Quantitative Analysis of Signaling Pathways: Imaging and Modeling of the Terminal Patterning System of the *Drosophila* Embryo

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Abstract

One of the key concepts of Systems Biology is that of a module, defined as a network with a function that is largely independent of its context. Based on the modular decomposition of large networks it may be possible to predict their dynamics and function from properties of constituent modules. Integral to such an approach is the ability to define individual modules and distinguish their core components from the rest of the network. While conceptually simple, this task can be highly nontrivial in reality.

In my thesis, I analyzed the mitogen activated protein kinase (MAPK) pathway, an essential regulator of cellular processes in all eukaryotes. The MAPK pathway is a three-tiered cascade of phosphorylation-dephosphorylation cycles. An input to the pathway can be provided by a cell surface receptor; its output is the activation level of MAPK, a kinase at the bottom of the cascade. MAPK is activated when it is phosphorylated by a MAPK kinase; this process is reversed by a MAPK phosphatase. Active MAPK controls cellular processes by phosphorylating its substrates. According to the current models, the dynamics of the MAPK cascade can be understood independently of MAPK substrates. This unidirectional view of MAPK signaling is consistent with a large body of work, but the reality is more complex: The enzymes that regulate MAPK and the substrates phosphorylated by MAPK have the potential to interact with the same domains on the MAPK protein. Thus, MAPK substrates can compete with each other, as well as with the MAPK regulators for binding to MAPK.
The experimental work in my dissertation demonstrates that this is indeed the case. Using the early *Drosophila* embryo as an experimental system, I have shown that the level of MAPK substrates can influence the activation status of this enzyme and its ability to distribute its activity among multiple substrates. In the first part of my thesis, I used a combination of genetic and imaging approaches to show that the spatial pattern of MAPK activation in the early embryo exhibits a striking asymmetry (Chapter 2 and Chapter 3). My subsequent genetic experiments revealed that this asymmetry is due to the spatially non-uniform distribution of the MAPK substrates that compete both among themselves and with the regulators of MAPK for access to this enzyme (Chapter 3 and Chapter 4). I have developed a chemical kinetics theory of the observed substrate competition effect and successfully tested this theory *in vivo*, using a large number of mutants in the MAPK pathway (Chapter 4). Finally, I was able to demonstrate that MAPK substrate competition is biologically significant and play a key role in the regulation of gene expression and cell differentiation in the embryo (Chapter 5 and Chapter 6). Going beyond the early *Drosophila* embryo, I propose that enzyme substrate competition is an important regulatory strategy in biomolecular networks where enzymes, such as MAPK, interact with their multiple regulators and substrates.
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Chapter 1

Introduction

1.1 Signaling pathways

The development and maintenance of living tissues relies on cell-cell communication by secreted proteins that bind to cell surface receptors and activate intracellular signaling pathways. To a first approximation, these signaling pathways can be viewed as large networks of biochemical reactions. Signaling pathways are conserved across species to such an extent that components of a pathway can be swapped between different organisms. Defects in signaling pathways can lead to severe developmental disorders and disease states. Consequently, a number of therapeutic strategies for diseases such as asthma and cancer target signaling pathways and their components [1, 2]. After a decade of trial and error approaches, it is recognized that the development of such therapies is impossible without quantitative models. Thus, quantitative models of signaling pathways holds the key to the fundamental understanding of embryonic development, elucidating the origins of developmental disorders, and designing manmade tissues. Successful models should integrate large amounts of data, test the feasibility of proposed modes of regulation, and suggest new mechanisms that can be subsequently tested experimentally.
1.2 MAPK signaling pathway

The mitogen activated protein kinase (MAPK) signaling pathway is a critical regulator of cellular processes in developing and adult tissues [3, 4]. In metazoans, this pathway frequently acts downstream of receptor tyrosine kinases (RTK) activated by extracellular ligands. The MAPK pathway is organized as a cascade of three phosphorylation-dephosphorylation stages and culminates in a double phosphorylation of MAPK, the serine/threonine kinase at the last stage of the cascade. Phosphorylated MAPK shuttles in and out of a nucleus and acts on both cytoplasmic and nuclear substrates; controlling, in this way, cellular processes such as cell division and differentiation [5].

The MAPK pathway regulates cell growth and differentiation, but when MAPK signaling goes awry, it often leads to malignant growth of many cell types [2, 6, 7]. For example, overexpression of the human Epidermal Growth Factor Receptor (EGFR) homolog ERBB2 (HER2), which signals via the MAPK cassette, is intimately linked with malignancy and poor prognosis in breast cancer [6]. Consequently, MAPK pathway is an important target of molecular therapies in a number of diseases [7]. In fact, treatments with antibodies directed against HER2 constitute a part of the standard and essential protocol for curing patients with breast tumors that overexpress HER2 [6, 8].

The MAPK pathway is one of the most extensively modeled eukaryotic signaling systems [9, 10]. The earlier models attempted to predict the dynamics of the signaling and develop a more fundamental understanding of information transmission in cells. For example, the first model of the MAPK cascade, developed by Huang and Ferrell, argued that the three-tiered structure of the MAPK cascade controls its steady-state input-output behavior [9]. Specifically, based on simulations with randomly generated parameter sets,
they found that the input-output map is ultrasensitive: the output can switch from “off” state to “on” state within a very narrow range of inputs.

Most of the models of MAPK signaling system are developed on the basis of experiments with purified molecules or cultured cells and have not been subject to rigorous experimental validation. In addition, there exist essentially no models that can adequately describe how this pathway operates in tissues. To address the need for such models, we use the terminal patterning of the Drosophila embryo as an experimental system to develop and experimentally test mechanistic models of MAPK signaling.

1.3 Early Drosophila embryo

When a Drosophila embryo is fertilized, zygotic nuclei divide sequentially without forming cell membranes between neighboring nucleus. After the eighth division, a majority of nuclei migrate to the cortex of the embryo, giving rise to the syncytial blastoderm embryo comprised of multiple nuclei with a shared cytoplasm (Figure 1.1) [11]. The first and the only cells to form at this early developmental stage are the posterior pole cells, the precursors of the germline. It is only during the fourteenth nuclear cycle, approximately three hours after fertilization, that the nuclei at the surface of the embryo are concurrently encompassed by cell membranes to form approximately 6,000 cells of the cellular blastoderm (Figure 1.1). By the time of cellularization, the embryo has been patterned along its anteroposterior (AP) and dorsoventral (DV) axes, under the control of four maternal coordinate systems [12]. These four systems are briefly described below (Figure 1.2).
Figure 1.1: Early *Drosophila embryo*. The first fourteen cell cycles of *Drosophila* embryogenesis can be divided into two distinct phases. During the first phase, a single nucleus, located near the center of the embryo, undergoes eight subsequent nuclear divisions. In the second phase, most of the nuclei migrate to the periphery where they undergo five additional nuclear divisions before cellularization [13].

Four maternal systems operate in *Drosophila* embryogenesis to transform the maternal asymmetric cues into differential zygotic gene expression patterns that specify distinct cell fates along the AP and DV axes. They are the anterior, posterior, dorsoventral, and terminal systems [12]. The head and thorax are patterned by the activity of the anterior morphogen Bicoid (Bcd), while abdomen formation is directed by a reciprocal posterior gradient of Nanos (Nos). Asymmetrical nuclear distribution of Dorsal (Dl) governs the DV axis formation, whereas the non-segmented termini of the embryo are patterned by the terminal system. The activities of these systems induce region specific transcription of zygotic downstream target genes, the products of which eventually assign the different body parts with their prospective identities [12].
Mutations in one maternal coordinate system seem to eliminate a specific body part without grossly affecting the rest of the embryonic pattern. Hence, the distinct maternal genetic systems were initially thought to act independently of each other to define discrete portions of the embryo. Several studies, however, have revealed interactions between the anterior, terminal, and DV systems at the most anterior region of the embryo [14], and between the posterior and terminal group genes at the posterior [15]. Most of these interactions have been inferred from genetic studies and molecular mechanisms are only partially understood.

### 1.4 Terminal patterning system

The first round of MAPK signaling in the Drosophila embryo is induced to pattern the non-segmented termini of the embryo; hence it is also known as the terminal patterning system [16]. The Torso RTK pathway is the core of the terminal patterning
system. Torso is uniformly distributed throughout the plasma membrane of the early embryo, but is activated by localized production of its ligand at the poles, where it transmits signal via the canonical MAPK cascade [17-19]. Signaling by this cascade leads to the restricted expression of two zygotic terminal gap genes, *tailless* (*tll*) and *huckebein* (*hkb*), at the poles of the embryo (Figure 1.3) [20, 21]. The Torso signal transduction cascade is one of the most studied models of RTK signaling in *Drosophila*, both in terms of identifying the molecular components of the RTK cascade as well as for studying the transcriptional regulation of pathway target genes [22-24].

**Figure 1.3: Terminal patterning system.** (A) Cuticle of a wild type (top) and a mutant embryo that lacks terminal signal (bottom). (B) Activation of Torso signaling by the localized processing of its ligand at the poles. (C) Localized activation of Torso signaling activates *tailless* (left) and *huckebein* (right).

Genetic and molecular studies revealed that the activation of *tll* and *hkb* expression by the Torso pathway is indirect. Torso signaling allows regional expression of these zygotic genes by counteracting transcriptional repression at the embryonic poles. It has been shown that expression of *tll* and *hkb* is blocked outside the termini by both the DNA-binding high mobility group (HMG) box repressor Capicua (Cic) and the global co-
repressor Groucho (Gro) [25-28]. Embryos deprived of the maternal contribution of these determinants display ectopic *tll* and *hkb* transcription in more central regions, even in the absence of the Torso activation [28, 29]. At the termini, activation of the Torso pathway induces expression of *tll* and *hkb* by locally inhibiting repression exerted by Cic and Gro. Specifically, phosphorylation of Cic by MAPK targets it for degradation, and Cic is thus cleared from the poles. Accordingly, Cic protein is discernible throughout the embryo except where the Torso pathway is active [28]. Additionally, phosphorylation-dependent attenuation of Gro-mediated repression also regulates the transcriptional output of the Torso pathway [27].

1.5 Thesis overview

The elucidation of the patterning hierarchy in the early fly embryo was a great triumph of genetic approach to study developing embryos. Within the last two decades, the field had gone from the identification of patterning genes in experiments that used the embryonic cuticle phenotypes as their main assay, to visualizing the distribution of transcripts and proteins encoded by the identified genes and developing network-type description of pattern formation. The structure of this network, formed by maternal signals, gap, pair-rule, and segment polarity genes can be found in essentially every textbook on genetics and development. We are now in a position to ask increasingly quantitative questions about the dynamics, robustness, and evolvability of this network. These questions are most naturally explored within the framework of quantitative models.

Rigorous tests of these models require quantitative information about maternal gradients. Over the past couple of years, several groups have reported quantitative measurements of Bcd and DI gradients, analyzed their dynamics in live imaging
experiments, and proposed biophysical models for their transcriptional interpretation [30-32]. At the same time, no quantitative models have been proposed for the terminal system until Shvartsman lab’s work on analyzing the dynamics of MAPK phosphorylation gradient [33], published at the onset of this thesis project. Thus, the main goal of this thesis is to formulate, analyze, and experimentally test models of the terminal patterning system.

The chapters of this thesis are organized as follows. In Chapter 2, quantitative characterization of signal transduction in the MAPK signaling pathway is described with focus on the steps between the activated MAPK and spatial expression patterns of \( tll \) and \( hkb \). The following three chapters discuss AP asymmetries in MAPK signaling, its mechanism, and developmental function with respect to positioning gene expression boundaries. Chapter 6 presents a model of regulation of \( tll \) at the anterior region by the maternal inductive signals. The final chapter describes the conclusions of these studies and presents future directions.

1.6 References


Chapter 2

Signal transduction in terminal patterning of the

*Drosophila* embryo

This chapter is based on a collaborative work with Antonina Iagovitina, Keisuke Ishihara, Jitendra S. Kanodia, Bart Deplancke, and Stanislav Y. Shvartsman. The authors thank G. Jiménez and other members of Shvartsman lab for helpful discussions and comments. We are indebted to G. Jiménez and C. Berg for flies and reagents.

2.1 Introduction

The mitogen activated protein kinase/extracellular-signal regulated kinase (MAPK/ERK) signaling pathway is a critical regulator of cellular processes in developing and adult tissues [1-3]. In metazoans, this pathway frequently acts downstream of receptor tyrosine kinases (RTK) activated by extracellular ligands. The MAPK pathway is organized as a cascade of three phosphorylation-dephosphorylation stages and culminates in a double phosphorylation of MAPK, the serine/threonine kinase at the last stage of the cascade. The phosphorylated MAPK accumulates inside a nucleus and catalyzes phosphorylation of cytoplasmic and nuclear substrates; controlling, in this way, cellular processes such as cell division and differentiation.

The MAPK pathway is one of the most extensively modeled eukaryotic signaling systems [4-6]. The first model of MAPK cascade was developed by Huang and Ferrell, who found that the input-output relation in MAPK cascade is ultrasensitive [7]: The output of the
system switches from “off” state to “on” state within a very narrow range of inputs. This model prediction for intrinsic ultrasensitivity of the MAPK cascade was validated experimentally in *Xenopus* oocyte extracts where MAPK activation shows all-or-nothing response to continuously varying levels of progesterone [8]. In contrast, an analogous study of MAPK signaling in mammalian fibroblast cells showed that MAPK is activated in a graded fashion in response to increasing hormone concentrations [9]. At the same time, the target gene of MAPK signaling, *c-Fos*, showed switch-like behavior in response to this graded MAPK activation, suggesting that the all-or-nothing cell fate of mammalian fibroblast proliferation occurred at the level of gene induction [9].

The above studies, however, only provided analysis of MAPK signaling on the level of individual cells as they used single cells (*Xenopus* oocyte or mammalian fibroblast) as their experimental systems. Here, we used the terminal patterning system of the *Drosophila* embryo as our experimental to characterize MAPK pathway in a multicellular tissue. Specifically, we quantitatively analyzed the spatial gradients of multiple components within the MAPK signaling cascade: from activated MAPK, to its substrate, and to target genes of the signaling. We found that MAPK is activated in a graded manner from the embryonic poles and establishes graded spatial gradient of nuclear Capicua (Cic), a transcriptional repressor that acts downstream of MAPK [10, 11]. However, two identified target genes of MAPK signaling, *tailless* (*tll*) and *huckebein* (*hkb*) show switch-like expression patterns, suggesting that similar to mammalian fibroblast cells, ultrasensitive response occurs at the level of gene induction.
2.2 Results

2.2.1 Graded spatial activation of MAPK is converted to switch-like target gene expressions

In the early *Drosophila* embryo, the terminal structures of future larva are first specified by the localized activation of MAPK. In this context, MAPK acts downstream of Torso RTK whose ligand is present only at the poles [12-14]. Activated MAPK phosphorylates transcriptional repressors such as Cic and in this way, controls expression of at least two gap genes *tll* and *hkb* (Figure 2.1A) [10, 11, 15, 16]. To characterize the regulation of these two genes by MAPK, we first visualized the spatial pattern of MAPK activation using an antibody that specifically recognizes the double phosphorylated/activated form of MAPK (dpERK) [17].

We note that both *tll* and *hkb* are expressed at both poles of the embryo, reflecting their activation by MAPK in these regions. However, anterior expressions of both genes receive additional spatial signals such as Bicoid which is present in an anterior gradient and patterns the anterior half of the embryo [18, 19]. On the other hand, MAPK signaling is essential for the posterior expressions of *tll* and *hkb* [20, 21]. Indeed, in the absence of MAPK activation, the posterior expressions of *tll* and *hkb* are abolished while their anterior expressions remained due to direct activation by Bcd [18, 19]. Thus, in this study, we focused only on the posterior half of the embryo to minimize the effects from other signaling systems.

Using the previously developed image analysis approach [22], we quantified the spatial pattern of dpERK from the posterior tip to the mid-body of the syncytial blastoderm.
embryo (Figure 2.1C). We assumed that dpERK activation is symmetric along the dorsoventral axis [22] and thus, each embryo yields two gradients of dpERK. As shown in Figure 2.1C, dpERK pattern is distributed in a graded manner in the posterior half of the embryo. To examine the pattern of gene expression in response to this graded dpERK signal, we visualized mRNAs of the two target genes of MAPK signaling using fluorescent in situ hybridization (FISH). This technique allows simultaneous detection of dpERK protein, tll mRNA, and hkb mRNA in a single embryo (Figure 1B). Quantification of their spatial patterns reveals that the boundaries of the two genes are clearly distinct from each other with tll boundary always extending further toward the center of the embryo compared to that of hkb (Figure 2.1D). In addition, both genes show very sharp transition from “on” state to “off” state.

To characterize the sharpness of these gene expression patterns, we constructed input-output maps between dpERK and the two genes. We ignored the posterior 10% of the embryo due to imaging artifact associated with the pole cells, whose presence result in arbitrarily lower signal as depicted in Figure 2.1D. We found that the input-output maps resemble sharp sigmoidal curves, suggesting switch-like behavior. To quantify the sharpness of the response curves, we fitted these curves with a hill function shown below:

\[
Output(x) = \frac{\left(\text{dpERK}(x)\right)^n}{K^n + \left(\text{dpERK}(x)\right)^n},
\]

where Output is spatial pattern of either tll or hkb

Both curves are well represented by the hill function with hill coefficient (n) of approximately 5 (Figure 2.1E). Our results suggest that the two target genes of the terminal patterning system shows all-or-nothing behavior in response to graded MAPK activation.
Figure 2.1: A graded dpERK is converted to switch-like gene expressions. (A) Representation of signal transduction from RTK ligand Trunk to the activation of terminal genes tll and hkb. (B) Using fluorescent in situ hybridization, we can simultaneously visualize spatial pattern of dpERK, tll, and hkb in a single embryo. (C) Quantification of dpERK intensity from the posterior pole (x=0) to mid-body of the embryo (x=1; see inset). The expression boundaries of tll and hkb are shown by red and green arrows respectively. (D) Normalized spatial gradient of tll and hkb mRNA. (E) Input-output maps of tll and hkb as a function of dpERK. Average gradients shown in panels C and D are used to generate the plot. The solid lines represent fitted hill functions.

2.2.2 Phosphorylation of Cic by dpERK

Activated MAPK, however, does not regulate gene expressions directly, but it acts through phosphorylation of multiple transcription factors [23]. One identified direct transcriptional regulator of tll and hkb is an HMG box protein Cic. Cic is a transcriptional repressor whose mRNA is maternally deposited in uniform profile during oogenesis [11]. Previous biochemical and genetic studies have shown that Cic directly interacts with dpERK
and is phosphorylated by dpERK at the termini of the embryo, which leads to its cytoplasmic accumulation and subsequent degradation [10, 11]. Hence, MAPK regulates expression of \textit{tll} and \textit{hkb} by establishing spatial gradient of Cic (Figure 2.2A). We thus investigated formation of Cic gradient by dpERK and asked whether the switch-like gene expressions is initiated at the level of Cic regulation.

We used an antibody that recognizes Cic protein and quantified the spatial gradient of nuclear Cic along the anteroposterior (AP) axis (Figure 2.2B). The AP gradient of nuclear Cic exhibits two minima at the poles, corresponding to the two peaks of MAPK activation (Figure 2.2C). To characterize MAPK mediated downregulation of Cic, we first quantified the fraction of Cic protein removed at the poles by MAPK signaling.

Assessing the fractional removal of Cic requires quantifying the background intensity of the gradient which can be achieved by simultaneously staining wild-type embryos with embryos derived from \textit{cic}\textsuperscript{1} flies. \textit{cic}\textsuperscript{1} is a strong hypomorphic allele of \textit{cic} resulted from insertion of a transposon in the 5’ untranslated region of the \textit{cic} locus [11]. Embryos derived from \textit{cic}\textsuperscript{1} homozygous flies exhibit expansion of terminal structures in expense of segmented trunk regions [11].

By quantifying Cic gradients in these mutant embryos, we found that the maximum level of Cic determined at the mid-body of these embryos is lower than the minimum Cic level from wild-type embryos (Figure 2.2E). This result suggests that the residual level of Cic in the \textit{cic}\textsuperscript{1} embryos is very low. We thus assumed that nearly all of Cic is removed by dpERK at the poles of \textit{cic}\textsuperscript{1} embryos and used Cic intensity at the pole as the background level. We then subtracted this background level from the wild-type gradients and divided the gradients by its maximum intensity, obtained from the mid-body of the embryo. We found
that in the anterior region of the embryo, Cic is removed to about 50% of the maximum while in the posterior region, 80% of the protein is downregulated by dpERK (Figure 2.2F).

Figure 2.2: Characterization of Cic gradient established by dpERK. (A) During the terminal patterning, MAPK signaling regulates the expression of \textit{tll} and \textit{hkb} by downregulating transcriptional repressor Cic. (B) Simultaneous detection of dpERK (cyan) and Cic (orange) proteins. (C) Quantification of AP gradient of dpERK and Cic. Average of 84 gradients are plotted and error bars are s.e.m. (D) Input-output map of Cic as a function of dpERK. (E) Quantification of Cic gradients in wild-type embryos (orange) and embryos derived from \textit{cic} \textsuperscript{1} flies (black). (F) Cic levels at the poles of the \textit{cic} \textsuperscript{1} embryos are used to determine the background intensity which is then used to determine the fractional downregulation of Cic by dpERK at the poles.
2.2.3 Graded Cic pattern is converted to switch-like target gene expressions

Next in the signaling cascade, we investigated the regulation of gene expression by Cic. Biochemical evidences clearly showed that Cic controls expression of \( tll \) and \( hkb \) by directly binding to their cis-regulatory regions (Figure 2.3A) [24]. To quantitatively characterize the input-output relation between Cic and the two genes, we used FISH to simultaneously visualize Cic, \( tll \), and \( hkb \) and examined the spatial patterns of \( tll \) and \( hkb \) as a function of nuclear Cic gradient (Figure 2.3B-D). Since Cic acts as a transcriptional repressor, we fitted the input-output maps with the inverse hill equation (below) and determined the hill coefficients (Figure 2.3D).

\[
\text{Output}(x) = \frac{K^n}{K^n + \{Cic((x))^n}, \text{ where Output is spatial pattern of either } tll \text{ or } hkb
\]

We found that the response curves are extremely sharp with hill coefficient (\( n \)) ~15. Hence, our results clearly show that the all-or-nothing patterns of \( tll \) and \( hkb \) occurred at the level of gene induction.

Our quantification of nuclear Cic gradient reveals that it is surprisingly shallow between the boundaries of \( tll \) and \( hkb \) (Figure 2.3C). This is consistent with the input-output map between dpERK and Cic which shows that the downregulation of Cic by dpERK is subsensitive (Figure 2.2D). Assuming dpERK mainly signals through Cic, the observed gradient suggests that only a small change in the amount of Cic is sufficient in positioning two distinct gene expression boundaries. Thus, we investigated whether this shallow gradient of Cic has a potential to provide enough positional information to specify the boundaries of \( tll \) and \( hkb \).

We estimated the boundary of the gene expression as the location at which the transcript level drops to 50% of the maximum (Figure 2.4). Under this definition, \( tll \) and \( hkb \)
boundaries are located at 17% and 15% embryo length from the posterior pole respectively. If Cic can position these two boundaries, then Cic levels must be different from each other at these two points and from the Cic levels at the mid-body of the embryo. We found that this is indeed the case. The levels of Cic at the two boundaries are statistically different from each other and from the Cic level at the mid-body of the embryo (Figure 2.3E). Interestingly, applying the same analysis on dpERK gradient, we found that the difference in the dpERK levels between the two gene expression boundaries is greater than that of Cic (Figure 2.3F). Combined, our data suggest that the enzymatic conversion of Cic by dpERK is subsensitive, resulting in a shallow Cic gradient in the posterior half of the embryo. Nevertheless, Cic levels at the boundaries of tll and hkb are statistically different, and Cic can potentially position two distinct gene expression boundaries in a wild-type embryo.
Figure 2.3: All-or-nothing response occurs at the level of gene induction. (A) Regulatory mechanism of the expression of \( \text{tll} \) and \( \text{hkb} \) by localized MAPK activation. (B) Simultaneous detection of Cic protein and \( \text{tll} \) and \( \text{hkb} \) mRNAs in a single embryo using FISH. (C) Quantification of nuclear Cic gradient in the posterior half of the embryo. The boundaries of \( \text{tll} \) and \( \text{hkb} \) are indicated by red and green arrows respectively. (D) Input-output plot of \( \text{tll} \) and \( \text{hkb} \) as a function of nuclear Cic. The solid lines represent fitted inverse Hill function for \( \text{tll} \) (red) and \( \text{hkb} \) (green). (E, F) Bar graph of average intensities of Cic (E) and dpERK (F) at the mid-body of the embryo (blue) and at the boundary of \( \text{tll} \) (red) and \( \text{hkb} \) (green). Error bars are s.e.m.
Figure 2.4: Determining the gene expression boundaries. Expression boundaries of \textit{tll} and \textit{hkb} are determined as the locations at which the transcript level reaches 50\% of the maximum (top). These locations are then used to determine the dpERK (middle) and Cic (bottom) levels at these gene expression boundaries.
2.3 Discussion

2.3.1 Ultrasensitivity in the terminal patterning system

The first model of MAPK signaling pathway developed by Huang and Ferrell predicted intrinsic ultrasensitivity within the structure of the pathway that converts a graded input into all-or-nothing output [7]. In contrast, our characterization of MAPK signaling in the posterior patterning of the early Drosophila embryo reveals that MAPK is activated in a graded fashion. Our results do not exclude the possibility of intrinsic ultrasensitivity in the MAPK signaling cascades as we cannot visualize the spatial pattern of the input to the pathway, the ligand of Torso RTK. However, our results clearly indicate that MAPK activation is still graded in the posterior half of the embryo and that switch-like response occurs only at the level of gene induction. This result is consistent with the previous characterization of MAPK signaling in mammalian fibroblast cells that the MAPK activation is graded and the switch-like behavior is initiated at the level of DNA [9].

Interestingly, we found that the fitted hill coefficients for both tll and hkb in response to Cic are approximately the same. In the simplest scenario, this result indicates that the regulatory regions of these two genes have similar number of Cic binding sites, but the affinities of these binding sites are different, resulting in two distinct gene expression boundaries. Alternatively, it is possible that the affinities of Cic binding sites are also similar, but the two genes are activated by two different sets transcription factors [25-27]. For example, the embryos that lack Lilliputian show weak expression of only hkb while the expression of tll remained unaffected, suggesting Lilliputian can only activate hkb [25]. In the future, we plan to complement these experiments by identifying the number of Cic binding sites and estimating their affinities in the cis-regulatory regions of tll and hkb.
2.3.2 Model for the regulation of terminal gap genes

Our results can be summarized into a model of the regulation of posterior *tll* and *hkb* by MAPK signaling (Figure 2.5). In the model, locally activated Torso signals through MAPK which establishes spatial gradient of a transcriptional repressor by catalyzing its phosphorylation. The model also takes into consideration that these two genes are activated by different set of transcription factors.

![Figure 2.5: Model for the regulation of *tll* and *hkb*.](image)

**Figure 2.5: Model for the regulation of *tll* and *hkb*.** During the terminal patterning of the embryo, localized activation of MAPK phosphorylates Cic and de-represses *tll* and *hkb*. In addition, the two genes are activated by different group of transcription factors such as Zelda (Zld), STAT, and Lilliputian (Lilli).

We note that this model is a simplified picture of a complex system and requires further assessment. For instance, the model ignores the fact that MAPK regulates *tll* and *hkb* by phosphorylating multiple transcription factors in addition to Cic. It has been shown that MAPK also interacts with Groucho, a global co-repressor whose mRNA is also deposited uniformly in the embryo by the mother fly and is downregulated by MAPK through direct
phosphorylation [28]. Although our results suggest that Cic alone has potential to position expression borders of tll and hkb, they do not exclude the possibility that it is the combinatorial effort of multiple repressors that together specify the expression patterns of tll and hkb [28-30].

2.3.3 Concluding remarks

Our work provides the first quantitative tissue level analysis of signal transduction in the MAPK pathway. We note that our analysis is still mainly focused on static picture and largely ignores the dynamic behavior of these patterning signals. We have previously shown that MAPK phosphorylation gradient sharpens over the last five nuclear cycles before gastrulation [22]. In addition, our preliminary analysis suggests that Cic level in the mid-body of the embryo is continuously increasing during this time (data not shown). Understanding the dynamics of Cic gradient formation and its effect on the dynamic expression patterns of tll and hkb will greatly enhance our understanding of the tissue patterning by the MAPK signaling pathway.

2.4 Experimental procedures

Fly Strain. The following fly strains were used in this study: OreR, cic1, and P[cic-HA] (cic4s). Flies were grown and embryos were collected at 25°C.

Fluorescent in situ hybridization and immunohistochemistry. Fluorescent in situ hybridization was performed as described previously [31]. Embryos were fixed in 8% formaldehyde for 20mins and treated with 90% xylene for 1hr and 80% acetone for 10mins at -20°C. Embryos were then hybridized overnight at 60°C with antisense probes labeled
with digoxigenin (DIG) or Biotin (BIO). Embryos with labeled probes were visualized using standard immunofluorescence technique. The following primary antibodies were used: sheep anti-DIG (Roche; 1:200), mouse anti-BIO (Jackson ImmunoResearch; 1:200), rabbit anti-dpERK (Cell Signaling; 1:100), and rabbit anti-Cic (1:2000). Alexa fluor conjugated secondary antibodies (Invitrogen; 1:500) were used to label the primary antibodies and DAPI (1:10,000; Vector Laboratories) was used to detect nuclei.

**Microscopy and Image Processing.** Imaging was done on a Zeiss LSM510 confocal microscope, with a Zeiss C-Apo 20x objective (NA=0.6). High-resolution images (512x512 pixels, 12 bits depth) were obtained from a focal plane in the mid-horizontal cross section of the embryo. Images of individual embryos were automatically extracted from raw confocal files and re-oriented and processed as described elsewhere [22].

**Statistical Analysis.** All calculations were done using MATLAB (MathWorks) functions. A paired t-test was used to test whether the Cic or dpERK levels at the boundaries of *tll* and *hkb* are statistically different.

### 2.5 References


Chapter 3

MAPK substrate competition integrates patterning signals in the *Drosophila* embryo

This chapter is based on a study published with Mathieu Coppey, Rona Grossman, Leiore Ajuria, Gerardo Jiménez, Ze’ev Paroush, and Stanislav Y. Shvartsman. The authors thank E. Wieschaus, T. Schüpbach, M. Lemmon, R. Steward, A. Ninfa, S. Merajver, M. Levine, A. Veraksa, and M. Tipping for helpful discussions and comments on the paper. We are indebted to E. Wieschaus, O. Grimm, N. Dostatni, S. Small, S. Hanes, and J. Dubuius for flies and reagents. We are grateful to N. Yakoby and J. Zartman for help with molecular characterization of the Bcd-A9 flies and to A. Boettiger for his contribution to the development of image processing tools.

### 3.1 Introduction

Anteroposterior (AP) patterning of the *Drosophila* embryo depends on three inductive signals [1]: the head and thorax are specified by the anterior gradient of Bicoid (Bcd), a homeobox transcription factor; abdomen formation is directed by Nanos (Nos), a translational repressor; and the non-segmented termini are patterned by the localized activation of the mitogen activated protein kinase/extracellular-signal regulated kinase (MAPK/ERK) pathway. MAPK signaling is induced by a uniformly expressed receptor tyrosine kinase Torso (Tor), which is activated by its ligand produced at the poles of the embryo [2].
Activated Tor promotes the expression of tailless (ill) and huckebein (hkb), zygotic gap genes required for the development of the terminal structures. This depends on MAPK-mediated phosphorylation of the transcriptional repressors Capicua (Cic) and Groucho (Gro), both of which are initially distributed uniformly throughout the embryo [2-5]. Phosphorylation of Cic and Gro relieves their repression of ill and hkb and enables expression of these genes at both poles of the embryo. At the anterior pole, MAPK also phosphorylates Bcd [6, 7]. Thus, MAPK is activated in a localized pattern and phosphorylates substrates that can be either uniformly (Cic and Gro) or non-uniformly (Bcd) distributed along the AP axis of the embryo (Figures 3.1A-3.1C).

We characterized the spatial pattern of MAPK phosphorylation and found that it exhibits a strong asymmetry at the two poles of the embryo. Based on the quantitative analysis of MAPK signaling in multiple mutants, we argue that this asymmetry is resulted from the non-uniform distribution of MAPK substrates and their competition for binding to MAPK. In addition, we identify Hunchback as a novel target of MAPK phosphorylation that can account for the previously described genetic interaction between the posterior and terminal systems. Thus, a common enzyme-substrate competition mechanism can integrate the actions of the anterior, posterior, and terminal patterning signals.

3.2 Results

3.2.1 MAPK phosphorylation exhibits AP asymmetry

The spatial pattern of MAPK activation can be visualized with an antibody that recognizes the double phosphorylated form of MAPK (dpERK) [8, 9]. We found that this pattern is strongly asymmetric: the anterior levels of dpERK are significantly higher than the
posterior levels (Figure 3.1). Because the early *Drosophila* embryo is highly polarized, multiple factors can potentially contribute to the observed asymmetry. These include the differences in the anterior and posterior levels of the extracellular ligand that activates Tor [10] and/or in the intracellular localization of maternal determinants.

![Figure 3.1: Spatial asymmetries of MAPK phosphorylation.](image)

We hypothesized that the higher levels of dpERK at the anterior pole reflect the presence of an anteriorly localized maternal factor. One candidate for such a factor is Bcd, which is localized to the anterior of the embryo and is one of the phosphorylation targets of MAPK [6]. To test whether Bcd can affect the AP asymmetry of the dpERK pattern, we
examined this pattern in embryos with different levels of Bcd. We found that the anterior levels of dpERK are significantly decreased in embryos from *bcd* null or heterozygous mothers and increased in embryos with two extra copies of *bcd* (Figure 3.2A and 3.2B). Importantly, the posterior levels of dpERK are unaffected in these embryos, reflecting the anterior localization of Bcd. Thus, changing the level of Bcd, a substrate of MAPK, influences the level of MAPK phosphorylation.

Previous studies have shown that overexpressing MAPK substrates in a heterologous cell culture system leads to a higher level of MAPK phosphorylation [11]. Furthermore, experiments with purified components revealed that MAPK substrates can directly compete with the MAPK phosphatases for binding to the common docking domain of MAPK [11-15]. Thus, the level of MAPK substrates can increase the level of MAPK phosphorylation by outcompeting the MAPK phosphatases. Correspondingly, a similar effect can be responsible for the observed dose-dependent control of MAPK phosphorylation by Bcd: the total concentration of MAPK substrates at the anterior pole is higher than at the posterior because of the anterior localization of Bcd, resulting in a stronger interference with MAPK dephosphorylation in this region of the embryo. This can account for the higher level of dpERK at the anterior pole.

3.2.2 Transcriptional activity of Bcd is dispensable for its effect on MAPK

The fact that the anterior level of dpERK is sensitive to dose of Bcd is consistent with the proposed competition model. This, however, does not exclude the possibility of a more indirect transcriptional effect, whereby one or more transcriptional targets of Bcd would affect the levels of MAPK phosphorylation. To distinguish between these two alternatives, we used the *bcd* transgene (*Bcd-A9*), which encodes a Bcd variant that has an impaired ability
to bind its DNA recognition sequence [16], but still contains all of the sites required for binding to and phosphorylation by MAPK (Figure 3.2C). We first confirmed that the expression of *hunchback (hb)*, a direct target of Bcd, was not affected in embryos with two copies of wild-type *bcd* and two copies of the *Bcd-A9* transgene (data not shown). Remarkably, the anterior level of dpERK was significantly increased in these embryos (Figure 3.2D). In fact, the anterior level of dpERK was indistinguishable from that in embryos with two extra copies of wild-type *bcd* (*bcd*). Taken together, these results imply that Bcd can affect the level of MAPK phosphorylation independently of its transcriptional activity.
Figure 3.2: The anterior level of dpERK responds to changes in the level of Bcd. (A) The AP gradient of dpERK in progenies of females with different bcd copy number. Each line indicates an average gradient of dpERK for 20–25 individual embryos of the same genotype. Note that the anterior level of dpERK changes as the amount of maternal bcd present is altered, whereas posterior levels are not affected. (B) Changes in the anterior and posterior levels of dpERK as a function of maternal bcd copy number. Each bar represents an average of MAPK phosphorylation for 20–25 individual embryos of the same genotype, with standard error (SE) indicated. The data are normalized such that the values of wild-type (bcd+/+, embryos marked with Histone-GFP) are set at 1. Only the anterior level shows an increasing trend as a function of bcd copy number (generalized linear model: p_{anterior} = 2.9 \times 10^{-8}, p_{posterior} = 0.30). (C) Schematics of Bcd and a Bcd variant (Bcd-A9) used in the experiments. Gray boxes show the following domains in the Bcd protein: the proline-histidine repeat (H/P), the DNA-binding domain (homeodomain, HD), the serine-threonine (S/T)-rich domain, a glutamine-rich domain (Q), an alanine-rich domain (A), and an acidic domain (C). Putative MAPK phosphorylation sites (based on [6]) are indicated by yellow bars. Bcd-A9 encodes a Bcd variant with a single lysine-to-alanine amino acid substitution at position 50 of the homeodomain (K9 to A9, shown in red). (D) Embryos expressing two wild-type bcd and two copies of the bcd-A9 transgene show increased phosphorylation of MAPK at the anterior poles, similar to embryos with four copies of wild-type bcd (bcd4x). Mean ± SE of 20–30 embryos for each genotype is shown; * denotes a statistically significant difference from the wild-type (t-test, p < 0.01).
3.2.3 MAPK mediated downregulation of Cic also exhibits AP asymmetry

The results presented so far are consistent with the idea that Bcd makes phosphorylated MAPK less available for binding to and dephosphorylation by the MAPK phosphatase. According to the same model, Bcd can make the phosphorylated MAPK less available for its other substrates. To explore this possibility, we examined the distribution of Cic, which is downregulated at the poles as a result of its direct phosphorylation by MAPK (Figures 3.3A-3.3C). Statistical analysis revealed that, similar to the wild-type pattern of dpERK, the wild-type pattern of Cic is asymmetrical, with the levels of Cic significantly higher at the anterior pole (Figure 3.3D). Because of the distribution of Cic is uniform in the absence of MAPK signaling, the wild-type pattern of Cic downregulation suggests that, although the level of MAPK phosphorylation is higher at the anterior pole, its activity directed toward Cic is actually lower.

We emphasize that this observation argues against the possibility that the AP asymmetry of the wild-type MAPK phosphorylation pattern is generated only by the asymmetry in the extracellular activation of Tor. If this were true, then higher levels of MAPK phosphorylation at the anterior pole would lead to a higher level of Cic downregulation, which is contrary to what we observed. Based on this, we argue that the AP asymmetry of the wild-type MAPK signaling pattern is generated mainly by the intracellular asymmetries of the early embryo. Upon quantifying the spatial pattern of Cic downregulation in embryos with varying levels of Bcd, we established that this asymmetry is increased in embryos with an extra copy of \( bcd \) and reduced in embryos with progressively lowered levels of \( bcd \) (Figure 3.3E). These effects are consistent with the model, in which
anteriory localized Bcd acts as a competitive inhibitor of MAPK-mediated Cic downregulation.

Figure 3.3: Asymmetry of MAPK mediated downregulation of Cic. (A–C) Co-immunostaining reveals the spatial patterns of dpERK (green) and Cic (red). (D) Averaged AP gradient of nuclear Cic at cell cycle 14 from 40 embryos. The dashed line indicates reference level of Cic repression. Similar to MAPK phosphorylation gradient, Cic pattern also exhibits a clear AP asymmetry. (E) The dose of bcd affects the asymmetry of the spatial pattern of Cic downregulation in the early embryo. Each bar represents an average asymmetry of nuclear Cic gradient for 40–90 embryos of the same genotype. As the amount of maternal bcd mRNA is lowered, the anterior and posterior levels of Cic become more symmetric (generalized linear model: \( p = 2.5 \times 10^{-12} \)).

3.2.4 Hunchback is a substrate of MAPK

If Bcd is the only source of the AP asymmetries of MAPK phosphorylation and Cic downregulation, then both of these patterns should become symmetric in bcd null embryos. Surprisingly, however, we found that both MAPK phosphorylation and Cic downregulation exhibited a significant amount of AP asymmetry even in the bcd mutant embryos (Figures 3.4A and 3.4B). It is possible that this residual asymmetry reflects the presence of another
additional MAPK substrate that is non-uniformly distributed in bcd null embryos and contributes to the AP asymmetry of MAPK signaling in this genetic background.

One candidate for this residual asymmetry is Hunchback (Hb), a transcription factor that plays a key role in the embryonic AP patterning [17, 18]. The distribution of Hb protein depends on both maternal and zygotic inputs [17, 19]. Briefly, maternal hb transcript is deposited uniformly, but its translation is repressed in the posterior region of the embryo by Nos, resulting in an anterior gradient of maternal Hb protein. In the anterior half of the embryo, hb is also zygotically activated by Bcd, providing an additional gradient of zygotic Hb protein (Figure 3.4C). As a consequence of its dual control by Bcd and Nos, the pattern of Hb protein is not symmetric in bcd null embryos: the levels of Hb are still higher at the anterior pole. This led us to test whether, similar to Bcd and Cic, Hb acts as a hitherto-unrecognized MAPK substrate that modulates the patterns of MAPK phosphorylation and Cic downregulation along the AP axis.

Indeed, in a proteomics screen aimed at identifying potential MAPK substrates in the early blastoderm embryo, we found that Hb is phosphorylated by MAPK in vitro. Like Bcd and Cic, but unlike Runt and other segmentation gene products, Hb displays a MAPK-dependent electrophoretic mobility shift on SDS polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 3.4D). To test whether Hb can affect the level of MAPK phosphorylation in vivo, we examined MAPK phosphorylation in embryos with uniform maternal expression of Hb. We found that Hb expression leads to a statistically significant increase in the posterior dpERK signal, as would be expected if Hb was acting as a MAPK substrate in the early embryo (Figure 3.4E).
Figure 3.4: Hb is a new substrate of MAPK in the embryo. (A) Comparison of the dpERK gradients in wild-type (Histone-GFP) embryos and embryos from bcd null mothers. Although the anterior levels of dpERK in bcd null embryos are lower than that in the wild-type, the gradient is still asymmetric along the AP axis (arrow). (B) Averaged AP gradient of nuclear Cic at cell cycle 14, obtained from 27 embryos laid by bcd null flies. Similar to the dpERK gradient, the Cic pattern is still asymmetric along the AP axis (arrow). (C) Simplified description of Hb regulation in a wild-type embryo. Maternal hb mRNA is deposited uniformly throughout the embryo (green), but its translation is repressed in the posterior half by Nos (dashed green), resulting in anterior gradient of maternal Hb protein (blue). In the anterior half of the embryo, Bcd activates zygotic hb transcription, which further adds to the preexisting Hb gradient (red). Note that even in the absence of Bcd, Hb protein is still present in anterior gradient (blue). (D) Radiolabeled Hb migrates with slower mobility on SDS polyacrylamide gel electrophoresis following incubation in the presence (+), but not in the absence (-), of activated ERK2, indicating that Hb is phosphorylated by MAPK. Similar mobility shift is observed for known targets of MAPK, such as Cic and Bcd, but not for Runt. Note that Hb contains several consensus MAPK phosphorylation sites. (E) Ubiquitous expression of hb with a maternal Gal4 driver leads to an increase in MAPK phosphorylation at both the anterior and posterior poles of the embryo (t-test: $p_{anterior} = 0.03$, $p_{posterior} = 0.01$). (F) Quantifying the dpERK gradient in embryos mutant for oskar (osk) shows an increase in MAPK phosphorylation at the posterior region. In osk null embryos, maternal hb mRNA is translated at the posterior of the embryo.
3.3 Discussion

To summarize, the proposed substrate competition model accounts for the experimentally observed AP asymmetry of the wild-type patterns of MAPK phosphorylation and the activity toward its substrates, predicts how this asymmetry responds to multiple genetic perturbations, and identified Hb as a novel potential target of MAPK phosphorylation. In addition, the proposed model can account for the previously unexplained genetic interaction between the posterior and terminal systems. It was established that removal of posterior patterning determinants (Nos or Oskar (Osk)) increases the posterior level of Cic and consequently reduces the posterior expression domains of its targets, *tll* and *hkb* [20]. In light of our results, these effects can be interpreted by the fact that, in *nos* or *osk* mutant embryos, ectopic Hb acts as a competitive substrate of MAPK, reducing its ability to downregulate Cic. This would be analogous to the effect at the anterior pole, where downregulation of Cic is antagonized by Bcd. In partial confirmation to this model, we found that removal of either *nos* or *osk* increases the posterior level of dpERK, indicating that increased amounts of Hb, like Bcd, can influence the level of MAPK phosphorylation (Figure 3.4F).

Thus, a common substrate competition can provide a basis for the modulation of MAPK signaling by the anterior and posterior systems. Going beyond the early fruit fly embryo, we propose that competitive interactions can provide a general control strategy in signaling networks where enzymes, such as MAPK, interact with their multiple regulators and targets [11, 21-33].
3.4 Experimental procedures

**Drosophila strains and whole-mount immunostaining.** The following stocks were used in this study: Histone-GFP (a gift from E. Wieschaus), bcd\(^{d}\), bcd\(^{d+}\), osk\(^{166}\), nos\(^{BN}\), Cic-HA; cic\(^{d}\), Cic-HA; cic\(^{d}\) bcd\(^{E1}\), UAS-hb (a gift from N. Dostatni), and Bcd-A\(_{9}\). All flies were raised and embryos were collected at 25°C. Antibody stainings were performed as described in [8]. Monoclonal mouse anti-dpERK (1:100, Sigma) and polyclonal rabbit anti-HA (1:500, Roche) were used as primary antibodies. Alexa Flour and Oregon Green (1:500, Invitrogen) conjugated secondary antibodies were used to label the primary antibodies.

**Microscopy and image processing.** Imaging was done on a Zeiss LSM510 confocal microscope, with a Zeiss 20x (NA 0.6) A-plan objective. High resolution images (512x512 pixels, 12bits depth) were obtained from the focal plane in the mid-horizontal cross section of the embryo. Images of individual embryos were automatically extracted from raw confocal images and re-oriented for gradient quantification as described elsewhere [8].

**In vitro MAPK/ERK2 phosphorylation assays.** Proteins were synthesized and labeled using the Quick TNT-coupled rabbit reticulocyte lysate system (Promega), in the presence of [35S]-methionine. Labeled proteins were then incubated with (or without) 0.2 micrograms of active ERK2, in a total volume of 50 microliter of kinase reaction buffer (20 mM HEPES, 0.1 mM benzamidine, 25 mM beta-glycerophosphate, 0.1 mM DTT, 1 mM Na\(_{3}\)VO\(_{4}\), 10 mM MgCl\(_{2}\) and 0.1 mM ATP) for 30 minutes at 30°C. Reactions were stopped by adding SDS sample buffer x3 (0.25M Tris pH 6.8, 6% SDS, 30% Glycerol, B-mercapto-ethanol and a few grains of Bromo-phenol-blue). The phosphorylation state of the proteins was subsequently
analyzed by SDS-PAGE and autoradiography. To activate ERK2, a HIS-tagged ERK2 fusion protein was expressed in *Escherichia coli*, purified on nickel beads (Qiagen) and activated using active MEK1 (Upstate).

**Statistical Analysis.** An unpaired t-test was used to compare the mean levels of both anterior and posterior dpERK between wild-type and mutant embryos of interest. For this analysis, dpERK gradients were extracted from 20-30 wild-type and mutant embryos. To examine the correlation between the amount of Bcd and level of MAPK phosphorylation, a generalized linear model (GLM) was employed with copies of *bcd* as the independent variable and either the anterior or posterior dpERK levels as the response variable. Similarly, GLM was also used to show the correlation between the *bcd* copy number and the Cic asymmetry where Cic asymmetry is defined as the ratio of anterior to posterior repressions of Cic.

### 3.5 References


Chapter 4

Substrate-dependent control of MAPK phosphorylation in vivo

This chapter is based on a study published with Ze’ev Paroush, Knud Nairz, Ernst Hafen, Gerardo Jiménez, and Stanislav Y. Shvartsman. The authors thank E. Wieschaus, O. Grimm, M. Coppey, R. Seger, K. Dalby, M. Levine, and all members of the Shvartsman lab for helpful discussions. We also thank K. Ishihara and K. Fitzgerald for technical assistance with experiments.

4.1 Introduction

The mitogen activated protein kinase/extracellular-signal regulated kinase (MAPK/ERK) pathway is a three-tiered cascade of phosphorylation-dephosphorylation cycles found in all eukaryotes [1, 2]. An input to the pathway can be provided by a cell surface receptor; its immediate output is the phosphorylation of MAPK, a serine-threonine kinase at the bottom of the cascade. Active MAPK controls cellular processes by phosphorylating its multiple intracellular substrates. According to the current models of MAPK signaling, control of MAPK phosphorylation is independent of MAPK substrates, which are viewed as mere sensors of MAPK activity [3-6]. While this modular view of MAPK signaling is consistent with a large body of biochemical evidence, interactions can be more complex in vivo, as substrates phosphorylated by MAPK can interfere with the
processes that control the phosphorylation of MAPK itself, by direct competitive interactions or by affecting subcellular location of MAPK [7-11]. Substrate-dependent control of MAPK phosphorylation has been demonstrated in studies with heterologous expression systems [7], but the extent to which any given substrate controls MAPK phosphorylation in vivo is an open question. We explore this question in the Drosophila embryo, a powerful system that offers the possibility to genetically manipulate the levels of MAPK substrates and quantify MAPK phosphorylation. We found that the genetic removal of any one of the four known MAPK substrates in this system leads to a significant decrease of MAPK phosphorylation. These changes can be interpreted in terms of a model, whereby MAPK substrates counteract MAPK dephosphorylation by phosphatases.

4.2 Results

4.2.1 Removal of endogenous MAPK substrates reduces MAPK phosphorylation in vivo

Drosophila uses its MAPK/ERK pathway throughout embryonic development [12]. This pathway is activated for the first time in the syncytial blastoderm to specify the terminal regions of the embryo. In this case, a locally activated receptor tyrosine kinase establishes a two-peaked pattern of MAPK phosphorylation, which controls the expression of tailless (tll) and huckebein (hkb), two genes essential for the specification of the non-segmented terminal structures (Figures 4.1A and 4.1B) [13, 14]. In the absence of MAPK signaling, tll and hkb are repressed by the ubiquitously expressed transcriptional repressors Capicua (Cic) and Groucho (Gro) [15, 16]. At the termini, their action is counteracted by MAPK, which phosphorylates both Cic and Gro and thus de-represses tll and hkb [17, 18]. MAPK also
phosphorylates Bicoid (Bcd) and Hunchback (Hb), two other transcription factors [19, 20]. In contrast to Cic and Gro, Bcd and Hb are localized to the anterior of the embryo (Figure 4.1C).

We have previously shown that removal of a single MAPK substrate, Bcd, leads to a significant reduction of MAPK phosphorylation in the embryo [19]. To test whether this effect is limited to Bcd or more general, and can be induced by other substrates, we genetically removed Cic, Gro, or Hb and quantified the resulting pattern of MAPK phosphorylation, assayed using the antibody that recognizes the double phosphorylated form of the MAPK/ERK (dpERK) [21, 22]. In all cases, the level of dpERK was significantly reduced (Figures 4.1D-K). As expected, based on their wild-type spatial expression patterns, the effects of Bcd and Hb were limited to the anterior pole of the embryo (Figures 4.1H-K), whereas removal of either Cic or Gro influenced both the anterior and posterior levels of dpERK (figures 4.1D-G). Furthermore, removal of multiple substrates has a cumulative effect, as removal of both Bcd and Hb leads to a stronger reduction in dpERK levels compared with the effect induced by removing Bcd or Hb alone (Figures 4.1H-M). Thus, the level of MAPK phosphorylation is reduced by removing any one of the four known MAPK substrates in the early embryo.
4.2.2 MKP3 negatively regulates MAPK signaling in the early embryo

What can be the mechanism of substrate-dependent control of MAPK phosphorylation? Previous studies with heterologous expression systems suggested that MAPK substrates can increase the level of MAPK phosphorylation by counteracting MAPK dephosphorylation by phosphatases [7-11, 23]. In theory, a substrate can inhibit MAPK dephosphorylation directly by competing with MAPK phosphatase for binding to MAPK. Alternatively, a nuclear substrate can protect MAPK from the action of the cytoplasmic phosphatases by increasing the nuclear residence time of phosphorylated MAPK.
As a first step toward testing this mechanism, we set out to identify the phosphatase that acts during the terminal patterning of the embryo. One strong candidate is MAPK phosphatase-3 (MKP3), a highly conserved cytoplasmic threonine/tyrosine phosphatase that is expressed in the early embryo [24-26]. Previous genetic analysis of MKP3 in Drosophila development used the mkp3\(^1\) and mkp3\(^2\) loss-of-function alleles, both of which were generated by the P-element transposons inserted in the 5’ untranslated region (UTR) of the mkp3 gene [27]. The mkp3\(^1\)/mkp3\(^2\) transheterozygous flies have extra wing veins and rough eye phenotypes, which demonstrates that MKP3 negatively regulates MAPK signaling during wing and eye development [27]. We used these alleles to investigate the role of MKP3 in the terminal system.

We found that the MAPK phosphorylation was significantly increased in embryos derived from the mkp3\(^1\)/mkp3\(^2\) females (Figures 4.2A and 4.2B). This increase was accompanied by the expansion of expression domains of tll and hkb, consistent with the notion that MKP3 is a functionally significant negative regulator of MAPK signaling at this stage of development (Figures 4.2I and 4.2J). This conclusion is further supported by the results of ectopic expression experiment, in which we overexpressed MKP3 in the early embryo using a maternal Gal4 driver. This led to a significant reduction of MAPK phosphorylation, with greater effect in the posterior region (Figure 4.2C and 4.2D). On the basis of these results, we conclude that MKP3 negatively regulates MAPK phosphorylation in the early embryo.
Figure 4.2: MKP3 negatively regulates MAPK phosphorylation and signaling in the terminal system. (A–H) Quantified average dpERK gradients and peak levels in wild-type embryos (blue) and mutant embryos with lower level of MKP3 (mkp3/mkp3) (A, B), ectopic overexpression of MKP3 (C, D), ectopic overexpression of MKP3 in bcd null background (E, F) and ectopic overexpression of both MKP3 and Bcd (G, H). (I, J) Altering the level of MKP3 is accompanied by changes in the expression of the downstream gene tll. The posterior expression of tll is expanded toward the center in the embryos derived from mkp3 transheterozygous mothers, while it shrinks toward the pole when MKP3 was overexpressed. Quantification of the position of the posterior boundaries indicates that these shifts are statistically significant (J). * indicates p<0.01.

4.2.3 A model for substrate-dependent control of MAPK phosphorylation

Thus, the phosphorylation level of MAPK is negatively regulated by MKP3, a cytoplasmic phosphatase [26, 28], and positively regulated by the four nuclear substrates. We then asked whether nuclear localization of MAPK substrates is important for their ability to control MAPK phosphorylation in vivo. To address this question, we used a mutant version of Cic that is predominantly cytoplasmic [17]. This protein retains a MAPK-docking site, which is essential for its interaction with and downregulation by MAPK. Expression of this protein led to a marginal increase of MAPK phosphorylation (Figure 4.3). At the same time, we found that adding extra genomic copies of the wild-type cic, which is predominantly
localized to the nucleus, lead to a strong increase of MAPK phosphorylation (Figure 4.4A and 4.4B). On the basis of these observations, we favor a model where substrates control MAPK phosphorylation by affecting its subcellular localization.

**Figure 4.3: Nuclear localization is important for Cic-dependent control of MAPK phosphorylation.** (A) Cic variant that lacks its HMG domain (Cic\(^{ΔHMG}\)) localizes to cytoplasm (right) whereas wild type Cic is nuclear (left). (B) Quantified dpERK gradients of wild type embryos and embryos expressing Cic\(^{ΔHMG}\). Expressing cytoplasmic Cic has a mild effect on MAPK phosphorylation. (C) Statistical comparison shows that the effect of Cic\(^{ΔHMG}\) on MAPK phosphorylation is not significant (p\(_{anterior}\) = 0.23; p\(_{posterior}\) = 0.09).

Our results can be compactly summarized by a simple mathematical model (Box 1), in which an enzyme (E) is controlled by a phosphorylation-dephosphorylation cycle in the cytoplasm. The active form of the enzyme (E\(^{*}\)) shuttles between cytoplasm and nucleus, where it phosphorylates multiple substrates (S\(_i\)). We analyzed this model at steady state, assuming that the concentrations of substrates (S\(_i\)) and regulators (A and D) are kept constant. We also assumed that the concentrations of regulators are significantly less than the concentrations of the enzyme and its substrates. Finally, we assumed that phosphorylated substrates do not interact with the enzyme and thus do not affect the state of the network. These assumptions were made to simplify the algebra and are not essential for the model to exhibit substrate-dependent control of enzyme phosphorylation.
The total amount of the enzyme is denoted by \([E_{\text{tot}}]\). Let \(K_{M,i}\) denotes the Michaelis constant of the \(i\)-th phosphorylation reaction: \(K_{M,i} \equiv (k_{r}^{s} + k_{r}^{s})/k_{b}^{s}\), and let \(K_{M,A}\) and \(K_{M,D}\) denote the Michaelis constants for the activation and deactivation reactions, respectively. Using the conservation law for the enzyme, one can show that the fraction of the enzyme in the phosphorylated state is given by the following expression:

\[
P = \frac{\alpha(1 + \beta \gamma)}{1 + \alpha(1 + \beta \gamma)}
\]

where \(\alpha = \frac{k_{r}^{A}K_{M,D}[A]}{K_{r}^{D}K_{M,A}[D]}, \beta = \frac{k_{i}}{k_{e}}, \gamma = \left(1 + \sum_{i=1}^{n} \frac{[S_{i}]}{K_{M,i}}\right)\).

Thus, \(P\) depends on three dimensionless groups: The first of them, \(\alpha\) can be interpreted as the level at which the phosphorylation-dephosphorylation cycle is driven by the balance of kinase and phosphatase activities, \(\beta\) is the nucleocytoplasmic concentration ratio of the phosphorylated enzyme, and \(\gamma\) characterizes the extent to which the phosphorylation-dephosphorylation cycle is “loaded” by substrates.

Differentiating this expression, we obtain the differential sensitivity of the level of phosphorylated enzyme to changes in the level of network activation:
\[
\frac{\alpha \partial P}{P \partial \alpha} = \frac{1}{1 + \alpha(1 + \beta \gamma)} > 0
\]

This differential sensitivity quantifies the fractional change in the level of enzyme phosphorylation that is induced by the fractional change in the level of network activation, such as the change in the level of kinase or phosphatase activity. For example, a uniform increase in the phosphatase level would decrease the value of \( \alpha \) and, consequently, decrease the observed value of \( P \). The magnitude of this change, however, is modulated by substrates, which is characterized by the value of \( \gamma \): As the total concentration of substrates increases, the effect of increasing the phosphatase concentration gets smaller. This can explain why overexpression of \( mkp3 \) does not affect the level of MAPK phosphorylation at the anterior pole, where the total concentration of MAPK substrates is higher (Figure 4.2D).

The differential sensitivity of enzyme phosphorylation to changes in the concentration of any given substrate is given by the following expression:

\[
\frac{\left[ S_i \right] \partial P}{P \partial \left[ S_i \right]} = \frac{\beta \left[ S_i \right]}{K_{M,i} \left( 1 + \beta \gamma + \alpha(1 + \beta \gamma)^2 \right)} > 0
\]

Once again, the magnitude of the effect induced by a substrate is a decreasing function of \( \gamma \), which implies that a change in the level of MAPK phosphorylation that is induced by any given substrate is buffered by competing substrates. Thus, the model can explain why uniformly expressed exogenous substrates, such as Yan, increase the MAPK phosphorylation only at the posterior pole (Figure 4.2G and 4.2H).

To introduce spatial component to the model, we assumed that the catalytic activation of \( E \) by \( A \) (\( k_A^A \)) occurs in a graded manner from the poles which results in a spatial gradient of \( \alpha \) with maxima at the poles (Figure 4.5A). We assumed that the diffusion is slow in this system. We used an exponential curve to depict the spatial distribution of anteriorly
localized substrates which results in non-uniform profile of \( \gamma \) along the x-axis (Figure 4.5B). The model is then used to predict the spatial profile of the total fraction of activated enzyme (P) and how it is affected by changes in deactivator (D) or substrates (S). Note that the model successfully predicts that these perturbations always have stronger effect at the posterior pole (Figure 4.5C-F).

Importantly, this model readily accounts for the region-specific effect of MKP3 overexpression in a wild-type embryo: The fact that the resulting reduction of dpERK levels is weaker at the anterior pole can be explained by the effect of the anteriorly localized Bcd and Hb, which increase the total concentration of MAPK substrates at the anterior and provide a more efficient protection of phosphorylated MAPK from phosphatases (Figure 4.5). Following the same reasoning, the effect of spatially uniform changes in the level of any given MAPK substrate should be always smaller at the anterior pole. Consistent with this expectation, we found that the effect of the spatially uniform increase in the level of a MAPK substrate is always smaller at the anterior pole (Figure 4.4A, 4.4B, and 4.4H). Finally, the model predicts that a similar effect can be induced by substrates that are not normally expressed at this point of development. Indeed, ectopic expression of Yan, a MAPK substrate that is not expressed in the syncytial blastoderm [29, 30], significantly increased the dpERK level at the posterior pole, whereas the anterior level was indistinguishable from that of the wild-type (Figure 4.4G and 4.4H).
Figure 4.4: Cic-dependent control of MAPK phosphorylation. (A) dpERK gradients in embryos with different cic copy number; each gradient is an average of ~20 embryos of the same genotype. (B) The anterior and posterior peak levels of dpERK in embryos with different cic copy number. Each bar indicates averaged gradient of ~20 embryos with standard error indicated as error bars; the values are normalized such that the wild-type data are set at 1. (C) Schematic representation Cic variants used in the experiments. (D) Expression of CicΔC2 in wild-type embryos decreases the expression domains of tll and hkb. In contrast, addition of CicΔC1 does not affect the gene expression. (E) Averaged dpERK gradients in wild-type embryos (blue) and embryos expressing CicΔC2 (red). (F) Quantitative comparison of posterior dpERK levels in the wild-type embryos (blue) and embryos overexpressing wild-type Cic (cic4x), CicΔC2 or CicΔC1. (G) Immunostaining of Bcd (top) and Yan (bottom) in embryos with ubiquitous maternal expression of bcd or yan. (H) Quantification of posterior peak dpERK levels in wild-type embryos (blue) and embryos with uniform overexpression of Bcd or Yan (red). * indicates p<0.01.
Figure 4.5: Model predictions of substrate-dependent control of MAPK phosphorylation. (A) Anteroposterior (AP) spatial profile of $\alpha$ which characterizes the ratio of activation and deactivation of the enzyme. All the plots are generated such that the x-axis represents the AP axis of the embryo with anterior tip at x=0. (B) Spatial profile of $\gamma$, which characterizes the effect from the substrates. (C) Spatial profile of the total fraction of enzyme activated, $P$ in a wild-type embryo. Due to the non-uniform distribution of substrates, the profile of $P$ exhibits a strong asymmetry at the poles. (D) Profile of $P$ is affected by changes in the level of the deactivator, $D$. The model predicts that the effect is greater at the posterior compared to the anterior. (E) Comparison of $P$ in wild-type (blue) and embryos that lack substrates ($\gamma = 1$, red). Note that fraction of activated enzyme is significantly decreased in the absence of substrates. (F) Upon ectopic overexpression of a uniform substrate, the level of $P$ is increased (red) with stronger effect at the posterior.
Thus, a model in which substrates positively regulate the level of MAPK phosphorylation successfully predicts the effect of multiple genetic perturbations. At the same time, the results presented so far do not exclude the possibility that the weaker effect in the anterior region in response to perturbing the level of MKP3 might reflect differences in signaling between the two poles. In particular, MKP3 might not be the relevant phosphatase in the anterior region of the embryo. To explore this possibility, we examined how the level of MAPK phosphorylation is affected when MKP3 is uniformly overexpressed in the absence of Bcd, an anteriorly localized substrate. According to our model, overexpressing MKP3 will have greater effect in this background due to lower level of substrates. Consistent with this prediction, we found that uniform overexpression of MKP3 in bcd null background significantly decreased the anterior level of MAPK phosphorylation (Figure 4.2E and 4.2F).

In addition, we examined the level of MAPK phosphorylation in embryos with uniform overexpression of Bcd and MKP3. Note that ectopic expression of either one of these factors did not affect the anterior level of dpERK, but had an opposite effect at the posterior pole: The level of dpERK was increased by ectopic expression of Bcd (Figure 4.4H), but reduced by ectopic expression of MKP3 (Figure 4.2D). When Bcd and MKP3 were overexpressed together, the effect was intermediate (Figure 4.2G and 4.2H). Thus, the level of MAPK phosphorylation in vivo can be affected both by the direct regulators of MAPK phosphorylation, such as MKP3, and by MAPK substrates, such as Bcd.

4.2.4 Transcriptional activity of Cic is not essential for Cic-dependent control of MAPK phosphorylation

All of the MAPK substrates analyzed in our experiments are transcription factors and can potentially affect the level of MAPK phosphorylation indirectly, via transcriptional
feedback. For example, in the developing *Drosophila* wing disc, Cic acts as a repressor of *argos*, which encodes an inhibitor of the epidermal growth factor receptor that signals through the MAPK pathway [31]. In the early embryo, we found that increasing the gene copy number of *cic* leads to a clear increase of MAPK phosphorylation (Figure 4.4A and 4.4B). This change was accompanied by a reduction in the expression domains of *tll* and *hkb* (data not shown). Thus, we tested whether the experimentally observed Cic-dependent control of MAPK phosphorylation is direct, or mediated by transcriptional repression of negative regulators of MAPK signaling.

To address this question, we used a mutant derivative of Cic that lacks the MAPK-docking domain (CicΔC2) and is therefore insensitive to MAPK-dependent downregulation, but is otherwise active as a repressor [17]. As expected, adding the MAPK-insensitive mutant Cic has a strong effect on the expression of *tll* and *hkb* (Figure 4.4D). If the effect of Cic overexpression is mainly transcriptional, then expression of the CicΔC2 derivative should elicit a response similar to that induced by the wild-type Cic protein. On the other hand, if the effect does not depend on transcriptional activity of Cic, then the level of MAPK phosphorylation should be unaffected. Our experiments established that the dpERK levels were indistinguishable from those in the wild-type embryos (Figures 4.4E and 4.4F). Thus, even though CicΔC2 is transcriptionally active and represses MAPK target genes, it cannot significantly change the level of MAPK phosphorylation.

On the basis of these observations, we conclude that direct interaction between MAPK and Cic is essential for inducing the experimentally observed increase in dpERK levels, whereas its transcriptional activity is not. This conclusion is further supported by the experiments with a mutant Cic that lacks a domain important for its transcriptional activity
(Cic\textsuperscript{ΔC1}). This mutant protein still interacts with and is downregulated by MAPK, but it is functionally weaker [17]; for instance, its expression does not alter the expression boundaries of \textit{tll} and \textit{hkb} (Figure 4.4D). At the same time, the level of MAPK phosphorylation was significantly increased upon expressing the Cic\textsuperscript{ΔC1} derivative (Figure 4.4F). Thus, despite the weaker activity as a transcriptional repressor, Cic\textsuperscript{ΔC1} still controls the level of MAPK phosphorylation.

Our previous experiments, using a Bcd derivative with impaired DNA binding, support a similar model for the Bcd-dependent control of MAPK phosphorylation [19]. We note that while our studies of the effects of Cic and Bcd do not rule out the importance of transcriptional effects, they strongly suggest that they do not have a dominant role. Although we have not yet investigated the mechanism of the experimentally observed control of MAPK phosphorylation by Hb and Gro, we currently favor a common, non-transcriptional model, which is intrinsic to the phosphorylation-dephosphorylation module that controls MAPK. This model parsimoniously explains changes in the level of MAPK phosphorylation induced by five different transcription factors (Bcd, Cic, Hb, Gro, and Yan).

4.3 Discussion

MAPK phosphorylation is controlled by multiple enzymes, adaptors and scaffolds that regulate its catalytic activity, interaction partners, and subcellular localization [2, 32-35]. Our study demonstrates that MAPK substrates can be equally important in controlling the level of MAPK phosphorylation \textit{in vivo}. Similarly, the level of MAPK activity toward any one of its substrates can be significantly affected by other co-expressed substrates [19]. As
shown in Figure 4.6, this effect is not limited to Bcd: Cic downregulation is increased upon removal of Gro, another substrate of MAPK.

**Figure 4.6: MAPK signaling activity is modulated by MAPK substrates.** Quantified Cic gradients in wild-type (blue) and embryos lacking maternal gro (red) are plotted with standard error of the mean.

To the best of our knowledge, substrate-dependent control of MAPK phosphorylation in the *Drosophila* embryo is the first *in vivo* demonstration of a recently introduced systems biology concept called retroactivity, defined as the ability of a downstream target of a module to induce a change in the internal state of the module [36, 37]. Retroactivity is a form of feedback, but it is different from more conventional types of feedback control where a target of the pathway directly interacts with its upstream components [6, 33, 38-40]. In the case of retroactivity, feedback cannot be removed genetically or pharmacologically without affecting the input-to-output connection.

In some systems, retroactive effects can be small [36]. For example, MAPK signaling could operate in a regime where the activated MAPK is in excess of its substrates and thus would be unaffected by changes in the levels of its substrates. Retroactive effect
will also be insignificant when a substrate occupies only a small fraction of the total amount of substrates. Our experiments with five different MAPK substrates provide clear counterexamples and reveal that even a single MAPK substrate can exhibit strong retroactivity and have an appreciable effect on the internal state of the MAPK signaling module.

### 4.4 Experimental procedures

**Drosophila strains and germline clones.** Histone-GFP, OreR, cic, FRT82B gro, bcd, FRT82B bcd, hb nos (a gift from E. Wieschaus), FRT82B hb spz (a gift from E. Wieschaus), cic, cic, cic, MKP3, MKP3, UAS-BcdGFP (a gift from E. Wieschaus), UAS-MKP3, mata4-Gal-VP16 (a gift from E. Wieschaus), and UAS-Yan. All flies were raised and embryos were collected at 25°C.

The maternal Gal4 driver contains the DNA binding domain of Gal4 fused to VP16 activation domain and is expressed from α4 tubulin promoter [41]. The hb spz double mutant flies were used to analyze the effect of removing maternal Hb. We have analyzed that removing Dorsal signaling alone has no effect on the level of MAPK phosphorylation and thus, the observed effect of the hb spz double mutant embryos can be attributed to the removal of hb.

To make germline clone of gro, bcd hb nos, and hb spz standard FLP-FRT technique was used. Males with hsFLP and FRT82B ovo were crossed with females carrying the mutant of interest. The resulting progenies were heat-shocked at 37.5°C for 1 hour for 2 consecutive days at 3rd instar larva stage.
Immunostaining and in situ hybridization. Primary antibodies used in this study were: Monoclonal mouse anti-dpERK (1:100, Sigma), monoclonal mouse anti-Yan (1:100, DBHS), polyclonal rabbit anti-HA (1:500, Roche), polyclonal rabbit anti-GFP (1:500, Chemicon), and polyclonal rabbit anti-Cic (1:2000, a gift from C. Berg). Alexa Flours (1:500, Invitrogen) conjugated secondary antibodies were used. Embryos were mounted in Aqua PolyMount and kept in 4°C. To detect *tlb* and *hkb*, embryos were hybridized overnight at 60°C with Digoxigenin (DIG) labeled anti-sense probes. Embryos were then incubated with alkaline phosphatase conjugated anti-DIG (Roche, 1:200) antibody for 1hr at room temperature and developed in NBT/BCIP solution for 20mins.

Imaging was done on a Zeiss LSM510 confocal microscope, with a Zeiss 20x (NA 0.6) A-plan objective. Images of individual embryos were automatically extracted from raw confocal images and quantified as described elsewhere [21].

Statistical analysis of dpERK patterns and quantification of gene expression boundaries. A paired t-test was used to compare the mean levels of both anterior and posterior dpERK between wild-type and mutant embryos of interest. For this analysis, dpERK gradients were extracted and anterior and posterior expressions were independently fitted with a Gaussian curve. The maximum of this fitted curve was used as the dpERK peak level. The expression boundaries of *tlb* and *hkb* were determined using automated image analysis program in Matlab, which finds the boundary of the embryo and then averages staining intensity along the dorsoventral axis. This was done for 1,000 points uniformly spaced along the AP axis, generating an AP expression profile of the gene. The locations of the half maximum level were used as boundaries of the expression domains.
4.5 References


Chapter 5

Gene regulation by MAPK substrate competition

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5.1 Introduction

Similar to other multicellular organisms, *Drosophila* uses its MAPK/ERK pathway throughout embryogenesis. It is first used downstream of Torso receptor tyrosine kinase (RTK), to specify the non-segmented terminal regions of the future larva. Locally activated Torso establishes a two-peaked pattern of activated, double phosphorylated MAPK/ERK (dpERK) [1, 2]. One MAPK substrate is an HMG-box transcriptional repressor Capicua (Cic), which is uniformly expressed in the embryo [3]. In a process essential for the development of the terminal structures, Cic is degraded at the poles in direct response to its phosphorylation by MAPK [3-5]. Another substrate of MAPK is an anteriorly localized homeodomain protein Bicoid (Bcd), which determines the anterior pattern of the embryo [6, 7].
Together with another anteriorly localized MAPK substrate, Hunchback (Hb), Bcd antagonizes MAPK-dependent downregulation of Cic [8]. Our earlier work supports a model whereby MAPK substrates indirectly affect each other by competing for their common regulator [8]. Competitive effects of this type should be common in large-scale biomolecular networks where one regulator controls a large number of targets. Computational studies of mass-action networks demonstrate that, in general, competitive effects decay with distance (the number of interaction edges) from perturbed nodes [9]. In other words, competitive effects could be localized to a specific node and thus might not significantly affect more distant network components. In the context of the terminal patterning system, anteriorly localized MAPK substrates can decrease the level of Cic downregulation, but the effect may be too small to impact Cic-dependent gene regulation.

Here, we present evidence to the contrary and propose that MAPK substrate competition controls gene expression in the early embryo. This conclusion is based on the analysis of the expression pattern of zerknüllt (zen), which encodes a homeodomain transcription factor and is expressed in a complex pattern that depends on the joint action of the terminal and dorsoventral (DV) patterning systems (Figure 5.1). zen expression is broadly activated by Zelda and possibly other maternal factors, and repressed in the ventral and lateral regions by Dorsal (Dl), a transcription factor that patterns the DV axis (Figure 5.1A and 5.1B) [10-13]. At the poles, Dl-mediated repression of zen is antagonized by Torso signaling [14].

The precise mechanism of zen de-repression by the terminal system has been unclear, but it is likely to depend on MAPK-dependent downregulation of Cic [3, 5, 15]. We demonstrate that the anti-repressive effect of MAPK signaling on zen is modulated by the
anteriorly localized MAPK substrates. Specifically, removing anterior MAPK substrates makes zen expression symmetric along the anteroposterior (AP) axis. Our results support a mechanism whereby MAPK substrate competition controls spatial patterns of gene expression by coordinating the actions of the anterior, dorsoventral, and terminal systems. We formalize this mechanism in a mathematical model and demonstrate that this model correctly predicts zen expression in multiple genetic backgrounds.

5.2 Results

5.2.1 Cic downregulation is required for zen expression at the poles

zen expression pattern displays a pronounced AP asymmetry: zen transcripts are present at the posterior but not at the anterior pole. This asymmetry is particularly clear when embryos are imaged in an upright position (Figure 5.1C). As shown in Figure 5.1D and 5.1E, zen transcript levels are markedly different at the anterior versus the posterior pole of the embryo. Below, we argue that AP asymmetry of zen expression can be explained by a mechanism that relies on MAPK substrate competition. Briefly, we propose that MAPK-dependent downregulation of Cic is responsible for zen de-repression at the poles. Anteriorly localized MAPK substrates inhibit Cic downregulation, increasing the level of Cic, and thus the strength of DI-dependent zen repression at the anterior pole.
Figure 5.1: Expression of *zen* mRNA exhibits AP asymmetry. (A and B) Spatial patterns of Dl protein (green) and *zen* mRNA (red) in a wild-type embryo. *zen* transcripts were detected by FISH. The image is oriented with anterior left and dorsal side up. (C) Schematic of end-on imaging. (D) Optical sections of an embryo stained with *zen* mRNA at ~70 µm (D), ~7 µm (D’), and 0 µm from the anterior pole (D’’). These images are oriented with the dorsal side up. (E) *zen* expression at the posterior pole: optical sections at ~70 µm (E), ~7 µm (E’), and 0 µm from the posterior pole (E’’). (F) AP gradient of nuclear Cic in wild-type embryos. The nuclear level of Cic is significantly higher at the anterior pole, suggesting weaker MAPK enzymatic activity in this region (p < 0.001). (G) Enzymatic activity of MAPK can also be assessed by quantifying the spatial pattern of ectopically expressed Yan. Similar to Cic, Yan downregulation by MAPK is significantly weaker in the anterior region (p < 0.001). (H–K) *zen* mRNA in a wild-type embryo (H), *trk* embryo (I), *cic* embryo (J), and *trk* *cic* double mutant embryo (K). *zen* transcripts are detected using alkaline phosphatase staining.

As a first step in evaluating this model, we asked whether Cic downregulation by Torso signaling contributes to *zen* expression at the poles of the embryo. Previous studies [3, 5] suggested that Cic is involved in Dl-dependent ventral repression of *zen* (Figure 5.1H-J). To test whether downregulation of Cic is also involved in Torso-dependent control of *zen* at the poles, we examined *zen* expression in embryos derived from *trunk* (*trk*) and *cic* double mutant females. *trk* encodes a Torso ligand, which is required for MAPK activation at the poles [16] as well as the posterior expression of *zen* (Figure 5.1I). If the latter effect is due to the lack of Cic downregulation, then posterior expression of *zen* should be restored in embryos that lack both Trk and Cic. Consistent with this prediction, we found that *zen* is significantly de-repressed in embryos derived from *trk* *cic* double mutant flies (Figure 5.1K).
Taken together with the results of previous studies, our data support a model where Cic downregulation is necessary for *zen* expression at the poles. Based on the previously characterized AP asymmetry of Cic downregulation (Figure 5.1F), we propose that higher levels of Cic at the anterior pole are responsible, at least in part, for reduced expression of *zen* in this region. We found that both of these asymmetries are seen in other *Drosophilids* (Figure 5.2), suggesting that the proposed mechanism is conserved and might be functionally significant.

**Figure 5.2:** Spatial patterns of the terminal MAPK signaling and *zen* mRNA are conserved in different *Drosophila* species. (Left) Pattern of Cic protein in *D. willistoni* (A), *D. yakuba* (B), *D. melanogaster* (C), *D. borealis* (D), and *D. virilis* (E). (middle) Quantified AP profile of nuclear Cic gradients in five *Drosophila* species. Note that in all five species, nuclear levels of Cic are higher at the anterior pole, suggesting weaker MAPK signaling activity in this region. (Right) AP asymmetric spatial patterns of *zen* are also conserved in different fly species.
5.2.2 **Mathematical model describes *zen* expression**

Based on the above results, we formulated a mathematical model of *zen* regulation: *zen* is activated by spatially uniform activators, such as Zelda (U), and repressed by the ventral-to-dorsal nuclear gradient of Dl. Repression of *zen* by Dl requires a cofactor (R), which is downregulated at the poles by the terminal signal (E, which models locally activated MAPK). At the anterior pole, the enzymatic activity of E towards R is inhibited by the anteriorly localized substrates of E (B, which models the combined effects of Bcd and Hb) (Figure 5.4B). In the simplest case, R can be viewed as Cic, which is clearly involved in Dl-dependent *zen* repression (Figure 5.1).

We note that our model does not rely on the assumption that Cic is the only MAPK substrate required for Dl-dependent repression of *zen* [3, 11, 17]. Thus, R can represent a collective effect of spatially uniform MAPK substrates that are involved in Dl-dependent *zen* repression. We expect that these substrates, some of which are yet to be identified, will also be subject to competitive effects of anteriorly localized MAPK substrates. This idea is supported by the following experiment, which used nuclear accumulation of ectopically expressed Yan as a Cic-independent reporter of MAPK activity. Similar to Cic, Yan is degraded in response to MAPK phosphorylation (Figure 5.3) [18, 19]. We found that the spatial pattern of Yan downregulation is also asymmetric: nuclear levels of Yan at the anterior pole were higher than at the posterior pole (Figure 5.1G). This suggests that MAPK activity towards all of its uniformly distributed substrates is lower in this region of the embryo.
Figure 5.3: Yan and Cic expression in syncytial blastoderm embryos. (A) Expression pattern of Cic in the early stage-5 embryo. (B) Expression pattern of Yan in an embryo with ectopic expression of Yan using a maternal Gal4 driver. Yan is expressed throughout the embryo and is downregulated at the poles by MAPK. (C) Expression pattern of Yan in a wild-type embryo at the same stage as panel (B). The absence of nuclear signal suggests that Yan is not active at this stage of embryogenesis.

According to our model, zen repression requires a critical value of both Dl, distributed in a ventral-to-dorsal pattern, and R, distributed in an AP pattern. To find this pattern, we used an enzyme kinetics model where anteriorly localized substrates of E competitively inhibit R downregulation at the poles. Starting from the experimentally observed shape of the nuclear Dl gradient [20, 21], the AP asymmetry of Cic downregulation [8], and the expression boundary of zen in the mid-body of the embryo [20], we derived an analytical expression that describes zen expression boundary on the surface of a spheroid that approximates the three-dimensional shape of the embryo (see Experimental Procedures for a more detailed presentation of the model).
Figure 5.4: Mathematical model of *zen* regulation by MAPK substrate competition. (A) A model for the early pattern of *zen* expression: *zen* is induced by uniform activators (U) and repressed in the ventral region of the embryo by the nuclear DI gradient (DI-R). DI-mediated repression is antagonized at the poles by MAPK. (B) Revised model: the anteriorly localized substrates of MAPK (Bcd, Hb) competitively inhibit MAPK-mediated *zen* de-repression. (C) Schematic representation of the spatial distribution of maternal factors contributing to the regulation of *zen*: Bcd+Hb (green), dpERK (cyan), and DI (blue). (D–F’) The model successfully predicts *zen* expression in mutants. The boundary of the wild-type pattern is indicated by a solid red line. *zen* in wild-type embryos (D), embryos with no DI (E), and no terminal signaling (F). (G and H) The model also predicts ectopic expression pattern of *zen* in the absence of anterior substrates of MAPK (G) and posterior expression in embryos with uniform nuclear of DI (H).
Remarkably, this simple model can account for changes of *zen* expression in response to multiple perturbations of maternal patterning systems (Figure 5.4D-H). For instance, removal of anterior MAPK substrates leads to ectopic *zen* expression at the anterior pole (Figure 5.4G), making the pattern symmetric along the AP axis. This model also successfully predicts the persistence of *zen* expression at the posterior pole of embryos with uniform nuclear Dl (Figure 5.4H), in agreement with *zen* expression in embryos derived from *cactus* mutant females [22]. Thus, this model parsimoniously explains how *zen* expression changes in multiple mutant backgrounds. Finally, this model makes a number of predictions, which motivated our experiments presented below.

5.2.3 Experimental tests of model predictions

A substrate competition model for *zen* regulation predicts that *zen* expression should become symmetric in embryos that lack anteriorly localized MAPK substrates (Figure 5.4G). To test this prediction, we examined *zen* expression in embryos that lack both Bcd and maternally contributed Hb, which is also distributed in an AP pattern and is phosphorylated by MAPK. In these embryos, both MAPK phosphorylation and signaling activity, reported by Cic downregulation, are essentially symmetric (Figure 5.5C and 5.5D). Furthermore, a target gene of MAPK signaling, *tailless* (*tll*), is expressed as two symmetric caps at the poles (Figure 5.5F). Thus, MAPK signaling and its transcriptional effects are indeed symmetric in these embryos. Importantly, *zen* expression also becomes symmetric (Figure 5.6).
Figure 5.5: Symmetric MAPK signaling in the absence of Bed and Hb. (A and B) Quantified gradient of MAPK phosphorylation (A) and Cic downregulation (B) in wild-type embryos. (C and D) Quantified gradients of MAPK phosphorylation (C) and Cic downregulation (D) in embryos derived from bcd hb nos germline clone flies. The anterior and posterior levels of both MAPK phosphorylation and nuclear Cic are nearly symmetric (p > 0.1). (E and F) Expression of tll in wild-type (E) and bcd hb nos (F) embryos.
Figure 5.6: Anterior repression of *zen* requires Bcd and Hb. (A and B) Dl protein and *zen* mRNA in a *bcd hb nos* embryo. In the absence of Bcd, Hb, and Nos, *zen* is expressed throughout the anterior pole (arrow). (C and D) Optical images of *zen* at the anterior (C) and posterior (D) poles. (E and F) Dl protein and *zen* mRNA in a wild-type embryo. (G and H) End-on images of *zen* mRNA at the anterior (G) and posterior (H).

These observations are consistent with the notion that the AP asymmetry of the wild-type *zen* expression pattern is generated by anteriorly localized MAPK substrates. Furthermore, these observations suggest that Bcd and Hb constitute a significant fraction of all MAPK substrates at the anterior pole. Moreover, loss of *bcd* alone increases *zen* expression at the anterior pole (Figure 5.8E-H). Thus, removal of a single binding partner of MAPK can generate a significant downstream effect in a larger patterning network.

Another prediction of our model is that the inhibitory effects of Bcd on MAPK signaling and *zen* expression do not depend on its transcriptional activity. To test this prediction, we used Bcd-A<sub>o</sub>, a (K50L) form of Bcd that does not bind to Bcd’s DNA recognition sequences and thus, cannot activate endogenous transcriptional targets of Bcd.
We found that expression of two copies of this construct, driven by Bcd endogenous promoter in the bcd homozygous embryos (Bcd-A9;bcd) does not activate several bcd targets (Figure 5.7E-H), yet does rescue both MAPK phosphorylation and Cic downregulation gradients (Figure 5.7A-D). Importantly, zen expression is greatly reduced at the anterior pole in these embryos (Figure 5.8A-D). Thus, transcriptionally inert Bcd can inhibit MAPK-dependent downregulation of Cic and this effect propagates to the DI-dependent gene expression.

Another argument supporting our model of gene regulation by MAPK substrate competition is based on the analysis of the expression of hb, which is transcriptionally regulated by both anterior and terminal systems. In embryos derived from bcd homozygous flies, hb is expressed at the poles, reflecting its activation by the terminal system [24]. However, the anterior domain of this pattern is eliminated by expressing two copies of Bcd-A9 (Figure 5.7F). This suggests that transcriptionally inert Bcd weakens terminal signaling at the anterior region, leading to a drastic reduction of the anterior hb expression. This conclusion is further strengthened by the fact that, in the same background, the anterior expression of hkb and tll, both of which depend on MAPK signaling, is also attenuated, even when compared to their expression in embryos derived from bcd homozygous females (Figure 5.7H; data not shown).
Figure 5.7: Characterization of Bcd-A₉. (A and B) Expression of two copies of Bcd-A₉ construct driven by Bcd promoter in the bcd null embryos (Bcd-A₉;bcd) results in MAPK phosphorylation (A) and nuclear Cic (B) gradients that are indistinguishable from those in wild-type embryos. (C and D) Quantified gradients of MAPK phosphorylation (C) and Cic downregulation (D) in bcd null embryos. (E–H) Expression of otd (E), hb (F), kni (G), and hkb (H) in embryos derived from bcd homozygous (first column), bcd heterozygous (second column), wild-type (third column), and bcd homozygous with Bcd-A₉ (fourth column) flies. Anterior expression of these genes is significantly reduced in Bcd-A₉;bcd embryos.
Figure 5.8: Anterior repression of *zen* does not require transcriptional activity of Bcd. (A and B) Nuclear Dl and *zen* mRNA in a *Bcd-A9;bcd* embryo: similar to wild-type embryos, *zen* is repressed at the anterior pole. (C and D) *zen* expressions at the anterior (C) and posterior (D) poles also show that *zen* is not expressed at the anterior pole in this mutant background. (E and F) Nuclear Dl and *zen* mRNA in a *bcd* null embryo. (G and H) *zen* expression at the anterior (G) and posterior (H) poles of embryos from *bcd* null flies.

Taken together with the wild-type pattern of *zen* expression and its changes in mutant backgrounds, our results support a non-transcriptional mechanism, according to which the anterior repression of *zen* depends on competitive inhibition of Cic downregulation by anteriorly localized MAPK substrates. While our proof of this mechanism is not exhaustive, we cannot formulate a viable alternative that would have a similar explanatory and predictive power. For instance, we could rule out the alternative models that rely on the AP asymmetry of the Dl gradient or on the more indirect, transcriptional, effect of the terminal system (Figure 5.9).
Figure 5.9: Alternative mechanisms for the anterior repression of zen. (A-C) Spatial pattern of zen mRNA and Hkb protein in a wild-type embryo. The two expression patterns partially overlap in the dorsal-anterior region (C), suggesting that Hkb is unlikely to be the anterior specific repressor of zen. (D-F) Expression pattern of zen mRNA and Hkb protein in embryos that lack Hkb. Even in the absence of Hkb, zen is still repressed at the anterior pole. (G) Surface image of an embryo stained with anti-Dl antibody. The two dashed lines represent the approximate locations at which we obtained the optical sections to compare anterior and posterior nuclear Dl gradients. (H) Quantified ventral-to-dorsal nuclear gradient of Dl protein at the poles. Note that the two gradients are indistinguishable from each other, suggesting that differences in the nuclear Dl gradient cannot account for the asymmetry in zen expression pattern.
Patterning of the anterior region of the *Drosophila* embryo depends on the concentration gradient of Bcd, the nuclear localization gradient of Dl, and the phosphorylation gradient of MAPK [25-28]. Previous studies revealed several pairwise interactions between these signals [7, 14, 29]. In particular, Bcd and Dl synergistically regulate a large number of genes by binding to common *cis* regulatory regions of those genes [30]. Similarly, the concentration gradient of Cic, established by MAPK signaling, controls the posterior expression borders of genes activated by Bcd [31].

We discovered an additional, ternary interaction of the AP, DV, and terminal systems. This mechanism cannot be reduced to a particular regulatory region on DNA or to a single protein. Instead, it is based on competitive effects in a network formed by MAPK and its substrates. Based on the asymmetry of the wild-type pattern of zen mRNA, we propose that the anteriorly localized substrates of MAPK act as competitive inhibitors in the process of MAPK-dependent gene de-repression. The same mechanism can regulate other genes that are repressed by the Dl gradient. For example, *decapentaplegic (dpp)*, another gene expressed in the dorsal region, is regulated by the joint action of the DV and terminal systems [32, 33]. Similar to zen, the expression of dpp is weaker at the anterior pole than at the posterior (data not shown).

At this point, the main evidence supporting the notion that MAPK substrate competition controls gene expression in the embryo is provided by experiments that perturb the expression levels of MAPK substrates. In the future, we plan to complement these experiments by perturbations of the docking domains involved in MAPK interactions with its binding partners. For instance, according to our model, the spatial pattern of zen should
become symmetric in embryos in which the wild-type Bcd protein is replaced by a Bcd variant that lacks MAPK docking domain. To test this prediction, we plan to identify the docking domains involved in MAPK/Bcd and other MAPK-dependent interactions in the early embryo. Identification of these domains should shed light on the functional significance of MAPK substrate competition in the early embryo and other stages of development.

Competitive effects are likely to be a general feature of biomolecular networks, which are commonly organized around hubs, regulators that can interact with a large number of targets. For instance, in post-transcriptional regulation by microRNAs, different targets of a given microRNA can compete for their common regulator. Indeed, a number of recent studies suggest that mRNA competition in microRNA networks can lead to indirect inhibitory interactions between transcripts [34]. As a consequence, a given mRNA can control translation of other transcripts by regulating activity of their common microRNAs, in a way that is independent of its protein-coding function [35]. Exploring network-level consequences of such effects requires a quantitative approach, similar to the one used in our study of MAPK signaling in Drosophila embryo.

5.4 Experimental procedures

**Drosophila Stocks.** The following stocks were used in this study: OreR, trk\(^1\), gd\(^7\), bcd\(^6\), cic\(^1\), cic\(^{D49}\), Histone-GFP, UAS-Yan, mata4-Gal4-VP16, Bcd-A\(^9\), and hkb\(^{XM9}\). FRT82B bcd\(^6\) hb\(^{15}\) nos\(^{BN}\) and FRT82B bcd\(^6\) hb\(^{15}\) tsi\(^{4}\) flies were kindly provided by E. Weischaus. Flies were grown and embryos were collected at 25°C.
To make germline clone of bcd hb nos and bcd hb tsl, standard FLP-DFS technique was used. Males with hsFLP and FRT82B ovoD were crossed with females carrying the mutation of interest. The resulting progenies were heat shocked at 37.5°C for 2 hours at 3rd instar larva stage.

**In situ hybridization and immunohistochemistry.** Fluorescence in-situ hybridization (FISH) was performed as described elsewhere [36]. Embryos were dechorionated in 50% bleach and fixed in 8% formaldehyde in PBS for 20mins. Embryos were then incubated in 90% xylene for 1hr and then treated with 80% acetone for 10mins at -20°C. Next, embryos were hybridized overnight at 60°C with anti-sense probes labeled with digoxigenin (DIG) or fluorescein (FITC). Embryos with labeled probes were visualized using standard immunofluorescence technique. The following primary antibodies were used in this study: sheep anti-DIG (Roche, 1:200), mouse anti-dpERK (Sigma, 1:100), mouse anti-Dorsal (DSHB, 1:100), rabbit anti-Cic (1:2000), mouse anti-Yan (DSHB, 1:100), rat anti-Hkb [37], and rabbit anti-FITC (Invitrogen, 1:200). The Alexa Flour conjugated secondary antibodies were from Invitrogen (1:500). DAPI (1:10,000, Vector Laboratories) was used to visualize nuclei.

**Anti-Capicua Antibody.** DNA near the 5’ end of the capicua gene was used to screen an ovary cDNA library [38]. One cDNA clone, K6, was full-length and matched the intron-exon boundaries of the cic-RA transcript described by FlyBase. A 1.4-kb BglII fragment from the 3’ portion of the cDNA was subcloned into the pRSETc expression vector (Invitrogen) to create plasmid RCB1.4. The cloned fragment contains coding DNA from K6.
(cic-RA) between the genomic identifiers 3R AE014297 16122211....16125976, including 10 nucleotides of exon five, a small portion of exon six, all of exons 7—11, and part of exon 12. Importantly, this region does not include the SOX DNA-binding domain. The expressed peptide was purified using the 6XHis tag and sent for injection into rabbits to R&R Rabbitry (Stanwood, WA). The specificity of the antibody was tested by antibody stainings of the egg chambers, where the spatial pattern of Cic expression is known [5]. Immuno-histochemistry of Drosophila egg chambers using anti-Cic sera revealed nuclear stain that was absent in clones lacking the cic gene (data not shown).

**Microscopy and image processing.** Imaging was done on a Zeiss LSM510 confocal microscope, with a Zeiss C-Apo 20x objective (NA=0.6). High resolution images (512x512 pixels, 12bits depth) were obtained from a focal plane in the mid-horizontal cross section of the embryo. Images of individual embryos were automatically extracted from raw confocal files and re-oriented as described elsewhere [1]. For end-on imaging, embryos were oriented using microfluidic device described in Appendix A [20]. Zeiss LSM510 confocal microscope with a C-Apo 40x water immersion objective (NA=1.2) was used for end-on imaging.

**Gradient quantification and statistical analysis.** Quantification of spatial gradients of a protein of interest (dpERK, Cic, or Yan) was performed as described previously [1]. Confocal images of embryos stained with antibodies detecting the protein of interest were analyzed using custom-made Matlab image processing program. A Student’s t-test was used to determine the statistical significance in the asymmetry of Cic and Yan gradients. Briefly,
a quantified gradient from a single embryo was first separated into anterior and posterior regions; these two regions were then independently approximated by an inverse Gaussian (constant minus a Gaussian). The minima of the two fitted curves provide the anterior and posterior levels of Cic (or Yan) in this embryo. This process was repeated for gradients extracted from 20-30 of embryos. A t-test was used to test whether the mean of this sample is significantly different from 1.

Mathematical model of zen regulation. We explain our model using a rectangular embryo, where the x and y directions represent the AP and DV axes of the actual embryo. We assume that the AP and terminal signals are uniform along the DV axis. The rectangular case is presented here for simplicity because it contains all of the essential features of our model and leads to a simple algebraic expression that describes the boundary of zen expression. The same model applies for an embryo shaped as a prolate spheroid and was used to generate images in Figure 5.4.

Let R denote a factor, such as Cic, that acts together with Dl to repress zen. The joint repressive action of Dl and R can be modeled by the product of Dl and R concentrations, Dl*R. In the model, zen is expressed when this product is below some threshold, denoted by Θ. Thus, the level of zen expression, denoted by Z, is given by $Z(x, y) = H(\Theta - Dl(y) \times R(x))$, where $H(u)$ is the Heaviside step function: $H(u) = 1$, when $u > 0$ and zero otherwise. The boundary of zen expression, denoted by $y_z(x)$ is then given by the implicit function: $\Theta = Dl(y) \times R(x)$. This equation can be related to the experimentally observed profiles of the Bcd, Dl, and Cic gradients.
We assume that repressor $R$ is produced at a constant rate, denoted by $S_R$, and degraded via two parallel channels: constitutive degradation throughout the embryo and faster degradation that depends on the terminal signal. A steady state model for repressor level then becomes $S_R = k_c R + V(R)$, where $k_c$ is the rate constant of constitutive degradation, and $V(R)$ is the rate law for the enzymatic degradation that depends on the terminal signal. We assume that the spatial pattern of active enzyme, denoted by $E(x)$ (phosphorylated MAPK) is uniform along the DV axis. The spatial pattern of enzyme distribution $E(x)$ is given by the product of the amplitude $E_0$ and a symmetric function $f_E(x)$ that is equal to one at the poles and close to zero in the middle of embryo.

We assume that degradation of $R$ follows Michaelis-Menten kinetics, with constants $k_{cat}$ and $K_M$. We also assume that Bcd competitively inhibits MAPK-dependent repressor degradation. This leads to the following expression for the rate of signal-induced repressor degradation: $V(R) = \frac{k_{cat} E(x) R(x)}{(K_M + R(x) + K_M B(x) / K_I)}$, where $K_I$ is the equilibrium constant of enzyme-Bcd interaction.

To simplify the algebra, we assume that the enzyme is not saturated by $R$. Under this assumption, $V(R) = \frac{k_{cat} E(x) R}{K_M (1 + B(x) / K_I)}$. Substituting this into the mass balance for repressor levels we get the following expression for the spatial pattern of $R$:

$$R(x) = \frac{S / k_c}{\left(1 + \alpha f_E(x) / (1 + f_B(x) \beta)\right)},$$

where $\alpha$ and $\beta$ are defined as: $\alpha = k_{cat} E_0 / k_c K_M$, $\beta = B_0 / K_I$. These parameters can be estimated from the wild-type pattern of Cic downregulation: At the posterior pole, where $f_E(x) = 1$, but the concentration of anteriorly localized Bcd is zero ($f_B(x) = 0$), Cic is
downregulated to 10% of its level in the mid-body of the embryo, where \( f_E(x) = 0 \). From this result, we find that \( \alpha \approx 9 \). At the same time, at the anterior of the embryo, where \( f_B(u) = 1 \), Cic is downregulated only to \( \sim 50\% \) of its mid-body level. Based on this result, and on the estimate for \( \alpha \), we get that \( \beta \approx 4 \). Combining these estimates with the shape of the Bcd gradient and the shape of the terminal signal, we can predict how \( R \) is distributed throughout the AP axis of the embryo.

The equation for the boundary of \( zen \) expression then takes the following form:

\[
D_0 f_D(y) \times \frac{S/k_c}{(1 + \alpha f_E(x)/(1 + f_B(x)\beta))} = \Theta
\]

where \( D_0 \) is the amplitude of the nuclear Dl gradient and \( f_D(y) \) is the shape that characterizes its distribution along the embryo.

Introducing one more dimensionless group \( \gamma = \Theta k_c / (S \times D_0) \), we get the following equation for the \( zen \) expression boundary:

\[
\frac{f_D(y)}{1 + \alpha f_E(x)/(1 + \beta f_B(x))} = \gamma
\]

The remaining parameter of the model, \( \gamma \), can be estimated from the location of the \( zen \) boundary in the mid-body region of the embryo, where \( f_E(x) \approx 0 \), which implies that \( \gamma = f_D(y_{Z,m}) \), where \( y_{Z,m} \) denotes the position of the \( zen \) boundary at the mid-body region of the embryo. Based on our previous imaging results [20, 21], we estimate that \( \gamma \approx 0.1 \).

Putting everything together we get the following equation for the expression boundary of the wild-type pattern:

\[
\frac{f_D(y)}{(1 + 9f_E(x)/(1 + 4f_B(x)))} = 0.1
\]
Note that the values of the three dimensionless groups in the model were obtained from the asymmetries of the wild-type pattern of Cic downregulation, the location of the *zen* expression boundary in the mid-body region of the embryo, and the spatial distribution of the nuclear Dl gradient in the mid-body region.

We can now combine the values of \( \alpha, \beta, \) and \( \gamma \) with the empirically determined distributions for the patterning signals, \( f_D(y), f_E(x), \) and \( f_B(x), \) to plot the wild-type pattern of *zen* expression. This model predicts how the *zen* expression boundary “bends” in response to variations in the levels of anterior, terminal, and dorsoventral signals. For example, removing Bcd makes MAPK more available for \( R, \) lowering a co-repressor that acts together with Dl in *zen* repression, and results in ectopic *zen* at the anterior pole (Figure 5.4G).

### 5.5 References


Chapter 6

A model for the regulation of *tailless* by three maternal signals in the early embryo

This chapter is based on work done with Kate M. Fitzgerald, Gerardo Jiménez, Dmitri Papatsenko, and Stanislav Y. Shvartsman. We are indebted to E. Wieschaus, O. Grimm, and Z. Paroush for flies and reagents. We are grateful for the members of Shvartsman lab for their discussions and comments on the work.

6.1 Introduction

The execution of complex biological functions requires interpretation of a collection of signals. Known as crosstalk between signaling pathways, this feature is often a characteristic and a necessary signature of biological systems. For patterning events during development, gene regulation, and metabolic/enzymatic pathways, the integration of multiple signals through crosstalk is required for complex multidimensional patterns, cell differentiation, and protein regulations [1-4].

Patterning of the *Drosophila* embryo is initiated by four maternal signals and requires crosstalk between these inductive cues [5, 6]. Traditional view of pattern formation by these systems is essentially one-dimensional: The anterior, posterior, and terminal systems initiate the formation of bands and stripes of gene expression along the anteroposterior (AP) axis. Independently, the orthogonal nuclear localization gradient of Dorsal (Dl) controls a different set of genes along the dorsoventral (DV) axis. However, genes that exhibit a combination of
AP and DV polarities cannot be explained by just one system and require interactions of multiple spatial signals.

The anterior expression of terminal gap gene *tailless (tll)* is an example of a complex 2-dimensional spatial pattern that requires interaction of multiple signaling systems [7-11]. *tll* encodes a transcription factor with high similarities to steroid hormone receptors [8]. Tll activity is required to pattern the acron, defined as a future brain and a part of head skeleton, and telson, the last segment of the abdomen [8]. *tll* transcript is first detected in the syncytial blastoderm embryo during nuclear cycle 11 [9]. Initially, *tll* is expressed as cap-like domains at the poles, reflecting its de-repression by MAPK signaling in these regions. However, in nuclear cycle 14, anterior expression of *tll* undergoes a dramatic change that it is repressed at the anterior-most and at the anterior-ventral regions, resulting in a horse-shoe like expression pattern in the dorsal region of the embryo (Figure 6.1A-C) [9].

Previous studies have proposed a model for the regulation of *tll* by three maternal signals [7, 9]. According to this model, expression of anterior *tll* requires both de-repression by MAPK and direct activation by Bicoid (Bcd), anterior morphogen that patterns the head and thorax of the embryo (Figure 6.1D, and 6.1E) [12-14]. In the ventral side of the embryo, *tll* expression is repressed by Dl, a Rel-like protein that is localized in the nucleus in the ventral half of the embryo and subdivides the embryo into three germ layers [15-17]. The regions exposed to high, medium, and low levels of Dl contribute to the formation of the muscle tissue, nervous system, and skin of the embryo, respectively. Indeed, it has been shown that *tll* is expressed ventrally in the absence of nuclear Dl and reciprocally, its expression is abolished in embryos with uniform nuclear Dl (Figure 6.1F and 6.1G). However, previous genetic experiments suggested that this Dl-mediated repression also
requires Bcd because it is not observed in the posterior region of wild-type embryos (where there is no Bcd) and even in the anterior region of the embryos that lack Bcd [9] (Figure 6.1B and 6.1C).

Figure 6.1: *tll* expression pattern requires interaction of three maternal signals. (A-C) *tll* expression a wild-type embryo in nuclear cycle 14 (A). Image is oriented with anterior left and dorsal side up. (B, C) Optical images of *tll* at the anterior (B) and posterior (C) poles. Embryos are oriented with dorsal side up. (D-G) *tll* mRNA pattern in *bcd* mutant embryo (D), *trunk* (*trk*) mutant embryo that lacks terminal signaling, *gastrulation defective* (*gd*) mutant embryo that lacks Dl signaling, and *toll* gain-of-function mutant embryo with uniform nuclear Dl (G).

The above model, however, is still a phenomelogical model that only provides the minimal signaling systems in the regulation of *tll*. Consequently, it fails to provide mechanistic basis of the gene regulation and identify the key molecular players. For instance, based on the model, it is not clear how Bcd is able to both repress and activate *tll*, given that Bcd mainly functions as a transcriptional activator. Here, we use a combination of genetic
experiments and modeling approaches to develop a mechanistic model of the regulation of the anterior \textit{tll}. We propose that \textit{tll} is regulated through two incoherent feed-forward loops where Bcd activates both \textit{tll} and its repressor at the anterior-most region of the embryo. Our subsequent experiments suggest that Huckebein (Hkb) as an anterior repressor of \textit{tll}, and its ventral repression is mediated by both DI and Knirps (Kni). Our results provide a mechanistic regulatory model for the anterior \textit{tll} and suggest a mode of interaction of Bcd, MAPK, and DI that might be commonly used in patterning the anterior region of the embryo.

6.2 Results

6.2.1 Anterior repression of \textit{tll} requires both Bcd and MAK signaling

Previous genetic and biochemical studies of \textit{tll} regulation showed that repression of \textit{tll} at the anterior-most region requires both high levels of Bcd and strong MAPK signaling [9]. Indeed, in the absence of either one of the two signaling systems, \textit{tll} is expressed at the anterior-most region of the embryo (Figure 6.1D and 6.1E). To further analyze the necessary conditions for the anterior repression, we examined the effect of perturbing the levels of Bcd or strength MAPK signaling on the spatial extent of the repression. Consistent with the previous studies, our results show that the anterior boundary of \textit{tll} shifts posteriorly in response to increased level of Bcd (Figure 6.2A).

We found that this boundary also moves posteriorly in response to decreased level of Capicua (Cic), a transcriptional repressor that is downregulated by MAPK at the poles of the embryo [18-20] (Figure 2B). Since MAPK activates \textit{tll} by downregulating Cic, decreasing Cic level can be understood as increased strength of MAPK signaling. Interestingly, we observed that in the embryos from \textit{cic}^{l} homozygous mothers where the level of Cic is very
low, the spatial extent of the anterior repression expanded posteriorly (Figure 6.2B). Since Cic binds to the regulatory region *tll* and directly represses its transcription [18], our results suggest that in these *cic* mutant embryos, the repressive activity of Bcd is enhanced such that it can overcome the decreased level of Cic.

Figure 6.2: The effect of Bcd and MAPK signaling on the anterior repression of *tll*. (A) *tll* mRNA patterns in embryos with different levels of Bcd (left) and the quantified boundaries of the anterior *tll* (right). Both anterior and posterior boundaries shift posteriorly with increased level of Bcd. (B) *tll* pattern in embryos with different levels of Cic (left) and the quantified boundaries (right). Both anterior and posterior boundaries shift anteriorly with increased level of Cic.

One mode of interaction between Bcd and MAPK signaling, which acts through Cic, occurs on DNA as many of Bcd target genes contain Cic binding sites in their regulatory regions [18, 21]. Indeed, posterior boundaries of these genes shift posteriorly in embryos that lack Cic [21]. Thus, the above results can be explained if Bcd does not directly repress *tll*, but acts through a repressor that are controlled together by Bcd and Cic.

6.2.2 Hkb is a candidate anterior repressor of *tll*

In search for the anterior repressor of *tll*, we noticed that the posterior boundary of a terminal gap gene *huckebein* (*hkb*) matches closely with the anterior boundary of *tll* in wild-type embryos (Figure 6.3D-F). *hkb* is expressed as cap-like domains at both poles, reflecting its de-repression by MAPK signaling (Figure 6.3A and 6.3B). Previous studies have shown that *hkb cis*-regulatory elements contain multiple Cic binding sites, and removal of Cic at the
poles is essential for expression of *hkb* [18, 19]. In the anterior region, *hkb* is also directly activated by Bcd and Dl [22, 23], suggested by the residual *hkb* expression in the anterior-ventral region in the absence of MAPK signaling (Figure 6.3C). Importantly, this expression pattern closely matches the anterior-ventral repression of *tll* in the same background (Figure 6.1E and 6.3C).

**Figure 6.3: hkb expression complements tll mRNA pattern in multiple genetic backgrounds.** (A-C) *hkb* mRNA expression pattern in a wild type embryo (A), *bcd* mutant embryo (B), and *trk* mutant embryo (C). (D-F) Quantification of the boundary of *hkb* reveals that *hkb* expression complements *tll* pattern in wild type embryos. (G) The boundaries of two transcripts are within experimental variation in multiple genetic backgrounds. Each bar indicates mean location of boundaries of *tll* (gray) and *hkb* (black) of greater than 50 embryos for each background. Error bars represent standard deviation.
As a step toward testing whether hkb encodes a repressor of tll, we used in-situ hybridization and quantitatively compared the posterior boundary of hkb to that of the anterior boundary of tll in wild type embryos. We found that the two boundaries are within the experimental variations of ±1.5 percent embryo length (Figure 6.3F). This is consistent with the idea that Hkb is a repressor of tll. We further compared the two boundaries in multiple genetic backgrounds with perturbed Bcd, MAPK, or Dl signaling. We analyzed total of 21 genetic backgrounds and found that in 16 of them, the spatial extent of hkb complements the anterior repression of tll (Figure 6.3G). Interestingly, the five mutants where the two genes are expressed in overlapping domains can be categorized into either embryos that lack Bcd or Groucho (Gro), a global co-repressor whose activity is downregulated through phosphorylation by MAPK [24]. The latter result can be understood by the fact that Hkb requires Gro to repress tll. This is similar to Hkb mediated repression of other genes such as hunchback and snail [25, 26].

We further examined the possibility of Hkb as a repressor of tll by using fluorescent in-situ hybridization to visualize Hkb protein with tll transcript simultaneously in a single embryo. If Hkb is a transcriptional repressor of tll, then the Hkb protein domain, not its mRNA pattern, should complement the tll expression pattern. We found that in the dorsal side of wild-type embryos, Hkb protein expression nearly perfectly matches the domain of the tll anterior repression (Figure 6.4A and 6.4B). In addition, in the absence of nuclear Dl, Hkb protein expression complements the tll pattern throughout the DV axis (data not shown).

Lastly, we examined tll pattern in the absence of functional Hkb as a more direct test of our hypothesis. We observed that anterior tll expression expands anteriorly, but it is still repressed in the ventral and the anterior-most region (Figure 6.4D). This result supports the
notion that Hkb can repress \textit{tll} in the anterior region, but at the same time, also suggests that Hkb is not solely responsible for the anterior repression of the transcript.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure6_4.png}
\caption{Hkb as a candidate repressor of \textit{tll}. (A, B) Simultaneous detection of Hkb protein and \textit{tll} mRNA in a wild-type embryo. Cross-sectional (A) and surface (B) views both suggest that Hkb protein domain closely matches the domain of \textit{tll} repression. (C, D) \textit{tll} mRNA pattern in a wild-type embryo (C) and \textit{hkb} mutant embryo (D).}
\end{figure}

6.2.3 Ventral repression of \textit{tll} is mediated by Dl and Kni

A straightforward explanation for the lack of \textit{tll} expression at the anterior tip of \textit{hkb} mutant embryos is that \textit{tll} is directly repressed by nuclear Dl in this region. As shown previously, Dl is required for the ventral repression of anterior \textit{tll} [9]. Indeed, Dl is expressed throughout the anterior pole of the embryo [27], making it a possible repressor of \textit{tll} in this region even in the absence of Hkb (Figure 6.5A). To test whether Dl is responsible for the lack of \textit{tll} expression in \textit{hkb} mutant embryos, we generated a mutant embryo that lacks Dl signaling in addition to functional Hkb. We found that \textit{tll} pattern expands both anteriorly and ventrally to cover nearly the entire anterior pole of the embryo (Figure 6.5C).

Next, we asked whether the repression of \textit{tll} by Dl is direct or mediated by a downstream target of Dl signaling. The nuclear localization gradient of Dl initiates a complex patterning network that subdivides the DV axis into three germ layers [17, 28]. One possible candidate is \textit{kni}, which is expressed in the anterior-ventral region, reflecting its
activation by Bcd and Dl (Figure 6.5B) [29]. Thus, we asked whether Kni can repress tll in the anterior region of the embryo by examining tll pattern in embryos that lack both Kni and Hkb. We found that tll pattern resembles that of Dl and Hkb double mutant embryos; tll is expressed nearly throughout the anterior pole (Figure 6.5D). In addition, we used a recently developed microfluidics device to compare the expression pattern of kni and tll in optical sections [30]. As expected from our mutant data, kni is expressed in complementary pattern as tll, further supporting that Kni is responsible for ventral-repression of tll (Figure 6.5E-G).

However, a number of experiments suggest that the ventral repression of tll is also mediated by Dl itself. First, the Dl Hkb double mutant embryos show stronger phenotype than Kni Hkb double mutant embryos (Figure 6.5C and 6.5D). In addition, we observed that embryos in early nuclear cycle 14, at the stage where the expression of kni is low, tll mRNA clearly exhibits ventral repression (Figure 6.5H-J). Considering the delay between the appearances of kni transcript to accumulation of Kni protein, it is likely that Kni might not be responsible for the ventral repression observed at this stage. Thus, we argue that both Dl and Kni repress tll in the ventral region of the embryo.

6.2.4 A model for the regulation of anterior tll

Our results can be summarized by a model for the regulation of anterior tll (Figure 6.6A). tll is directly activated by Bcd and de-repressed by MAPK signaling which acts through phosphorylation of transcriptional repressors such as Cic and Gro. In the anterior most region, Bcd and MAPK also activate hkb, which encodes a transcriptional repressor that, in association with Gro, blocks transcription of tll. At the same time, tll is repressed in the ventral region by Dl, which also requires Gro to repress its target genes. Lastly, Dl, together with Bcd, activates kni whose encoded protein also interacts with Gro to repress tll [29, 31].
Figure 6.5: Ventral repression of *tll* is mediated by Dl and Kni. (A, B) Expression pattern of Dl protein and *kni* mRNA in wild-type embryos. (C) *tll* mRNA pattern in embryos that lack Dl signaling and functional Hkb. (D) *tll* pattern in embryos that lack functional Kni and Hkb. (E-G) Simultaneous detection of Dl protein (E), *kni* mRNA (F), and *tll* mRNA (G) in a wild-type embryo in late cell cycle 14. (H-J) Simultaneous detection of Dl protein (H), *kni* mRNA (I), and *tll* mRNA (J) in an early cell cycle 14 embryo. Note the ventral repression of *tll* despite the low level of *kni* transcript.
Figure 6.6: A model for the regulation of \textit{tll}. (A) A regulatory network of \textit{tll} by the three maternal signals. (B-D) The model successfully predicts anterior \textit{tll} pattern in mutants. Predicted \textit{tll} pattern (left) in a wild-type embryo (B), embryos with no terminal signaling (C), and embryos with no Dl signaling (D).

The inputs to the model are the spatial gradients of the three maternal signaling systems that regulate \textit{tll}: Bcd, nuclear Dl, and MAPK phosphorylation gradients. The spatial pattern of Cic and Gro can be determined using the Bcd and terminal signals as described in Chapter 5. We also used previously proposed regulatory models for \textit{hkb} and \textit{kni} to determine their spatial expression patterns [23, 29]. Specifically, we assumed that \textit{hkb} expression strongly depends on Bcd and MAPK signaling while it also can be activated by Dl [23, 32]. For \textit{kni}, we assumed its expression requires high levels of both Bcd and Dl [29]. Hkb and Kni then interact with Gro and repress \textit{tll} in the anterior-most region of the embryo.
Given the shape of Bcd, nuclear Dl, and MAPK phosphorylation gradients, this model successfully recapitulates the horse-shoe like pattern of \textit{tll} mRNA in the anterior region of the embryo (Figure 6.6B). Furthermore, the model can predict how this pattern changes in mutants with perturbed spatial pattern of the three maternal signaling systems (Figure 6C and 6D).

\section*{6.3 Discussion}

One common mode of crosstalk between multiple inductive cues occurs on the DNA where multiple transcription factors bind to common regulatory regions to control expression of their shared target genes. The anterior expression of \textit{tll} is regulated by the three spatial signals that pattern the anterior region of the embryo \cite{9}. Here, we developed a mechanistic regulatory model for \textit{tll} by Bcd, Dl, and MAPK signaling systems. Specifically, this model can be broken down into two incoherent feed-forward loops. First, Bcd, together with MAPK, activates \textit{tll} and its repressor, Hkb. Bcd also participates in a second incoherent feed-forward loop where it activates \textit{tll} and together with Dl, also activates Kni which represses \textit{tll} at the anterior-most and anterior-ventral regions.

We note that the same mechanism is used to specify spatial patterns of other genes that are expressed in similar pattern as \textit{tll}. For example, \textit{orthodenticle (otd)} is expressed as a dorsal stripe pattern in the anterior region of the wild-type embryo (Figure 6.7A). Previous genetic studies have shown that \textit{otd} is directly activated by Bcd and its repression at the anterior-most and anterior ventral regions require Dl and MAPK signaling \cite{33, 34}. Considering the similarities between \textit{otd} and \textit{tll} patterns, we tested whether \textit{otd} is regulated by the same mechanism that specifies \textit{tll} expression pattern. Thus, we examined \textit{otd} pattern
in the embryos that lack both nuclear DI and Hkb and found that *otd* is expressed throughout the anterior region of the embryo (Figure 6.7B). In addition, similar pattern was observed in embryos that lack Kni and Hkb, suggesting that like *tll*, ventral repression of *otd* is at least partially mediated by Kni (Figure 6.7C).

![Figure 6.7: Regulation of orthodenticle.](image)

**Figure 6.7: Regulation of orthodenticle.** Expression pattern of *orthodenticle* (*otd*) in a wild-type embryo (A), in an embryo with no nuclear DI and functional Hkb (B), and in an embryo with no functional Kni and Hkb.

Our results show that Hkb is one of the repressors responsible for the anterior repression of *tll*. Interestingly, Hkb only represses *tll* in the anterior region although it is present in nearly equal levels at both poles (Figure 6.3A) [35]. One possible explanation is that anterior and posterior *tll* are expressed from two different enhancers and that only the anterior enhancer contains Hkb binding sites. Consistent with this argument, recent studies identified an enhancer in *tll* locus whose reporter line recapitulates only the anterior expression of wild-type *tll* [36, 37]. Our preliminary bioinformatics analysis on this enhancer predicts putative binding sites for Hkb, DI, and Kni in addition to those for Cic and Bcd (Figure 6.8). Thus, we argue that *tll* is activated by at least two different enhancers, which allow the transcript to be expressed in two distinct patterns in two different regions of the embryo.
Figure 6.8: Analysis of the anterior enhancer of \textit{tll}. Preliminary analysis of the anterior enhancer of \textit{tll} suggests possible binding sites for Hkb, Dl, and Kni in addition to Bcd and Cic.

One outstanding question is the mechanism through which the two enhancers of \textit{tll} are activated. How does the embryo know to use only the anterior enhancer in the anterior region while silencing the posterior enhancer? We have previously shown that Bcd competitively inhibit MAPK mediated downregulation of Cic, resulting in an asymmetric Cic gradient with higher levels of nuclear Cic in the anterior region of the embryo [38, 39]. Since removal of Cic is essential for the posterior expression of \textit{tll}, one possibility is that the posterior enhancer is silenced due to higher level of nuclear Cic in the anterior region. However, this is unlikely the case because in embryos derived from \textit{cic}^l flies, \textit{tll} is still repressed at the anterior-most region, suggesting that even in the embryos with very low levels of Cic, posterior enhancer is silenced in the anterior region.

Alternatively, the two enhancers can compete for the same promoter and only the stronger one is chosen to be expressed in the anterior region of the embryo. Consistent with this idea, when we expressed \textit{lacZ} using the posterior enhancer [18], we observed weak \textit{tll} expression in the anterior region of the embryo (data not shown), suggesting that when expressed alone, posterior enhancer is not silenced in the anterior region of the embryo.
Such enhancer competition has been shown to play a key role in specifying complex gene expression patterns in the fly embryo [40]. In this context, one possibility is that high levels of nuclear Cic and high levels of Bcd ensure the anterior enhancer to “win” the competition and consequently, silence the posterior enhancer.

The role of Bcd and its effect on inhibiting MAPK mediated Cic downregulation on regulation of $tll$ require further analysis in the future. Specifically, examining the anterior $tll$ patterns in mutants expressing Bcd transgene with perturbed MAPK docking domains will provide valuable information. In these embryos, we expect that the spatial profiles of MAPK substrates such as Cic and Gro will be symmetric along the AP axis, and thus these embryos can be used to test whether the asymmetric pattern of Cic and Gro plays a role in specifying asymmetric pattern of $tll$.

We note that regulatory connections and the direct interaction between the transcription factors and the cis-regulatory modules of $tll$ still need to be verified experimentally. Furthermore, the anterior enhancer of $tll$ should be further dissected using various reporter constructs with mutated transcription factor binding sites. Nevertheless, the model proposed in this work will provide a valuable starting point in understanding a common gene regulatory mechanism used in patterning a complex tissue such as the *Drosophila* embryo.

6.4 Materials and methods

**Fly Strain.** The following fly strains were used in this study: *OreR, bcd* $^6$, *trk* $^1$, *gd* $^7$, *cic* $^1$, *Toll* $^{10b}$, *P[Bcd-GFP] (bcd* $^{dx}$), *P[Cic-HA] (cic* $^{dx}$), *hkb* $^{XM9}$, and *kni* $^9$. Flies were grown and embryos were collected at 25°C.
**in situ hybridization and immunohistochemistry.** Fluorescent *in situ* hybridization was performed as described previously [41]. Embryos were fixed in 8% formaldehyde for 20mins and treated with 90% xylene for 1hr and 80% acetone for 10mins at -20°C. Embryos were then hybridized overnight at 60°C with antisense probes labeled with digoxigenin (DIG) or Biotin (BIO). Embryos with labeled probes were visualized using standard immunofluorescence technique. The following primary antibodies were used: sheep anti-DIG (Roche; 1:200), mouse anti-BIO (Jackson ImmunoResearch; 1:200), rabbit anti-dpERK (Cell Signaling; 1:100), mouse anti-Dorsal (Developmental Studies Hybridoma Bank, 1:100), and rat anti-Hkb [35]. Alexa fluor conjugated secondary antibodies (Invitrogen; 1:500) were used to label the primary antibodies and DAPI (1:10,000; Vector Laboratories) was used to detect nuclei.

**Microscopy and Image Processing.** Imaging was done on a Zeiss LSM510 confocal microscope with Zeiss C-Apo 20x (NA=0.6) or C-Apo 40x water-immersion (NA = 1.2) objectives. High-resolution images (512x512 pixels, 12 bits depth) were obtained from a focal plane in the mid-horizontal cross section of the embryo. Images of individual embryos were automatically extracted from raw confocal files and re-oriented and processed as described elsewhere [42].

**Quantification of gene expression boundaries.** The expression boundaries of *tll* and *hkb* were determined using automated image analysis program in Matlab, which finds the boundary of the embryo and then averages staining intensity along the DV axis. This was performed for 1,000 points uniformly spaced along the AP axis, generating an AP expression
profile of the gene. The locations of the half maximum level were used as boundaries of the expression domains.

6.5 References


Chapter 7

Conclusion

7.1 Introduction

Large scale biomolecular regulatory networks are organized around highly connected nodes, or hubs [1, 2]. Hubs, by definition, can interact with a large number of targets, creating a possibility of target-target competition. By competing for access of their common hub, targets can potentially act as inhibitors of the hub. In addition, targets can also compete with the regulators of the hub and in this way, control its abundance. As a physicochemical consequence of biomolecular interactions, these effects of target competition are always present, but whether they are strong in magnitude and biologically significant is unclear.

The studies presented in this thesis demonstrated that removal of a single target can generate a strong perturbation in the overall state of the network in vivo. Using the terminal patterning of the Drosophila embryo as the experimental system, we showed that MAPK substrates can control the phosphorylation status of MAPK and its ability to distribute its catalytic activity among its multiple substrates (Chapter 3 and Chapter 4). We further demonstrated that these competitive effects are important in positioning multiple gene boundaries, thus providing biological function of the competitive effects (Chapter 5 and Chapter 6). Hence, we argue that MAPK substrate competition is an additional mechanism of spatial regulation of gene expression by coordinating the actions of multiple inductive cues.
7.2 Future directions

The experimental results presented in this dissertation clearly show that the competitive effects can be strong and functionally significant in vivo. However, it is not clear under what conditions such effects will become significant. Future research should be directed to analyze and identify the conditions under which these competitive effects may be observed and functionally significant in vivo.

To further characterize the terminal patterning system, the total number of substrates present at this developmental stage needs to be identified using bioinformatics and in vitro assays such as co-immunoprecipitation. Other important players in the competitive effects are the phosphatases. We have shown that MKP3 is present in the early embryo and can regulate MAPK in terminal patterning (Chapter 4). However, it is likely that MKP3 is not the only phosphatase acting on the terminal system. Indeed, multiple phosphatases including protein phosphates 2A, protein phosphatase 2C, and PTP-ER are reported to target fly MAPK [3]. At the same time, it is unknown whether these phosphatases are present in the early embryo at the stage when terminal system is active. The presence and the biological function of these phosphatases need to be examined.

Another key challenge in studying competitive effect is to distinguish this phenomenon from the conventional feedback. Numerous studies have shown that MAPK is regulated through negative feedback as MAPK signaling induces multiple inhibitors such as Argos, Kekkon, and Sprouty [4, 5]. Negative feedback regulation of MAPK signaling is known to play an important role in other patterning systems such as in the follicle cells of the Drosophila egg chambers [5]. However, it is currently unknown whether this regulatory mechanism is also present in the terminal system. Combination of computational modeling
and molecular genetics experiments on MAPK inhibitors and their potential interactors will be helpful in analyzing the presence and regulatory roles of the feedback in the terminal system.

Going beyond the terminal system, the presence and the biological function of competitive effects should be analyzed in other patterning systems. For example, MAPK later becomes active as two lateral stripes in the embryo and is used to specify the future nervous system [6]. Similar to that found in the terminal system, the specification of the nervous system relies on MAPK mediated phosphorylation of its multiple transcription factors. Thus far, three transcription factors have been identified to be present at this stage, which are shown to be phosphorylated by MAPK in vitro [6, 7]. Hence, there exists potential for the competitive effects to be present and functional in this system.

### 7.3 Competitive effects in other biomolecular networks

Competitive effects are not unique to MAPK signaling pathway, but are also present in other systems where hubs such as MAPK interact with their multiple targets. For example, similar effects are also observed in post-transcriptional regulatory network formed by microRNA (miRNA) and its target transcripts. Below, we briefly review the competitive effects in the miRNA regulatory network.

miRNAs belong to a family of small non-coding RNAs that induces post-transcriptional regulation of gene expression through translational repression or degradation of target transcripts (reviewed in [8]). Although miRNAs are only 21-24 nucleotides long, they comprise one of the largest gene families in higher eukaryotes [8]. miRNAs are used in a broad range of processes such as cell differentiation, proliferation, metabolism, and
apoptosis, and deregulation of miRNAs are often involved in developmental disorders and diseases such as cancer [9-11].

Similar to protein kinases, miRNAs also interact with a large number of targets in a given cellular context. Thus, similar to the MAPK substrates, targets of a given miRNA will inevitably compete with each other for access to their common regulating miRNA and this way, they can control the effective activity of the miRNA. Indeed, several recent studies showed that removal of even a single transcript can affect the state of the miRNA regulatory network in both Caenorhabditis elegans and mammalian cell cultures [12-17].

These studies suggest the two main systems level effects of a miRNA’s ability to interact with a large number of targets: 1) Different targets compete with each other and negatively regulate the miRNA activity; 2) Targets sequester the miRNA to RNA-induced silencing complex and prevent its interaction with exoribonuclease, which results in increased abundance of the miRNA by protecting it from degradation.

7.4 Concluding remarks

The competitive effects in MAPK and miRNA regulatory networks are strikingly similar. In both cases, targets can negatively control the activity of their regulator and at the same time, increase the abundance of the regulator by protecting it from either degradation (for miRNA) or dephosphorylation (for MAPK). These competitive effects are also functionally significant in fine tuning the activity of the regulator toward its targets.

Given the generality of competitive effects, we expect that target competition is an important regulatory strategy in biomolecular networks and quantitative studies will provide valuable insights toward systems level understanding of these regulatory networks.
7.5 References


Appendix A

A microfluidic array for large-scale ordering and orientation of embryos

This chapter is based on a study published with Kwanghun Chung, Jitendra S. Kanodia, Emily Gong, Stanislav Y. Shvartsman, and Hang Lu. The authors acknowledge A. Boettiger and M. Levine for the antibody to Twist, M. Zhan for technical assistance, A. Boettiger, A. Ervies, M. Levine, J. Lippincott-Schwartz, C. Rushlow, M. Serpe, and R. Steward for helpful discussions, and M. Osterfield for assistance with live imaging.

A.1 Introduction

Cell differentiation in embryos can be spatially controlled by the graded distribution of morphogens, chemical signals that act as dose-dependent regulators of gene expression. Some of the first morphogen gradients had been identified in the *Drosophila* embryo, in which dorsoventral patterning is initiated by the nuclear localization gradient of Dorsal (Dl). Dl is an NF-κB transcription factor, which subdivides the embryo into three germ layers [1-3]; the dorsoventral pattern of the embryo defines the dorsoventral pattern of the adult (Figure A.1A and A.1B). The regions exposed to high, medium, and low levels of Dl contribute to the formation of the mesoderm, the nervous system, and the skin of the embryo, respectively.

Quantitative analysis of developmental systems controlled by morphogens requires information about both the regulatory regions of genes comprising the network and the
spatial distribution of patterning signals. The dorsoventral patterning system in *Drosophila* is arguably one of the best understood systems with regard to its sequence-specific transcriptional regulation. However, information about the distribution of patterning signals is currently lacking, mainly because of technical difficulties associated with imaging the spatial distribution of proteins and transcripts along the dorsoventral axis of the embryo [4, 5]. When imaged on a regular microscope slide, embryos are oriented with their major axis parallel to the coverslip, and their dorsoventral orientation is essentially random. As only a small fraction of embryos can be used for quantitative imaging, previous analyses of signals in the dorsoventral system relied on data collected from about ten embryos [6, 7]. To enable high-throughput analysis of the dorsoventral patterning signals, we developed a microfluidic embryo-trap array, a device in which hundreds of embryos are oriented vertically in a few minutes. Such ‘end-on’ orientation allows for dorsoventral axis data to be easily collected for multiple embryos. Previously, end-on imaging has been possible only for very small numbers of embryos, which had to be individually and manually placed into an upright position [5, 6].

Here we describe the design and the physical principles of the embryo-trap array, and use it to quantify morphogen gradients in fixed embryos and to monitor nuclear divisions in live embryos. The device enables high-throughput analysis of the dorsoventral patterning system at the level of the inductive cues and their signaling and transcriptional targets in multiple genetic backgrounds. Using this device to image a large number of embryos, we resolved an outstanding issue regarding the spatial extent of the DI morphogen gradient.
A.2 Results

A.2.1 Design of the embryo-trap array

The array is a single-layer microfluidic device fabricated from poly-dimethylsiloxane (PDMS), an optically transparent elastomer widely used in biological microfluidics [8, 9]. To allow for imaging of a large number of embryos, the array needs to have traps that are densely packed, which is an engineering challenge. Conventional approaches using hydrodynamics for cell trapping typically do not achieve such high packing density [10, 11] mostly owing to the requirement to properly balance flow resistance, resulting in a relatively large space between neighboring traps. The mechanism used in our design, in contrast, does not rely on resistance change upon the occupation of traps and therefore allows for dense arraying of ~700 traps in the space of a microscope slide (Figure A.1C and A.1D).

Our design consists of a serpentine fluid-delivery manifold and an array of cross-flow channels (Figure A.1C and A.1D). The 700μm wide serpentine channel is wider than the major axis of the embryo (~500μm), allowing embryos of any orientation to move easily through it. This feature is particularly important for robust handling of non-spherical objects such as Drosophila embryos. Each cross-flow channel includes a truncated cylindrical trap where the embryo is located for imaging; the trap is connected to a narrowing channel and a long and narrow resistance channel (Figure A.1D and A.1E). When an embryo approaches an empty trap, flow through the trap guides it into the trap (Figure A.1F). The shape of the trap dictates that the embryo is in an upright position for imaging (Figure A.1G) such that each embryo on every device is oriented with its dorsoventral plane positioned horizontally. Oriented embryos, which appear round when viewed from the top, are thus arrayed on the device (Figure A.1H).
Figure A.1: Microfluidic embryo trap array (META) for high-throughput arraying of vertically-oriented *Drosophila* embryos. The dorsoventral polarity of the adult *Drosophila* (a) is specified in the early embryo (b), by the nuclear localization gradient of Dl, an NF-κB transcription factor, visualized using an anti-Dl antibody staining. Both images are oriented with anterior left and dorsal side up as shown by the two arrows on the right. The scale bar is 100 μm. (c) Left, photograph of the device; right, optical micrograph of the region in the box of the left photograph, showing array of the embryo traps. The scale bar is 500 μm. (d) Drawings and details of META design (top view). Numbers have units of μm unless otherwise stated. (e) Scanning electron micrograph image showing details of the trap structure. The scale bar is 100 μm. (f) Schematic diagrams showing the embryo trapping process: top, an embryo is guided into the cylindrical trap by the cross flow; middle, the flow around the embryo orients it vertically; bottom, the trap contracts after loading is finished and secures the embryo inside the trap. The yellow plane represents the focal plane where images are obtained. (g) Schematic drawing showing the imaging setup. Inset: a representative confocal image of an embryo stained with Dl, Twist, and phosphorylated MAPK. (h) An optical image of a section of META with embryos trapped in the packed array. The dark circular object in each trap is a successfully oriented and trapped embryo. The scale bar is 500 μm.

Figure A.2: Flow profile from numerical simulations. (a) Geometry of the channels and finite element mesh used in numerical simulations. To simplify the numerical simulations, only six columns of the serpentine channel are used, and the trap is assumed as a rectangular rod. (b) Flow profile at the vertical middle plane of the device (280 μm from the bottom of the channel).

Using a computational fluid dynamics approach, we engineered the hydrodynamic resistances of the cross-flow channels. A simplified smaller array in a three-dimensional computational model (Figure A.2) demonstrated that our design satisfied the following criteria. First, all the traps were exposed to similar flow rates (Figure A.4A). If the flow in the array has large variations in different rows or columns, the trap occupancy will be
severely compromised; optimal design, however, yields highly repeatable near-perfect occupancy, as we found experimentally (Figure A.3). Second, the bulk of the embryo suspension flows along the serpentine manifold (Figure A.4A, and A.4B). The bulk flow through the main channel efficiently sweeps out extra and improperly trapped embryos. Additionally, too low a cross-flow through the traps prevents embryos from being introduced into the traps, resulting in inefficient trapping, but too-high cross-flow causes embryos to accumulate near traps and clump together (Figure A.4B). Thus, optimal design of an array that works well with *Drosophila* embryos requires proper parameter choice, including geometry and operating pressure range.

![Figure A.3: Characterization of trapping efficiency.](image)

(a) Optical image showing trapped embryos through the entire device. (b) Trapping efficiency in each column. (c) Trapping efficiency in each row. The error bars represent standard deviation. $n = 4$ independent trials.

Another important mechanism for orientation of embryos in our device is Dean flow (with a Dean number greater than 100 throughout the device), an effect in which curvature of
the channel induces a secondary non-axial flow [12]. This hydrodynamic effect was apparent in the stream-line trace (Figure A.4C). The Dean flow and the diverging and converging flow through the cross-flow channels focus the embryos toward the traps (as opposed to embryos distributing in random locations in the bulk flow) and increases the frequency with which embryos contact the traps and are loaded into them. The presence of the secondary Dean flow at the bends of the channel not only greatly improves trap occupancy but also maximizes loading efficiency because an embryo has many opportunities to be in contact with an empty trap. Essentially every single embryo entering the device was trapped, a feature that will be very useful for studies in which one has to work with small numbers of embryos in complex genetic backgrounds. When imaging, we filled about 90% of the traps with embryos.

During loading, the entire device is under a slight positive pressure. Because PDMS is an elastomer, the pressure can expand the trap opening to facilitate loading (Figure A.1F and A.4E-J) [13]. Confocal microscopy characterization of trap behavior under different pressures (Figure A.4E and A.4H) demonstrated that at ambient condition (0 pounds per square inch (psi)), the traps had smaller openings (not enough for an embryo to be loaded or released) as compared to their size under 6 psi of positive pressure. When operating the device, we first connected the device at the outlet to a pressure-drop tube to raise the average pressure of the device to ~6 psi to open the traps. Then we introduced the embryo suspension into the device using a syringe or a pressure source (for example, compressed air).
Figure A.4: META’s operating principles. (a-d) Mechanisms of META’s high-efficiency trapping. Dummy columns are the columns at the edge of the device. (a) Volumetric flow rate in the serpentine main channel at each trap. (b) Volumetric flow rate through the cross-flow channels at each trap. (c-d) Dean flow and the converging and diverging flow along the cross-flow channels focus the embryos towards the traps. (c) Numerical model showing flow stream line in the turn. (d) Optical images showing an embryo migrates along the wall of the serpentine channel where the traps are located. The scale bar is 800 μm. (e-j) Three-dimensional (3D) reconstruction of the trap imaged by confocal microscopy. Each trap can be expanded by positive pressure while loading and contract in order to hold embryos in the vertical position while imaging. (e-g) 0 psi; (h-j), 6 psi (loading pressure). (e, h) (f, i) Single frame from the middle of the device. Dotted red circle represents dorsoventral plane of an embryo. (g, j) Confocal images showing cross-sectional profiles of the trap opening. Dotted red ellipse represents vertically oriented embryo. The scale bars are 100 μm.

Under flow conditions, embryos at the traps experience non-uniform pressure and shear by the surrounding fluid; the resulting force flips the embryo vertically, inserting it into the cylindrical trap (Figure A.1F and A.5). This is achieved entirely passively by hydrodynamics, without user intervention or control. Upon completion of loading, we reduced the injection pressure, and the trap opening contracted, securing the embryo inside in
an upright position (with dorsoventral axis parallel to the coverslip; Figure A.1E and A.1F). This lock-in feature allows the device to be disconnected from the rest of the hardware, to transport it for imaging or to store it with the embryos embedded. Operation of the device consists of two simple steps and does not require a computer, valves or other off-chip components except a pressure source, and thus non-experts can use it easily. Because the embryos have different sizes and shapes and because antibody staining can be highly variable, many embryos are needed for statistical analysis. We quantified 4,6-diamidino-2-phenylindole (DAPI) staining in many embryos in the trap array, which revealed that the variability of the supposedly uniform DAPI staining signal along the dorsoventral axis was negligible compared to morphogen gradients that we typically quantify (Figure A.6). We conclude from these data that the device did not introduce illumination bias.

**Figure A.5: Mechanism of embryo flipping into vertical orientation.** (a) Numerical simulation showing hydrodynamic force on the surface of the embryo simplified as a rectangular rod. (b) Torque generated by hydrodynamic force when an embryo is located inside of the trap with various angles.
A.2.2 Quantitative imaging of pattern formation

We used the embryo-trap array to analyze the distribution of nuclear Dl. The ventral-to-dorsal distribution of nuclear Dl is induced by localized activation of the Toll receptor on the ventral side of the embryo [14]. Before Toll activation, Dl is sequestered in the cytoplasm, in a complex with its binding partner Cactus [15]. In response to Toll signaling, Cactus is degraded, and Dl moves into the nucleus, where it binds the regulatory regions of its target genes.

One of the outstanding questions regarding dorsoventral patterning is the spatial extent of the Dl gradient [15]. More specifically, it is not clear over what part of the dorsoventral axis the Dl gradient is ‘flat’, and where it therefore cannot act as a patterning signal. This has been a matter of intense debate in recent publications [6, 7, 16, 17]. The disagreements in the literature may be traced to current methodological limitations in quantification of the Dl gradient. Although end-on imaging provides information about the
entire dorsoventral axis, it has only been possible to apply it to a few embryos until now [5, 6, 18]. Lateral imaging, in contrast, can be applied to analyze more embryos and to image more gradients, but it is limited to only a fraction of the dorsoventral axis [7, 17]. Our platform substantially increases the statistical power of end-on imaging, allowing us to investigate the spatial extent of the DI gradient.

The lowest level of nuclear DI is at the dorsal-most point of the embryo, which corresponds to the least Toll activation. If the amount of nuclear DI at an arbitrary position $x$ along the dorsoventral axis is statistically indistinguishable from the nuclear DI level at the dorsal side of the embryo, then the DI gradient can be considered ‘flat’ between the position $x$ and the dorsal-most position. We compared the distribution of nuclear DI along the dorsoventral axis to the nuclear DI levels at the dorsal-most point of the embryo. In each of over ten independent experiments, we collected at least 50 DI gradients 70$\mu$m from the poles of embryos during the last nuclear division cycle before cellularization (Figure A.6A-E). For each of these datasets, we used a pairwise statistical test to find the dorsoventral position at which the mean of the nuclear DI level became indistinguishable from the value at the dorsal side of the embryo; we considered the differences significant for $p<0.01$.

Based on this analysis, we estimate that the spatial range of the DI gradient reaches ~60% of the dorsoventral axis. Thus, any gene expression boundary located outside of this range cannot be explained by a model based on the direct control side of the embryo. As an example, we consider the regulation of zerknült (zen), a transcription factor expressed on the dorsal side of the embryo [19]. The gene encoding zen is expressed in a dynamic pattern that first covers the dorsal half of the embryo (Figure A.7F-H). The expression boundary is well within the estimated range of the DI gradient, consistent with previous studies suggesting that
DL represses *zen*. At a later time point, *zen* expression boundary moves to ~90% of the dorsoventral axis (Figure A.7F and A.7G), outside the estimated range of the DL gradient, which suggests a more complex mode of regulation. Indeed, previous studies revealed that the later phase of *zen* expression depends on DL only indirectly (summarized in Figure A.6H) [20].

Quantitative analysis of dorsoventral patterning requires systematic analysis of multiple transcriptional and signaling targets of DL, in both wild-type and mutant backgrounds. The embryo-trap array can be readily used to statistically compare spatial patterns across multiple genetic backgrounds [21]. For instance, DL gradients in wild-type embryos and embryos derived from mothers with only a single copy of the *dl* gene (Figure A.7I-K) show that the nuclear DL levels in the latter are reduced throughout the dorsoventral axis. Note that the nuclear DL levels in these mutant embryos were only 80% of that in wild-type embryos (Figure A.7K).
Figure A.7: Using META to quantify the spatial extent of the Dl gradient. (a) Images of Dl and nuclei in a vertically oriented embryo. (b-e) Average gradients of nuclear Dl from four representative experiments. The gradients are shown with standard errors of the mean along the dorsoventral axis. The number of embryos analyzed in each experiment is indicated. The arrows denote the dorsoventral position beyond which the spatial pattern of nuclear Dl can be considered flat. (f-g) Early (f) and late (g) spatial patterns of Dl and zen expression. (h) Schematics of regulatory models that can be used to account for the two phases of zen expression. Schematic for early expression pattern of zen is shown on top. (i, j) Nuclear Dl gradients from the wild type (i) and embryos from dl heterozygous females (j). (k) Average gradients for both genetic backgrounds shown with standard error of mean.
We analyzed the distribution of other regulators of dorsoventral patterning. This system is dominated by feedforward loops, a network motif in which a gene is controlled both by the primary input, such as DI, and by one of its more proximal targets [22] (summarized in Figure A.8A). For instance, Snail (Sna), a transcription factor expressed in the future mesoderm, is activated both by DI and by Twist (Twi), a transcription factor that is directly activated by DI. Patterning of the neurogenic ectoderm requires a two-peaked pattern of signaling through the mitogen-activated protein kinase (MAPK) cascade. This in turn reflects localized expression of components of the epidermal growth factor receptor pathway, which are activated by DI and Twi and repressed by Sna. Finally, the dorsal ectoderm is patterned by the gradient of signaling through the bone morphogenetic protein (BMP) pathway, which is spatially regulated by DI and its multiple targets.

We used the embryo-trap array to characterize Twi expression gradients as well as gradients of MAPK and BMP signaling (Figures A.8B-G). Twi and BMP signaling gradients we observed were consistent with the ones reported previously [6, 7, 17, 23], but we quantified the gradient of phosphorylated MAPK (dpERK) here to our knowledge for the first time. We observed MAPK phosphorylation at the ventral-most region of the embryo (Figure A.8G). Furthermore, we found that Cic, a transcriptional repressor that is degraded as a consequence of its phosphorylation by MAPK [24], is substantially downregulated at the ventral side of the embryo (data not shown), supporting the notion that the ventrally activated MAPK contributes to dorsoventral patterning.

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Figure A.8: Signal transduction in dorsoventral patterning and morphogen gradients quantitatively characterized using META. (a) A simplified view of the dorsoventral patterning network, showing the feedforward loops activated by DI. (b-d) Confocal images of embryos stained with antibodies that recognize DI and Twi (b), DI and phospho-MAPK (dpERK) (c), and DI and phospho-MAD (pMAD) (d). The scale bar represents 25 μm. (e-g) Averaged gradients of pMAD (e), Twist (f), and dpERK (g) are plotted with standard error. The numbers of embryos used in the analysis are 32, 20, and 19 respectively.

A.3 Discussion

Until now, end-on imaging was not ideally suited for quantitative and statistical studies of pattern formation [5, 6, 18]. Using our microfluidic embryo-trap array, hundreds of embryos can be oriented in an upright position in a matter of minutes. Data for dozens of embryos are sufficient for statistical analysis of spatial patterns in both wild-type and mutant backgrounds.

In the future, the temporal resolution of end-on imaging can be increased by grouping the images collected from fixed samples into distinct temporal classes. This could be based on cytological markers, such as the nuclear density in the syncytium or the extent of
membrane invagination during cellularization. In preliminary experiments, we established
that live embryos can be loaded into and imaged in the device as well. We obtained videos
of cell divisions in the early embryo as well as in an embryo undergoing gastrulation (Figure
A.9). We observed that some of the embryos hatched in the device, but we did not confirm
whether those that hatched were the ones that we imaged. The timing of nuclear divisions
observed in our device was very close to that observed in the classical studies of the early
Drosophila embryo [25]. This suggests that our imaging platform can be used to study the
real-time dynamics of embryonic development.

Unlike the anteroposterior patterning system, which has been a subject of extensive
mathematical modeling and computational analysis [26, 27], comprehensive quantitative
models of the dorsoventral system have yet to be developed [28, 29]. This is now a feasible
goal, enable by the efficiency of end-on imaging in our platform. Furthermore, embryo-trap
array-based imaging is not limited to the analysis of pattern formation in the early embryo.
Other related developmental events, such as gastrulation, could be readily analyzed using this
system. Devices for related fly species can be readily designed by modifying the trap size for
embryos that are smaller or larger than those of Drosophila melanogaster. Finally, because
we demonstrated a general method for handling non-spherical objects, which is more
difficult than handling cells, we expect that similar microfluidic designs can be used to image
pattern formation and morphogenesis in other model organisms of developmental genetics.
Figure A.9: Live imaging of embryos in META. Live embryos expressing nuclear Histone-GFP can be imaged for extended periods. (a) Frames of an embryo undergoing nuclear divisions. (b) Frames of an embryo undergoing ventral invagination. For both videos, images were taken 70 μm from the anterior pole. The scale bar represents 25 μm.
A.4 Experimental procedures

Microfluidic device fabrication. A mold was first fabricated by photolithographic processes. In the first step, a 250µm thick negative photoresist (SU8-2100, Microchem) was spin-coated twice at 400-600 rpm onto a silicon wafer to form a 500 µm-thick layer. Features on a transparency mask were transferred to the SU-8 coated wafer by standard UV photolithography. The mold was then treated with tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane vapor (United Chemical Technologies) in a vacuum desiccator to prevent adhesion of PDMS during the molding process.

For fabricating the PDMS devices, a mixture of PDMS (part A and B in 15:1 ratio) was poured onto the mold to give a ~1mm-thick layer and partially cured at 70ºC for 20mins. A mixture of PDMS (part A and B in 10:1 ratio) was then poured on top to form ~ 4mm thick layer and cured at 70ºC for two hours. After peeling off the 5mm PDMS layer, the individual devices were cut out, and access holes were punched in the PDMS. The devices were then treated with oxygen plasma and bonded to a cover glass.

Microfluidic device operation. Drosophila embryos suspended in 100ml PBS buffer were contained in a glass bottle. The tubing from the glass bottle was connected to the inlet of the microdevice. The outlet of the device was connected to 5m long PE90 tubing. The high resistance of the long PE90 tubing makes the pressure drop along the device less than 20% of the total pressure drop. This allows the traps expand uniformly throughout the device. To load the embryo suspension into the device, a constant pressure source (6 psi) was applied to the glass bottle to drive the flow into the device. After loading, the injection pressure was slowly decreased to 0 psi. Then, all the tubing were disconnected from the device.
**Fly strain and whole-mount immunostaining.** *OreR* flies were used as a wild type strain and *dl^6* flies were used as *dl* heterozygous mutant strain in this study. Flies were raised and embryos were collected at 25°C. Antibody stainings were performed as described in [21]. The following primary antibodies were used: rabbit anti-dpERK (1:100, Cell Signaling), mouse anti-Dorsal (1:100, Developmental Studies Hybridoma Bank), guinea pig anti-Twist (1:40, a gift from M. Levine), and rabbit anti-phospho-SMAD (1:3500, a gift from D. Vasiliauskas, S. Morton, T. Jessell and E. Laufer). DAPI (1:10,000) was used to stain nuclei and Alexa Fluors (1:500, Invitrogen) conjugated secondary antibodies were used.

To visualize *zen* transcript, fluorescent in-situ hybridization was used as described elsewhere [30]. Embryos were hybridized with DIG-labeled antisense probe to *zen* mRNA for overnight at 60°C. Sheep anti-DIG (1:200, Roche) was used as primary antibody and Alexa Fluors (1:500, Invitrogen) were used as secondary antibodies.

**Microscopy and gradient quantification.** Imaging was done on a Zeiss LSM510 confocal microscope with a Zeiss 20x (NA 0.6) A-plan objective. High-resolution images (1024 x 1024 pixels, 12 bits depth) were obtained from the focal plane ~70μm from anterior or posterior pole. For live-imaging, LEICA SP5 confocal microscope was used with 63x (NA 1.3) glycerin objective. Images were obtained every 7 seconds from the focal plane ~70μm from the anterior pole.

Protein gradients were extracted from confocal images by using a Matlab program described in [21]. DAPI staining was used to determine the positions of nuclei, which were then used to quantify the nuclear gradient of the protein of interest. The embryos were also co-stained with Dl in order to determine the ventral most point of the embryo. Briefly, the
extracted nuclear DI gradient was fitted with a Gaussian curve and the raw data were oriented such that the maximum of the Gaussian fit was set as the ventral most point of the embryo, i.e. $x = 0$.

**Characterization of flow profile in the microdevice by numerical simulation.** Simulations were performed using a commercial finite element package, COMSOL® (Stockholm, Sweden). The three-dimensional geometry of the section of the device is shown in Figure A.2A. The actual geometry was simplified to reduce the number of mesh elements. Incompressible steady-state Navier-Stokes equations were used. The pressure at the outlet was fixed at atmospheric pressure, and the pressure at the inlet was set to obtain a volumetric flow rate equal to the measured value ($\sim 7.5 \times 10^{-8} \text{ml/s}$).

**Characterization of hydrodynamic force on an embryo by numerical simulation.** The simulation described above was utilized to calculate hydrodynamic force on an embryo located in the trap. The embryo were simplified as a rectangular rod as described in Figure A.6. The rod was located in the trap with various values of angle. The total forces on the surface 1 and 2 were calculated using the post processing feature of COMSOL®. These values were then used to calculate the torque.

**A.5 References**


