidence for binding between Nodal-GFP and Lefty-RFP \textit{in vivo}. FCCS experiments. Secreted GFP- or RFP-tagged proteins were expressed in zebrafish, and extracellular fluorescence fluctuations were observed. Autocorrelation and cross-correlation curves were calculated and fitted. Shown are the ratios between the amplitudes of the fitted cross-correlation curves and the autocorrelation curves (relative cross-correlation amplitude, RCA). Secreted GFP and RFP do not interact and have a low RCA value, but the secreted GFP-RFP fusion has a much higher RCA. When Sqint-GFP is co-expressed with Lefty1- and Lefty2-RFP, the high RCA values are consistent with direct binding between. Error bars = standard error.
CHAPTER 5
Lefty is a global dampener of Nodal signaling

PREFACE

I designed, performed, and interpreted all experiments. James A. Gagnon and the Joung lab generated the TALENs used to create the *lefty* mutants. Laila Akhmetova and Julien Dubrulle ran the NanoString experiments with RNA that I generated. Julien also provided guidance on using the NanoString software. Alexander F. Schier helped conceive, design, and support the project.

ABSTRACT

The secreted protein Lefty acts as a feedback inhibitor during mesendoderm patterning: the TGFβ ligand Nodal forms a signaling gradient and induces expression of mesendodermal genes in an exposure-dependent manner, and also induces expression of Lefty, a long-range, highly mobile Nodal inhibitor. To better understand the function of Lefty during vertebrate embryogenesis, I generated and analyzed zebrafish *lefty* mutants. Although patterning is highly robust to *lefty* gene dosage, complete loss of *lefty* causes upregulation of mesendodermal genes during gastrulation, aberrant cell internalization, and severe patterning defects, consistent with excess Nodal signaling. Mutations in *nodal* partially suppress patterning defects caused by *lefty* loss, and *lefty* mutants can be fully rescued by ectopic expression of *lefty-gfp* or by treatment with low levels of a Nodal inhibitor drug. Further, *lefty* expression is highly responsive to Nodal signaling. My results demonstrate that Lefty acts as a fast-responding, global dampener of Nodal signaling that restricts mesendoderm specification.
INTRODUCTION

During vertebrate embryogenesis, graded signaling by the TGFβ ligand Nodal patterns the germ layers. In zebrafish, two Nodal ligands, Cyclops and Squint, are expressed from a localized region in the embryo and induce mesendodermal gene expression in an exposure-dependent manner. Nodal signaling also induces expression of Lefty1 and Lefty2, which are secreted, highly diffusive long-range Nodal inhibitors (Chen & Schier 2002, Müller et al. 2012) (Fig. 5.1A). In the absence of Nodal signaling, embryos do not produce head and trunk mesendoderm, cells fail to internalize, and embryos are incorrectly patterned.

Lefty was first identified in zebrafish in a large-scale in situ hybridization screen of random cDNA clones (Thisse & Thisse 1999), three years after its initial discovery in mice (Meno et al. 1996). Injection of lefty mRNA into zebrafish embryos causes loss of mesendoderm that phenocopies Nodal loss of function, demonstrating that Lefty is an inhibitor of Nodal signaling (Bisgrove et al. 1999, Meno et al. 1999, Thisse & Thisse 1999). Lefty can inhibit Nodal target gene expression at a distance when expressed in a clone of cells (Chen & Schier 2002), and Lefty has a high effective diffusion coefficient in vivo (Müller et al. 2012), suggesting that it can act as a secreted, long-range global Nodal dampener.

lefty1 and lefty2 are 70% identical, and their expression patterns overlap but are non-identical (Bisgrove et al. 1999). Whereas injection of morpholinos targeting either lefty1 or lefty2 results in subtle phenotypes such as notochord kinking, co-injection of lefty1 and lefty2 morpholinos causes expansion of mesendodermal gene expression domains, and death of ~97% of embryos following gastrulation (Agathon et al. 2001,
Chen & Schier 2002, Feldman et al. 2002). Rare escapers that survive until 24 hours post-fertilization (hpf) exhibit severe patterning defects, including loss of tail, excess tissue protruding from the posterior trunk, hypertrophic axial mesoderm, and an excess of mesodermal tissues such as the notochord and hatching gland (Agathon et al. 2001). Dramatic upregulation of mesendodermal genes in double morphants occurs prior to gastrulation, shortly after Nodal signaling commences (Agathon et al. 2001).

Many developmental patterning systems, including the Nodal/Lefty mesendoderm patterning system, exhibit feedback inhibition. It has been proposed that feedback inhibition can buffer fluctuations in signaling in order to maintain an appropriate signaling gradient (Barkai & Shilo 2009, Eldar et al. 2003, Lander et al. 2009). To gain insight into patterning systems that feature feedback inhibition and determine the consequences of losing inhibition or decoupling feedback and inhibition, I used TALENs to generate null alleles of lefty1 and lefty2 in zebrafish. In the absence of Lefty-mediated inhibition, excess mesendoderm is specified, cell internalization is abnormal, and patterning is severely disrupted. Patterning defects due to lefty loss could be partially suppressed by mutations in the Nodal ligands cyclops or squint. Further, lefty loss could be fully rescued by ectopic expression of lefty-gfp or by treatment with low levels of a Nodal inhibitor drug. However, whereas even a single lefty allele is sufficient for normal patterning, only a narrow range of Nodal inhibitor drug concentrations can rescue lefty double mutants. Finally, lefty itself responded more rapidly to excess Nodal signaling than all other known Nodal target genes. Together, my findings suggest that Lefty is a fast-responding feedback inhibitor that acts as a global Nodal dampener and may buffer fluctuations in Nodal signaling.
lefty1\(^{-/-}\);lefty2\(^{-/-}\) mutants exhibit severe patterning defects

To better understand the role of Nodal feedback inhibition by Lefty, I used TALEN-mediated genome editing to generate putative null alleles of lefty1 and lefty2 (Fig. 5.1) (Sanjana et al. 2012). Whereas injection of wild-type lefty mRNA phenocopies EGF-CFC coreceptor mutants that are insensitive to Nodal signaling (Bisgrove et al. 1999, Meno et al. 1999, Thisse & Thisse 1999), injection of mRNA encoding mutant leftys does not result in Nodal loss-of-function phenotypes, suggesting that the mutant lefty alleles represent non-functional genes (Fig. 5.1D-I’).

Patterning is highly robust to lefty gene dosage: embryos containing four (wild-type), three (lefty1\(^{+/+}\) and lefty2\(^{+/+}\)) two (lefty1\(^{-/-}\), lefty2\(^{-/-}\), and lefty1\(^{+/+}\);lefty2\(^{+/+}\)), or one (lefty1\(^{+/+}\);lefty2\(^{-/-}\) and lefty1\(^{-/-}\);lefty2\(^{+/+}\)) functional lefty allele exhibit normal gross morphology and are viable (Fig. 5.1J-Q,S), although lefty1\(^{-/-}\) mutants display partially penetrant heart laterality defects (Fig. 5.S1). In contrast, lefty1\(^{-/-}\);lefty2\(^{-/-}\) double homozygous embryos typically die by 2 days post-fertilization (dpf) from severe patterning defects, but undergo complete gastrulation and generate clear anterior-posterior and dorsal-ventral axes (Fig. 5.1R, Fig. A.30). At 1 dpf mutants lack structures such as tail, eyes, and heart (Fig. 5.S1), and do not develop a well-formed brain. Double lefty mutants often exhibit excess tissue protruding along the posterior trunk (Fig. 5.1R, Fig. A.30). I also observe a clutch-dependent, partially penetrant (~3-40\% of embryos) axis bifurcation (Fig. A.30).
Figure 5.1: Complete lefty loss causes severe patterning defects. A) Nodal activates mesendodermal genes as well as itself and the secreted Nodal inhibitor, Lefty. B) A 13-base-pair deletion at the 5’ end of lefty1 removes the translational start site and part of the signal sequence. C) An 11-base-pair deletion at the 5’ end of lefty2 removes part of the signal sequence. D-1’’) Testing the activity of lefty mutant mRNA. All images were obtained at 24-28 hours post-fertilization (hpf). Wild-type embryos at the one-cell stage were injected with wild-type lefty1 (F-F’) or mutant lefty1 (G-G’) mRNA. Embryos expressing wild-type lefty1 mRNA exhibit Nodal loss-of-function phenotypes, similar to maternal-zygotic mutants for the zebrafish EGF-CFC coreceptor one-eyed pinhead (oep) (D), which are insensitive to Nodal signaling. Embryos expressing mutant mRNA do not exhibit Nodal loss-of-function phenotypes. Similar results were obtained for wild-type lefty2 (H-H’) and mutant lefty2 (I-I’) mRNA. E) Uninjected embryo. J-R) lefty mutant phenotypes. All images were obtained at 24-29 hpf. A single functional lefty allele is sufficient for grossly normal patterning (P,Q). Q,R) lefty1;lefty2 double homozygous mutants have severe patterning defects and lack eyes, heart, and tails, and excess tissue along the posterior trunk. S) Percentage of embryos with normal gross morphology at 1 day post-fertilization (dpf). Number normal / total embryos: wild-type = 50/50, lefty1+/– = 49/50, lefty2+/– = 46/46, lefty1+/– = 50/50, lefty2+/– = 46/46, lefty1+/–;lefty2+/– = 50/50, lefty1+/–;lefty2+/– = 43/43, lefty1+/–;lefty2+/– = 24/24 lefty1+/–;lefty2+/– = 0/55.
Importantly, most *lefty* double mutants undergo gastrulation and survive past 24 hours post-fertilization (hpf) (Fig. A.31), whereas ~97% of double morphants die by 24 hpf and undergo strongly disrupted gastrulation (Agathon et al. 2001, Feldman et al. 2002). The ~3% morphant escapers that survive past 24 hpf exhibit phenotypes similar to double mutant phenotypes, although axis bifurcation has not been reported in morphants. Differences in morphant and mutant phenotypes are unlikely to be due to inhibition of maternally contributed *lefty* by the morpholinos, since I have failed to observe a requirement for maternal *lefty* (Fig. A.31), consistent with the low/undetectable levels of *lefty* transcripts prior to sphere stage (Pauli et al. 2012). Off-target morpholino effects and morpholino toxicity have been previously shown to confound studies of gene function (Kok et al. 2014, Schulte-Merker & Stainier 2014). Thus, because *lefty* morpholinos apparently cause massive toxicity independently of *lefty* knockdown, care must be taken when interpreting *lefty* morphant phenotypes.

Excessive internalization of mesendoderm precursor cells during gastrulation in *lefty*1\(^{-/-}\);*lefty*2\(^{-/-}\) mutants

Nodal signaling is required for the internalization of prospective mesendodermal cells during gastrulation (Carmany-Rampey & Schier 2001, Feldman et al. 2000). Previous work with double *lefty* morphants suggested that in the absence of Lefty activity, increased Nodal signaling leads to excessive internalization during gastrulation due to an extended period of rapid cell internalization (Feldman et al. 2002). To determine whether similar aberrant internalization could explain some of the morphogenetic defects in *lefty* double mutants, I labeled nuclei in wild-type and *lefty*
double mutants with H2B-Dendra2 and performed time-lapse imaging using Selective Plane Illumination Microscopy (SPIM) (Huisken 2004, Keller et al. 2008).

In lefty double mutants, more cells internalize and the internalization period is extended compared to wild-type embryos (Fig. 5.2). Excessive internalization is consistent with the increase in Nodal signaling and subsequent increase in the number of mesendodermal precursor cells that is expected in lefty double mutants.

![Figure 5.2: Excessive cell internalization in lefty double mutants. Nuclei in wild-type and lefty double mutant embryos were labeled by injection of 20 pg H2B-Dendra2 mRNA at the one-cell stage. Embryos were mounted at sphere stage and imaged using Selective Plane Illumination Microscopy (SPIM). Z-stacks encompassing most of the embryo were acquired every 90 s for ~7 hours during gastrulation as in (Pauli et al. 2014). A - H) Maximum intensity projections of 20 z-slices in representative wild-type (A-D) and lefty double mutant (E-H) embryos starting at the onset of gastrulation (0 min). Dorsal is to the right. In lefty double mutants, excess internalization of dorsal cells is visible (F-H).](image)

lefty1\(^{+/−}\);lefty2\(^{+/−}\) mutants generate excess mesendoderm during gastrulation

In double lefty morphants, upregulation of the mesoderm marker no tail and the endoderm marker casanova is evident as early as the late blastula stage (30% epiboly),
shortly after nodal expression commences (Agathon et al. 2001). no tail and casanova are both direct Nodal target genes (Dubrulle et al., submitted), and their upregulation in double morphants was interpreted as a response to excess Nodal signaling in the absence of the Nodal inhibitor Lefty. However, given the differences between morphant and mutant phenotypes, I wanted to systematically examine Nodal target gene expression in lefty double mutants to determine the consequences of lefty loss on mesendoderm induction in the absence of possible morpholino artifacts.

To assess expression of Nodal target genes in lefty double mutants, I used two strategies: in situ hybridization to provide information about spatial changes in gene expression, and a NanoString assay to quantitatively measure changes in transcript levels. NanoString assays quantify transcripts present in a sample using transcript-specific fluorescent probes, and do not require amplification or conversion of RNA into cDNA (Geiss et al. 2008). Our lab created a NanoString array containing probes targeting all 47 known direct Nodal target genes (Dubrulle et al., submitted), which I used to analyze RNA levels in samples collected from wild-type and lefty double mutant embryos at late blastula (sphere stage and 30% epiboly) and gastrulation stages (50% epiboly, shield stage, and 75% epiboly). I obtained RNA counts high enough to calculate fold changes for 20 genes. I also used in situ hybridization to assess the spatiotemporal expression of ten direct Nodal target genes (no tail, casanova, floating head, draculin, lefty1, lefty2 (Fig. 5.3A-F), spadetail, squint, cyclops, and one-eyed pinhead (Fig. A.32)) and one indirect Nodal target gene (sox17 (Fig. A.32)) in lefty double mutants as well as single mutants (Fig. A.32). I assessed gene expression using in situ hybridization at the same five developmental stages used for NanoString analysis.
Figure 5.3: Nodal target gene expression is upregulated in lefty double mutants. A-F) *In situ* hybridization in wild-type and lefty double mutant embryos at sphere stage, 30% epiboly, 50% epiboly, shield stage, and 75% epiboly using the indicated probes. A, E, and F are lateral views; B, C, and D are views from the animal pole, dorsal to the right where evident. G) NanoString analysis of expression of 20 direct Nodal target genes in wild-type versus lefty double mutant embryos at the indicated developmental stages. Log2 fold change between wild-type and lefty double mutants is plotted. Colors correspond to genes shown in (A-F); gray lines represent the remaining 14 Nodal target genes, see Table A.10 for details. In general, upregulation of Nodal target genes is evident starting after gastrulation begins at 50% epiboly. *lefty1* (E) and *lefty2* (F) themselves are among the most dramatically upregulated Nodal target genes in lefty double mutants.

In contrast to what is observed in double lefty morphants, no tail and casanova are not obviously upregulated until after gastrulation begins (50% epiboly or shield stage) in lefty double mutants (Fig. 5.3A,B,G). Other Nodal target genes also typically exhibited normal expression patterns in double mutants at late blastula stages, but were
dramatically upregulated by shield or 75% epiboly (Fig. 5.3; Fig. A.32, Table A.10).

Thus, the expression of most mesendodermal genes is normal in lefty double mutants until after the start of gastrulation, several hours after nodal expression commences. It is unclear why gene expression differs between lefty double morphants and double mutants, but one possibility is a potential developmental delay in morphants due to morpholino toxicity.

leftys are dramatically upregulated prior to gastrulation in lefty1−/−;lefty2−/− mutants

An effective feedback inhibition system should act on a time scale that is fast enough to buffer effects of aberrant signaling before it is converted into misexpression of target genes and therefore mis-specification of cells. In contrast to mesendodermal genes, both in situ hybridization and the NanoString assay demonstrate that leftys themselves are strongly upregulated prior to gastrulation in lefty1−/−;lefty2−/− double mutants (Fig. 5.3E,F,G). lefty1 and lefty2 were the most strongly upregulated Nodal target genes in lefty double mutants out of the 20 genes I was able to assess, and they were the first Nodal target genes to reach 2-fold upregulation in lefty double mutants (Fig. A.33, Table A.10). Mutant transcripts did not appear to be targeted for nonsense-mediated decay.

This increase in lefty transcript number could result from two mechanisms: mutant lefty transcripts could be more stable than wild-type transcripts, or lefty expression may be highly responsive to excess Nodal signaling. If mutant lefty transcripts are more stable than wild-type transcripts, then similar “upregulation” should be observed in lefty single mutants; e.g., lefty1 transcripts should appear to be upregulated in lefty1−/− single mutants. However, the extent of upregulation in single mutants is much less
dramatic than that observed in double mutants (Fig. A.32A-D). In addition, recent work from our lab found that leftys have the highest apparent transcription rates of all 47 known Nodal target genes (Dubrulle et al., submitted). Together, this supports the idea that transcript accumulation results from greater production of lefty rather than enhanced stability of lefty transcripts, and further suggests that Nodal signaling activity is in excess starting at the late blastula stage. Excess Nodal signaling strongly upregulates lefty expression before obviously affecting expression of most other Nodal target genes.

Mutations in the Nodal ligands squint or cyclops partially suppress lefty1−/−;lefty2−/− mutant defects

Two zebrafish Nodal ligands, squint and cyclops, are expressed during gastrulation and specify head and trunk mesendodem (Schier 2009). If the defects observed in lefty double mutants are caused by excess Nodal signaling, we reasoned that reducing Nodal levels using compensatory loss-of-function mutations in squint or cyclops might suppress double lefty mutant phenotypes. I therefore generated squint+/−;lefty1+/−;lefty2+/− and cyclops+/−;lefty1+/−;lefty2+/− fish and incrossed these respective lines. Heterozygosity of either squint or cyclops suppressed multiple aspects of the lefty double mutant phenotype—squint+/−;lefty1−/−;lefty2−/− and cyclops+/−;lefty1−/−;lefty2−/− embryos exhibit long tails, no axis bifurcation, and no tissue protruding along the posterior trunk, in contrast to lefty1−/−;lefty2−/− embryos (Fig. 5.4E,K). However, patterning was still strongly disrupted and embryos were non-viable.

nodal homozygosity provided even stronger suppression of the gross morphological defects resulting from lefty loss: nodal+/−;lefty1+/−;lefty2+/− embryos have obvious eyes and tails (Fig. 5.4F,L) at 1 dpf, structures that are missing in lefty1−/−;lefty2−/−
mutants. However, $\text{nodal}^{+/+};\text{lefty}1^{-/-};\text{lefty}2^{-/-}$ embryos do not develop into viable adults. This demonstrates that compensatory mutations in $\text{squint}$ or $\text{cyclops}$ can partially suppress the patterning defects due to $\text{lefty}$ loss.

Figure 5.4: Mutations in the Nodal ligands $\text{squint}$ or $\text{cyclops}$ partially suppress $\text{lefty}$ double mutant defects. $\text{nodal}^{+/+};\text{lefty}1^{-/-};\text{lefty}2^{-/-}$ adult fish were incrossed, and the resulting progeny were imaged at 27-30 hpf and subsequently genotyped. A-F) Progeny from a $\text{sqt}^{+/+};\text{lefty}1^{-/-};\text{lefty}2^{-/-}$ incross at 27 hpf with the indicated genotypes. $\text{lefty}$ double mutants exhibit typical double mutant phenotype when $\text{squint}$ is wild-type (D), but multiple aspects of the double $\text{lefty}$ mutant phenotype are partially suppressed when $\text{squint}$ is heterozygous (E). Triple homozygous mutants (F) exhibit grossly normal patterning. G-L) Progeny from a $\text{cyc}^{+/+};\text{lefty}1^{-/-};\text{lefty}2^{-/-}$ incross at 27 hpf with the indicated genotypes. $\text{cyclops}$ heterozygosity (K) partially suppresses multiple aspects of the double $\text{lefty}$ mutant phenotype, and $\text{cyclops}$ homozygosity strongly suppresses double $\text{lefty}$ mutant phenotypes (L).
Ectopic expression of lefty-gfp from the animal pole rescues lefty loss

Based on the high in vivo effective diffusion coefficient of Lefty-GFP (~15 \( \mu \text{m}^2/\text{s} \)), the ability of Lefty-GFP to form long-range gradients (Müller et al. 2012), and the fact that Lefty can inhibit Nodal signaling over a long distance (Chen & Schier 2002), it has been proposed that Lefty diffuses far from its localized source at the margin and acts as a global dampener of Nodal signaling (Bisgrove et al. 1999, Thisse & Thisse 1999). In this case, Lefty expressed outside of its endogenous expression domain at the opposite end of the embryo—the animal pole—should be sufficient to rescue lefty loss.

To test this idea, I injected mRNA encoding active Lefty-GFP fusions (Müller et al. 2012) into “donor” lefty double mutant embryos at the one-cell stage. At sphere stage (when Nodal expression commences), I transplanted ~50 Lefty-GFP-expressing mutant cells into the animal pole of uninjected “host” lefty double mutant embryos (Fig. 5.5A). At 1 dpf, ~30-50% of host embryos that received Lefty-GFP-expressing cells exhibited grossly normal patterning and morphology (Fig. 5.5D,E), whereas mutant embryos that did not receive transplanted cells exhibited the typical double lefty mutant defects. This demonstrates that Lefty can inhibit Nodal over long distances, that the localization of lefty expression at the margin is dispensable for normal patterning, and that Lefty does not provide positional information.
Figure 5.5: Expression of lefty-GFP in the animal pole rescues lefty loss. A) Diagram of experiment. A lefty1⁺;lefty2⁻ donor is injected with mRNA encoding either lefty1-gfp (200 pg) or lefty2-gfp (60 pg) mRNA at the one-cell stage. At sphere stage, GFP⁺ cells from the donor are transplanted into the animal pole of a lefty1⁺;lefty2⁻ host. All images obtained at 28 hpf. B) Wild-type embryo. C) Untreated lefty1⁺;lefty2⁻ embryo. D) A host lefty1⁺;lefty2⁻ embryo that received Lefty1-GFP-positive cells. E) A host lefty1⁺;lefty2⁻ embryo that received Lefty2-GFP-positive cells. Both embryos that received cells from Lefty-GFP-expressing donors appear normally patterned; roughly 30-50% of host embryos were rescued. Host embryos that were not fully rescued typically exhibited phenotypes similar to partially rescued lefty double mutants (e.g., see Fig. 5.6D).

Artificial, uniform Nodal inhibition rescues lefty loss by reducing mesendodermal gene upregulation

When is Lefty-mediated Nodal inhibition required during normal germ layer patterning? Nodal target gene expression appears to be relatively normal in lefty double mutants until after gastrulation begins (Fig. 5.3, Fig. A.32). Is Lefty required to abruptly shut off Nodal signaling starting at gastrulation, or does Lefty restrict mesendoderm specification by providing continuous, low levels of Nodal inhibition?

If Lefty normally diffuses throughout the embryo and acts as a global Nodal inhibitor, then artificial, uniform Nodal inhibition using a Nodal inhibitor drug should recapitulate lefty activity and rescue lefty loss. In this case, it should be possible to determine when Lefty activity is required by inhibiting Nodal in lefty double mutants at different developmental stages. SB-505124 is a drug that interferes with ATP binding to
the TGFβ receptors Alk4 and Alk5, and selectively inhibits TGFβ and Activin signaling (DaCosta Byfield et al. 2004). Exposure of wild-type embryos to 30-50 μM SB-505124 starting at 2.75 hpf—prior to the onset of nodal expression—is sufficient to fully block Nodal signaling and phenocopy Nodal loss-of-function mutants (Hagos & Dougan 2007). To determine whether uniform dampening of Nodal signaling can rescue lefty loss and when inhibition is required, I exposed wild-type and lefty double embryos to five concentrations of SB-505124 ranging from 1.25 – 6.25 μM starting either at the onset of nodal expression (sphere stage) or at the onset of gastrulation (50% epiboly – shield). lefty double mutant defects were rescued over a narrow range of SB-505124 concentrations in both cases, and rescued embryos developed into viable, fertile adults.

About 90% of double lefty mutant embryos exposed to 2.5 or 3.75 μM SB-505124 starting at sphere stage exhibited normal gross morphology at 1 dpf (Fig. 5.6J,K). At higher concentrations, these embryos exhibited typical Nodal loss-of-function defects such as cyclopia (Fig. 5.6L,M). Wild-type embryos exposed to > 2.5 μM SB-505124 starting at sphere stage also exhibited phenotypes indicative of Nodal loss-of-function (Fig. 5.6F,G,H). About 80% of double lefty mutant embryos exposed to 3.75, 5, or 6.25 μM SB-505124 starting at the onset of gastrulation exhibited normal gross morphology at 1 dpf (Fig. 5.6V,W); wild-type embryos exposed at this stage appeared normal at all concentrations (Fig. 5.6N-W).
Figure 5.6: Artificial, uniform Nodal inhibition rescues lefty loss. Dechorionated wild-type and lefty double mutant embryos were exposed to five concentrations of the Nodal inhibitor drug SB-505124 starting either at sphere stage (D-M) or 50% epiboly - shield stage (gastrulation) (N-W) and ending at 24 hpf. Embryos were imaged and gross morphology was scored scored at 1 dpf (C). Exposure to higher concentrations of Nodal at sphere stage causes Nodal loss-of-function phenotypes (e.g. G, H, M). lefty double mutants were rescued by exposure to 2.5 or 3.75 μM SB-505124 starting at sphere (J,K) or by exposure to 5 or 6.25 μM starting at gastrulation (V,W).
I hypothesized that the rescue of *lefty* double mutants by exposure to SB-505124 was due to a decrease in the upregulation of mesendodermal genes. To test this and examine mesendodermal gene expression in drug-rescued mutants over time, I exposed wild-type and *lefty* double mutants to 5 µM SB-505124 starting at shield stage and fixed embryos 30, 120, 210, and 300 min post-exposure (Fig. 5.7). By 210 min, upregulation of the mesoderm marker *floating head* and the endoderm marker *casanova* was less pronounced in drug-treated *lefty* double mutants compared to untreated mutants (Fig. 5.7Q,II). After 300 min of exposure to the drug, expression of these genes in *lefty* double mutants was similar to their expression in wild-type embryos (Fig. 5.7R,JJ); in contrast, both genes were dramatically upregulated in untreated mutants (Fig. 5.7N,FF). Thus, within 5 hours of exposure to 5 µM SB-505124 starting at shield stage, the expression of mesendodermal genes in *lefty* double mutants is relatively normal.

The rescue of *lefty* double mutants by uniform, artificial inhibition of Nodal signaling is consistent with the idea that Lefty can diffuse throughout the embryo and globally dampen Nodal, and it demonstrates that the patterning defects observed in double *lefty* mutants are caused by excess Nodal signaling. I also show that both constant, moderate Nodal dampening as well as stronger Nodal dampening at later stages is sufficient for normal patterning, suggesting that the timing of Nodal inhibition is flexible, as long as the strength of inhibition is properly adjusted.
Figure 5.7: Nodal inhibitor drug reduces upregulation of mesendodermal genes in lefty double mutants. Dechorionated wild-type and lefty double mutant embryos were exposed to 5 µM Nodal inhibitor drug SB-505124 starting at shield stage and fixed at the indicated times post-exposure. All views are dorsal. In situ hybridization with a probe targeting the Nodal target genes floating head (mesoderm, A-R) or casanova (endoderm, S-JJ). A-E and S-W are untreated wild-type embryos; F-I and X-AA are wild-type embryos treated with 5 µM SB-505124; J-N and BB-FF are lefty double mutant embryos, and O-R and GG-JJ are lefty double mutant embryos treated with 5 µM SB-505124. Whereas untreated lefty double mutants exhibit upregulation of floating head (J-N) and casanova (BB-FF), upregulation is reduced in mutants treated with SB-505124 after 210 min (Q,II), and by 300 min expression of these genes in drug-treated double mutants is similar to their expression in wild-type embryos (compare E to R and W to JJ).

DISCUSSION

My analysis of zebrafish lefty mutants supports previous morpholino experiments demonstrating partially overlapping roles of lefty1 and lefty2 in regulating the extent of the mesendoderm by inhibiting Nodal signaling (Agathon et al. 2001, Chen & Schier 2002, Feldman et al. 2002). Whereas complete knockdown of lefty results in excess mesendoderm (Fig. 5.3, Fig. A.32), aberrant cell internalization (Fig. 5.2), and severe patterning defects (Fig. 5.1, Fig. A.30), even a single functional lefty allele is sufficient to generate viable fish (Fig. 5.1).
The ability of the Nodal/Lefty patterning system to handle dramatic reductions in *lefty* gene dosage may result from the high sensitivity of *lefty* expression to Nodal signaling. I show that expression of *leftys* is more dramatically and rapidly upregulated in response to excess Nodal signaling than mesendodermal genes (Fig. 5.3, Fig. A.32). This is consistent with a recent study from our lab that demonstrated that *lefty1* and *lefty2* have the highest transcription rates in response to Nodal signaling of all Nodal target genes (Dubrulle et al., submitted). Effective feedback inhibition should act on a relatively rapid timescale in order to buffer changes in signaling gradients before they are converted into inaccurate gene expression and therefore inaccurate patterning. In many developmental patterning systems, negative feedback occurs when a signal induces transcription of an inhibitor, creating a delay during which the feedback inhibitor must be generated. The kinetics of feedback inhibitor expression compared to other target genes has not been systematically characterized for any gradient-based patterning system, but an effective buffering system might be expected to exhibit relatively fast expression kinetics of feedback inhibitors compared to target genes. In the event of excess Nodal signaling, one of the first genes to respond is *lefty*, which should allow signaling to be rapidly dampened back to normal levels before expression of other target genes is affected.

The internalization defects I observed in *lefty* double mutants also support previous morpholino-based experiments suggesting that Lefty restricts the number of cells that internalize during gastrulation (Fig. 5.2) (Feldman et al. 2002). However, I find several striking discrepancies between *lefty* double mutant and double morphant phenotypes, and my results demonstrate the importance of validating morpholino phenotypes using mutants. New genome editing technologies such as CRISPR and
TALENs provide straightforward gene knockout strategies (Bedell et al. 2013, Gagnon et al. 2014, Hwang et al. 2013b, Montague et al. 2014, Sander et al. 2011), and since morpholino artifacts are a known risk it has been suggested that all morphant phenotypes should be validated by generating and assessing mutants (Schulte-Merker & Stainier 2014). Consistent with this suggestion, a recent study in zebrafish found that as many as 80% of tested morphant phenotypes were not observed in mutant embryos (Kok et al. 2014). While rare *lefty* double morphant escapers exhibited many of the phenotypes seen in *lefty* double mutants (Agathon et al. 2001), the premature death of morphants and strong, early upregulation of mesendodermal genes in morphants are likely due to morpholino toxicity. I show that the phenotypic consequences of *lefty* loss manifest later and are less severe than previously thought based on morpholino knockdown experiments. Analyses of Nodal target gene expression in *lefty* double mutants (Fig. 5.3, Fig. A.33, Fig. A.32, Table A.10) demonstrate that mesendodermal gene expression is not immediately affected by increased Nodal signaling, and that the time available to correct excessive Nodal signaling is longer than previously thought.

Cell culture studies have suggested that Lefty can activate MAPK signaling (Ulloa et al. 2001), and both overexpression and morpholino knockdown studies in *Xenopus* provided evidence that Lefty might inhibit Wnt or BMP signaling (Branford & Yost 2002, Meno et al. 1997). However, my experiments with the Nodal inhibitor drug SB-505124 support a primary role for Lefty in Nodal inhibition. SB-505124 specifically inhibits Nodal and Activin signaling, but does not disrupt BMP signaling (DaCosta Byfield et al. 2004), and is not predicted to directly affect Wnt or MAPK signaling. Treatment with SB-505124 can fully rescue *lefty* double mutants by reducing the
upregulation of mesendodermal genes (Fig. 5.6 and 5.7), suggesting that the defects observed in lefty double mutants are caused by excess Nodal signaling, and that Lefty’s principle function is to inhibit Nodal signaling. This is also consistent with the ability of squint and cyclops mutations to partially suppress lefty double mutant defects.

The ability of Lefty to inhibit Nodal over a long distance (Chen & Schier 2002) together with the relatively high effective diffusivity of Lefty-GFP (~15 µm²/s) and its ability to form long-range gradients in vivo (Müller et al. 2012) suggests that Lefty diffuses away from its localized source, spreads throughout the embryo, and globally dampens Nodal signaling. Argos, the secreted, long-range inhibitor of Spitz, is thought to act similarly and possibly bathe its patterning field (the developing Drosophila eye) in a “sea” of inhibitor (Freeman 1997). Lefty and Argos can therefore act non-cell-autonomously to inhibit their respective signals. I found that uniform, artificial Nodal inhibition by the Nodal inhibitor drug SB-505124 (Figs. 5.6 and 5.7) and Lefty-GFP expressed far from the presumptive mesendoderm (Fig. 5.5) can recapitulate Lefty activity and rescue lefty double mutants to adulthood, consistent with the idea that Lefty can spread throughout the embryo and globally dampen Nodal. My results are among the first examples of the rescue of a structural congenital defect by a drug treatment, and also show that it is possible to generate correctly patterned mesendoderm when feedback is decoupled from inhibition. However, whether this is a robust developmental strategy remains to be determined.

It has been suggested that the primary role of Lefty during embryogenesis is specifically to inhibit Squint rather than Cyclops (Chen & Schier 2002, Feldman et al. 2002). In previous studies, removal of squint, but not cyclops, was sufficient to reduce
expansion of Nodal target gene expression and mitigate morphological defects observed in double *lefty* morphants. This predicts that in *lefty* double mutants 1) removal of *squint* should alleviate mesendoderm expansion, but 2) removal of *cyclops* should not alleviate mesendoderm expansion. My results are consistent with the first, but not the second prediction: both *squint<sup>−/−;lefty<sup>1<sup>−/−;lefty<sup>2<sup>−/−*; and *cyclops<sup>−/−;lefty<sup>1<sup>−/−;lefty<sup>2<sup>−/−*; mutants were relatively normally patterned at 1 dpf compared to *lefty* double mutants and therefore likely experienced less severe mesendoderm expansion (Fig. 5.4). Thus, during normal embryogenesis, inhibition by Lefty probably affects signaling by both Squint and Cyclops.

We and others have suggested that Nodal and Lefty act as a reaction/diffusion system to pattern the germ layers (Chen & Schier 2002, Duboc et al. 2004, 2008; Kondo & Miura 2010, Meinhardt 2009, Müller et al. 2012, Saijoh et al. 2000, Schier 2009, Shen 2007, Shiratori 2006). The Nodal/Lefty patterning system has characteristics consistent with classical reaction/diffusion systems (Meinhardt & Gierer 2000): Nodal is a short-range activator that promotes its own production as well as that of a long-range, more mobile inhibitor (Lefty). In this paradigm, the feedback between activator and inhibitor shapes their respective distributions and allows the system to respond robustly to fluctuations in activator or inhibitor levels, as well as to changes to the size of the patterning field. Such a mechanism could help ensure that the Nodal signaling gradient is correctly established, robustly generating the appropriate proportions of endoderm, mesoderm, and ectoderm during the potentially noisy process of embryogenesis. Interestingly, I found that germ layer patterning can still occur when feedback and inhibition are decoupled (Fig. 5.5, 5.6, 5.7), and occurred to a certain extent in the total
absence of Lefty when nodals were mutated to compensate (Fig. 5.4). This demonstrates that patterning can proceed without a fine-tuned balance between signaling by Nodal and negative feedback by Lefty.

My analysis of lefty mutants provides a basis for future research aimed at understanding why feedback inhibition is a common feature of developmental patterning systems. It has been proposed that feedback inhibition can buffer fluctuations in signal production and help maintain an appropriate signaling gradient (Barkai & Shilo 2009, Eldar et al. 2003, Lander et al. 2009). Consistent with this idea, lefty expression responds rapidly to Nodal signaling (Fig. 5.3, Fig. A.32), patterning is robust to the gene dosage of both nodal (Dougan 2003, Feldman et al. 1998, Pei et al. 2007) and lefty (Fig. 5.1), and only a narrow range of Nodal inhibitor drug concentrations can rescue lefty double mutants (Fig. 5.6). Further research will be required to determine whether lefty-mediated feedback inhibition enhances developmental robustness by adjusting or holding steady the shape of the Nodal signaling gradient.
CHAPTER 6
Conclusions and prospects

My work provides support for the synthesis, diffusion, and clearance (SDC) model of morphogen gradient formation (Rogers & Schier 2011) (Chapter 1): We found that Nodal- and Lefty-GFP form in vivo gradients whose shapes are consistent with the measured half-lives and diffusion coefficients of Nodal and Lefty, respectively (Müller et al. 2012) (Chapter 2). We also discovered that the differences in the activity ranges of Nodal and Lefty are likely caused by differences in their diffusivity, demonstrating that control of diffusivity is a major determinant of the size of the mesendodermal domain. I established a novel Fluorescence Decay After Photoconversion (FDAP) assay in zebrafish to measure the half-lives of secreted proteins in vivo, which should be adaptable to other taggable proteins in other optically accessible organisms (Chapter 3). I uncovered some of the factors that regulate Nodal diffusivity, and found that, surprisingly, interactions with receptors or HSPGs do not appear to be solely responsible for the low diffusion coefficient of Nodal (Chapter 4). Finally, I confirmed a role for Lefty as a global, long-range Nodal dampener, demonstrated that patterning is highly robust to changes in lefty gene dosage, the localization of lefty expression, and the timing of Nodal inhibition; I have also generated novel reagents that will be useful for future studies assessing the purpose of feedback inhibition during developmental patterning (Chapter 5).

My work raises interesting questions that I hope will be explored in the future: What factors regulate Nodal diffusivity? Is Lefty-mediated enhancement of Nodal diffusivity biologically relevant, and if so, how? How and why do Cyclops clusters form?
What is the role of feedback inhibition in the Nodal/Lefty patterning system? What determines the transcription rates of Nodal target genes? How is the reduction of mesendodermal gene expression in drug-rescued lefty double mutants achieved? And finally, what are the spatiotemporal in vivo distributions of Nodal and Lefty during normal and perturbed embryogenesis? Below, I discuss these open questions in more detail.

WHAT FACTORS REGULATE NODAL DIFFUSIVITY?

Although interaction with HSPG sugar chains appears to have only a modest effect on Nodal diffusivity (see Chapter 4), it is possible that interactions with HSPG core proteins themselves, rather than their sugar side chains, play a role in inhibiting Nodal mobility. For example, the core protein of the Drosophila HSPG Dally-like can interact directly with the ligand Wingless in the absence of sugar chains (Yan et al. 2009). Interactions with HSPG core proteins could explain why Knypek-GFP localization is altered by the presence of Cyclops, but disruption of HS-Nodal interactions by extracellular heparin injection or blocking HS synthesis using the UDP-glucose dehydrogenase mutant jeekyll apparently has minimal effects on Nodal-GFP mobility (see Chapter 4). Multiple glypican, syndecan, HSPG, and CSPG core proteins are encoded in the zebrafish genome, all of which may have redundant roles in regulating Nodal mobility or stability. It may be possible to use CRISPR/Cas9-mediated multiple knockdown methods to simultaneously remove all of these factors (James Gagnon, personal communication) and determine the effects on Nodal signaling, but such extensive knockdown could lead to toxicity or create other artifacts.
Another possibility is that some of the factors that I have already identified as minor diffusion regulators act redundantly, and the full extent of their effects might only be obvious when both regulators are experimentally removed. For example, what is the diffusivity of Nodal-GFP in an MZoep embryo that has received an extracellular injection of heparin?

One tantalizing possibility is that the diffusion regulator/s of Nodal may be factors that are not currently known to interact with signaling molecules, which could explain why our biased, candidate-based approach to find Nodal diffusion regulators so far has not revealed a key regulator. Identifying such novel regulators would provide additional insight into the control of signal movement, and might have implications for other patterning systems as well. Future studies utilizing alternative, non-biased approaches will be required to identify any novel diffusion regulators.

WHAT IS THE BIOLOGICAL SIGNIFICANCE OF LEFTY-MEDIATED ENHANCEMENT OF NODAL DIFFUSIVITY?

A central question in developmental biology is how the growth and patterning of organs are coordinated (Crickmore & Mann 2008). Patterning must scale to the size of the tissue, and in some cases the size of the tissue undergoing patterning changes dramatically or can be experimentally altered without deleterious effects to patterning. For example, the Drosophila wing disc increases in size as it is patterned by the morphogen Dpp (Crickmore 2006, Wartlick et al. 2011), the dorsal half of a bisected Xenopus embryo develops into a normally proportioned tadpole (Cooke 1981, Barkai & Shilo 2009, Ben-Zvi et al. 2008, Reversade & De Robertis 2005), and zebrafish embryos that have had ~30% of the blastoderm surgically removed prior to gastrulation also
recover and develop into normal-appearing adults (Patrick Müller, personal communication). This demonstrates the existence of compensatory mechanisms that allow scaling over a large range of size differences.

Multiple mechanisms might help explain scaling during embryogenesis. “Shuttling” of BMP by the secreted antagonist Chordin (discussed in Chapter 1) could account for the scaling observed in the *Xenopus* embryo (Ben-Zvi et al. 2008). In this scenario, uniformly expressed BMP is “shuttled” to the ventral side of the embryo upon binding to Chordin, which inhibits and mobilizes BMP. Cleavage of Chordin by an extracellular protease then releases and disinhibits BMP. Mathematical modeling demonstrates that such a system could support the scaling that has been observed in *Xenopus* embryos, but it has not been directly demonstrated that Chordin enhances BMP diffusivity. Similarly, a related “expansion-repression” mechanism has been proposed to explain scaling in the *Drosophila* wing disc (Ben-Zvi et al. 2011). In this model, Dpp represses the expression of a diffusible expander molecule—possibly Pentagone (Hamaratoglu et al. 2011, Vuilleumier et al. 2010)—that binds to and enhances the diffusivity or stability of Dpp. This size-sensing mechanism would ensure that Dpp signaling reaches the distal-most edge of the patterning field. Again, a direct effect of Pentagone on Dpp mobility has not been demonstrated.

I have shown that the Nodal inhibitor Lefty may bind to Nodal and enhance its diffusivity, similar to what has been proposed for BMP/Chordin and Dpp/Pentagone in the above models. Could the Nodal/Lefty activator/inhibitor pair act as a size-sensing patterning system, similar to those proposed for BMP/Chordin or Dpp/Pentagone? There are several differences between the Nodal/Lefty patterning system and the scaling-
capable systems described above. Whereas Nodal induces expression of the feedback inhibitor Lefty, BMP does not induce Chordin expression, and Dpp represses Pentagone. The geometry of these systems also differ: Nodal and Lefty are expressed in largely overlapping domains; the re-distribution of BMP to the ventral side by Chordin is effective because Chordin expression is localized to the dorsal side, and Pentagone is expressed at the distal end of the Dpp gradient. Thus, the Nodal/Lefty system does not appear to be an example of a shuttling or expansion-repression system. It could be interesting to explore in more detail whether such a system could give rise to scaling or could theoretically possess other interesting properties that could be tested experimentally.

Further, although FCCS experiments suggest that Nodal and Lefty may bind in the extracellular space (see Chapter 4), it is unclear whether this binding is reversible. If binding is reversible, then Lefty may act (somewhat counter-intuitively) to expand the range of Nodal signaling by transporting and depositing active Nodal ligand farther from its source than it would otherwise move. Consistent with that idea, I was able to demonstrate that Cyclops-GFP forms a longer-range gradient when Lefty is overexpressed compared to a non-overexpression scenario. On the other hand, if binding is irreversible, Lefty binding to Nodal could be viewed as a mechanism to remove or “degrade” Nodal—bound Nodal would be functionally inert and effectively dead. In this case, the apparent enhanced diffusivity of Nodal in the presence of Lefty may not have biological relevance, and may merely reflect higher diffusivity of biologically inert molecules. In either case, Lefty-mediated enhancement of Nodal diffusivity should
reduce the concentration of active Nodal near the Nodal source, as bound Nodal would move away from the source more rapidly than unbound Nodal.

HOW AND WHY DO CYCLOPS CLUSTERS FORM?

Many ligands and receptors important for developmental patterning, including Wg, Dpp, FGF, and Cyclops, form membrane-associated clusters or puncta (Entchev et al. 2000, Müller et al. 2012, Strigini & Cohen 2000, Yu et al. 2009). Some of these puncta represent endocytosed ligand/receptor complexes that are internalized to prolong signaling, recycle receptor back to the membrane, and/or degrade signals (Chen 2009). Other puncta represent extracellular clusters of signaling molecules bound to receptors. Such clustering has been proposed to enhance the bioactivity of ligands (Conway et al. 2013), facilitate retention of ligand by reducing the effective dissociation rate (Gopalakrishnan et al. 2005), or have more complex effects on signaling (Mugler et al. 2012).

In the case of Cyclops-GFP, it is not clear how or why membrane-associated clusters form, or even whether these clusters represent extracellular, receptor-bound Cyclops, or endocytosed Cyclops en route to degradation. The fact that the introduction of extracellular, exogenous heparin causes the appearance of diffuse, extracellular Cyclops-GFP (Fig. 4.1) suggests that Cyclops-GFP clusters are extracellular. The clustering of an HSPG core protein fused to GFP (Knypek-GFP) induced by Cyclops overexpression (Fig. 4.3) suggests that HSPGs may be involved in the clustering of Cyclops. In addition, Cyclops overexpression also induces clustering of Squint-, Lefty1-, and Lefty2-GFP (Fig. 4.1), but the biological relevance of this clustering is not yet clear.
The factors responsible for the clustering of Cyclops are also unknown. I have found that Cyclops-GFP still forms clusters in maternal-zygotic oep mutants, in which the Nodal coreceptor that is required for Nodal binding to the receptor complex is missing. Indeed, the only treatment I found that fully dissolved Cyclops-GFP puncta was extracellular injection of collagenase, which tended to fully dissolve the embryo as well.

Recent advances in high-resolution light microscopy such as STORM/PALM imaging (Bates et al. 2007, Betzig et al. 2006) may help resolve questions about why and how Cyclops-GFP clustering occurs. We have already generated functional Cyclops-Dendra2 fusions (Müller et al. 2012) that could be utilized in PALM studies. It will likely be informative to co-label candidate molecules involved in Cyclops clustering (e.g. HSPGs) and observe clusters at nanometer resolution to learn more about the substructure of these puncta. In addition, experiments using standard confocal microscopy in live embryos could be performed to examine the turnover within puncta: individual Cyclops-Dendra2 clusters could be photoconverted, and the recovery of the green signal monitored over time. Clusters could also be tracked embryo-wide using Selective Plane Illumination Microscopy (SPIM) (Huiskken 2004, Keller et al. 2008) in order to determine how clusters develop, degrade, and move. Some reports suggest that signaling molecules may be transported through tissues in lipoprotein particles (Eaton 2008) (reviewed in Rogers & Schier 2011, Chapter 1); it is possible that Cyclops-GFP clusters represent packaged molecules diffusing through the tissue. Careful studies of Cyclops clusters can provide insight into how and why ligand clusters form.
WHAT IS THE ROLE OF FEEDBACK INHIBITION?

A common patterning mechanism employed during embryogenesis is the formation of a signaling gradient within a tissue that is eventually translated into distinct cell fates as a function of signal exposure. However, a simple signaling gradient is insufficient to explain the observed precision, robustness, and reproducibility of embryogenesis (Rogers & Schier 2011). Signaling gradients alone cannot explain the ability of some patterning mechanisms to adjust to changes in the size of the patterning field, a developmental phenomenon known as scaling (see Chapter 1). In addition, given the noise associated with gene expression and protein production, the expected fluctuations in signal production should cause patterning disruptions, which are not observed. Patterning systems must also deal with environmental perturbations like temperature fluctuations, changes in oxygen availability, or injury during development. Tissue inhomogeneity, growth, cell movement, and cell-to-cell variability in sensing and responding to signaling all present further challenges to signaling gradient-based patterning systems.

Feedback inhibition is a common feature of gradient-based patterning mechanisms, and may help explain the robustness and reproducibility of embryogenesis. Signaling often activates expression of negative regulators that inhibit signaling via a variety of mechanisms, which ultimately helps stabilize or adjust the shape of the signaling gradient. The Wnt ligand Wingless induces expression of a secreted Wingless inhibitor, Wingful/Notum, as well as a cytoplasmic signaling inhibitor called Naked cuticle that are thought to shape the range and slope of the Wingless signaling gradient (Gerlitz & Basler 2002, Piddini & Vincent 2009). The Dpp ligand represses expression of
its receptor Tkv, desensitizing cells to signaling while simultaneously allowing Dpp to move farther from its source (Lecuit & Cohen 1998). Signaling by retinoic acid induces the expression of an intracellular enzyme, Cyp26a1, that degrades retinoic acid; this patterning mechanism is remarkably insensitive to absolute retinoic acid levels and distribution (reviewed in Schilling et al. 2012). The long-range inhibitor Argos is induced by the TGF-α-like ligand Spitz; remote inhibition by Argos ensures proper fate specification in the developing Drosophila eye (reviewed in Freeman 1997). The ability of the dorsal half of bisected frog embryos to develop normally—that is, to exhibit scaling—is thought to involve feedback inhibition of the BMP ligand Admp on its own expression (Ben-Zvi et al. 2008) (see Chapter 1). Finally, modeling demonstrates that self-enhanced degradation resulting from feedback inhibition can buffer fluctuations in signal production and help maintain an appropriate signaling gradient (Barkai & Shilo 2009, Eldar et al. 2003, Lander et al. 2009). Thus, feedback inhibition is a common component of gradient-mediated patterning and has been proposed to enhance developmental robustness, often by adjusting or holding steady the shape of a signaling gradient.

SHH signaling induces the expression of extracellular, membrane-bound factors (Ptc1 and Hhip1) that dampen the ability of cells to sense to SHH, limit its spread, and cause degradation of SHH; further, SHH signaling represses the expression of other membrane-bound extracellular factors (Cdo, Boc, and Gas1) that enhance sensitivity to SHH (reviewed in Ribes & Briscoe 2009). This feedback inhibition plays a role in the temporal adaptation to SHH signaling that is required for precise patterning of the neural tube. In Ptc1 mutants, the ventral domain (normally specified by high SHH levels) is
dramatically expanded. Ptc1 mutants can be rescued to a large extent by ubiquitously expressing low levels of Ptc1 from a non-SHH-responsive promoter, thus decoupling feedback and inhibition. However, the normally sharp borders between gene expression domains are less sharp when Ptc1 expression is decoupled from SHH signaling, suggesting that feedback inhibition is required for robust border formation. (Note that other feedback inhibition mechanisms that are independent of Ptc1 also regulate SHH signaling (e.g. Hhip1 expression and repression of Cdo, Boc, and Gas); therefore, some of the roles of feedback inhibition may be masked in these experiments.)

The presence of feedback inhibition is initially counterintuitive—why employ a complicated system of signals and inhibitors when a single signal should suffice? However, although a single signaling gradient is technically sufficient for patterning, this method may fail under real-world conditions. It is possible that rapid feedback inhibition by Lefty acts as a developmental “thermostat” to regulate Nodal signaling levels and ensure that the appropriate amount of Nodal signaling is maintained without requiring absolute Nodal levels to be fixed. This is consistent with the fact that lefty expression is much more responsive to Nodal signaling than other Nodal target genes (Fig. 5.3), that patterning is highly robust to nodal and lefty gene dosage (Fig. 5.1) (Dougan 2003, Feldman et al. 1998, Pei et al. 2007), and that only a narrow range of Nodal inhibitor drug concentrations rescues loss of lefty (Fig. 5.6). This suggests that the relatively complicated Squint/Cyclops/Lefty1/Lefty2 patterning system could provide hidden robustness benefits that require feedback mechanisms between activator and inhibitor. Experiments exploring the consequences of different stresses in the absence of negative feedback will be necessary to test the idea that feedback inhibition improves robustness
against fluctuations in Nodal signaling levels, size differences, or environmental insults (e.g. temperature, osmolarity, oxygen content, etc.). In addition, future experiments assessing mesendodermal gene expression in nodal<sup>−/−</sup>;lefty1<sup>−/−</sup>;lefty2<sup>−/−</sup> mutants and drug- or transplantation-rescued double lefty mutants could provide insights into how these treatments compensate for lefty loss.

It is also unclear whether fluctuations in Nodal signaling are actually a significant challenge during normal embryogenesis. Is it difficult for embryos to consistently produce a specific amount of Nodal, and therefore easier to create a system in which a feedback inhibitor adjusts signaling? Do Nodal levels frequently deviate from the average in wild-type embryos—i.e., is Nodal production highly variable from embryo to embryo? Temperature appears to affect Nodal signaling activity (Pei et al. 2007), and embryos could reasonably be expected to face fluctuations in temperature during embryogenesis in the wild. Further observation and experimentation will be required to test the variability of Nodal signaling during embryogenesis.

**WHAT DETERMINES THE TRANSCRIPTION RATES OF NODAL TARGET GENES?**

*leftys* are upregulated early and dramatically in *lefty* double mutants (Fig. 5.3). In contrast, upregulation of other Nodal target genes in *lefty* double mutants is usually not observed until later. This is consistent with the recent finding that the estimated transcription rate of *leftys* in response to Nodal signaling is higher than those of other Nodal target genes (Dubrulle et al., submitted). The relatively robust expression kinetics of *lefty* may allow inhibition to proceed on a timescale that is rapid enough to adjust Nodal levels before inappropriate signaling is converted into aberrant gene expression. It
might be informative to swap the responsive *lefty* promoters with those of less responsive Nodal target genes to examine how this might affect germ layer patterning. Would less responsive feedback inhibition result in problems during embryogenesis, or the inability to respond to fluctuations in Nodal signaling?

The mechanisms by which differences in target gene responses to Nodal signaling are achieved remain elusive. *leftys* are not short genes compared to other Nodal targets, and do not contain more or higher-affinity binding sites for pSmad2 or FoxHI, transcriptional effectors of Nodal signaling (Laila Akhmetova, personal communication). Understanding how the transcription rates of Nodal target genes are determined has broader implications for other aspects of germ layer patterning: it has been proposed that the different apparent ranges of Nodal target genes is due to differences in target gene transcription rates (Dubrulle et al., submitted). There are many mysteries remaining at the level of interpretation of Nodal signaling.

**HOW IS THE REDUCTION OF MESENDOERMAL GENE EXPRESSION IN DRUG-RESCUED LEFTY DOUBLE MUTANTS ACHIEVED?**

*lefty* double mutants can be rescued to adulthood by exposure to the Nodal inhibitor drug SB-505124 during early embryogenesis (Fig. 5.6 and 5.7). Rescue by drug exposure is most likely due to a reduction in the upregulation of mesendodermal genes in *lefty* mutants (Fig. 5.7). Expression of mesendodermal genes appears to be normal in *lefty* double mutants after ~5 hours of exposure to 5 µM SB-505124 starting at gastrulation, although these genes are upregulated in treated mutants prior to this (Fig. 5.7). How is the number of mesendodermal cells adjusted in this scenario? Below I propose three possible mechanisms.
One possibility is that all cells specified as mesendoderm in *lefty* double mutants retain their identity, even cells that would not normally have acquired mesendodermal fates at earlier stages (similar to the “ratchet” mechanism discussed in Chapter 1). In this case, the rate at which new cells are specified as mesendoderm would be lower in drug-treated mutants compared to wild-type embryos, compensating for the early increase in specification. A second possibility is that the cells that were inappropriately specified as mesendoderm in *lefty* mutants lose their mesendodermal identity a few hours after application of the drug. These cells may not have received sufficient Nodal signaling to maintain expression. Here, the rate at which new cells are specified as mesendoderm would be similar to the wild-type rate. A third possibility is that cells that were inappropriately specified as mesendoderm in *lefty* mutants die a few hours after application of the drug. These cells may somehow be recognized as aberrant and encouraged to undergo apoptosis. Again, the rate at which new cells are specified as mesendoderm would be similar to the wild-type rate in this scenario.

To distinguish between these possibilities, it would be useful to have a dynamic live reporter of mesendodermal gene expression. Live imaging could show when each cell first starts (or stops) expressing mesendodermal genes. In addition, TUNEL staining could show whether there are more apoptotic cells in drug-rescued double mutants compared to wild-type embryos, which would support the third scenario. Whatever the correction mechanism, it happens within 5 hours of drug exposure. It could be interesting to remove the drug at different points to determine when quenching of Nodal signaling is no longer necessary. Understanding how this adjustment is made may provide insight
into the strategies used during normal embryogenesis to ensure correct germ layer patterning.

WHAT ARE THE IN VIVO DISTRIBUTIONS OF NODAL AND LEFTY?

Many open questions about the Nodal/Lefty patterning system, including several of those discussed above, could be addressed or informed by direct observation of in vivo Nodal and Lefty distributions. Although the prevailing model of Nodal-mediated germ layer patterning postulates an animal-vegetal gradient of Nodal, this proposed endogenous gradient has never been directly observed. Previous efforts by our lab to generate antibodies targeting Nodal and Lefty have been unsuccessful. I therefore attempted to generate transgenic embryos carrying GFP-tagged Nodal and Lefty under the control of endogenous expression elements using a genome editing technique called OBLIGARE (Maresca et al. 2013). However, I was unable to recover usable zebrafish lines due to sloppy insertion and low efficiency. As genome editing techniques improve, however, I am optimistic that transgenics will eventually be generated. We have already validated the functionality of Nodal/Lefty-GFP fusions (Müller et al. 2012), and I have observed even better activity with superfolder GFP (sfGFP) (Pèdelacq et al. 2005) Nodal/Lefty fusions. Nodal/Lefty-sfGFP transgenics could open up new and exciting avenues of research in the field of patterning biology.
APPENDIX

Human *lefty2* variants associated with atrial fibrillation are weak Nodal inhibitors

PREFACE

This work was done in collaboration with Diane Fatkin at the Victor Chang Cardiac Research Institute in Australia and Gunjan Trivedi, a postdoctoral fellow in the Fatkin lab. Gunjan Trivedi cloned the human *lefty2* variants. I designed, performed, and interpreted the zebrafish experiments in the lab of Alexander F. Schier.

INTRODUCTION

Dysregulation of Nodal signaling is implicated in multiple human diseases such as cancer and situs inversus (mirroring or mis-positioning of bilaterally asymmetric internal organs). Certain allelic variants of the Nodal inhibitor *lefty* and down-regulation of *lefty* and have been associated with congenital heart disease in humans (Deng et al. 2014, Su et al. 2013). The Fatkin lab at the Victor Chang Cardiac Research Institute in Australia uncovered several non-synonymous SNPs in human *lefty2/EBAF/LEFTYA* that are over-represented in populations that manifest adult-onset atrial fibrillation (AF), a heart arrhythmia that can result in deleterious symptoms for affected individuals.

RESULTS

It is unclear why these *lefty* variants are associated with AF, but it is possible that their ability to inhibit Nodal signaling differs from wild-type *lefty*, affecting Nodal
signaling levels and leading to altered cardiac development and subsequent pathology. To determine whether Leftys encoded by these variants have different Nodal inhibitory activity than variants not associated with disease, I collaborated with the Fatkin lab to test their activity in zebrafish embryos. I previously established assays to test the activity of various Lefty-FP fusions (see Chapter 2 (Müller et al. 2012)), which I adapted to test the activity of the human lefty variants.

Although human and zebrafish lefty are only 32-35% identical (Bisgrove et al. 1999), overexpression of the wild-type human lefty2 in zebrafish embryos results in repression of Nodal target genes and phenocopies Nodal loss of function, though equimolar amounts of zebrafish lefty2 mRNA are more potent in this context (Fig. A.1). (Although both zebrafish and humans have two lefty genes, it is thought that they are the result of independent gene duplication events in ray-finned fish and mammals, respectively (Hashimoto et al. 2007).) I then tested the ability of the human lefty variants associated with AF to inhibit Nodal signaling in this assay. Both AF-associated lefty variants were weaker Nodal inhibitors than wild-type human lefty (Fig. A.1), implicating excess Nodal signaling in the pathology of some cases of AF. The mechanisms connecting possible excess Nodal signaling and congenital heart defects, however, remain obscure.
Figure A.1: Human \textit{lefty2} variants associated with atrial fibrillation are weak Nodal inhibitors. Zebrafish embryos were injected at the one-cell stage with the indicated amounts of human or zebrafish \textit{lefty2}. P286L and S92L are human \textit{lefty2} variants that the Fatkin lab identified as associated with atrial fibrillation. At 50\% epiboly, embryos were snap-frozen in liquid nitrogen and processed for qRT-PCR. Expression of the Nodal target gene \textit{goosecoid} was assessed as in Müller et al. 2012. The P286L and S92L variants are weaker inhibitors of \textit{goosecoid} expression than wild-type \textit{lefty2}; however, zebrafish \textit{lefty2} was the strongest \textit{goosecoid} inhibitor in this context. Error bars represent standard error.
Lefty2 is a stronger Nodal inhibitor than Lefty1

Zebrafish lefty1 and lefty2 are 70% identical, and their expression patterns overlap but are non-identical (Bisgrove et al. 1999). It is not clear whether lefty1 and lefty2 have distinct or merely overlapping roles during embryogenesis (although lefty1, but not lefty2 mutants exhibit heart laterality defects, see Fig. A.29), and whether their inhibitory activities differ.

To determine the relative abilities of lefty1 and lefty2 to inhibit Nodal, I compared the ability of Lefty1-GFP and Lefty2-GFP to inhibit the expression of Nodal target genes. I injected embryos at the one-cell stage with Alexa546-dextran and different amounts of mRNA encoding either Lefty1-GFP or Lefty2-GFP, measured extracellular fluorescence intensity at sphere stage (using the intracellular Alexa546 signal to mask intracellular fluorescence, similar to the masking technique described in Chapter 3), then snap-froze sibling embryos in liquid nitrogen at 50% epiboly. I then performed qRT-PCR with probes targeting the Nodal target gene no tail. At identical extracellular fluorescence intensities, Lefty2-GFP consistently inhibited no tail more strongly than Lefty1-GFP (Fig. A.2). Assuming that the average extracellular GFP intensity reflects the average concentration of extracellular Lefty molecules, this demonstrates that zebrafish Lefty2 is a stronger Nodal inhibitor than zebrafish Lefty1.
Figure A.2: Lefty2-GFP is a stronger Nodal inhibitor than Lefty1-GFP. (A) Diagram of experiment. Embryos were injected at the one-cell stage with different amounts of lefty1- or lefty2-gfp mRNA. Extracellular GFP intensity was quantified at 5 hpf, and sibling embryos were collected at 50% epiboly. qRT-PCR using primers targeting the Nodal target gene no tail (ntl) was used to assess inhibitory activity. (B) Average ntl expression is plotted against average extracellular intensity. At similar intensities, Lefty2-GFP consistently repressed ntl expression more effectively than Lefty1-GFP.
Supplementary materials for Chapter 2: Differential Diffusivity of Nodal and Lefty Underlies a Reaction-Diffusion Patterning System

PREFACE

Our study measures the distribution, diffusivity and clearance kinetics of the two Nodal signals Squint and Cyclops and the two Lefty proteins Lefty1 and Lefty2 in living zebrafish embryos. This supplementary document is divided into eight sections and contains detailed methods and discussions regarding 1) the influence of clearance and diffusion on pattern formation in reaction-diffusion systems, 2) Nodal and Lefty as a reaction-diffusion patterning system, 3) the generation and characterization of active fusion proteins, 4) measurements of distribution profiles, 5) measurements of clearance rate constants, 6) measurements of effective diffusion coefficients, 7) modeling of gradient formation, and 8) comparison of the Nodal/Lefty system to other reaction-diffusion systems.

1. INFLUENCE OF CLEARANCE AND DIFFUSION ON PATTERN FORMATION IN REACTION-DIFFUSION SYSTEMS

Summary

Classical reaction-diffusion models postulate that spatial patterning can be mediated by short-range activators and long-range inhibitors. One of the central tenets of reaction-diffusion models is that pattern formation depends critically on the relative diffusivities of the activator and inhibitor: the activator must be several fold less diffusive than the inhibitor for patterning to occur. Here, we illustrate the contributions of diffusivity and clearance on pattern formation using the Meinhardt-Gierer activator/inhibitor system as an example. Differential diffusivity, not clearance, of
activator and inhibitor is absolutely required for pattern formation to occur, but both diffusion and clearance can influence the probability of patterning.

Reaction-diffusion models describe how chemical reactions and dispersal by diffusion lead to spatial and temporal concentration changes (Gierer & Meinhardt 1972, Kondo & Miura 2010, Turing 1952, Mendez et al. 2010). The ability of a reaction-diffusion system to generate a spatial pattern depends on the parameter values of the system. These parameters include activator and inhibitor diffusion coefficients, clearance rates, cross-reaction kinetics, a measure of the geometry, as well as initial and boundary conditions (Murray 2003, Arcuri & Murray 1986). Murray stated that “[...] it is the orchestration of several effects which produce pattern, not just one, since we can move into the pattern formation regime by varying one of several parameters. Clearly we can arrive at a specific point in the space by one of several paths. The concept of equivalent effects, via parameter variation, producing the same pattern is an important one in the interpretation and design of relevant experiments associated with any model. It is not a widely appreciated concept in biology." (Murray 2003). In particular, it has been demonstrated that the values of the diffusion coefficients and clearance rate coefficients both strongly affect the ability of a reaction diffusion system to generate patterns (Granero et al. 1977, Koch & Meinhardt 1994, Murray 1982).

The general two-component reaction-diffusion system is described by

\[
\frac{\partial U}{\partial t} = D_U \nabla^2 U + F(U, V) \tag{1}
\]

\[
\frac{\partial V}{\partial t} = D_V \nabla^2 V + G(U, V) \tag{2}
\]
Several biologically reasonable reaction systems have been considered, including the Schnakenberg (Schnakenberg 1979), Thomas (Thomas 1976) and Meinhardt-Gierer (Gierer & Meinhardt 1972, Granero et al. 1977, Koch & Meinhardt 1994) systems. Here, we consider the classical Meinhardt-Gierer activator/inhibitor system. Note that this is one of many reaction-diffusion systems (e.g. Christley et al. 2007, Kondo & Asal 1995, Lengyel & Epstein 1991, Nakamura et al. 2006, Sick et al. 2006, Yamaguchi et al. 2007), and the domains in parameter space in which patterns are obtained depend on the equations that describe the reactions. However, all systems absolutely require differential diffusivity of activator and inhibitor for pattern formation, as demonstrated below. The reactions in the classical Meinhardt-Gierer system are described by the equations

\begin{align}
F(U, V) &= \rho_U \frac{U^2}{V} - k_U U + \sigma_U \\
G(U, V) &= \rho_V U^2 - k_V V
\end{align}

where $k_U$ and $k_V$ are the clearance rate coefficients and $\rho_U$ and $\rho_V$ are the cross-reaction coefficients of the activator $U$ and the inhibitor $V$. $\sigma_U$ is a constant production term for the activator.

In order to determine which conditions lead to patterns, we consider the one-dimensional system with no-flux boundary conditions and some initial conditions. We non-dimensionalize the system following the analysis by Koch and Meinhardt (Koch & Meinhardt 1994). By forming dimensionless groups, we define the new dimensionless variables:
Substituting these into the original equations gives the dimensionless form of the system
\[
\frac{\partial u}{\partial t} = D\tilde{\nabla}^2 u + f(u, v) \\
\frac{\partial v}{\partial t} = \tilde{\nabla}^2 v + g(u, v)
\]
where
\[
f(u, v) = \frac{u^2}{v} - u + \sigma \\
g(u, v) = k(u^2 - v)
\]
with \( D = \frac{D_u}{D_v} \), \( k = \frac{k_v}{k_u} \), \( \sigma = \frac{\rho_v \sigma_u}{k_v \rho_u} \), and \( \tilde{\nabla}^2 = \frac{\partial^2}{\partial z^2} \).

If we ignore diffusion and only consider the reactions, the system reaches a steady state with uniform concentrations of the two species. The system has a single steady state \((u_0, v_0)\) at
\[
u_0 = 1 + \sigma \\
v_0 = (1 + \sigma)^2 = u_0^2
\]
To determine its stability, we linearize the system about the steady state. The steady state is stable when the real part of the eigenvalues \( \lambda \) of the linearized system is less than zero, i.e. \( \text{Re}(\lambda) < 0 \). Given the form of the characteristic polynomial of the system and the requirement that it be equal to zero
\[
\lambda^2 - (f_u + g_v)\lambda + (f_u g_v - f_v g_u) = 0
\]
the steady state is guaranteed to be stable when

\[
\begin{align*}
tr(J) &= f_u + g_v < 0 \\
det(J) &= f_u g_v - f_v g_u > 0
\end{align*}
\]  

(7) (8)

where the Jacobian is

\[
J = \begin{bmatrix}
\frac{\partial f}{\partial u} & \frac{\partial f}{\partial v} \\
\frac{\partial g}{\partial u} & \frac{\partial g}{\partial v}
\end{bmatrix} = \begin{bmatrix}
f_u & f_v \\
g_u & g_v
\end{bmatrix}
\]

For our choice of reaction system in Equations 5 and 6, the components of the Jacobian are

\[
\begin{align*}
f_u &= \frac{2u}{v} - 1 \\
f_v &= -\frac{u^2}{v^2} \\
g_u &= 2k u \\
g_v &= -k
\end{align*}
\]

and the stability conditions from Equations 7 and 8 become

\[
\begin{align*}
tr(J) &= \frac{2u}{v} - k - 1 < 0 \\
det(J) &= \frac{k(-2uv + v^2 + 2u^3)}{v^2} > 0
\end{align*}
\]

Turing demonstrated that spatially inhomogeneous patterns can arise from diffusion-driven instabilities (Turing 1952). The conditions which give rise to these instabilities are determined by considering the full linearized reaction-diffusion system. For the reactions only, we determined the stability of the system when \( Re(\lambda) < 0 \). For spatial patterns caused by diffusive instabilities, we need to find \( Re(\lambda) > 0 \) for the full system. Here, \( \lambda = \lambda(q) \), where \( q \) is any of the wavenumbers of the eigenfunctions on the domain, implying that certain modes can drive diffusive instabilities. The modes that do so are found by computing when the determinant of the linearized system is equal to zero. Given the requirement on the characteristic polynomial of the system.
instabilities arise if either of the following conditions are true:

$$\lambda(q)^2 + \lambda(q)[q^2(1 + D) - f_u - g_v] + \left[Dq^4 - (f_u + Dg_v)q^2 + f_u g_v - f_v g_u\right] = 0$$

where $q$ is not equal to 0. Given Equations 7 and 8 and the positive values of $D$ and $q$, the first of these two conditions cannot be satisfied. Thus, the only way that $\text{Re}(\lambda(q)) > 0$ for some $q$ is if the second of these conditions is satisfied, which is only possible if

$$f_u + D g_v > 0$$

In conjunction with Equation 7, it follows that is necessary that $D < 1$ for this to be satisfied. Thus, the diffusivity $D_Y$ of the inhibitor must be greater than the diffusivity $D_U$ of the activator. This difference in activator and inhibitor diffusivities is a necessary but not sufficient condition for pattern formation; in addition, the minimum of $h(q^2)$ must be negative. This minimum is obtained by differentiating $h(q^2)$ and setting it equal to zero. Solving for $q^2$, we find that

$$q^2 = \frac{f_u + D g_v}{2D}$$

which substituted into the original expression for $h(q^2)$ gives the minimum $h$ at

$$f_u g_v - f_v g_u - \frac{(f_u + D g_v)^2}{4D}$$

For the above minimum of $h$ to be negative

$$f_u g_v - f_v g_u < \frac{(f_u + D g_v)^2}{4D}$$

In summary, from Equations 7, 8, 9 and 10, the four conditions that have to be fulfilled by a reaction-diffusion system to give rise to patterns are thus (Murray 2003):
Written in terms of the dimensionless Meinhardt-Gierer system that we consider, we have

\[
\begin{align*}
    f_u + g_v < 0 \\
    f_u g_v - f_v g_u > 0 \\
    f_u + D g_v > 0 \\
    \frac{(f_u + D g_v)^2}{4D} - (f_u g_v - f_v g_u) > 0
\end{align*}
\]  

(Equations 11, 12, 13, and 14)

Evaluated at the steady state \((u_0, v_0)\), these conditions become

\[
\begin{align*}
    \frac{2u}{v} - 1 - k < 0 \\
    \frac{k(-2uv + v^2 + 2u^3)}{v^2} > 0 \\
    \frac{2u - v}{Dv} - k > 0 \\
    \left( \frac{2u - v}{Dv} - k \right)^2 - \frac{4k(-2uv + v^2 + 2u^3)}{Dv^2} > 0
\end{align*}
\]

(Equations 15, 16, 17, and 18)

Pattern formation can occur for values of the parameters \(D\), \(k\) and \(\sigma\) that satisfy the above conditions. Using the dimensionless groups defined above, \(D\) is the ratio of the diffusion coefficients and \(k\) is the ratio of the clearance rate constants, whereas \(\sigma\) is a more complicated ratio involving the cross-reaction kinetics, the production of the activator, and the degradation of the inhibitor. Given the complexity of \(\sigma\) and the unknown values of the quantities it involves, we chose to explore the pattern forming capacity of \(D\) and \(k\) over reasonable ranges of values. To do so, we tested the above four conditions (Equations 15, 16, 17 and 18) over a linearly spaced sampling of floating point values of parameter space of \(D\), \(k\), and \(\sigma\). The positive \((= 1)\) or negative \((= 0)\) outcome of the
testing of these conditions at each value triplet was averaged over all values of \( \sigma \), and projected into a two dimensional representation of parameter space, interpreted as a probability of pattern forming capacity of the system (Fig. A.3). Importantly, patterns in this system cannot be formed with equal diffusivities of activator and inhibitor; the inhibitor must be at least \(~6\)-fold more diffusive than the activator. As the ratio of \( D \) decreases, the probability that a system is capable of pattern formation increases. This probability also increases as the ratio of \( k \) approaches unity (assuming \( D \leq 0.2 \)). Interestingly, even a system with a very low \( D \) value may not form a Turing pattern if the value of \( k \) is very high or very low.

In conclusion, the ability of a reaction-diffusion system to form patterns is absolutely dependent on an inhibitor that is more diffusive than the activator, but both diffusion and clearance can influence the probability of patterning. To test the central tenet of reaction-diffusion models postulating differential diffusivity, we measured both the diffusion coefficients of the activator and inhibitor as well as the clearance rate constants. We found that the inhibitor Lefty has a much higher diffusion coefficient than the activator Nodal, whereas clearance is similar for both activator and inhibitor. Our findings therefore experimentally support the mathematical predictions of reaction-diffusion models of pattern formation.
2. NODAL AND LEFTY AS A REACTION-DIFFUSION PATTERNING SYSTEM

Summary

Reaction-diffusion systems involving auto-regulatory short-range activators and activator-induced long-range inhibitors can generate patterns ranging from simple gradients to stripes and spots depending on initial conditions, parameter values and boundary conditions (Gierer & Meinhardt 1972, Kondo & Miura 2010, Meinhardt & Gierer 2000). In this section, we describe the previously identified properties of the Nodal/Lefty system that have led to its designation as a reaction-diffusion patterning system (Chen & Schier 2002, Duboc et al. 2004, 2008; Kondo & Miura 2010, Meinhardt 2009, Saijoh et al. 2000, Schier 2009, Shen 2007, Shiratori 2006).

2006, Wright 2001, Yaguchi et al. 2008). For example, Meinhardt has highlighted the Nodal/Lefty system as one of the first biological examples of an activator/inhibitor reaction-diffusion system (Meinhardt 2009), Kondo and Miura have stated that the Nodal/Lefty interaction “[...] indicates that this system fulfills the fundamental requirements for Turing pattern formation [...]” (Kondo & Miura 2010), Hamada and colleagues used reaction-diffusion models to simulate left-right patterning by Nodal/Lefty (Nakamura et al. 2006), and Horsthemke has stated that “Nodal and Lefty fulfill Turing's requirement of local self-activation and long-range inhibition.” (Mendez et al. 2010).

Closer analysis of the Nodal/Lefty reaction-diffusion patterning system in developmental contexts shows that it is influenced by additional constraints: Nodal and Lefty expression is biased by pre-patterns, and the tissue response is restricted by size and time scales. These constraints likely allow the generation of highly reproducible patterns during embryogenesis rather than the complex de novo self-organizing patterns found in some other incarnations of the reaction-diffusion model. For example, during germ-layer formation, Nodal signals act as short- to mid-range inducers of endodermal and mesodermal fates, whereas Lefty signals act as long-range antagonists to prevent Nodal signaling and promote ectoderm formation at a distance. The zebrafish Nodal signals Cyclops (short-range) and Squint (mid-range) are expressed at the blastula margin and induce mesendodermal target genes (Chen & Schier 2001, Schier 2009). At the blastula margin, Nodal signals also induce their own expression as well as the expression of Lefty1 and Lefty2 (Chen & Schier 2002). Lefty signals inhibit mesendoderm induction by blocking Nodal signaling (Chen & Shen 2004, Cheng et al. 2004) (Fig. 2.1A). Loss of
Nodal signaling leads to the transformation of mesendodermal progenitors into ectodermal progenitors, whereas loss of Lefty leads to the transformation of presumptive ectoderm into mesendoderm (Feldman et al. 1998, 2002; Schier 2009). Although Nodal and Lefty display local self-activation and long-range inhibition in this context, the reaction-diffusion system is constrained by maternal transcription factors that activate Nodal expression at the blastula margin (Shimizu et al. 2000), by the short time period during which cell fates can be allocated (cells only respond to Nodal signaling for a few hours (Dougan 2003)), and the short length scale of the embryo (~500 µm) compared to the range of the signals (~150 µm) (Chen & Schier 2001, Faure et al. 2000, Gritsman et al. 2000, Harvey & Smith 2009, Lee et al. 2001, Schier 2009). The regulatory and inductive interactions of Nodal and Lefty can be recapitulated at the animal pole of zebrafish embryos. Clones expressing Nodals and Leftys at the animal pole recapitulate the major aspects of patterning induced by endogenous sources: High- and low-threshold target genes are induced in and around Nodal-expressing clones (Chen & Schier 2001), the different ranges of Cyclops, Squint and Lefty proteins are maintained (Chen & Schier 2001, 2002), cell internalization associated with gastrulation can be induced (Carmany-Rampey & Schier 2001, David & Rosa 2001), and cross-regulation of Nodals and Leftys is preserved (Chen & Schier 2002, Feldman et al. 2002, Meno et al. 1999).

Constraints also exist during Nodal-mediated patterning of the left-right axis (Nakamura et al. 2006). During embryogenesis, both the left and the right lateral plate mesoderm initially express low levels of Nodal, but cilia-induced flow in the node generates a pre-pattern that is thought to result in slightly higher expression of Nodal on the left. This initial asymmetry is amplified by Nodal auto-regulation and the induction of
Lefty. The long-range activity of Lefty then suppresses Nodal signaling in the right lateral plate mesoderm. Thus, Nodal/Lefty interactions appear to amplify small differences between left and right lateral plate mesoderm using short-range activation and long-range inhibition. In contrast to mesendodermal patterning, in which graded Nodal signaling specifies multiple cell fates, Nodal signaling during left-right patterning controls the binary decision between left and right (Schier 2009). Similar to mesendodermal patterning, the Nodal/Lefty system during left-right patterning is constrained by pre-patterns, the length scale of the system and the rapid assignment of cell fates.

3. GENERATION AND CHARACTERIZATION OF FLUORESCENT NODAL AND LEFTY PROTEINS

Rationale and summary

To visualize Nodal and Lefty signals in living embryos, we generated GFP and Dendra2 fusion proteins (Fig. 2.2 and Figs. A.4-13). A major concern in studies of signaling molecules is whether the position or the size of the fluorescent tag alters the signaling activity, clearance or dispersal characteristics of the protein. We therefore systematically analyzed dozens of constructs to identify fusion proteins that were active and properly processed. Western blots indicated that the fluorescent fusion proteins were processed and present as mature ligands in the extracellular space (Fig. A.4 and Fig. A.8). qRT-PCR (Fig. A.5) and RNA in situ hybridization analyses (Fig. A.6) indicated that tagged Nodals were potent inducers of Nodal target gene expression, whereas tagged Leftys repressed Nodal target genes (Figs. A.9-A.11). To examine the ranges of the fusion proteins, we generated clones of cells expressing the constructs of interest similar
to previous studies (Chen & Schier 2001, Yu et al. 2009). Reflecting the properties of their untagged counterparts, Cyclops and Squint fusions had short- and mid-range activity (Fig. A.7), respectively, whereas Lefty fusions had long-range inhibitory activity (Fig. A.12).

**Cloning of fusion constructs**

All enhanced GFP (referred to as GFP throughout the text) and Dendra2 fusion constructs were generated by PCR-based methods (Horton et al. 1990) and cloned into the pCS2(+) vector. Briefly, fragments encoding fluorescent proteins or Nodal and Lefty domains were amplified individually and spliced together by PCR using overlapping overhangs. The untagged constructs were cloned into the same restriction sites in pCS2(+) as the fusion constructs. All constructs contain the consensus Kozak sequence gccacc 5' of the start codon.

Cyclops fusions: Sequences encoding fluorescent proteins or the FLAG tag (DYKDDDDK) were inserted two amino acids downstream of the Furin cleavage site (RRGRR) between the pro- and mature domains of Cyclops. To generate Cyclops-FLAG-GFP, the sequence encoding DYKDDDDKLG was inserted between the pro-domain and GFP two amino acids downstream of the Furin cleavage site. The fusion constructs were cloned into the pCS2(+) vector via ClaI and EcoRI restriction sites.

Squint fusions: Sequences encoding fluorescent proteins or the FLAG tag were inserted between the pro- and mature domains of Squint 10 amino acids downstream of the Furin
cleavage site (RRHRR) with a GSTGTT linker separating the prodomain and the fluorescent protein and a GS linker separating the fluorescent protein from the mature domain. To generate Squint-FLAG-GFP, the sequence encoding GSTGTTDYKDDDDKLG was inserted between the prodomain and GFP 10 amino acids downstream of the Furin cleavage site. The fusion constructs were inserted into the pCS2(+) vector via ClaI and EcoRI restriction sites.

Lefty1 fusions: Sequences encoding fluorescent proteins or the FLAG tag were inserted at the C-terminus of full-length Lefty1. An LG linker was used to separate Lefty1 from the FLAG tag, and an LGDPPVAT linker was used between Lefty1 and the fluorescent proteins GFP and Dendra2. To generate Lefty1-GFP-FLAG, the FLAG tag was fused to the C-terminus of GFP separated by an LG linker. The fusion constructs were inserted into the pCS2(+) vector via ClaI and XhoI restriction sites.

Lefty2 fusions: Sequences encoding fluorescent proteins or the FLAG tag were fused to the C-terminus of full-length Lefty2. An LG linker was used to generate Lefty2-FLAG and Lefty2-GFP, and an LGDPPVAT linker was used between Lefty2 and Dendra2. To generate Lefty2-GFPFLAG, the FLAG tag was fused to the C-terminus of GFP separated by an LG linker. The fusion constructs were inserted into the pCS2(+) vector via ClaI and XhoI restriction sites.

Secreted GFP and Dendra2: Sequences encoding fluorescent proteins were fused to the
pro-domain of Squint 10 amino acids downstream of the Furin cleavage site (RRHRR) with a GSTGTT linker following the Furin cleavage site. The fusion constructs were inserted into the pCS2(+) vector via ClaI and XhoI restriction sites.

Global proteome-wide studies in cell culture suggest that fluorescent proteins in general do not affect the degradation dynamics of fusion partners (Eden et al. 2011, Yen & Elledge 2008, Yen et al. 2008). Indeed, the secreted Dendra2 control construct reported here had a significantly higher extracellular half-life than the Nodal fusions (see “Measurement of Clearance Rate Constants”, Fig. A.15, and Table A.3).

Given that the Nodal and Lefty fluorescent fusion proteins have a molecular mass of up to three times higher than the untagged ligands, it can be expected that the tagged ligands are less mobile than the untagged ligands. The Einstein-Stokes equation relates the diffusion coefficient $D$ to the radius $r$ of spherical particles diffusing through liquid with low Reynolds numbers:

$$D = \frac{k_B T}{6\pi \eta r}$$

where $k_B$ is the Boltzmann constant, $T$ is temperature and $\eta$ is the viscosity (Müller & Schier 2011, Berg 1993). With the simplifying assumption that the proteins are perfect spheres and that the volume of the fusion constructs is approximately threefold larger than the volume of the untagged proteins, the radii of the fusion constructs will be $\sim$1.4-fold larger than those of the untagged proteins. Therefore, using the Einstein-Stokes equation, the diffusion coefficients of the GFP fusion proteins is expected to be only $\sim$30% smaller than those of the untagged ligands. This lower diffusivity may be reflected in the slightly reduced activity ranges observed for the fusion constructs (Fig. A.7 and Fig. A.12). However, it is unlikely that the proteins fold into perfect spheres, and
differences in protein conformation and tertiary structure might lead to additional deviations from the ideal behavior described by the Einstein-Stokes equation.

*mRNA synthesis, embryo injections and in situ hybridization*

Capped mRNAs were synthesized using the mMessage mMachine Kit (Ambion) with SP6 RNA polymerase according to the manufacturer's protocol. Vectors were linearized by digestion with NotI. Embryos were dechorionated using 1 mg/ml Pronase (Protease type XIV from Streptomyces griseus, Sigma) prior to injection and subsequently cultured in agarose-coated dishes.

For *in situ* hybridization, embryos were fixed overnight at 4°C using 4% formaldehyde in PBS. *In situ* hybridization and anti-sense probe synthesis for *fascin, no tail* and *goosecoid* probes (Chen & Schier 2001, Choi et al. 2007) was carried out according to standard protocols (Thisse & Thisse 2008).

*Preparation of extracellular protein fractions for western blots*

If degradative processes generate free extracellular fluorescent species by cleaving fusions between the mature ligand and the fluorescent protein, the measurements of the half-lives would likely be overestimates given that free Dendra2 is cleared relatively slowly (Fig. A.15 and Table A.3). Furthermore, if there were significant amounts of free GFP, the recovery dynamics in the FRAP experiments could be dominated by the smaller and highly diffusive free GFP, thereby increasing the apparent diffusion coefficients of the fusion proteins. To determine whether the Dendra2 and GFP fusion proteins were processed correctly (i.e. without releasing free fluorescent species),
extracellularly enriched fractions were purified and analyzed by immunoblotting using anti-Dendra2 and anti-GFP antibodies. As shown in Fig. A.4 and Fig. A.8 no significant amounts of free extracellular Dendra2 or GFP were detected, and the majority of the species fused to Dendra2 or GFP was processed correctly. Therefore, the majority of the extracellular signal in the clearance assay and FRAP experiments likely originated from fusion proteins rather than free Dendra2 or GFP.

Embryos at the one- or two-cell stage were injected with the mRNAs encoding GFP or Dendra2 fusion proteins indicated in Fig. A.4 and Fig. A.8 with mRNA amounts equimolar to 250 pg Squint-GFP mRNA (Cyclops-GFP/Dendra2: 284 pg, Squint-GFP/Dendra2: 250 pg, Lefty1-GFP/Dendra2: 234 pg, Lefty2-GFP/Dendra2: 236 pg, secreted GFP/Dendra2: 209 pg). mRNA encoding FLAG-tagged and FLAG-GFP-tagged constructs were injected at the one- or two-cell stage at equimolar amounts for each protein species (Cyclops-FLAG: 360 pg, Cyclops-FLAG-GFP: 500 pg; Squint-FLAG: 85 pg, Squint-FLAG-GFP: 125 pg; Lefty1-FLAG: 330 pg, Lefty1-GFP-FLAG: 500 pg; Lefty2-FLAG: 330 pg, Lefty2-GFP-FLAG: 500 pg). Embryos were grown at 28°C and manually de-yolked between sphere and dome stages in embryo medium (250 mg/l Instant Ocean salt, 1 mg/l methylene blue in reverse osmosis water adjusted to pH 7 with NaHCO3) in agarose-coated dishes using forceps. The resulting blastoderm caps were washed three times in embryo medium to remove excess yolk. Approximately twenty caps (60 caps for FLAG-tagged constructs) were transferred into 50 or 100 µl deyolking buffer (Link et al. 2006) (a quarter of a protease inhibitor cocktail tablet (Complete Mini, Roche) per 2 ml deyolking buffer was used for the FLAG-tagged constructs) and shaken at 1,100 rpm using an Eppendorf shaker cooled to 4°C. Cells were
then spun down at 300 g for 30 seconds at 4°C, and 40 or 80 µl of the supernatant was transferred to a new tube that was frozen immediately in liquid nitrogen. Protein samples mixed with Laemmli buffer were denatured by incubation for 10 min at 98°C, resolved by SDS-PAGE using 10-12% polyacrylamide gels and transferred to PVDF membranes (GE Healthcare). Membranes were blocked with 5% (3% for FLAG-tagged constructs) non-fat milk (BioRad) in TBST. The membranes were incubated with primary antibodies in 5% (3% for FLAG-tagged constructs) non-fat milk in TBST at 4°C overnight. Anti-GFP (Molecular Probes/Invitrogen) and anti-Dendra2 (obtained from Evrogen and antibodies-online Inc.) antibodies were used at a concentration of 1:5,000, anti-FLAG antibody (Sigma) at a concentration of 1:1,000, and monoclonal anti-β-tubulin antibody (Sigma) at a concentration of 1:25,000. Proteins were detected using HRP-coupled secondary antibodies (goat anti-rabbit and donkey anti-mouse (Jackson ImmunoResearch Labs)) at a 1:25,000 (1:5,000 for FLAG epitope detection) dilution. Chemiluminescence was detected using ECL Plus reagent (Amersham) and imaging film (Kodak BioMax Light). The purification protocol yields an enriched extracellular fraction that contains residual amounts of β-tubulin.

**Analysis of fusion protein activity**

Quantitative reverse transcription PCR: Quantitative reverse transcription PCR (qRT-PCR) was used to assess the activity of the fusion constructs. Embryos were injected with two different amounts of mRNA per construct (see Fig. A.5 and Fig. A.10 for amounts) to assess dose-dependent activation or repression of the Nodal target gene *goosecoid (gsc)* (Bennett et al. 2007, Choi et al. 2007, Gritsman et al. 1999). To correct
for the length differences between constructs, equimolar amounts of tagged and untagged constructs were injected. Ten embryos per sample were frozen in liquid nitrogen, and three samples were obtained per construct. Uninjected embryos and embryos injected with Lefty1 and Lefty2 constructs were frozen at 50% epiboly, at the end of blastula stage. Embryos injected with Nodal constructs arrest during epiboly and were frozen when uninjected siblings reached 50% epiboly. Total RNA was extracted using Trizol reagent (Invitrogen). cDNA was generated using the iScript cDNA Synthesis Kit (Bio-Rad). The zebrafish elongation factor 1-α (eF1α) transcript was used as a normalization control (Tang et al. 2007). qRT-PCR was performed using either the Qiagen QuantiTect SYBR Green PCR Kit or Promega Go-Taq qPCR Master Mix on a Stratagene MX3000p qPCR machine. C\textsubscript{T} values were determined using MxPro software. Fold changes in gsc relative to eF1α levels were calculated using the \textit{ΔΔ}C\textsubscript{T} method (Livak & Schmittgen 2001).

Primer sequences used:

\textit{eF1α} forward: agaaggaagccgctgagatgg
\textit{eF1α} reverse: tccgttcttgagataccagcc
\textit{gsc} forward: gagacgacaccgaaccattt
\textit{gsc} reverse: cctctgacgacgaccttttc

Whole mount \textit{in situ} hybridization: Embryos were injected with the mRNA amounts indicated in Fig. A.6 and Fig. A.11. To correct for the length differences between the constructs, equimolar amounts of tagged and untagged constructs were injected. Uninjected embryos and embryos injected with Lefty1 and Lefty2 constructs were fixed
at 50% epiboly, at the end of blastula stage. Embryos injected with Cyclops and Squint constructs were fixed when uninjected siblings reached 50% epiboly. To assess ectopic induction or repression of Nodal target genes, a probe against the Nodal target gene *goosecoid* (*gsc*) (Bennett et al. 2007, Choi et al. 2007, Gritsman et al. 1999) was used. Using a dissecting microscope, embryos were scored according to the scoring classes shown in Fig. A.6 and Fig. A.11, similar to (Tian et al. 2008). Representative embryos were imaged using an Axio Imager.Z1 microscope (Zeiss). Images were cropped according to the diameter of the imaged embryos.

*Analysis of fusion protein activity range*

Cyclops and Squint constructs: Donor embryos were co-injected with mRNA amounts equimolar to 250 pg of Squint-GFP mRNA (Cyclops-GFP: 284 pg, Squint-GFP: 250 pg, Lefty1-GFP: 234 pg, Lefty2-GFP: 236 pg) and 0.25 mg/ml 10 kDa biotinylated dextran (Molecular Probes) in a volume of 1 nl at the one-cell stage. Embryos developed at 28°C. At sphere stage, embryos were transferred to modified Danieau's medium (0.2 μm filtered solution of 58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO₄, 0.3 mM CaCl₂, 5 mM HEPES pH 7.2, 5% Penicillin/Streptomycin). Approximately 40 to 50 cells from donor embryos were transplanted into the animal pole of wildtype host embryos at sphere stage. Embryos were fixed one hour later and processed for in situ hybridization using *fascin* (Choi et al. 2007) or *no tail* (Chen & Schier 2001) probes. The presence of biotinylated dextran in donor cells was detected using the Elite Vectastain ABC kit (VECTOR Laboratories) and DAB substrate. Stained embryos were imaged using an Axio Imager.Z1 microscope (Zeiss). The area occupied by donor cells and the area occupied by *fascin or no tail* gene
expression was quantified in animal pole views using ImageJ (Abràmoff et al. 2004). The activity range was calculated as the ratio of the *fascin*- or *no tail*-positive area divided by the clone area as shown in Fig. A.7.

Lefty1 and Lefty2 constructs: In order to assess the activity range of the Lefty fusion proteins, clonal sources were generated by injecting mRNAs encoding the Lefty constructs along with biotinylated dextran as an injection tracer in a volume of 100 pl into a single blastomere at the 64- to 128-cell stage. The suppression of the Nodal target gene fascin was assessed by *in situ* hybridization using embryos that were fixed at 50% epiboly stages. Embryos were categorized into classes of *fascin* suppression as shown in Fig. A.12.

*Assessment of fusion proteins*

Cyclops fusions: Western blots of extracellular fractions indicate that the Cyclops fusions are processed to mature ligands (Fig. A.4). Equimolar injections of mRNA encoding Cyclops-FLAG and Cyclops-FLAG-GFP yield similar protein levels (Fig. A.4). The amount of recovered Cyclops protein on the western blot is lower than the amounts recovered for Squint, Lefty1 and Lefty2, potentially owing to its higher clearance (Table A3) or its punctate membrane-associated localization (Fig. A.13). The Cyclops fusions with GFP and Dendra2 had activity similar to untagged Cyclops (Fig. A.5 and Fig. A.6), and their activity range was slightly reduced (Fig. A.7).

Squint fusions: The Squint fusions were processed and present as mature ligands in the
extracellular space (Fig. A.4). Equimolar injections of mRNA encoding Squint-FLAG and Squint-FLAG-GFP yields similar protein levels (Fig. A.4). The Squint fusions had similar activity as untagged Squint (Fig. A.5 and Fig. A.6) and an approximately 30% decreased activity range, consistent with their larger size (Fig. A.7).

Lefty1 fusions: The Lefty1 fusions were present as mature ligands in the extracellular space (Fig. A.8). Equimolar injections of mRNA encoding Lefty1-FLAG and Lefty1-GFP-FLAG yield lower Lefty1-GFP-FLAG protein levels compared to Lefty1-FLAG (Fig. A.8). The Lefty1 fusions were potent long-range repressors of Nodal target gene expression (Figs. A.9-12). The decreased activity and activity range of the Lefty1 fusions compared to untagged Lefty1 at equimolar mRNA amounts is likely due to differences in protein levels (Fig. A.8), since Nodal target gene expression can be suppressed to a similar extent by injecting higher mRNA amounts of the Lefty1 fusions (Figs. A.9-11).

Lefty2 fusions: The Lefty2 fusions were present as mature ligands in the extracellular space (Fig. A.8). Equimolar injections of mRNA encoding Lefty2-FLAG and Lefty2-GFP-FLAG yield lower Lefty2-GFP-FLAG protein levels compared to Lefty2-FLAG (Fig. A.8). The Lefty2 fusions were very potent repressors of Nodal signaling and had long-range activity (Figs. A.9-12). The slightly decreased activity of the Lefty2 fusions compared to untagged Lefty2 at equimolar mRNA amounts is likely due to differences in protein levels (Fig. A.8), since Nodal target gene expression can be suppressed to a similar extent by injecting higher mRNA amounts of the Lefty2 fusions (Figs. A.9-11).
4. MEASUREMENT OF DISTRIBUTION PROFILES

Rationale and summary

Previous embryological and genetic studies have shown that Cyclops, Squint, Lefty1 and Lefty2 have very different ranges of activity during mesendoderm induction, despite similar molecular weights: Cyclops has short-range activity, Squint has mid-range activity, and Leftys have long-range inhibitory activity (Chen & Schier 2001, 2002) (Fig. 2.1A, Fig. A.7, and Fig. A.12). Analogous studies in left-right patterning suggest that Lefty has a longer range than Nodal (Marjoram & Wright 2011, Nakamura et al. 2006). To analyze the in vivo distributions of Cyclops-GFP, Squint-GFP, Lefty1-GFP and Lefty2-GFP, we expressed the fusion proteins (Figs. A.4-13) from a local source in blastula-stage embryos (Fig. 2.2). Fluorescence quantification revealed that Cyclops-GFP formed a short-range gradient, Squint-GFP a mid-range gradient, Lefty1-GFP a long-range gradient and Lefty2-GFP a super-long-range shallow gradient (Fig. 2.2). The distance from the source at which the concentration dropped to 50% of the value at the source boundary was ~20 µm for Cyclops-GFP, ~40 µm for Squint-GFP, ~80 µm for Lefty1-GFP, and ~100 µm for Lefty2-GFP. These distribution profiles are in good agreement with the activity ranges deduced from embryological and genetic studies.

Experimental setup

In order to characterize the protein distribution profiles of Nodals and Leftys, clonal sources of GFP fusion proteins were generated by transplantation. Such clones recapitulate the major aspects of patterning induced by endogenous sources: High- and low-threshold target genes are induced in and around Nodal-expressing clones (Chen &
Schier 2001), the different ranges of Cyclops, Squint and Lefty proteins are maintained (Chen & Schier 2001, 2002), cell internalization associated with gastrulation can be induced (Carmany-Rampey & Schier 2001, David & Rosa 2001), and cross-regulation of Nodals and Leftys is preserved (Chen & Schier 2002, Feldman et al. 2002, Meno et al. 1999).

Donor embryos were injected with mRNA at amounts equimolar to 250 pg of Squint-GFP mRNA at the one-cell stage (Cyclops-GFP: 284 pg, Squint-GFP: 250 pg, Lefty1-GFP: 234 pg, Lefty2-GFP: 236 pg). At sphere stage, approximately 40-50 cells were explanted, left briefly in modified Danieau's medium to allow residual extracellular fluorescent proteins to dissipate, and then transplanted into wildtype host embryos (Fig. 2.2). Embryos were mounted in 1% low-melting point agarose in glass-bottom Petri dishes (MatTek Corporation) with the animal pole facing the coverslip. The dishes were then filled with embryo medium in order to hydrate the agarose during imaging. Embryos were imaged 30, 60 and 120 min post transplantation using a Pascal confocal microscope (Zeiss) with a 25X objective. Images (z-stacks) were acquired in 5 different confocal slices separated by 5 µm at each time point. Imaging earlier than 30 min post-transplantation was not possible due to the handling times involving transplantation, embryo immobilization and sample mounting.

To test whether ectopic expression of the constructs causes saturation of binding sites on cell surfaces that influence the diffusion properties of Nodals and Leftys, 30 pg of mRNA encoding untagged Squint were injected into the host embryos (similar to the concentration used in the FRAP experiments, see “Measurement of Effective Diffusion Coefficients" section), and the transplantation and imaging conditions were as described
above. No significant difference in the Squint-GFP gradients was observed in the absence or presence of untagged Squint, indicating that saturation of binding sites does not occur under these conditions (Fig. A.19).

*Image analysis*

Images were analyzed in ImageJ (Abramoff et al. 2004) as follows: Maximum intensity projections were generated for individual z-stacks comprising five confocal slices, similar to (Kicheva et al. 2007). To measure the fluorescence intensity as a function of distance from the clone, a rectangular region of interest (ROI) with a height of 36.56 µm (corresponding to 52 pixels) abutting the clone was drawn. The width of the ROI differed depending on the size of the embryo. The average intensity in 0.7 µm strips within the ROI was calculated. To subtract background due to auto fluorescence, average intensity profiles were calculated that were extracted from four medial positions each within three ungrafted wildtype embryos, where the z-position and developmental time were matched. These background datasets were truncated at each end to clip off regions where intensities deviate from the baseline due to embryo curvature. The average baseline intensity for background subtraction was then calculated as a single value. The background-subtracted experimental intensity profiles were truncated in the same way as the background data sets. After background subtraction and truncation, an average of 7 µm was binned using a sliding window similar to previous studies (Gregor et al. 2007b, 2008; Grimm et al. 2010, He et al. 2010). The resulting data was normalized to the value closest to the clonal source boundary, and the normalized data was sampled every 3.5 µm as shown in Fig. 2.2. Embryos with low signal-to-noise ratios were excluded from the
5. MEASUREMENT OF CLEARANCE RATE CONSTANTS

Rationale and summary
The clearance kinetics of extracellular signaling molecules can be a major determinant of their distribution: the more quickly a signal is cleared from the extracellular space, the shorter its range (Kicheva et al. 2007, Müller & Schier 2011, Wartlick et al. 2011, Yu et al. 2009). To determine whether the differences between the distributions of Nodal and Lefty signals are due to differences in their clearance kinetics, we measured extracellular half-lives. We developed a pulse-labeling assay to monitor the extracellular clearance of fluorescent fusion proteins (Fig. 2.3 and Fig. A.14). Nodal and Lefty signals were fused to the photoconvertible protein Dendra2 (Hamer et al. 2010, Zhang et al. 2007) (Figs. A.4-13), uniformly expressed in blastula embryos and photoconverted throughout the entire embryo with a short UV pulse. Observation of changes in the photoconverted extracellular signal over time allowed measurement of extracellular protein half-lives (Fig. 2.3 and Fig. A.14). Control experiments indicated that non-uniform photoconversion (Fig. A.16) and photobleaching (Fig. A.18) did not alter measurements, and that the extracellular photoconverted Dendra2 signal was significantly above background (Fig. A.17). Extracellular half-lives between 95 and 218 min were obtained for Nodal- and Lefty-Dendra2 fusion proteins.
Two previous studies have examined the clearance kinetics of Nodal and Lefty signals. Jing et al. used autoradiography to characterize the decay dynamics of radioactively pulse-labeled Cyclops- and Squint-GFP expressed in COS7 tissue culture cells (Jing et al. 2006). Half-lives of two (Cyclops-GFP) and eight hours (Squint-GFP) were identified, but it is unclear whether clearance in a mammalian tissue culture system reflects clearance in zebrafish embryos. Marjoram and Wright introduced tissue grafts expressing Myc-tagged *Xenopus* Lefty and Xnr1 (a *Xenopus* Nodal homolog) into *Xenopus* lateral plate mesoderm, then removed the grafts and used quantitative immunohistochemistry to follow the decrease in Myc signal over time. This analysis revealed half-lives of 45 min for Lefty and 25 min for Xnr1 (Marjoram & Wright 2011), but the observed decrease in signal intensity might be caused by diffusion of labeled protein into deeper layers of the embryo, leading to an apparent shortening of half-lives (see “Uniform Photoconversion” section). Moreover, protein clearance may differ between species (*Xenopus* versus zebrafish) and tissues (lateral plate mesoderm versus blastula), and the inability to distinguish between intra- and extracellular fractions prevents assessment of extracellular clearance. We therefore developed an alternative approach for the measurement of extracellular protein half-lives *in vivo*: Pulse-labeling of photoconvertible fusion proteins allowed clearance kinetics to be examined in live zebrafish embryos.

**Experimental setup**

Since secreted proteins predominantly spread through tissues by extracellular movement (Müller & Schier 2011, Schwank et al. 2011a), extracellular, not intracellular,
clearance is an important determinant of signal range. For example, a signal that is slowly cleared intracellularly but quickly cleared extracellularly will have a shorter range than a signal that is slowly cleared both intra- and extracellularly. To avoid these potential pitfalls, we measured the clearance of photoconverted fusion proteins intracellularly and extracellularly. No significant differences were found between intra- and extracellular half-lives for Nodal-and Lefty-Dendra2 constructs (Table A.3).

To determine intra- and extracellular half-lives, embryos were injected at the one-cell stage with 60 pg of mRNA encoding the Dendra2 fusion constructs along with 0.4-1.9 ng of a 3 kDa Alexa488-dextran conjugate (Invitrogen). The total injection volume was 1 nl. The Alexa488 signal was used during image analysis (see below) to mask cells in order to analyze extracellular or intracellular signal only. Several different tracers were tested, and Alexa488-dextran was selected because it was found to be non-toxic and bright. Injected embryos developed at 28°C and were kept in the dark until mounting between dome stage and 30% epiboly (~5 hours post fertilization) to prevent inadvertent photoconversion. Mounting was as described previously (see “Measurement of Distribution Profiles” section), except that embryos were mounted in 1% low-melting point agarose in modified Danieau's medium, and Petri dishes were filled with modified Danieau's medium rather than embryo medium. Modified Danieau's medium was used because embryo medium contains methylene blue, which produces background red fluorescence that can obscure relevant signal.

Experiments were performed on an inverted Pascal confocal microscope (Zeiss). Embryos were maintained at 28°C during the experiments using a heated stage. Images of size 512 X 512 pixels were acquired with a 40X objective, while photoconversion was
performed using a 10X objective. Immediately after mounting, a “pre-photoconversion" image was taken from a single confocal plane corresponding to a thickness of less than 3.3 µm, at a depth of about 30 µm from the animal pole. The 543 nm laser output was 20% (for imaging of red fluorescence), and the 488 nm laser output was between 0.25 and 1.0% (for imaging green fluorescence). Because Cyclops-Dendra2 signal is often concentrated in bright membrane-associated clusters that are saturated under the gain settings used for the other constructs, lower gain settings were used for acquisition of Cyclops-Dendra2 images in the red channel.

Embryos were photoconverted using a two-minute pulse of UV light from a mercury lamp at 100% output, while constantly manually shifting the focal plane. The 10X objective was used to photoconvert multiple embryos simultaneously. After photoconversion, a multitime imaging macro was used to image each embryo on the dish sequentially over a total period of 300 min post-photoconversion with intervals of either 10 or 20 min between images (see “Controls for clearance assay” section). The imaging conditions for the post-photoconversion images were identical to those described above for the pre-photoconversion images.

Controls demonstrated little drift in x, y or z over the duration of the time lapse experiments. Red fluorescent beads with 1 µm diameter (FluoSpheresNeutrAvidin labeled microspheres (580/605), Invitrogen) were embedded in 1% agarose. Four positions were imaged at 10 min intervals for 300 min, similar to the clearance assay experiments. We did not observe significant shifting of the beads from their original positions during the course of the experiment.
Image analysis

Custom macros in ImageJ (Abràmoff et al. 2004) were used to measure the change in average photoconverted Dendra2 signal intensity over time. Intensities were measured in three compartments: extracellular space, intracellular space, and in the entire optical slice (extracellular and intracellular combined, excluding extraembryonic regions). For each time point, the extracellular space was defined by thresholding the Alexa488 signal (which labels cells but not extracellular space) using the Otsu thresholding algorithm (which is based on the minimization of inter-class variance between two histogram classes (Otsu 1979)). Intracellular pixels defined by the Alexa488 mask were not considered for calculations of the extracellular signal in the red channel (Fig. A.15). Calculating the average intensity in a region of interest drawn around the embryo (to exclude extraembryonic space) is thus equivalent to summing the intensities of the extracellular pixels and dividing this sum by the number of extracellular pixels (i.e. the extracellular area $A_{ext}$ in units of pixels). To measure intracellular average intensities, the mask was inverted.

In summary, at each time frame $t_n$ the spatial average $I_{ext}$ in the extracellular area $A_{ext}$, $I_{int}$ in the intracellular area $A_{int}$, and $I_{sl}$ in the entire slice $A_{sl}$ was computed by

$$
\bar{I}_{ext}(t_n) = \frac{1}{A_{ext}} \sum_{i,j} I_{ext}(i, j, t_n)
$$

$$
\bar{I}_{int}(t_n) = \frac{1}{A_{int}} \sum_{i,j} I_{int}(i, j, t_n)
$$

$$
\bar{I}_{sl}(t_n) = \frac{1}{A_{sl}} \sum_{i,j} I_{sl}(i, j, t_n)
$$

where $i$ and $j$ represent the coordinates of pixels that fall inside of $A_{ext}$, $A_{int}$ and $A_{sl}$, respectively.
Embryos that died, produced very low levels of photoconverted Dendra2 signal after photoconversion, or whose position shifted significantly during the experiment were excluded from analysis. For Cyclops-Dendra2 experiments, only embryos in which clusters were uniformly distributed and highly abundant immediately post photoconversion were included in the analysis.

Data Fitting

Photoconversion led to a homogenous distribution of the photoconverted signal in the blastoderm (Fig. A.16). With the assumption that the photoconverted red fluorescent signal was cleared over time with the clearance rate constant $k_1$, without contributions from de novo production or diffusion to the change in signal over time, the change in concentration with respect to time in these experiments is described by the ordinary differential equation (ODE)

$$\frac{dc}{dt} = -k_1 c$$

The solution to this equation is an exponential function with $c_0$ as a starting value obtained from the initial condition (IC). For all constructs, a single exponentially decaying function with offset $y_0$

$$c(t) = c_0 e^{-k_1 t} + y_0$$

was therefore fitted to the experimental data $I(t_n)$ from individual embryos by minimizing the sum of squared differences (SSD)

$$SSD = \sum_1^n \left( I(t_n) - c(t_n) \right)^2$$
using a constrained optimization algorithm (Nelder-Mead, MATLAB), where the solution of the model \( c \) was evaluated at the \( n \) discrete time points \( t_n \). See Table A.1 for fitting constraints and initial parameter value guesses.

\( y_0 \) represents the background intensity. It is not necessarily equivalent to the preconversion value. Reasons that \( y_0 \) may deviate from the preconversion value for extracellular fits include secretion of photoconverted protein, the possible existence of a small amount of free photoconverted Dendra2 (Fig. A.4 and Fig. A.8), accidental photoconversion prior to acquisition of the pre-photoconversion image, and small fluctuations in background intensity over time. In addition, there seems to be a slight increase in background intensity subsequent to photoconversion (Fig. A.17). Therefore, for each embryo the upper \( y_0 \) limit was conservatively defined as the maximum average intensity from the intracellular fraction. The lower \( y_0 \) limit was adjusted to a percentage of the pre-photoconversion value based on fluctuations in background intensities and extraembryonic background (Table A.1, Table A.2, and Fig. A.17). Extraembryonic background for Cyclops-Dendra2 imaging conditions was \( \sim 60 \) a.u. For all other imaging conditions extraembryonic background was \( \sim 70 \) a.u.

The \( k_1 \) values determined for individual embryos were averaged for each construct (see Table A.3 for a summary of extracellular, embryo and intracellular clearance rate constants). Average half-lives \( \tau \) were calculated from average \( k_1 \) values \( k_1 \) using the relationship

\[
\tau = \frac{\ln(2)}{k_1}
\] (21)
**Statistical tests**

The Wilcoxon-Mann-Whitney test in R (R Development Core Team 2005) with a significance cutoff of 0.005 was used to determine whether k1 values significantly differed between constructs. See Fig. A.15 for a summary of the statistical analysis.

**Controls for clearance assay**

Uniform photoconversion: Non-uniform photoconversion could be a source of error when determining the half-lives of Dendra2 fusion proteins. For example, diffusion of photoconverted protein out of the imaging plane could be misinterpreted as clearance if photoconversion were biased towards the animal pole. To determine whether photoconversion is uniform along the animal-vegetal axis, embryos were co-injected with 60 pg of mRNA encoding secreted Dendra2 and 1.9 ng of Alexa488-dextran at the one-cell stage and imaged starting between dome stage and 30% epiboly. A z-stack comprised of 10 slices spaced 8 µm apart was taken every 20 min post-photoconversion for a total of 80 min. Photoconversion and imaging conditions were identical to those used in the clearance assay experiments. The average intensity in a small circular ROI (~35 µm diameter) in the center of each z-slice was determined and plotted as a function of depth for each time point (Fig. A.16; intensity decreases at deeper imaging planes due to light scattering and absorbance). If photoconversion were biased towards the animal pole, the red intensity profile should change over time, i.e. the (normalized) intensities near the vegetal end should increase due to diffusion. However, minimal differences were observed between the normalized red intensity profiles from early and late times. The same trend was observed for the intracellular Alexa488 signal, which was uniformly
distributed throughout the embryo. This suggests that photoconversion was likely uniform or near-uniform along the animal-vegetal axis.

Fluctuations in background intensity: To identify potential changes in background intensity over the five-hour imaging period that would require adjustment of data from the clearance assay experiments, embryos were co-injected with 1.9 ng of Alexa488-dextran and 40 pg of mRNA encoding untagged Cyclops, Squint, Lefty1 or Lefty2. These embryos were then mock photoconverted and imaged identically to the embryos used in the clearance assay described above, and the resulting average intensities in the extracellular space, intracellular space, and entire optical slice were determined (see “Image analysis”). No significant changes in intensity in any of these compartments during the five hours of imaging were detected (Fig. A.17). Because background intensities remained relatively constant over time, dynamic background adjustment of the data from the clearance assay experiments was not required.

Photobleaching and inadvertent photoconversion: If continuous time-lapse imaging results in photobleaching, the clearance kinetics of the Dendra2 fusion proteins would be artifactually modulated. Likewise, excitation by the 488 nm laser could cause inadvertent photoconversion of newly synthesized Dendra2 and also lead to artifactual clearance kinetics. Therefore, experiments were performed to determine whether significant photobleaching or inadvertent photoconversion occurred in the clearance assay. For each construct, experiments with imaging intervals of 10 or 20 minutes were performed. If significant photobleaching occurred, the half-lives extracted from the images collected
with 20 min intervals should be higher than those obtained from images collected with 10 min intervals, because the signal intensity should decrease less rapidly with less laser exposure. In contrast, if significant inadvertent photoconversion occurred, the half-lives extracted from the images collected with 20 min intervals should be lower than those obtained from images collected with 10 min intervals, because continuous photoconversion would increase the perceived signal intensity and counterbalance clearance. No statistically significant difference was observed between half-lives from data obtained with 10 or 20 min intervals, indicating that detectable photobleaching or inadvertent photoconversion did not occur in the clearance assay (Fig. A.18). Significance was determined using the Wilcoxon-Mann-Whitney test in R (R Development Core Team 2005) with a significance cutoff of 0.005.

6. MEASUREMENT OF EFFECTIVE DIFFUSION COEFFICIENTS

Rationale and summary

The diffusivity of extracellular signaling molecules can be a major determinant of their distribution: the more diffusive a signal is in the extracellular space, the longer its range (Müller & Schier 2011). To determine the effective diffusion coefficients \((D)\) of Nodals and Leftys, we developed a FRAP (Fluorescence Recovery After Photobleaching) assay in zebrafish (Fig. 2.4 and Fig. A.20). FRAP involves the irreversible bleaching of fluorescent molecules in a region of interest. The dynamics of re-appearance of fluorescence in the bleached region can be used to extract information about the mobility of the fluorescent species (Gregor et al. 2007b, Kicheva et al. 2007, Lippincott-Schwartz et al. 2003, Sprague & McNally 2005, Wartlick et al. 2009, 2011). Our FRAP data
support a diffusive process for the movement of Nodal and Lefty signals (Figs. A.25-27). Previous studies in Drosophila embryos and imaginal discs have used one- or two-dimensional models to analyze FRAP data (Gregor et al. 2007b, Kicheva et al. 2007, Wartlick et al. 2011). The more complex geometry of the zebrafish embryo made it necessary to develop a three-dimensional model for the analysis of our FRAP data (Figs. A.21-22). In addition, we also accounted for the effects of production and clearance (Fig. A.23) as well as time delays between the end of the bleaching and the beginning of the post-bleach imaging (Fig. A.24). We used this three-dimensional model to measure the effective diffusivity of Cyclops-GFP ($D = 0.7 +/- 0.2 \mu m^2/s$), Squint-GFP ($D = 3.2 +/- 0.5 \mu m^2/s$), Lefty1-GFP ($D = 11.1 +/- 0.6 \mu m^2/s$), and Lefty2-GFP ($D = 18.9 +/- 3.0 \mu m^2/s$) (Fig. 2.4, Figs. A.20-35, and Table A.6). The effective diffusion coefficients determined by FRAP reflect the distribution profiles of these proteins - the longer the range, the higher the effective diffusion coefficient. We also discuss alternative interpretations of FRAP experiments and potential mechanisms underlying differential diffusivity.

**Experimental setup**

A 1 nl injection mix composed of 30 pg of mRNA encoding the GFP fusion proteins with 0.05% phenol red (Sigma) as an injection tracer was injected at the one-cell stage. For FRAP experiments in which the diffusion coefficient of extracellular recombinant GFP in zebrafish embryos was determined, 100 pl of 0.5 mg/ml recombinant GFP protein (BioVision) were injected along with 0.05% phenol red at two to five different locations into the extracellular space of blastula stage embryos, similar to (Yu et al. 2009). Embryos with uniformly distributed fluorescence were selected between sphere
and dome stages and mounted as described above (see “Measurement of Distribution Profiles" section).

FRAP experiments were performed using LSM 510 and LSM 7 LIVE confocal microscopes (Zeiss). Two images separated by 10 s were acquired before photobleaching. These images exhibited a nearly homogeneous spatial distribution of fluorescence. Photobleaching in a square region (typically 147.4 µm X 147.4 µm, corresponding to approximately 8 X 8 cells) was performed through the depth of the blastoderm with 100% laser power and 100% transmission. Bleaching was completed in ~5 min. Imaging conditions used for the pre-bleach and post-bleach images were identical. Typically, recovery of fluorescence was monitored every 10 s for 50 min in one medial optical slice in the middle of the embryo. For some experiments with Cyclops-GFP, the time course was extended to 100 min.

No deleterious effects from constant laser scanning were observed during acquisition of the recovery profile. In addition, consecutive FRAP experiments were performed, in which embryos were allowed to recover total fluorescence after bleaching and then were subjected to up to two more bleach/recovery cycles. No major differences in the diffusion coefficients from these consecutive FRAP experiments were observed, indicating that there are no apparent phototoxic effects affecting diffusive processes.

*Image analysis*

Image analysis was performed using custom MATLAB scripts. Each FRAP experiment results in $I = I(i, j, t_n)$, a time series of n images of size 512 X 512 pixels, where $i$ and $j$ represent the pixel coordinates. All images were centered during
acquisition. The radius $r_{sl}$ of the blastoderm in the imaged slice did not change by a significant amount. $r_{sl}$ was measured in the first post-bleach image in each experiment (~230 µm on average, Fig. A.21). Immediately post-bleaching, the intensity decreases sharply between the unbleached and bleached region (Fig. 2.4, Fig. A.20, and Figs. A.24-25). This sharp gradient subsequently relaxes as fluorescent molecules diffuse into the bleached volume (Fig. 2.4, Fig. A.20, and Figs. A.24-25 S22). At each time frame, the spatial average intensity in the area $A_{win}$ inside ($I_{win}$), $A_{out}$ outside ($I_{out}$), and $A_{sl}$ for the entire slice ($I_{sl}$) was computed by

$$I_{win}(t_n) = \frac{1}{A_{win}} \sum_{i,j} I_{win}(i,j,t_n)$$
$$I_{out}(t_n) = \frac{1}{A_{out}} \sum_{i,j} I_{out}(i,j,t_n)$$
$$I_{sl}(t_n) = \frac{1}{A_{sl}} \sum_{i,j} I_{sl}(i,j,t_n)$$

where $i$ and $j$ represent the coordinates of pixels that fall inside of $A_{win}$, $A_{out}$ and $A_{sl}$, respectively.

**Mathematical modeling of FRAP experiments**

**Factors that affect recovery dynamics**

The spatial averages $I_{win}$, $I_{out}$ and $I_{sl}$ include both intra- and extracellular regions of the embryo. The observed changes in these spatial averages are the result of multiple physical phenomena that occur in both regions and at their boundaries, including molecular diffusion, cell movement, cell-packing geometry, binding interactions, and clearance and production. In the following, the factors that affect the recovery dynamics
and the interpretation of the experiments are discussed. The influence of reversible and irreversible binding on recovery dynamics is discussed in later sections.

Cell movement: Movement of unbleached cells into the bleached area might contribute to fluorescence recovery. Based on the maximal distance a cell has traveled in the FRAP experiments (t = 3000 s), its maximal mean-square displacement, $\gamma^2$, and its diffusion coefficient, $D_{\text{cell}}$, can be estimated using the relationship $\gamma^2 = tD_{\text{cell}}$ (Berg 1993). Non-dividing cells that stayed within the imaging plane over the entire time-course were manually tracked in embryos expressing cytoplasmic GFP to determine their maximal displacement from the origin. It was found that $D_{\text{cell}} = 0.22 \pm 0.05 \mu m^2/s$ (n = 12 cells from four embryos). The upper bound of the cellular “diffusion coefficient" is therefore much smaller than that of Cyclops-GFP, which had the smallest diffusion coefficient (0.7 $\mu m^2/s$, see below) of all of the constructs that were tested. In addition, FRAP experiments were performed in embryos uniformly expressing cytoplasmic GFP. The resulting “recovery curves" from these experiments were much flatter and lacked the characteristic initial steep recovery observed for the Nodal- and Lefty-GFP fusions. These experiments demonstrate that the contribution of unbleached cells moving into the bleached region is negligible and only affects the edges of the analysis window.

Clearance: Proteins are removed from the diffusible pool with a clearance rate constant $k_1$. The half-lives of Nodals and Leftys determined using the clearance assay (see “Measurement of Clearance Rate Constants" section) are long (> 90 min) compared to the length of the FRAP experiment (~50 min). Although it can therefore be assumed that
contributions of clearance to the FRAP recovery curves are small, the reaction term \( k_i \) was included in the reaction-diffusion equations to ensure that any effects of clearance were accounted for in the FRAP analysis. Consistent with the idea that effects from clearance are mostly negligible in the case of Nodal and Lefty signals, it was found that the diffusion coefficients were similar regardless of whether \( k_i \) was set to zero or to the value measured in the clearance assay experiments (Table A.6).

Production: In the FRAP experiments, mRNA was injected at the one-cell stage and continues to generate protein during the experiment with a production rate \( k_2 \). A potential caveat for the analysis of the FRAP experiments is that new production from injected mRNA modulates the recovery profiles. To analyze the magnitude of this effect, protein production was blocked by applying the translation inhibitor cycloheximide. Embryos were injected at the one-cell stage with 60 pg of mRNA encoding the GFP fusion constructs. This is double the amount used for the regular FRAP experiments to ensure that embryos express sufficient levels of protein before translation is blocked. The embryos were incubated in 50 µg/ml cycloheximide (Sigma) in 1% DMSO starting at the 1,000-cell stage, similar to (Bennett et al. 2007). Embryos exhibiting uniform fluorescence were mounted at sphere stage in 1% low melting point agarose and covered in embryo medium containing 50 µg/ml cycloheximide to sustain suppression of protein translation. FRAP experiments with cycloheximide-treated embryos resulted in recovery curves similar in shape to the ones determined in untreated embryos but without a linear increase in fluorescence intensities for the entire slice. This indicates that the chemical block of translation was efficient and that production affects recovery curves. To account
for production, the reaction term $k_2$ was included in the subsequent FRAP data analysis model. We found that the diffusion coefficients determined by this model are robust to variations in the freely-fit $k_2$ term (see “Modeling of the three-dimensional reaction-diffusion system” section).

Combined effects of production and clearance: It is generally assumed that fast recovery after photobleaching is due to high diffusivity of fluorescent molecules. Indeed, Lefty1-GFP, Lefty2-GFP, secreted GFP, and extracellularly injected recombinant GFP do not show a strong delay in fluorescence recovery in the middle of the bleached window relative to the recovery in the entire bleached region (Fig. A.25E-G). In this scenario, it is conceivable that diffusivity is very high and clearance and production rates become the major determinants of fluorescence recovery. To determine whether the recovery kinetics of Lefty2-GFP, secreted GFP, and extracellularly injected recombinant GFP could be explained by production and clearance, the predicted half-lives were compared with the independently measured half-lives (see “Measurement of Clearance Rate Constants” section). Using Equation 29, the time point $\tau$ at which

$$c(\tau) = \frac{1}{2} \left( \frac{k_2}{k_1} - c_0 \right) + c_0$$

represents the half-life of the protein in this scenario, where $c_0$ is the initial post-bleach intensity, the ratio $k_2/k_1$ is the recovery plateau, $k_1$ is the clearance rate constant and $k_2$ is the production rate. If recovery were due to production and clearance instead of diffusion, the half-lives for Lefty2-GFP, secreted GFP, and extracellularly injected recombinant GFP would be less than 250 s, i.e. $\tau \approx 4$ min (Fig. A.25E-G). These ultra-short
half-lives are incompatible with the measured half-lives (see “Measurement of Clearance Rate Constants" section) and with the half-life measurements for GFP in an earlier study (Yu et al. 2009). These considerations indicate that the FRAP measurements in our study are based on diffusion and not dominated by production and clearance.

Embryo geometry: Using the simplest assumption for the FRAP experiments, the embryo was initially modeled as a two-dimensional disc (Fig. A.21), similar to previous geometry simplifications of the zebrafish blastoderm (Yu et al. 2009). In a three-dimensional representation, this two-dimensional geometry simplification would extend to an infinitely long cylinder (Fig. A.21). However, recovery profiles for species with large diffusivities in a three-dimensional dome-shaped geometry deviate from those generated in a two-dimensional geometry, because the two-dimensional model fails to account for diffusive flux through the imaging plane (Figs. A.21-22). The reaction-diffusion system was therefore modeled in a three-dimensional geometry, similar to previous studies (Sample & Shvartsman 2010, Umulis et al. 2010).

Bleach profile: There are small time delays between the end of bleaching and the start of post-bleach imaging in all FRAP experiments presented here. Consequently, recovery has already begun by the time the first post-bleaching image is acquired (Fig. A.24). Therefore, fitting the data with a model that assumes an initial condition of zero concentration at all points in the bleach window would result in erroneous diffusion coefficient measurements due to a failure to account for this time shift (Castle et al.
To circumvent this issue, the intensity profiles from the first post-bleaching image were directly used as the initial condition for the simulations.

**Modeling of the three-dimensional reaction-diffusion system**

Description of the reaction-diffusion system: Given the large number of cells (>>100), we homogenize the embryonic tissue by averaging spatially over both intra- and extracellular regions similar to previous studies (Kicheva et al. 2007, Wartlick et al. 2011), allowing for the use of a tractable and computationally efficient continuum model of the experimental system.

Effective diffusion, clearance and production were modeled by the linear partial differential equation (PDE)

\[
\frac{\partial c}{\partial t} = D \nabla^2 c + R(c)
\]  

(22)

where \(c\) is the concentration, \(D\) is the effective diffusion coefficient acting on the divergence of the concentration gradient

\[
(\nabla^2 c = \frac{\partial^2 c}{\partial x^2} + \frac{\partial^2 c}{\partial y^2} + \frac{\partial^2 c}{\partial z^2})
\]

and \(R(c)\) may indicate either production, clearance or a combination of the two. Without evidence for higher-order production or clearance reactions (see “Measurement of Clearance Rate Constants” section), four simple linear models for \(R(c)\) were considered with respect to Equation 22:
where \( k_1 \) is the clearance rate constant and \( k_2 \) is the production rate.

Embryo geometry: The average three-dimensional geometry of the blastoderm was approximated by the complement of two spheres of different radii (Fig. A.21). The smaller sphere had a radius \( r_b \) (corresponding to the radius of the embryo), which is determined from the blastoderm radius in the imaged slice \( r_{sl} \) and \( z_{sl} \) (the depth of the imaged slice with respect to the animal pole) by

\[
r_b = \frac{r_{sl}^2 + z_{sl}^2}{2z_{sl}}
\]

The depth of the optical slice (\( z_{sl} \)) relative to the animal pole was set to 80 \( \mu \)m, given the average slice radius \( r_{sl} \approx 230 \) \( \mu \)m and the average radius of the embryo \( r_b \approx 300-400 \) \( \mu \)m (Kimmel et al. 1995, Olivier et al. 2010). The radius of the second sphere \( r_{b2} \) was set to a value 10\% larger than \( r_b \), and the sphere centers were displaced by the maximal thickness of the blastoderm \( z_b \) (Figs. A.20-21):

\[
z_b = \sqrt{r_{b2}^2 - r_b^2}
\]

The bleached domain in the blastoderm was modeled using the dimensions of the bleached window. The intersection of this domain with the optical slice domain \( \Omega_{sl} \) (in which \( c_{sl} \) was calculated) defined the bleached analysis subdomain \( \Omega_{win} \) (in which \( c_{win} \) was calculated):
was calculated), and the remaining subdomain $\Omega_{\text{out}}$ (in which $c_{\text{out}}$ was calculated) was defined by $\Omega_{\text{out}} = \Omega_{\text{sl}} / \Omega_{\text{win}}$.

The initial intensity $I_0 = I(i, j, t_0)$ after photobleaching for each FRAP experiment was used to create an initial condition for the model. The eight-fold symmetry of the optical slice was utilized to compute the average octant from the image by

$$
\bar{I}_0(i, j) = \frac{1}{8} \sum_{k=1}^{8} Q_k
$$

where $Q_k = Q_k(i, j)$ are the eight octants in the image plane, and the indices $i, j$ are taken with respect to the $k$-th octant (Fig. A.21). Assuming uniform photobleaching through the depth of the blastoderm, $I_0$ was taken to be the same through the modeled embryo geometry for the regions that were captured in the FRAP experiment. The values of the initial condition in regions of the domain that fall outside of the acquired image were approximated by taking the average concentration in an annulus extending 100 pixels from the edge of the embryo in the imaged slice towards the center of the image. The resulting initial condition (IC) for the model was obtained by the extension of $I_0$ in three dimensions, giving

$$
c(x, y, z, t_0) = \bar{I}_0(i, j, t_0)
$$

The enveloping layer that covers the blastoderm and the yolk syncitial layer between blastoderm and yolk do not allow flux across their boundaries, and the resulting boundary conditions (BC) on the model were

$$
\nabla_n c |_{\partial \Omega} = 0
$$
where
\[ \nabla_n \]
is the gradient operator scaled by the outward normal vector n, and \( \partial \Omega \) is the boundary of the embryo.

This geometry, together with the PDE, IC and BC, leads to an inhomogeneous and time-dependent change in concentration in the optical slice after bleaching. Gradients in concentration evolve not only in the plane of the slice but also through the depth of the blastoderm. This configuration enables the model to capture not only the effects of in-plane diffusion and homogeneous reactions considered in previous analysis techniques for FRAP experiments (reviewed in Lippincott-Schwartz et al. 2003, Sprague & McNally 2005), but also the effects of diffusive flux through the slice boundaries.

Numerical simulations: The finite element method (FEM) was used to solve the model. All domains of the modeled embryo geometry were discretized using a tetrahedral meshing scheme, and the mesh was refined using several boundary layer elements at the boundary between the bleached window and the rest of the embryo (see Table A.4 for meshing and error parameters). The solution at each time step was determined using a sparse LU factorization algorithm (UMFPACK), and the time stepping was computed using a backward Euler step method (Comsol Multiphysics).

In order to fit diffusion coefficients, the solution of the model \( c(x, y, z, t) \) needs to be compared with the measured intensity \( I(i, j, k, t_n) \) or its spatial averages \( I_{\text{win}}, I_{\text{out}} \) and \( I_{\text{sl}} \). The spatial averages of \( c \) for the regions inside (\( c_{\text{win}} \)), outside (\( c_{\text{out}} \)), in the analysis slice (\( c_{\text{sl}} \)), or for the entire blastoderm (\( c_{\text{b}} \)) were calculated by integrating the solution over the
subdomains and dividing by their volume. With the assumption of homogenous clearance and production in, and the no-flux BC on the entire blastoderm volume, the spatial average of the changes in the entire blastoderm \( (c_b) \) is only due to the reactions \( R(c) \). For the entire blastoderm domain, the diffusion term from Equation 22 can therefore be dropped to give the ordinary differential equation (ODE)

\[
\frac{d\bar{c}_b}{dt} = R(\bar{c}_b)
\]

For the three cases where \( R \neq 0 \), the analytical solutions are

\[
\begin{align*}
\text{clearance only:} & \quad \bar{c}_b(t) = c_0 e^{-k_1 t} & (k_1 \neq 0, k_2 = 0) \\
\text{production only:} & \quad \bar{c}_b(t) = c_0 + k_2 t & (k_1 = 0, k_2 \neq 0) \\
\text{clearance and production:} & \quad \bar{c}_b(t) = c_0 e^{-k_1 t} - \frac{k_2}{k_1} e^{-k_1 t} + \frac{k_2}{k_1} & (k_1 \neq 0, k_2 \neq 0)
\end{align*}
\]

where \( c_0 \) is determined in each case using the IC.

The spatial averages of \( I \) were measured only in the optical slice, however, and therefore \( c_{sl}(t) \) needs to be compared with \( I_{sl}(t) \). Since \( I \) is computed in a volumetric slice, whose upper and lower boundaries are continuous with the rest of the blastoderm, the geometry imparts a non-zero and unequal diffusive flux through the slice boundaries, which results in an average change in concentration within the slice volume (Fig. A.22). This average concentration change is computed directly from the volume-averaged solution to Equation 23 with \( R(c) = 0 \), as seen by applying the divergence theorem:

\[
\frac{1}{V_{sl}} \int_{\Omega_{sl}} \nabla^2 c_{df} \, dV = \frac{1}{V_{sl}} \int_{\partial \Omega_{sl}} \nabla_{n} c_{df} \, dS = \frac{1}{V_{sl}} \int_{\Omega_{sl}} \frac{\partial c_{df}}{\partial t} \, dV = k_{df}(t; D)
\]

where \( V_{sl} \) is the volume of the slice, \( c_{df} \) is the concentration resulting from diffusive flux in the slice, \( \Omega_{sl} \) is the slice domain, \( \partial \Omega \) is the boundary of the slice, and \( k_{df} \) is a volume-averaged time- and diffusion-dependent “production” term due to diffusive flux.

The resulting model for the volume-averaged concentration change in the slice is
Given the above solutions for the reaction ODEs (Equations 27, 28 and 29), it can be seen that the term $k_{df}$ contributes to the rate of change of the solution exactly the volume-averaged-solution to the diffusion-only problem $c_{df}(t)$. Subtracting $c_{df}(t)$ from $c_{sl}$ therefore gives the change in concentration in the optical slice due purely to the reactions.

Non-dimensionalization and scaling: Dimensional analysis of the model results in scaling of a one-parameter family of curves, allowing the solution for any $D$ to be determined given one solution. From Equation 23, the following dimensionless variables were defined:

$$ t = T_0 \tilde{t}, x = X_0 \tilde{x}, c = C_0 \tilde{c} $$

The scalings of each variable were established by forming groups with the following parameters:

$$ X_0 = r_b, C_0 = c_0, T_0 = \frac{r_b^2}{D} $$

Substituting these groups into Equation 23 and rearranging results in the corresponding dimensionless PDE, BC and IC

$$ \frac{\partial \tilde{c}}{\partial \tilde{t}} = \tilde{\nabla}^2 \tilde{c} \quad \text{(in } \tilde{\Omega}) $$

$$ \tilde{\nabla} \cdot \tilde{c} = 0 \quad \text{(on } \partial \tilde{\Omega}) $$

$$ \tilde{c} = \tilde{c}_0 \quad \text{(at } \tilde{t} = 0) $$

where $\sim c_0$ is taken from $I_0$ as described above and normalized to the final time point in the data, and both the IC and BC are independent of the length and time scales of the problem.
The resulting solution

\[ \tilde{c} = \tilde{c}(\tilde{x}, \tilde{y}, \tilde{z}, \tilde{t}) \]

can then be rescaled back into the dimensional solution \( c \) for any choice of \( D \) and the specific \( r_b \) measured for each embryo, importantly noting that the time scales with \( r_b^2/D \) (Fig. A.23). This is done by substituting back in the dimensional variables using the dimensionless groups.

This scaling was utilized to efficiently fit the effective diffusion coefficient without simulation for each choice of \( D \). For each FRAP experiment, a scaling solution was found by FEM using a logarithmic distribution of a sufficiently large number of discrete time steps (4,000). This spacing allows the comparison of the scaled solution \( c \) with the measured intensities \( I \) by piecewise-linear interpolation of the scaled solution at the time points given by the data.

Combining the numerical “diffusion only” solution with the reactions: The solution in the entire blastoderm \( c_b \) only differs from the solution in the optical slice \( c_{sl} \) by the contribution due to the diffusive flux \( c_{df} \). The solution \( c_{sl} \) is thus assembled by adding \( c_b \) and \( c_{df} \) (Fig. A.22 and Fig. A.23), requiring solely the computation of the “diffusion only” model (Equation 23) using FEM, which is then added to the solution for the choice of reaction parameters. Using this approach, the experimental results \( I_{sl}, I_{win}, \) and \( I_{out} \) can be directly compared with the simulated results \( c_{sl}, c_{win}, \) and \( c_{out} \), respectively.

Data fitting: For each FRAP experiment, the combination of parameters \( D, k_f \) and \( k_2 \) was
found which minimizes the sum of squared differences (SSD) between the simulations of one of the four reactions models (Equations 23, 24, 25, and 26) and the experimental data. Given the analytical solutions for the reactions (Equations 27, 28, and 29), the diffusive flux only needs to be computed for a given choice of $D$. The minimization of the SSD over the three possible parameters $D, k_1$ and $k_2$ is thus reduced computationally to the solution of a single FEM problem and the computation necessary to perform the minimization steps.

To maximize our confidence and to exploit all the available data, the least-squares minimization problem was defined by summing the residuals inside the window with those in the entire optical slice, such that

$$SSD = \sum_n (\bar{I}_{win}(t_n) - \bar{c}_{win}(t_n))^2 + \sum_n (\bar{I}_{sl}(t_n) - \bar{c}_{sl}(t_n))^2$$

The minimization was performed numerically using a constrained optimization algorithm (Nelder-Mead, MATLAB), which converges on a minimum for a given range of parameters values. Given the approximated uniqueness of the solution to the model (see below), the minimum found by this approach is the global minimum within the bounds of the parameter space up to the propagated determinate and indeterminate error. See Table S5 for fitting constraints and initial parameter value guesses and Table A.6 for results from three-dimensional simulations.

Uniqueness of the solution: For the linear reaction and diffusion models considered, the spatial solution at every time point is uniquely determined to within the error bounds of the algorithm for the numerical solution. Given the linearity of the model, linear superposition of the solutions demonstrates the uniqueness of any solution for a particular
choice of parameters. Consider two solutions $c_1$ and $c_2$, each having a unique set of parameters. Then $c_3 = c_1 - c_2$ is also a solution, and if the two solutions are unique everywhere in space, then their difference is non-zero at every time point. Likewise, since any two solutions having the same parameters must be identical, their difference is zero. Substituting $c_3 = 0$ into the PDE, BC, and IC, it can be seen that there is no initial or boundary data, thus satisfying the PDE trivially for all parameters choices. Likewise, if $c_3 \neq 0$, the PDE, BC, and IC results in a non-zero solution, which can only be satisfied for $c_1$ and $c_2$ that have different parameter sets.

Accounting for the immobile fraction and changes in the volume fraction: In most previously reported FRAP experiments, there is a fraction of fluorescent molecules that does not recover after photobleaching. This fraction is referred to as the immobile fraction and in the experiments presented here could be due to bleaching of fluorescence inside cells that only recovers slowly (on a longer time scale than the experiment), or due to long-term occupancy of binding partners that prevent fluorescent molecules from re-populating the area. The presence of such an immobile fraction would lead to a recovery plateau in the experiments that is lower than what is predicted by the homogenous continuum model presented here. Furthermore, the experimental recovery plateau might be different from the simulations due to changes in the extracellular volume fraction $\phi_{ext}$. In the present experiments, $\phi_{ext}$ is on the order of $\sim 0.15$ as estimated by the extracellular area fraction for the optical slice, but for any given embryo $\phi_{ext}$ can increase or decrease over time. Hypothetically, assuming a constant number of molecules in the extracellular
space, a decrease in $\phi_{\text{ext}}$ would thus lead to an increased fluorescence intensity, whereas an increase in $\phi_{\text{ext}}$ would lead to a decreased fluorescence intensity. Given the maximal observed change in $\phi_{\text{ext}}$, the upper boundary is assumed to be $\Delta \phi_{\text{ext}} < 300\%$. The immobile fraction and any changes in the volume fraction ($\phi_{\text{ext}}$) are accounted for by minimizing the difference between the simulated curve and the experimental data at each iteration with the equalization factor $EQ$, with $EQ_{\text{max}} = 3$ as the upper limit of the change in $\phi_{\text{ext}}$, and $EQ_{\text{min}} = 0.1$ as the lower limit for the immobile fraction (Table A.5).

Goodness of fit: $R^2$ values were calculated from the minimizing SSD ($SSD_{\text{min}}$) to assess the goodness of the fits by

$$R^2 = 1 - \frac{SSD_{\text{min}}}{\sum_n \left( I_{\text{win}}(t_n) - \frac{1}{n} \sum_n I_{\text{win}}(t_n) \right)^2}$$

**Fast diffusion and slow immobilization**

We considered alternative interpretations of the FRAP data. It is generally assumed that slow recovery after photobleaching is due to low diffusivity of signaling molecules. However, it is conceivable that FRAP curves could be dominated by slow binding reactions (“trapping model”). In such a “trapping model”, also referred to as “diffusion-uncoupled recovery” (reviewed in Sprague & McNally 2005), diffusion across the bleached window is fast but binding reactions are slow. In this model, diffusion initially rapidly equilibrates the concentration gradients in the bleached window followed by slow binding and trapping of the molecules. In this scenario, the observed intensity increase is due to the slow accumulation of bound fluorescent species. In other words, the increase in
fluorescence does not reflect diffusion and should thus be spatially uniform in the bleached window. For example, Lander and colleagues have recently argued that the recovery of Dpp-GFP in FRAP experiments is diffusion-uncoupled (Zhou et al. 2012). They suggest that the fraction of mobile Dpp-GFP in the wing disc is very low (< 3%) and therefore too dim to contribute to the observed fluorescence recoveries. Instead, the fraction responsible for recovery is thought to be immobilized Dpp-GFP that has slowly accumulated to detectable levels inside cells. In this model, low levels of mobile Dpp-GFP immediately move into the bleach window and fill it uniformly shortly after photobleaching. The fast diffusion of Dpp-GFP ($D \approx 20 \, \mu m^2/s$) would not be detected, and the observed fluorescence recovery would be due to the slow accumulation of immobilized Dpp-GFP reflecting binding and degradation kinetics. Lander and colleagues argue that the small diffusion coefficient ($D \approx 0.1 \, \mu m^2/s$) deduced from FRAP data in previous studies (Kicheva et al. 2007, Wartlick et al. 2011) therefore does not reflect the mobility of Dpp-GFP but rather binding and degradation kinetics.

To determine whether our FRAP recovery curves may be dominated by fast diffusion and slow binding kinetics, we performed simulations by considering the following reaction:

$$ C + R \xrightleftharpoons[k_{off}]{k_{on}} CR $$

where the diffusible signaling molecule $C$ and the immobile binding molecule $R$ are transformed to the immobilized complex $CR$ with the on-rate constant $k_{on}$. The immobilized complex $CR$ can dissociate back into $C$ and $R$ with an off-rate $k_{off}$. The equations describing the full system including diffusion, binding, unbinding, clearance and production are thus
with a clearance rate constant $k_{\text{clear}}$ acting on both free $C$ and bound $CR$, and a production rate $k_{\text{prod}}$ that continuously generates more of the signaling molecule $C$.

FRAP experiments were modeled in two dimensions using a circular domain with radius $r_{\text{sl}} = 300$ µm and a bleach window with $h_{\text{win}} = 147.4$ µm. A smaller analysis window with $h_{\text{small}} = 100$ µm was nested within the bleach window, similar to the analysis windows used to analyze the FRAP experiments. The initial concentration $C$ outside of the bleach window was set to 0.05 nM and the concentration within the bleach and smaller nested windows to 0 nM at $t = 0$. Note that these concentrations were chosen for convenience, but that these are not important for the simulation conclusions and do not have to be on the same order in vivo. The initial concentration of the bound complex $CR$ outside of the bleach window was set to 0.95 nM to reflect that most signaling molecules are bound in this model, and the concentration within the bleach and smaller nested windows was set to 0 nM at $t = 0$. In order to make the concentration of $R$ not limiting for binding, $R$ was set to 1000 nM at $t = 0$. FRAP experiments were simulated for 3000 s and spatial averages of $C$, $CR$ or $C + CR$ calculated in the bleach and smaller nested windows.

We then measured the average intensity during recovery in our experimental data in a smaller window within the bleach window and compared it to the recovery measured in the entire window (Fig. A.25). In contrast to what would be expected if recovery was diffusion-uncoupled (Fig. A.26), recovery in the small window was delayed compared to
recovery in the large window for Nodal-GFP fusions. Our results are consistent with diffusion-coupled recovery, in which molecules must first move through the edge of the bleached window before reaching the middle, causing a delay in recovery in the center of the window (Fig. A.27; see “Potential mechanisms underlying differential effective diffusivity” below for further discussion).

Another possibility is that recovery dynamics are dominated by fast diffusion and irreversible binding. To test whether irreversible binding could explain our FRAP data, we performed simulations with a modification of the system described by Equations 30, 31 and 32 with $k_{off} = 0$. This prevents the dissociation of the complex $CR$ into $C$ and $R$ and therefore permanently immobilizes the ligand. In this case, free signaling molecules are permanently immobilized, and the recovery curve almost exclusively reflects the spatially homogenous production and degradation kinetics. This behavior is not consistent with our FRAP measurements (Fig. A.25). Therefore, fast diffusion in combination with fast irreversible binding is incompatible with the recovery curves that we observe in our FRAP experiments.

Potential mechanisms underlying differential effective diffusivity

Our FRAP experiments reveal that Nodals have lower effective diffusion coefficients than Leftys. However, Nodals and Leftys have similar molecular weights (Fig. A.4 and Fig. A.8) and based on the Einstein-Stokes equation (Equation 19) would therefore be expected to have similar diffusion properties. We speculate that the differences in diffusivities are caused by differential binding interactions with molecules in the extracellular space. For example, Nodals may bind extracellular molecules or
receptors localized to cell surfaces. Far from cell surfaces, in the absence of such binding partners, Nodals may diffuse freely on very short length- and time-scales with a high diffusion coefficient. However, continuous binding and unbinding near the cell surface could lead to a global retardation in mobility resulting in slow *effective* diffusion compared to Leftys, which may not bind with such high affinity. In contrast to the interactions discussed above, these interactions have to be reversible and fast relative to diffusion. Below, we review the definition of “effective diffusion” and simulate the effects of reversible binding on fluorescence recovery curves. We find that our FRAP data can be explained by models in which fast reversible binding combined with effects from the tissue architecture leads to a decrease in global diffusivity.

It has been shown mathematically (Crank 1983) and experimentally (Thorne et al. 2008) that reversible binding can influence the effective diffusivity of a molecule. The concept of effective diffusivity incorporating reversible binding reactions (Crank 1983) is outlined in the following. Consider the reaction of species $c$ forming the complex $c_{\text{bound}}$ with a forward reaction rate of $k_{\text{on}}$ and a reverse reaction rate of $k_{\text{off}}$:

$$c \xrightarrow{k_{\text{on}}} c_{\text{bound}} \xleftarrow{k_{\text{off}}}$$

Using the law of mass action, the change in concentration of the complex $c_{\text{bound}}$ can be determined as

$$\frac{dc_{\text{bound}}}{dt} = k_{\text{on}}c - k_{\text{off}}c_{\text{bound}}$$

(33)

Including diffusive processes, the change in the concentration of species $c$ can be described as

$$\frac{\partial c}{\partial t} = D \nabla^2 c - k_{\text{on}}c + k_{\text{off}}c_{\text{bound}}$$

(34)
Solving Equation 33 for $c_{\text{bound}}$, the following equation is obtained

$$c_{\text{bound}} = \frac{1}{k_{\text{off}}} \left( k_{\text{on}} c - \frac{dc_{\text{bound}}}{dt} \right)$$

and by inserting this expression into Equation 34 therefore

$$\frac{\partial c}{\partial t} = D \nabla^2 c - k_{\text{on}} c + k_{\text{off}} c_{\text{bound}} = D \nabla^2 c - \frac{dc_{\text{bound}}}{dt} \quad (35)$$

If $k_{\text{on}} c \gg dc_{\text{bound}}/dt$, binding is almost instantaneous and therefore

$$c_{\text{bound}} = \frac{1}{k_{\text{off}}} \left( k_{\text{on}} c - \frac{dc_{\text{bound}}}{dt} \right) \approx \frac{k_{\text{on}}}{k_{\text{off}}} c = \kappa c \quad (36)$$

where $\kappa$ is the dissociation constant. In other words, the concentration of the complex $c_{\text{bound}}$ is directly proportional to the concentration of the free species $c$. This is the case if the binding reaction is fast compared to diffusion, yielding a local equilibrium between $c$ and $c_{\text{bound}}$. Inserting the time derivative of Equation 36 into Equation 35, the change in concentration over time by diffusion and reaction (i.e. fast reversible binding) can be described as

$$\frac{\partial c}{\partial t} = D \nabla^2 c - \kappa \frac{dc}{dt} = \frac{D}{\kappa + 1} \nabla^2 c = D_{\text{eff}} \nabla^2 c \quad (37)$$

with $D_{\text{eff}}$ as the reaction-dependent effective diffusion coefficient.

To further illustrate that molecules with similar free, molecular diffusion coefficients can have different effective diffusion coefficients, we performed simulations that include free diffusion and reversible binding reactions. FRAP experiments were modeled based on Equations 30, 31 and 32, but with fast reversible binding kinetics compared to diffusion. The on-rate constant $k_{\text{on}}$ was set to 0.001/(nM s), similar to the on-rate constants that have been measured for BMP molecules (Umulis et al. 2009).
Diffusion models assuming a high free diffusion coefficient in combination with fast reversible binding kinetics yield a smaller effective diffusion coefficient and reflect our experimental observations (Fig. A.25 and Fig. A.27). In these homogenized models, most of the ligand would be bound at any given time point. Our data is therefore consistent with reversible binding reactions that transform local free diffusion to global effective diffusion.

It has been shown mathematically (Rusakov & Kullmann 1998, Tao et al. 2005, Thorne et al. 2008) and experimentally (Thorne & Nicholson 2006) that the tissue architecture (e.g. presence of cells, small extracellular volume fraction) can further decrease the effective diffusivity of a molecule, since molecules have to travel around cells and potentially through transient cavities in cellular membranes. The exact fold difference between geometrically hindered and unhindered diffusion varies in the literature based on the geometry used for simulations, but conservative estimates indicate an approximately two- to three-fold decrease in diffusivity in the presence of cells compared to unhindered diffusion in aqueous solution (Rusakov & Kullmann 1998, Thorne et al. 2008). Importantly, effective diffusion coefficients have been measured experimentally for fluorescent dextran molecules (Thorne & Nicholson 2006). These experiments show that, on average, effective diffusivities in cellular environments (such as the densely packed brain) may be reduced by four-fold compared to diffusion in aqueous solution. Indeed, in local measurements on short length- (femtoliter) and time-scales (seconds) using Fluorescence Correlation Spectroscopy (FCS), Yu et al. (Yu et al. 2009) found a diffusion coefficient for extracellular GFP in zebrafish embryos that is similar to the diffusion coefficient of GFP in water but approximately two times greater
than the one measured with FRAP in our study ($D_{\text{FCS}} = 86 \, \mu m^2/s$ (Yu et al. 2009), $D_{\text{FRAP}} = 34 \, \mu m^2/s$). These measurements suggest that extracellular FCS measurements predominantly detect freely diffusing molecules whereas FRAP measurements capture longer-range movements in the context of a tissue, where effective diffusivity is influenced by cell packing, cell movement, viscous effects at cell surfaces as well as potential binding effects exerted by extracellular molecules. As Grimm et al. (Grimm et al. 2010) have argued: “[...] the temporal window of observation can drastically influence an experimentally determined diffusion coefficient because the effective movement of a probe can include a mixture of mechanistically distinct transport events. On short time scales, such as a few seconds, measurements will reflect the movement of proteins within a cellular compartment. Over the course of minutes, measurements will include the effective diffusion within a compartment together with shuttling of molecules between neighbouring compartments. On the order of hours, molecular transport results from a number of events, including diffusion inside compartments [...] Moreover, a given time scale is intrinsically linked to a corresponding spatial scale, where short time scales correspond to short spatial scales and long time scales correspond to large spatial scales.”

As a preliminary test of this scenario, we performed FCS measurements and found similarly high diffusion coefficients for Nodal- and Lefty-GFP fusion proteins ($D \approx 40 \, \mu m^2/s$; Müller, Yu, Schier and Brand, unpublished results). These results and considerations of effective diffusion support the idea that FCS and FRAP measure different aspects of signal dispersal. FCS measures fluorescence fluctuations in very small volumes that likely reflect local unhindered diffusion within a small extracellular pool. Conversely, FRAP assays allow the measurement of the global effective diffusivity
over large volumes in a field covering multiple cell lengths. FRAP is therefore better suited than FCS to measure effective diffusion over the long temporal and spatial scales that are relevant for gradient formation and signal dispersal in embryonic tissues. Our simulations show that the differences in diffusion coefficients measured by FRAP and FCS can be explained by tortuosity and reversible binding (Fig. A.27).

7. MODELING OF THE DISTRIBUTION PROFILES

Rationale and summary

The quantitative biophysical analyses in the previous sections showed that Nodal and Lefty signals have different diffusivities (Fig. 2.4 and Fig. A.20), whereas their clearance rate constants are relatively similar (Fig. 2.3, Figs. A.14-15, and Table A.3). In contrast to previous studies (Gregor et al. 2007b, Grimm et al. 2010, Kicheva et al. 2007, Wartlick et al. 2011) that deduced clearance rate constants from diffusion coefficients and distribution profiles, our independent measurements of diffusion, clearance and distribution enabled us to test the validity of our approaches. We performed mathematical simulations in three-dimensional geometries using the experimentally determined values. The observed and predicted shapes of the distribution profiles were in good agreement, suggesting that the different diffusivities of Nodal and Lefty signals can largely account for their distinct distribution profiles and activity ranges (Fig. A.28).
Mathematical modeling

The geometry of the blastoderm was approximated by the complement of two spheres, one of which has a radius of 304 µm, and the second has a radius that is 10% larger than the other (similar to the geometry presented in “Measurement of Effective Diffusion Coefficients”). The centers of the two spheres were displaced by $z_b = 96$ µm, the maximum thickness of the blastoderm (Figs. A.20-21). In a small columnar subdomain placed off-center (to mimic actual experiments), a signal source region with continuous boundaries is defined by a non-zero, constant and homogeneous production rate $k_2$. Outside the source subdomain $k_2$ is zero (Fig. A.28). Excluding this difference, the source and surrounding blastoderm (target field) are assumed to otherwise behave similarly. The chemical species of interest diffuses with a diffusion coefficient $D$ and is cleared with a clearance rate constant $k_1$. In the target field, there is no de novo production, but molecules diffuse and are cleared uniformly with the same dynamics as in the source.

The model is thus governed by the PDE

$$\frac{\partial c}{\partial t} = D\nabla^2 c - k_1 c + \delta_s k_2$$

with

$$\delta_s = \begin{cases} 
1 & \text{in the source} \\
0 & \text{otherwise} 
\end{cases}$$

The initial condition (IC) is chosen to be zero and homogeneous in the entire blastoderm, approximating the moment at which the source is activated (by transplantation in the experiment) and production, clearance, and diffusion begin

$$c(x, y, z, t_0) = 0$$
The enveloping layer that covers the blastoderm and the yolk syncitial layer between blastoderm and yolk do not allow flux across the boundaries of the blastoderm domain $\delta \Omega$, and the resulting boundary conditions (BC) on the model are

$$\nabla n |_{\partial \Omega} = 0$$

Numerical solution of reaction and diffusion in embryo geometry: The model was solved in the described geometry using the Finite Element Method (FEM) using Comsol Multiphysics operated under the control of MATLAB scripts. All domains were discretized using a tetrahedral meshing scheme (see Table A.7 for meshing and error parameters). The spatial solution at each time step was determined using UMFPACK, and the transient solution was determined using the backward Euler step method.

The distribution profile data $I(x_n)$ was compared with the model $c(x)$ by performing steps analogous to those taken during image analysis, as follows: The solution resulting from the FEM was evaluated at the nodes of 5 regular planar grids of dimensions 512 X 512 pixels, having $DX$ steps between each node in both the x- and y-directions, and located at 5 $\mu$m intervals centered about the distance from the animal pole to the middle of the z-stack. $DX$ was chosen to be sufficiently small such that there was no difference larger than the absolute (element-wise) tolerance of the FEM solution at any point in space between the solution evaluated on this grid, and the same solution evaluated on a grid of higher resolution. The grid was constructed such that the nodes were placed exactly at the boundaries of the source and the boundary of the embryo. The maximum intensity projection of the 5 gridded slices was computed, and the profiles were extracted by taking only the values of the solution located on the line of pixels at
$y = 0$ (centered in the source in the y-direction). These curves were normalized to the value at the source boundary, and then were interpolated piecewise-linearly onto a one-dimensional grid whose node locations matches the data points of $I(x_n)$. The resulting solution $c(x_n)$ at each time allows for comparison directly with the data, as well as accurately accounting for the diffusive effects conferred by the real blastoderm geometry.

**Data fitting**

Parameter space search: Since $k_2$ is the only term that has units of concentration, all curves scale relative to it and the absolute values of $k_2$ are not important. All space-dependent concentrations were therefore normalized to the value at the source boundary. A logarithmically spaced parameter grid of 50 X 50 values for $D$ and $k_1$ was screened (Table A.7). To identify the best $D$ and $k_1$ that describe the observed distribution profiles $I(x_n)$, either $D$ or $k_1$ were fixed to the values in the parameter space closest to the experimentally determined values, and the parameter space was searched for the value of the other parameter that minimized the sum of squared differences (SSD) between the simulations $c(x_n)$ and the experimental data sets $I(x_n)$ using

$$SSD = \sum_n (\bar{I}(x_n) - \bar{c}(x_n))^2$$

as shown in Fig. A.28.

To determine whether simulations using both our experimentally determined $D$ and $k_1$ values generate gradients that are similar to those we observed in vivo, both $D$ and $k_1$ were fixed to the experimentally determined values and the resulting curve overlaid onto the experimentally measured gradient data (Fig. A.28). Curves generated in this way fall within the error of the measured gradient data, supporting the idea that the distribution shapes are governed by diffusion and clearance.
Goodness of fit: To assess the goodness of the fits, $R^2$ values were calculated from the minimizing SSD ($SSD_{min}$) by

$$R^2 = 1 - \frac{SSD_{min}}{\sum_n \left( \bar{f}(x_n) - \frac{1}{n} \sum_f(x_n) \right)^2}$$

8. COMPARISON OF THE NODAL/LEFTY SYSTEM TO OTHER REACTION-DIFFUSION SYSTEMS

Summary

Diffusion-driven instabilities form the basis of reaction-diffusion systems (Turing 1952). It has been shown mathematically that a higher diffusivity of the inhibitor compared to the activator is a necessary condition for pattern formation (Murray 2003). However, clearance of activator and inhibitor can also affect the pattern formation probability (see “Influence of Clearance and Diffusion on Pattern Formation in Reaction-Diffusion Systems”). The ratios of diffusion coefficients and clearance rates ($d = D/k_i$) between activators and inhibitors ($R = d_{inhibitor}/d_{activator}$ (Granero et al. 1977)) provide a convenient measure to compare different patterning systems. Based on mathematical modeling, it has been suggested that the inhibitor in reaction-diffusion systems must have at least a six-fold higher normalized diffusivity than the activator in order for pattern formation to occur (Granero et al. 1977). In the following, we summarize reaction-diffusion modeling studies and compare the predicted $R$ values to our $R_{Lefty/Nodal}$. All models have values $R >> 6$. Intriguingly, the experimentally determined $R_{Lefty/Nodal}$ is approximately 14.
In the following, we describe previous biological and chemical studies of reaction-diffusion models. In Table A.8, we list diffusion coefficients and $k_1$ values used in these studies, as well as the average values we determined experimentally for Nodals and Leftys. In each system, the difference between $d_{\text{activator}}$ and $d_{\text{inhibitor}}$ is caused by either differences in diffusivity or differences in clearance. Note that in all systems except for the zebrafish Nodal/Lefty patterning system (this study), the parameter values have not been experimentally determined.

Hydra patterning: The freshwater polyp *Hydra* is a classical model system for spontaneous pattern formation due to its capability to regenerate and to form an organism even after dissociation into single cells. Pattern formation in *Hydra* was modeled by Meinhardt and Gierer in 1972, assuming an inhibitor that is 15 times more diffusive than the activator (Gierer & Meinhardt 1972). The greater than 10-fold difference in activator and inhibitor ranges originally postulated was later experimentally confirmed in Hydra aggregates (Technau et al. 2000), but diffusion and clearance have not been directly measured.

Mouse left-right patterning: In the reaction-diffusion models for left-right patterning, the Nodal/Lefty system amplifies a small bias in differential gene expression between the left and right sides of the embryo. In this system, the inhibitor Lefty has been modeled as two times more diffusive and five times more rapidly cleared than the activator Nodal (Nakamura et al. 2006).
Patterning of zebrafish stripes: Zebrafish stripe formation was modeled as a reaction-diffusion system with an activator and an inhibitor of unknown identity. In this system, differential diffusivity is the major contributor to differences between $d_{inhibitor}$ and $d_{activator}$ (Yamaguchi et al. 2007). A recent study has suggested contact-dependent cell depolarization and repulsive movement of pigment cells as a mechanism to achieve the short-range interaction, but the details remain to be determined (Inaba et al. 2012).

Patterning of angelfish stripes: The continually changing patterns of stripes on angelfish skin were modeled as a reaction-diffusion system with an activator and an inhibitor of unknown identity. In this system, differential diffusivity contributes to differences between $d_{inhibitor}$ and $d_{activator}$ more than differential clearance (Kondo & Asal 1995).

Patterning of mouse hair follicle spacing: Wnt was suggested to function as a short-range activator and Dkk as a long-range feedback inhibitor to pattern the spacing of murine hair follicles (Sick et al. 2006). In this system, differential diffusivity contributes to differences between $d_{inhibitor}$ and $d_{activator}$ slightly more than differential clearance.

Limb skeletal patterning: Vertebrate limb skeletogenesis is one of the longest-standing candidates for patterning by a reaction-diffusion mechanism. In a study of limb bud precartilage condensation size and spacing, a value of $R_{inhibitor/activator} = 9$ best reproduces the experimental measurements (Christley et al. 2007).

Chlorite/iodide malonic acid system: The chemical iodide-chlorite-malonic acid system
can generate Turing patterns (Lengyel & Epstein 1991). In this system, a ~15-fold higher diffusivity of inhibitor over activator was postulated ($R_{\text{inhibitor/activator}} = 15$, assuming that iodide and chlorite have approximately similar reaction-independent half-lives). Pattern formation is thought to be achieved by the introduction of a matrix that binds to and hinders the diffusion of the activator, but not the inhibitor. It is tempting to speculate that interactions with the extracellular matrix also generate the differences in the diffusivities of Nodal and Lefty signals during embryogenesis.
Figure A.3: Influence of relative clearance and diffusivity on pattern formation. Parameter space exploration in the Meinhardt-Gierer model (see Supplementary Material for details). The ability of the Meinhardt-Gierer reaction-diffusion system to form patterns was tested by evaluating the four conditions in Equations 15-18 over a range of values for $D$, $k$ and $\sigma$ (0-1) using linearly spaced sampling with 1,000 values for each parameter. The parameter space was collapsed along $\sigma$ by calculating the fraction of parameter combinations that satisfied all four conditions. This fraction reflects the probability that pattern formation occurs for the given values of $D$ and $k$. The relative diffusivities and clearance of activators and inhibitors affect the probability that a reaction-diffusion system will generate patterns. The probability of patterning increases as $k$ approaches 1 and as $D$ approaches 0. For example, no patterning will occur at position A; however, decreasing either $D$ (resulting in A**) or $k$ (resulting in A*) by a factor of four generates systems capable of forming patterns with identical probabilities. Similarly, the probability of patterning can be increased to the same extent for a system that is already capable of pattern formation (B) by decreasing $D$ (resulting in B**) or $k$ (resulting in B*) by a factor of two. Note that this is one of many reaction-diffusion systems, and the domains in parameter space in which patterns are obtained depend on the equations that describe the reactions.
Figure A.4: Processing of tagged Nodal signals. (A-B) Detection of extracellular fusion proteins by western blot. Embryos were injected with amounts of mRNA encoding Cyclops- (Cyc), Squint- (Sqt), or secreted (Sec) GFP or Dendra2 fusion proteins equimolar to 250 pg Squint-GFP at the one- to two-cell stage (Cyclops-GFP/Dendra2: 284 pg, Squint-GFP/Dendra2: 250 pg, Secreted GFP/Dendra2: 209 pg) and deyolked and dissociated at late blastula stages. The extracellularly enriched fraction was probed for the presence of GFP or Dendra2 by immunoblotting with anti-GFP or anti-Dendra2 antibodies. For each blot the position of marker bands of known molecular weight (kDa) is indicated. Asterisks denote the location of the mature processed Nodal fusion proteins (purple) as well as the free fluorescent proteins (green). (C) Embryos at the one- to two-cell stage were injected with mRNA encoding FLAG-tagged and FLAG-GFP constructs at equimolar amounts for each protein species (Cyclops-FLAG: 360 pg, Cyclops-FLAG-GFP: 500 pg, Squint-FLAG: 85 pg, Squint-FLAG-GFP: 125 pg). The mature Cyclops fusions are indicated with orange asterisks; the mature Squint fusions are indicated with blue asterisks. The FLAG-GFP-tagged proteins are present at similar levels as the FLAG-tagged proteins. (D) Input control for (C). Total protein was stained with Coomassie blue.
Figure A.5: Quantitative reverse transcription PCR (qRT-PCR) to assess Nodal fusion construct activity. Embryos at the one-cell stage were injected with two different amounts of mRNA encoding the indicated constructs and collected when uninjected siblings reached 50% epiboly. Fold increase in expression of the Nodal target gene goosecoid (gsc) compared to uninjected embryos was determined by qRT-PCR and is shown for embryos injected with mRNA encoding tagged or untagged versions of Cyclops (A) or Squint (B). Black and gray bars, respectively, represent results from embryos injected with equimolar amounts of mRNA. The zebrafish elongation factor eF1α was used as a normalization control. Error bars indicate standard deviation.
Figure A.6: *In situ* hybridization to assess Nodal fusion construct activity. Embryos at the one-cell stage were injected with different amounts of mRNA encoding the indicated constructs equimolar to the untagged constructs and fixed when uninjected sibling embryos reached 50% epiboly. Different amounts of mRNA were injected in order to assess dose-dependent activation of the Nodal target gene *goosecoid* (*gsc*) using *in situ* hybridization. Nodal-GFP and -Dendra2 fusion proteins are biologically active. Embryos were scored according to the indicated classes. n indicates the number of embryos analyzed.
Figure A.7: Tagged and untagged Nodal signals have similar activity ranges. To assess the range of Nodal constructs, donor embryos were injected with equimolar amounts of mRNA encoding tagged and untagged constructs (Cyclops: 204 pg; Cyclops-GFP: 284 pg; Squint: 175 pg; Squint-GFP: 250 pg). At late blastula stages, approximately 40 cells (brown, marked by the presence of the injection tracer biotinylated dextran) were transplanted into the animal pole of wildtype hosts. Embryos were fixed one hour after transplantation and processed for in situ hybridization using probes against the Nodal target genes fascin or no tail (blue). (A) Representative embryo used to assess the range of Nodal constructs. Blue: no tail expression, brown: transplanted cells, red: analysis window. (B) Overview of activity ranges. (C-D) Quantification of the activity range of Nodal constructs. In situ hybridization was used to determine the area around the clone in which the Nodal target genes fascin and no tail were expressed. The induced area was divided by the area of the clone (as assessed by staining for the injection tracer biotinylated dextran) to normalize for differences in clone size between embryos. Error bars represent standard error. n indicates the number of embryos analyzed.
Figure A.8: Detection of extracellular Lefty fusion proteins by western blot. (A-B) Embryos were injected with amounts of mRNA encoding GFP or Dendra2 fusion proteins equimolar to 250 pg Squint-GFP at the one- to two-cell stage (Lefty1-GFP/Dendra2: 234 pg, Lefty2-GFP/Dendra2: 236 pg) and deyolked and dissociated at late blastula stages. The extracellularly enriched fraction was probed for the presence of GFP or Dendra2 by immunoblotting with anti-GFP or anti-Dendra2 antibodies. For each blot the position of marker bands of known molecular weight (kDa) is indicated. Asterisks denote the location of the mature processed Lefty fusion proteins (purple) as well as the free fluorescent proteins (green). (C) Embryos at the one- to two-cell stage were injected with mRNA encoding FLAG-tagged and FLAG-GFP constructs at equimolar amounts for each protein species (Lefty1-FLAG: 330 pg, Lefty1-GFP-FLAG: 500 pg, Lefty2-FLAG: 330 pg, Lefty2-GFP-FLAG: 500 pg). The Lefty1 fusions are indicated with orange asterisks; the Lefty2 fusions are indicated with blue asterisks. The FLAG-GFP-tagged proteins are present at lower levels compared to the FLAG-tagged proteins. (D) Input control for (C). Total protein was stained with Coomassie blue.
Figure A.9: Overexpression of Lefty fusion proteins phenocopies Nodal loss of function. Embryos at the one-cell stage were injected with equimolar amounts of the indicated amounts of mRNA encoding tagged or untagged Lefty constructs and imaged 24 - 27 hours post-fertilization. Loss of Nodal signaling results in failure to generate head and trunk mesendoderm, leading to cyclopia and the lack of a notochord and hatching gland. (A) Uninjected wildtype embryo, lateral view on the left, ventral view of head on the right. Blue arrow: notochord. Black arrows: eyes. Brown arrows: hatching gland cells. (B) Maternal-zygotic (MZoep) mutant for the Nodal co-receptor oep (one-eyed pinhead). All Nodal signaling is abolished in MZoep embryos, which exhibit cyclopia (black arrowhead) and loss of notochord (blue arrowhead) and hatching gland (brown arrowheads). (C-H) Overexpression of Lefty1 constructs. (I-N) Overexpression of Lefty2 constructs. (E, G, K) Note partial reduction of notochord (thin blue arrow) and hatching gland (thin brown arrow). Also note close proximity of eyes in (E) and (G), consistent with partial loss of Nodal function.
Figure A.10: Quantitative reverse transcription PCR (qRT-PCR) to assess Lefty fusion construct activity. Embryos at the one- to two-cell stage were injected with different amounts of mRNA encoding the indicated constructs and collected at 50% epiboly. Fold decrease in expression of the Nodal target gene goosecoid (gsc) compared to uninjected embryos was determined by qRT-PCR. Different mRNA amounts were used to assess dose-dependent repression. The zebrafish elongation factor eFla was used as a normalization control. Fold decrease in gsc expression in embryos injected with mRNA encoding tagged or untagged versions of Lefty1 (A) and Lefty2 (B) compared to uninjected embryos is shown. Error bars indicate standard deviation.
Figure A.11: *In situ* hybridization to assess Lefty fusion construct activity. Embryos at the one-cell stage were injected with different amounts of mRNA encoding the indicated constructs equimolar to the untagged constructs and fixed at 50% epiboly. Different amounts of mRNA were injected in order to assess dose-dependent repression of the Nodal target gene *goosecoid* (*gsc*) using *in situ* hybridization. Lefty-GFP and -Dendra2 fusion proteins are biologically active. Embryos were scored according to the indicated classes. n indicates the number of embryos analyzed.
Figure A.12: Tagged and untagged Lefty proteins have similar activity ranges. To assess the range of Lefty constructs, single cells in embryos at the 64- to 128-cell stage were injected with equimolar amounts of mRNA (Lefty1 and Lefty2: 6 pg; Lefty1-GFP and Lefty2-GFP: 9 pg). Biotin-dextran (brown) was used as an injection tracer. Embryos were fixed at 50% epiboly. Activity range was assessed by in situ hybridization against the Nodal target gene fascin (blue). fascin is normally expressed in a ring at the blastula margin (A, left panel). In embryos containing clones that secrete tagged or untagged Lefty proteins, the ring of fascin expression is reduced or absent. (B) Quantification of the activity range of Lefty constructs. Embryos were scored according to the classes shown in (A). Arrow indicates faint dorsal expression. Blue: fascin expression, brown: cells expressing Lefty constructs (as assessed by staining for the injection tracer biotinylated dextran).
Figure A.13: Localization of uniformly expressed Nodal- and Lefty-GFP fusion proteins. Embryos at the one-cell stage were co-injected with 30 pg of mRNA encoding the indicated fusion construct and 30-50 pg of mRNA encoding membrane-bound RFP to outline cellular membranes. Embryos were imaged between sphere stage and 30% epiboly. Cyclops-GFP exhibits weak diffuse extracellular localization as well as bright membrane-associated clusters. Membrane-associated clusters are less frequently found in embryos expressing Squint-GFP. Lefty1- and Lefty2-GFP are almost exclusively extracellular. The localization of Nodal- and Lefty-Dendra2 fusion constructs is similar to that of the corresponding GFP fusions (not shown).
Figure A.14: Measurement of extracellular clearance rate coefficients ($k_t$). (A) Clearance assay experimental overview. Uniformly expressed Dendra2 fusion proteins were photoconverted using a UV pulse. (B) Images were obtained every 10 or 20 minutes following photoconversion for a total of five hours. The intensity decrease in the extracellular space over time was used to determine the clearance rate coefficient of the extracellular fusion protein. (C-F) Embryos were co-injected at the one-cell stage with
0.4-1.9 ng Alexa488-dextran and 60 pg of mRNA encoding Cyclops-Dendra2 (C), Squint-Dendra2 (D), Lefty1-Dendra2 (E), or Lefty2-Dendra2 (F). Ubiquitously expressed Dendra2 fusion proteins were photoconverted at late blastula stages. The average extracellular photoconverted Dendra2 intensity was monitored over time (the Alexa488 signal was used to mask intracellular regions). Clearance rate coefficients ($k_1$) and half-lives ($\tau = \ln(2)/k_1$) were determined by fitting exponential functions to data from individual embryos from both 10 and 20 min interval experiments. The normalized average intensity from 10 min interval experiments (black) is shown fitted with exponential functions (red). Error bars indicate standard deviation. For 10 min interval experiments, Cyclops-Dendra2: n=6, Squint-Dendra2: n=11, Lefty1-Dendra2: n=7, and Lefty2-Dendra2: n=13. Cyclops-Dendra2 images are shown at 3x magnification to enhance visibility of punctate signal.
Figure A.15: Masking intracellular signal in the clearance assay. (A–E) Embryos were co-injected at the one-cell stage with 0.4-1.9 ng Alexa488-dextran and 60 pg of mRNA encoding Cyclops-Dendra2 (A), Squint-Dendra2 (B), Lefty1-Dendra2 (C), Lefty2-Dendra2 (D), or secreted Dendra2 (E). Uniformly expressed fusion proteins were photoconverted using a UV pulse at late blastula stages, and one medial optical slice was
imaged once every 10 or 20 min. The majority of the Alexa488-dextran remains inside cells. Using a thresholding algorithm and Alexa488 signal, masks were generated to define extracellular space (middle panel, white). This mask was then applied to the images from the red channel (top panel) in order to generate images in which only the extracellular red photoconverted Dendra2 signal is considered (bottom panel). (F) Summary of average extracellular clearance rate coefficient ($k_i$) values. Extracellular red fluorescence after photoconversion was monitored over time in individual embryos expressing the indicated Dendra2 fusion constructs. For each embryo, the resulting data set was fitted with an exponential function, and the clearance rate coefficient ($k_i$) was determined. Average $k_i$ values from 10 and 20 min interval experiments (Fig. A.18) are shown for each construct. Average $k_1$ values that are significantly different between different experimental groups (p-value < 0.005, Wilcoxon-Mann-Whitney test) are indicated by black lines. Higher $k_i$ values indicate higher protein clearance. Error bars indicate standard error. Cyclops-Dendra2: n=9; Squint-Dendra2: n=23; Lefty1-Dendra2: n=19; Lefty2-Dendra2: n=27; secreted Dendra2: n=22.
Figure A.16: Assessing uniformity of photoconversion. If photoconversion in the clearance assay were non-uniform, the measurements of clearance rate coefficients could be flawed due to diffusive flux. Therefore, the uniformity of photoconversion along the animal-vegetal axis was determined by measuring mean intensity at different depths in embryos containing photoconverted secreted Dendra2 over time. If protein near the animal pole were more likely to be photoconverted than protein near the vegetal pole, the observed normalized signal intensity near the vegetal pole would increase over time due to diffusion of photoconverted protein from the animal pole towards the vegetal pole. (A) Experimental overview. Embryos were co-injected with 1.9 ng Alexa488-dextran and 60 pg mRNA encoding secreted Dendra2. Fusion proteins were photoconverted in the same manner as in the clearance assay experiments. Z-stacks comprised of 10 slices spaced by a depth of 8 µm were taken every 20 min for a total of 80 min post-photoconversion, and the mean intensity in a 35 µm diameter circular ROI centered in each slice was determined. If photoconversion were uniform, the signal should be homogeneously distributed throughout the embryo. Light scattering causes average intensity to drop as a function of depth. (B, E) Mean raw Alexa488 signal intensity as a function of depth in the embryo (animal pole = 0 µm). Colors indicate time post-photoconversion. Results for two embryos are shown. (C, F) Mean raw photoconverted red Dendra2 signal intensity as a function of depth in the embryo. (D, G) Normalized mean Alexa488 and photoconverted...
red Dendra2 signal intensity as a function of depth in the embryo. For each time point, data was normalized by dividing all time points by the average intensity in the first (most shallow) z-slice, after background subtraction of average extraembryonic intensity (70 a.u. for photoconverted Dendra2 imaging conditions and 300 a.u. for Alexa488 imaging conditions). If photoconversion were nonuniform, the normalized intensity near the vegetal pole would increase over time as photoconverted protein diffused vegetally. However, no increases in normalized vegetal signal over time were observed, suggesting that photoconversion was uniform.
Figure A.17: Assessing background intensity changes in clearance assay experiments. To determine whether changes in background intensity (e.g. due to autofluorescence and bleed-through of the green Alexa488 signal into the red channel) affect clearance measurements, 1.9 ng Alexa488-dextran were co-injected with mRNA encoding untagged Cyclops (A), Squint (B), Lefty1 (C) and Lefty2 (D) into embryos at the one-cell stage, followed by mock clearance assay. The raw mean background intensity (gray) is shown for the extracellular compartments, along with data from representative individual embryos expressing Dendra2 fusions (black). Error bars indicate standard deviation of background intensity. For all constructs, changes in background intensity were negligible (the changes in the mean raw background intensity over time are comparable to error bars at each time point). For background data, Cyclops: n=7, Squint: n=8, Lefty1: n=4, and Lefty2: n=3.
Figure A.18: Assessment of photobleaching and inadvertent photoconversion. In the clearance assay, embryos are imaged multiple times over the course of five hours. To determine whether significant photobleaching occurs, or whether imaging using the 488 nm laser causes inadvertent photoconversion of Dendra2 fusion proteins, experiments were performed in which the interval between images was either 10 or 20 min. Significant photobleaching or inadvertent photoconversion would cause discrepancies in clearance kinetics observed in these two experiments. The normalized average intensity for 10 min (black) and 20 min (blue) interval data for each construct is shown. No significant differences were observed between the 10 and 20 min interval data for any construct, or for any compartment (i.e. extracellular (first column), intracellular (second row), total slice (third column)).
column), and total optical slice (third column); all p-values were much higher than 0.005 using the Wilcoxon-Mann-Whitney test to determine whether $k_1$ values differed significantly between 10 and 20 min interval experiments. Average clearance rate coefficients ($k_1$) and standard error values are shown for 10 min ($k_1 [10 \text{ min}]$) and 20 min ($k_1 [20 \text{ min}]$) interval experiments separately, as well as for 10 and 20 min interval experiments combined ($k_1 [\text{all}]$). Error bars indicate standard deviation. For 10 min interval experiments, Cyclops-Dendra2: n=6, Squint-Dendra2: n=11, Lefty1-Dendra2: n=7, Lefty2-Dendra2: n=13, and secreted-Dendra2: n=6. For 20 min interval experiments, Cyclops-Dendra2: n=3, Squint-Dendra2: n=12, Lefty1-Dendra2: n=12, Lefty2-Dendra2: n=14, and secreted-Dendra2: n=16.
Figure A.19: Overexpression of Squint does not alter Squint-GFP dispersal. If Nodal binding sites are saturated, the diffusivity of the molecules may be overestimated in FRAP experiments. In this scenario, overexpression of untagged Squint should saturate binding sites and thus increase the diffusivity and extend the range of Squint-GFP. To test this idea, we overexpressed 30 pg of untagged Squint (similar to levels of Squint-GFP used in FRAP experiments) and then analyzed the distribution of Squint-GFP generated from a local source. Importantly, co-expression did not result in a change in Squint-GFP distribution, arguing against the idea of saturation. Data points represent averages, and error bars indicate standard error of n experiments.
Figure A.20: Measurement of effective diffusion coefficients. (A-B) FRAP experimental overview. A cuboidal volume was bleached into embryos uniformly expressing secreted fluorescent fusion proteins (A). Medial optical slices were imaged every 10 s following bleaching. The average intensity in the bleached region recovers over time (B) and can be used to calculate the diffusion coefficient of the fluorescent species. (C-F) Embryos were injected at the one-cell stage with 30 pg of mRNA encoding Cyclops-GFP (C), Squint-GFP (D), Lefty1-GFP (E) or Lefty2-GFP (F). Uniformly
expressed Nodal- or Lefty-GFP fusion proteins were locally photobleached at blastula stages. Optical slices were acquired every 10 s after the bleach for a total of 50 min. The effective diffusion coefficient, $D$, was determined by fitting the resulting recovery profile (black) with simulated recovery curves (red) that were numerically generated using a model that includes diffusion, production and clearance in a three-dimensional embryo-like geometry. Results for individual embryos are shown normalized to the final time point, and average diffusion coefficients are listed here and in Table A.6.
Figure A.21: FRAP geometries. (A) Average embryo geometry during FRAP experiments. Embryos were injected with mRNA encoding GFP fusion constructs at the one-cell stage, generating embryos that uniformly express the GFP fusions (green). FRAP experiments were performed at late blastula stages, when the blastoderm forms a dome on top of the yolk. A cuboidal volume was bleached into the center of the embryo (black), and recovery of fluorescence was observed in a medial optical section (red). This geometry was used for three-dimensional simulations of FRAP experiments (3D analysis model) with the geometric parameters indicated in (B). (C) Medial optical section acquired during FRAP experiments (two-dimensional view of the red line in (A)). The
bleached window is indicated in black. This geometry was used for two-dimensional simulations of FRAP experiments (2D analysis model) as shown in (D). The analysis areas inside the bleach window ($A_{\text{win}}$), outside the bleach window ($A_{\text{out}}$) and the entire slice ($A_{\text{sl}}$) used to calculate spatial intensity averages are indicated. (E) Three-dimensional extension of the two-dimensional model shown in (C) and (D). The slice would extend to infinity above and below the depicted cylinder geometry. (F) Averaging of the eight octants (Q1-Q8) for the first image taken after photobleaching defines the initial condition $I_0$ in the imaging plane used for three-dimensional simulations of FRAP experiments. (G) Simulated FRAP recovery curves in two- and three-dimensional model geometries. FRAP experiments were simulated using the 3D or 2D analysis models for a range of diffusion coefficients, $D$, from 0.1 to 50 µm$^2$/s without clearance or production. For the initial condition, the concentration in the bleached region was set to a value of zero and to a value of one everywhere else. The resulting recovery curves in the bleached window were normalized to the concentration at the final time point $t = 3000$ s. Note that the recovery profiles in two- and three-dimensional geometries are similar for small values of $D$, but different for higher diffusivities. (H) The 3D analysis model was used to generate a recovery curve given a diffusion coefficient of 10 µm$^2$/s (red). This simulated curve was then fitted using the 2D model (blue). Consistent with (G), the 2D fit results in a lower diffusion coefficient, highlighting the differences between the 2D and 3D approaches and the importance of using the appropriate geometry when fitting FRAP data.
Figure A.22: Diffusive flux in the three-dimensional embryo geometry. FRAP experiments were simulated using the 3D analysis models for a range of 20 diffusion coefficients, $D$, logarithmically spaced between 0.1 and 100 $\mu$m$^2$/s without clearance or production. For the initial condition, the concentration in the “bleached volume” was assigned a value of zero and a value of one everywhere else. The average concentration was calculated for each time point in the bleached window (A), in the region outside of the window (B), in the analysis slice (C), and in the total embryo (D). Note that in a two-dimensional geometry, the concentration in the analysis slice (C) should be constant due to no-flux boundary conditions, whereas diffusive flux through the slice has a significant contribution in the three-dimensional model for certain diffusion coefficients. The concentration in the entire three-dimensional model (D) is constant and conserved due to no-flux boundary conditions.
Figure A.23: Simulation of FRAP recovery curves with diffusion, clearance and production. All simulations were performed using the three-dimensional analysis model. For the initial condition, the concentration in the bleached volume was set to a value of zero, and a value of one was assigned everywhere else. The average concentration was calculated for each time point in the bleached window. (A) Scaling of recovery curves with diffusion time in the “diffusion only” model. Note that the recovery curves scale with diffusion coefficient and time. For example, it takes a recovery curve with a diffusion coefficient of $D_1 = 1.0 \, \mu m^2/s$ twice as long to reach the same concentration as a recovery curve with $D_2 = 2 \times D_1 = 2.0 \, \mu m^2/s$ and four times as long as a recovery curve with $D_2 = 4 \times D_1 = 4.0 \, \mu m^2/s$. This relationship was used to simulate FRAP experiments in a non-dimensionalized model using a single reference diffusion coefficient to generate a one-parameter family of curves. (B) Constructing recovery curves with diffusion, clearance and production. It was assumed that all reactions (clearance and production) are linear and occur everywhere in the embryo. Therefore, contributions from diffusion, clearance and production are linearly superimposed on the recovery profile. To generate recovery curves with the appropriate reactions model, a family of curves was first generated based on diffusion only by numerical simulation as described in (A). The resulting recovery curve was scaled to a diffusion coefficient of choice (in this case $D = 10 \, \mu m^2/s$), and then clearance (in this case $k_1 = 0.0001/s$) or production terms (in this case $k_2 = 0.0002 \, \text{a.u./s}$) were added using the analytical solutions described in the Supplementary Materials.
Figure A.24: Intensity profiles post-photobleaching in FRAP experiments. A cuboidal volume was bleached into blastula-stage embryos injected with 30 pg of mRNA encoding GFP fusion constructs at the one-cell stage. (A-D) The horizontal bleach profile in the optical medial slice was determined immediately after the completion of photobleaching. Note that the bleach profile is sharp for Cyclops-GFP but is more relaxed for Squint-GFP and Lefty-GFP constructs indicating increasingly higher diffusivities. (B) Time-dependent evolution of the horizontal bleach profile for an embryo expressing Cyclops-GFP. Early times are indicated as shades of blue and later times by shades of red. (E-F) False-color representation of the averaged image acquired immediately after photobleaching for Squint-GFP and Lefty1-GFP, respectively. The raw intensities of the eight octants (Q1-Q8) comprising the imaged slice were averaged pixel-wise, and the resulting averaged octant is displayed as a quadrant with two mirror images to illustrate the relatively uniform bleach profile. Note that these averaged images were used to define the initial condition for the simulations to fit the FRAP experiments as described in the Supplementary Materials.
Figure A.25: **Diffusion-dominated recovery in FRAP experiments.** The average intensity in the bleached window (black) or in a smaller window (red) nested within the bleached window (A) was quantified for FRAP data from single embryos (B-G). The first 1500 s of recovery are shown. Inset: entire 3000 s recovery. Note that recovery in a smaller analysis window of the bleached region is slower than the recovery in the bigger bleached window for the majority of Cyclops-GFP and Squint-GFP embryos analyzed.
These results indicate that the FRAP recovery curves are dominated by diffusion and not by binding or other uniform reactions, which would lead to a uniform recovery independent of the analysis window dimensions (Supplementary Materials; Fig. A.26). The recovery delay for the Lefty1-GFP, Lefty2-GFP, and extracellular GFP experiments is less apparent, presumably due to higher diffusivities (consistent with simulations of effective diffusion in Fig. A.27) and the limited time resolution of the FRAP assay. (H) Signal recovery occurs from the surrounding unbleached regions. Snapshots of octant-averaged images after photobleaching are shown for Squint-GFP. Note that the fluorescence in the bleached window recovers first in regions adjacent to the unbleached domain.
Figure A.26: Examples of diffusion-uncoupled recovery curves. See Supplementary Materials for details. Diffusion-uncoupled recovery after photobleaching occurs when diffusion is fast compared to binding kinetics. In this scenario, diffusion rapidly equilibrates the concentration in the bleached window, and the subsequent concentration increase is due to spatially uniform accumulation of bound molecules. The recovery curves would therefore not yield direct information about diffusivity but rather about binding and degradation kinetics. Diffusion-uncoupled recovery can also occur with fast diffusion and fast irreversible binding. In this case, any free molecules are rapidly trapped and the recovery curves almost exclusively reflect the spatially homogenous increase due to production and degradation. To determine whether our FRAP data could be dominated by a diffusion-uncoupled process, we simulated FRAP experiments with fast diffusion and slow binding kinetics as well as experiments with fast diffusion and fast irreversible binding. (A) FRAP experiments were simulated using a two-dimensional embryo model, and recovery was analyzed in the entire bleached window (black) and in a smaller window (red) nested within. Simulated recovery curves when binding is slow and reversible (B), slow and irreversible (C), and fast and irreversible (D). All recovery curves lack the initial delay that we observe in our FRAP experiments (Fig. A.25), suggesting that fast diffusion combined with slow reversible binding, slow irreversible binding, or fast irreversible binding does not occur in our experiments. Parameter values used for all simulations: $D = 20 \, \mu m^2/s$, $k_{\text{Clear}} = 0.0001/s$, $k_{\text{Prod}} = 0.0002 \, nM/s$. The initial concentration of the diffusible ligand was set to 0 in the entire bleached window and to 0.05 nM everywhere else, whereas the initial concentration of the bound complex was set to 0.95 nM to reflect that in these models most ligand molecules would be bound. The initial concentration of the free binding partner was set to 1000 nM. The spatial averages of the sum of the diffusible species and the bound complex are shown.
Figure A.27: Potential mechanism underlying differential effective diffusivity. See Supplementary Materials for details. A potential mechanism underlying differential effective diffusivity is reversible binding to immobile extracellular molecules. Nodals and Leftys may have equally high free diffusivities, but Nodals may have a higher affinity for extracellular binding partners than Leftys. In this model, fast binding and unbinding would differentially hinder free diffusion and yield smaller effective diffusion coefficients. To test this model, we simulated FRAP experiments using a two-dimensional embryo model and analyzed recovery in the entire bleached window (black) and in a smaller window (red) nested within (A). (B-E) Expected recovery curves for Cyclops-GFP (B), Squint-GFP (C), Lefty1-GFP (D), and Lefty2-GFP (E) with the indicated effective diffusion coefficients, $D_{\text{eff}}$, as measured by FRAP experiments. Recovery in the smaller window is delayed, because molecules must move from the edge of the larger window to the center. The simulations recapitulate our experimental observations (Fig. A.25). (F-I) Expected recovery curves for Cyclops-GFP (F), Squint-GFP (G), Lefty1-GFP (H), and Lefty2-GFP (I) using equally high free diffusion coefficients, $D^* = 20 \mu m^2/s$, in the presence of fast reversible binding. For the chosen binding kinetics, recovery curves look identical to those produced by small effective diffusion coefficients. Free diffusion combined with fast binding kinetics could therefore explain the different diffusion coefficients of Nodals and Leftys. Production rates and clearance rate constants used for the simulations are as follows: (B, F) $k_{\text{Prod}} = 0.0002$ nM/s, $k_{\text{Clear}} = 0.000122$/s; (C, G) $k_{\text{Prod}} = 0.0002$ nM/s, $k_{\text{Clear}} = 0.0001$/s; (D, H) $k_{\text{Prod}} = 0.0001$ nM/s, $k_{\text{Clear}} = 0.000053$/s; (E, I) $k_{\text{Prod}} = 0.0001$ nM/s, $k_{\text{Clear}} = 0.000069$/s. The clearance rate constants correspond to our experimental measurements. The initial concentration of the diffusible species was set to 0 in the entire bleached window and to 1 nM everywhere else. The initial concentration of the binding partner was set to 1000 nM. The spatial averages of the diffusible species are shown in (B-E), whereas the spatial averages of the sum of the diffusible species and the bound complex are shown in (F-I).
Figure A.28: Modeling and simulations of gradient formation. (A) A parameter grid was generated in the indicated three-dimensional geometry, and equations in the source and in the target field using a combination of 50 logarithmically spaced diffusion coefficients \([D_{min} = 0.1 \, \text{um}^2/\text{s}, D_{max} = 50 \, \text{um}^2/\text{s}]\) and clearance rate constants \([k_{1_{min}} = 0.00001/\text{s}, k_{1_{max}} = 0.0005/\text{s}]\) to simulate the gradient formation experiments from Fig. 2.2. (B-E) This parameter space was searched for the best match with the least sum of squared differences between experimental data and simulated gradients using values in the parameter space closest to the experimentally determined diffusion coefficients (fixed \(D\)) or clearance rate constants (fixed \(k_{1}\)) for Cyclops (B), Squint (C), Lefty1 (D) and Lefty2 (E) constructs. Finally, profiles using the experimentally determined values of both \(D\) and \(k_{1}\) were generated and overlaid onto the experimental data (fixed \(k_{1}\) and \(D\)). Black error bars indicate the standard deviation for the indicated experiments, and red lines show the results of the simulations. R2 values indicate the goodness of fit. Yellow
indicates the range of expected gradients based on \( D \) and \( k_i \) measurements: the upper limit was determined by generating a gradient using the mean \( D \) plus standard error and the mean \( k_i \) minus standard error, whereas the lower limit was determined using the mean \( D \) minus standard error and the mean \( k_i \) plus standard error. All diffusion coefficients, \( D \), are reported in units of \( \mu \text{m}^2/\text{s} \), and all clearance rate coefficients (\( k_i \)) are reported in units of \( 1/\text{s} \).
Table A.1: Minimization parameters for clearance assay fitting.

<table>
<thead>
<tr>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial guess for $k_1$</td>
<td>0</td>
</tr>
<tr>
<td>Lower limit for $k_1$</td>
<td>0</td>
</tr>
<tr>
<td>Upper limit for $k_1$</td>
<td>$\infty$</td>
</tr>
<tr>
<td>Initial guess for $y_0$</td>
<td>Average intensity of first postconversion image $I_{post}(t_0)$ + $\bar{N}$, with $F$</td>
</tr>
<tr>
<td>Lower limit for $y_0$</td>
<td>$\bar{B}_{pre}$: preconversion background average intensity $\bar{N}$: average instrument noise</td>
</tr>
<tr>
<td>Upper limit $y_0$</td>
<td>Maximum value of $\bar{I}_{int}$</td>
</tr>
<tr>
<td>Initial guess for $c_0$</td>
<td>$I_{post}(t_0) - \bar{I}_{pre}$</td>
</tr>
<tr>
<td>Lower limit for $c_0$</td>
<td>0</td>
</tr>
<tr>
<td>Upper limit for $c_0$</td>
<td>$\infty$</td>
</tr>
</tbody>
</table>

Table A.2: F values for all constructs and compartments.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Extracellular</th>
<th>Slice (embryo)</th>
<th>Intracellular</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclops-Dendra2</td>
<td>0.9922</td>
<td>0.9705</td>
<td>0.9258</td>
</tr>
<tr>
<td>Squint-Dendra2</td>
<td>0.9597</td>
<td>0.8612</td>
<td>0.8281</td>
</tr>
<tr>
<td>Lefty1-Dendra2</td>
<td>0.9562</td>
<td>0.9791</td>
<td>0.9818</td>
</tr>
<tr>
<td>Lefty2-Dendra2</td>
<td>0.9461</td>
<td>0.9590</td>
<td>0.9799</td>
</tr>
<tr>
<td>Secreted Dendra2</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Table A.3: Summary of clearance rate constants $k_i$.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Extracellular $k_1$</th>
<th>Slice (embryo) $k_1$</th>
<th>Intracellular $k_1$</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$(10^{-4}/s)$</td>
<td>$(10^{-4}/s)$</td>
<td>$(10^{-4}/s)$</td>
<td></td>
</tr>
<tr>
<td>Cyclops-Dendra2</td>
<td>$1.22 \pm 0.13$</td>
<td>$1.43 \pm 0.15$</td>
<td>$1.41 \pm 0.14$</td>
<td>9</td>
</tr>
<tr>
<td>Squint-Dendra2</td>
<td>$1.00 \pm 0.06$</td>
<td>$0.81 \pm 0.04$</td>
<td>$0.77 \pm 0.05$</td>
<td>23</td>
</tr>
<tr>
<td>Lefty1-Dendra2</td>
<td>$0.53 \pm 0.05$</td>
<td>$0.56 \pm 0.17$</td>
<td>$0.70 \pm 0.05$</td>
<td>19</td>
</tr>
<tr>
<td>Lefty2-Dendra2</td>
<td>$0.69 \pm 0.07$</td>
<td>$0.67 \pm 0.14$</td>
<td>$0.93 \pm 0.14$</td>
<td>27</td>
</tr>
<tr>
<td>Secreted Dendra2</td>
<td>$0.51 \pm 0.05$</td>
<td>$0.47 \pm 0.07$</td>
<td>$0.91 \pm 0.14$</td>
<td>22</td>
</tr>
</tbody>
</table>

Values represent the mean ± standard error of $n$ experiments.
Table A.4: FEM parameters for numerical simulation of FRAP experiments.

<table>
<thead>
<tr>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative tolerance for solver</td>
<td>$1 \times 10^{-2}$</td>
</tr>
<tr>
<td>Absolute tolerance for solver</td>
<td>$1 \times 10^{-3}$</td>
</tr>
<tr>
<td>Average mesh element size</td>
<td>$\sim 10 \mu$m</td>
</tr>
<tr>
<td>Boundary layer mesh thickness</td>
<td>8 $\mu$m</td>
</tr>
<tr>
<td>Layers in the boundary layer mesh</td>
<td>8</td>
</tr>
<tr>
<td>Number of time steps for simulations</td>
<td>$4 \times 10^3$</td>
</tr>
<tr>
<td>Diffusion coefficient used to generate reference curves</td>
<td>10 $\mu$m$^2$/s</td>
</tr>
</tbody>
</table>

To generate the mesh, a value of 7 was used for the ‘hauto’ parameter globally in Comsol Multiphysics, which controls the element size in the generated mesh and sets several mesh parameters automatically.

Table A.5: Minimization parameters for FRAP data fitting.

<table>
<thead>
<tr>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Termination tolerance for SSD minimization</td>
<td>$1 \times 10^{-6}$</td>
</tr>
<tr>
<td>Termination tolerance on the function value</td>
<td>$1 \times 10^{-6}$</td>
</tr>
<tr>
<td>Lower bound for $D$ during SSD minimization</td>
<td>$1 \times 10^{-3}$ $\mu$m$^2$/s</td>
</tr>
<tr>
<td>Upper bound for $D$ during SSD minimization</td>
<td>100 $\mu$m$^2$/s</td>
</tr>
<tr>
<td>Initial guess for $D$ during SSD minimization</td>
<td>0 $\mu$m$^2$/s</td>
</tr>
<tr>
<td>Lower bound for $k_2$ during SSD minimization</td>
<td>0 units/s</td>
</tr>
<tr>
<td>Upper bound for $k_2$ during SSD minimization</td>
<td>$\infty$</td>
</tr>
<tr>
<td>Initial guess for $k_2$ during SSD minimization</td>
<td>0 units/s</td>
</tr>
<tr>
<td>Lower limit for the equalization factor $EQ$</td>
<td>0.1</td>
</tr>
<tr>
<td>Upper limit for the equalization factor $EQ$</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Table A.6: FRAP results from three-dimensional simulations.

<table>
<thead>
<tr>
<th>Protein</th>
<th>D “diffusion only” ($\mu$m$^2$/s)</th>
<th>D “diffusion and production” ($\mu$m$^2$/s)</th>
<th>D “diffusion, production and clearance” ($\mu$m$^2$/s)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclops-GFP</td>
<td>0.7 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>0.7 ± 0.2</td>
<td>22</td>
</tr>
<tr>
<td>Squint-GFP</td>
<td>3.8 ± 0.7</td>
<td>3.8 ± 0.7</td>
<td>3.2 ± 0.5</td>
<td>30</td>
</tr>
<tr>
<td>Lefty1-GFP</td>
<td>6.5 ± 0.3</td>
<td>11.6 ± 0.6</td>
<td>11.1 ± 0.6</td>
<td>21</td>
</tr>
<tr>
<td>Lefty2-GFP</td>
<td>25.9 ± 9.8</td>
<td>30.0 ± 8.4</td>
<td>18.9 ± 3.0</td>
<td>5</td>
</tr>
<tr>
<td>Secreted GFP</td>
<td>33.7 ± 16.8</td>
<td>36.7 ± 16.5</td>
<td>33.9 ± 15.5</td>
<td>5</td>
</tr>
<tr>
<td>Recombinant GFP</td>
<td>44.0 ± 8.5</td>
<td>44.0 ± 8.5</td>
<td>37.6 ± 6.9</td>
<td>10</td>
</tr>
</tbody>
</table>

For the model “diffusion, production and clearance”, the clearance rate constants ($k_2$) measured using the clearance assay were used. The $k_3$ for secreted Dendra2 was used for secreted and recombinant GFP. Recombinant GFP was injected into the extracellular space. Values represent the mean ± standard error of n experiments.
<table>
<thead>
<tr>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distance from origin to center of source</td>
<td>80 µm</td>
</tr>
<tr>
<td>Distance from animal pole to center of z-stack</td>
<td>50 µm</td>
</tr>
<tr>
<td>Slice radius</td>
<td>167 µm</td>
</tr>
<tr>
<td>Source radius</td>
<td>40 µm</td>
</tr>
<tr>
<td>Slice thickness</td>
<td>20 µm</td>
</tr>
<tr>
<td>Relative tolerance for spatial solver</td>
<td>$1 \times 10^{-6}$</td>
</tr>
<tr>
<td>Absolute tolerance for spatial solver</td>
<td>$1 \times 10^{-7}$</td>
</tr>
<tr>
<td>Average mesh element size</td>
<td>~10 µm</td>
</tr>
<tr>
<td>Maximum element size at the source boundary</td>
<td>20 µm</td>
</tr>
<tr>
<td>Grid spatial resolution $DX$</td>
<td>100</td>
</tr>
<tr>
<td>Lower bound for $D$ in parameter grid</td>
<td>0.1 µm²/s</td>
</tr>
<tr>
<td>Upper bound for $D$ in parameter grid</td>
<td>50 µm²/s</td>
</tr>
<tr>
<td>Lower bound for $k_1$ in parameter grid</td>
<td>$1 \times 10^{-5}$ /s</td>
</tr>
<tr>
<td>Upper bound for $k_1$ in parameter grid</td>
<td>$5 \times 10^{-4}$ /s</td>
</tr>
</tbody>
</table>

To generate the mesh, a value of 7 was used for ‘hauto’ globally in Consol Multiphysics, which controls the element size in the generated mesh and sets several mesh parameters automatically.

**Table A.7: FEM parameters for numerical simulations of distribution profiles.**
<table>
<thead>
<tr>
<th>System</th>
<th>Units</th>
<th>$D_a$</th>
<th>$D_i$</th>
<th>$k_a$</th>
<th>$k_i$</th>
<th>$R$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydra patterning</td>
<td>dimensionless</td>
<td>0.03</td>
<td>0.45</td>
<td>0.0035</td>
<td>0.0045</td>
<td>12</td>
<td>(2)</td>
</tr>
<tr>
<td>Mouse left-right patterning</td>
<td>dimensionless</td>
<td>20</td>
<td>40</td>
<td>0.5</td>
<td>0.1</td>
<td>10</td>
<td>(16)</td>
</tr>
<tr>
<td>Patterning of zebrafish stripes</td>
<td>dimensionless</td>
<td>0.01</td>
<td>0.2</td>
<td>1.2</td>
<td>1.0</td>
<td>24</td>
<td>(29)</td>
</tr>
<tr>
<td>Patterning of angelfish stripes</td>
<td>dimensionless</td>
<td>0.007</td>
<td>0.1</td>
<td>0.03</td>
<td>0.06</td>
<td>7</td>
<td>(28)</td>
</tr>
<tr>
<td>Patterning of mouse hair follicle spacing</td>
<td>arbitrary</td>
<td>0.005</td>
<td>0.2</td>
<td>0.005</td>
<td>0.015</td>
<td>13</td>
<td>(8)</td>
</tr>
<tr>
<td>Limb skeletal patterning</td>
<td>$D$: pixels/iteration</td>
<td>30</td>
<td>120</td>
<td>0.4615</td>
<td>n.a.</td>
<td>9</td>
<td>(38)</td>
</tr>
<tr>
<td></td>
<td>$k$: dimensionless</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorite/iodide malonic acid system</td>
<td>$D$: $\mu$m$^2$/s</td>
<td>50</td>
<td>750</td>
<td>n.a.</td>
<td>n.a.</td>
<td>15</td>
<td>(30)</td>
</tr>
<tr>
<td>Zebrafish Nodal/Lefty patterning system*</td>
<td>$D$: $\mu$m$^2$/s</td>
<td>2</td>
<td>15</td>
<td>0.00011</td>
<td>0.00006</td>
<td>14</td>
<td>this work</td>
</tr>
<tr>
<td></td>
<td>$k$: 1/s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

With the exception of zebrafish Nodal and Lefty signals, none of the diffusion coefficients and clearance rates were experimentally measured.
The subscripts $a$ and $i$ indicate activator and inhibitor, respectively.
$D$ represents the diffusion coefficient, and $k$ represents the clearance rate constant.

* $D_a$: mean of Cyclops and Squint $D$ values; $D_i$: mean of Lefty1 and Lefty2 $D$ values; $k_a$: mean of Cyclops and Squint $k_a$ values; $k_i$: mean of Lefty1 and Lefty2 $k_i$ values.

Table A.8: Parameter values used in reaction-diffusion models.
<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Abbreviations</strong></td>
<td></td>
</tr>
<tr>
<td>BC</td>
<td>Boundary condition</td>
</tr>
<tr>
<td>FCS</td>
<td>Fluorescence correlation spectroscopy</td>
</tr>
<tr>
<td>FEM</td>
<td>Finite element method</td>
</tr>
<tr>
<td>FRAP</td>
<td>Fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>IC</td>
<td>Initial condition</td>
</tr>
<tr>
<td>ODE</td>
<td>Ordinary differential equation</td>
</tr>
<tr>
<td>PDE</td>
<td>Partial differential equation</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>SSD</td>
<td>Sum of squared differences</td>
</tr>
<tr>
<td><strong>Variables</strong></td>
<td></td>
</tr>
<tr>
<td>$A$</td>
<td>Area</td>
</tr>
<tr>
<td>$c$</td>
<td>Concentration</td>
</tr>
<tr>
<td>$D$</td>
<td>Diffusion coefficient</td>
</tr>
<tr>
<td>$EQ$</td>
<td>Equalization factor</td>
</tr>
<tr>
<td>$I$</td>
<td>Intensity</td>
</tr>
<tr>
<td>$\kappa$</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>$k_1$</td>
<td>Clearance rate constant</td>
</tr>
<tr>
<td>$k_2$</td>
<td>Production rate</td>
</tr>
<tr>
<td>$\Omega$</td>
<td>Domain</td>
</tr>
<tr>
<td>$\partial\Omega$</td>
<td>Domain boundary</td>
</tr>
<tr>
<td>$\Phi$</td>
<td>Volume or area fraction</td>
</tr>
<tr>
<td>$r$</td>
<td>Radius</td>
</tr>
<tr>
<td>$\tau$</td>
<td>Half-life</td>
</tr>
<tr>
<td>$V$</td>
<td>Volume</td>
</tr>
<tr>
<td>$z$</td>
<td>Depth</td>
</tr>
<tr>
<td><strong>Variable subscripts</strong></td>
<td></td>
</tr>
<tr>
<td>$b$</td>
<td>Blastoderm</td>
</tr>
<tr>
<td>$df$</td>
<td>Diffusive flux</td>
</tr>
<tr>
<td>$ext$</td>
<td>Extracellular</td>
</tr>
<tr>
<td>$int$</td>
<td>Intracellular</td>
</tr>
<tr>
<td>$out$</td>
<td>Outside of bleached window</td>
</tr>
<tr>
<td>$post$</td>
<td>After photoconversion or bleaching</td>
</tr>
<tr>
<td>$pre$</td>
<td>Before photoconversion or bleaching</td>
</tr>
<tr>
<td>$sl$</td>
<td>Entire optical slice</td>
</tr>
<tr>
<td>$win$</td>
<td>Inside of bleached window</td>
</tr>
</tbody>
</table>

Table A.9: List of frequently used abbreviations and variables.
Supplementary material for Chapter 5: Lefty is a global dampener of Nodal signaling

Figure A.29: *lefty1* mutants exhibit partially penetrant heart laterality defects. A) *In situ* hybridization using a probe against the heart muscle marker *cmlc2* in 24 hpf wild-type and *lefty* mutant embryos. Most wild-type and *lefty2* mutants exhibit normal *cmlc2* expression on the left side. In contrast, some *lefty1* mutants exhibit bilateral *cmlc2* expression, and double *lefty* mutants express very little *cmlc2* and typically fail to generate hearts. B) Quantification of heart laterality defects in *lefty1* and *lefty2* mutants. Live embryos were scored at 30 hpf. Whereas *lefty2* mutants typically exhibit normal heart laterality, *lefty1* mutants frequently have misplaced hearts. Despite this heart laterality defect, *lefty1* mutants are homozygous viable.

Figure A.30: *lefty* double mutants exhibit partially penetrant axis bifurcation. A) Wild-type embryo at 24 hours post-fertilization (hpf). B) *lefty* double mutant at 24 hpf, lateral view. C) *lefty* double mutant at 24 hpf, dorsal view. Axis bifurcation is visible.
Figure A.31: No apparent maternal requirement for lefty. Adult fish of the indicated genotypes were mated. 50 progeny from each cross were isolated and raised in the chorion at 28C. Phenotypes and genotypes of the 50 progeny embryos were scored at 24-25 hpf. One embryo from the lefty1+/−; lefty2−/− incross died before 24 hpf, but all others underwent gastrulation and survived at least 24 hpf. A) Wild-type embryo. B,C) Embryos with a single functional lefty allele (genotype: lefty1+/−; lefty2−/−) from either a lefty1+/−; lefty2−/− incross (B) or a cross between an SB-505124-rescued lefty1+/−; lefty2−/− mother and a lefty1+/−; lefty2+/− father (C) are normally patterned and develop into fertile adults. The embryo in (C) received no maternal lefty, but is still normally patterned, indicating that maternal lefty is not required during development (maternal lefty transcripts have not been previously detected). D,E) Embryos with no functional lefty (genotype: lefty1−/−; lefty2−/−) from either a lefty1−/−; lefty2−/− incross (D) or a cross between an SB-505124-rescued lefty1+/−; lefty2−/− mother and a lefty1+/−; lefty2−/− father (E) exhibit typical lefty double mutant phenotypes (Fig. 1M,N). Phenotypes are not exacerbated in progeny that lack maternal lefty (E), indicating that the greater severity of lefty morphant phenotypes...
compared to lefty mutant phenotypes is not due to inhibition of maternal lefty by morpholinos. F) At 24-25 hpf, phenotypes of progeny were scored. Individual embryos were then genotyped (note that a few of the genotyping PCRs failed). All embryos with wild-type phenotypes were either lefty1+/−;lefty2−/− or lefty1+/−;lefty2−/−, whereas all embryos with abnormal phenotypes were lefty1−/−;lefty2−/−, except for one abnormal embryo from the lefty1−/−;lefty2−/− x lefty1+/−;lefty2−/− incross, which was lefty1−/−;lefty2−/−. This embryo may possibly have been incorrectly genotyped, or its abnormal phenotype may have been due to injury.
Figure A.32: **In situ** hybridization of Nodal target genes in *lefty* mutants shows upregulation of most targets during gastrulation. Wild-type embryos, *lefty*1/− embryos, *lefty*2/− embryos, and progeny from *lefty*1/+:*lefty*2/− crosses or *lefty*1/−;*lefty*2/− x *lefty*1/+;*lefty*2/− crosses were fixed in 4% formaldehyde at the indicated stages. In situ hybridization was performed using the following probes: *lefty*1 (A,B), *lefty*2 (C,D), *squint* (E,F), *cyclops* (G,H), *no tail* (I,J), *casanova* (K,L), *one-eyed pinhead* (M,N), *sox17* (O,P), *floating head* (Q,R), and *spadetail* (S,T). After imaging, progeny from *lefty*1+/−;*lefty*2−/− incrosses or *lefty*1/−;*lefty*2/− x *lefty*1/+:*lefty*2/− crosses were genotyped. A,C,E,G,I,K,M,O,Q,S) Animal pole views. Dorsal is to the right where evident. B,D,F,H,J,L,N,R,T) Lateral views, dorsal in front where evident. Upregulation is most dramatic in double *lefty* mutants, though *lefty*1/− mutants tend to exhibit slight upregulation as well. (Figure continued on subsequent pages.)
Figure A.32 (Continued)
Figure A.32 (Continued)
Figure A.32 (Continued)
Figure A.32 (Continued)
Figure A.33: *leftys* are upregulated earlier and more dramatically in *lefty* double mutants than other direct Nodal target genes. RNA collected from wild-type and *lefty* double mutant embryos at sphere stage, 30% epiboly, 50% epiboly, shield stage, and 75% epiboly was subjected to NanoString analysis. The log2 fold change in expression of the indicated direct Nodal target genes between wild-type and *lefty* double mutants was calculated. A) The maximum log2 fold change for each Nodal target gene is plotted. *leftys* are the most dramatically upregulated. B) The time to reach and maintain 2-fold upregulation is shown. Not all analyzed genes reached or maintained 2-fold upregulation over the course of this study. *leftys* reach and maintain 2-fold upregulation earlier than other Nodal target genes.
Table A.10: NanoString analysis of 20 direct Nodal target genes. RNA from wild-type and double lefty mutant embryos was collected at the indicated stages and subjected to NanoString analysis. Log2 fold changes were calculated and are reported here.

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