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Report

MAPK Substrate Competition Integrates Patterning Signals in the *Drosophila* Embryo

Yoosik Kim,[1] Mathieu Coppey,[1] Rona Grossman,[2] Leiore Ajuria,[3] Gerardo Jiménez,[3] Ze'ev Paroush,[2] and Stanislav Y. Shvartsman[1,*]

[1]Department of Chemical Engineering and Lewis-Sigler Institute for Integrative Genomics, Princeton University, Princeton, NJ 08544, USA

[2]Department of Developmental Biology and Cancer Research, Institute for Medical Research Israel-Canada, The Hebrew University-Hadassah Medical School, P.O. Box 12272, Jerusalem 91120, Israel

[3]Institut de Biologia Molecular de Barcelona-CSIC and Institució Catalana de Recerca i Estudis Avançats, Parc Científic de Barcelona, Barcelona 08028, Spain

Summary

Terminal regions of the *Drosophila* embryo are patterned by the localized activation of the mitogen-activated protein kinase (MAPK) pathway [1]. This depends on the MAPK-mediated downregulation of Capicua (Cic), a repressor of the terminal gap genes [2, 3]. We establish that downregulation of Cic is antagonized by the anterior patterning morphogen Bicoid (Bcd). We demonstrate that this effect does not depend on transcriptional activity of Bcd and provide evidence suggesting that Bcd, a direct substrate of MAPK, decreases the availability of MAPK for its other substrates, such as Cic. Based on the quantitative analysis of MAPK signaling in multiple mutants, we propose that MAPK substrate competition coordinates the actions of the anterior and terminal patterning systems. 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 [4]. Thus, a common enzyme-substrate competition mechanism can integrate the actions of the anterior, posterior, and terminal patterning signals. Substrate competition can be a general signal integration strategy in networks where enzymes, such as MAPK, interact with their multiple regulators and targets [5–10].

Results and Discussion

Anteroposterior (AP) patterning of the *Drosophila* embryo depends on three inductive signals [11]: the head and thorax are specified by the anterior gradient of Bicoid (Bcd), a homeobox transcription factor; abdomen formation is directed by the reciprocal gradient of Nanos (Nos), a translational repressor; and the nonsegmented termini are patterned by the localized activation of the extracellular signal-regulated/mitogen-activated protein kinase (ERK/MAPK) pathway [6, 12]. 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 [1].

*Correspondence: stas@princeton.edu

Activated Tor promotes the expression of *tailless* (*tll*) 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 [1–3, 13]. Phosphorylation of Cic and Gro relieves their repression of *tll* and *hkb* and enables expression of these genes at both poles of the embryo. At the anterior pole, MAPK also phosphorylates Bcd [14, 15]. Thus, MAPK is activated in a localized pattern and phosphorylates substrates that can be either uniformly (Cic and Gro) or nonuniformly (Bcd) distributed along the AP axis of the embryo (Figures 1A–1C).

The spatial pattern of MAPK activation can be visualized with an antibody that recognizes the double phosphorylated form of ERK (dpERK) [16, 17]. We found that this pattern is strongly asymmetric: the anterior levels of dpERK are significantly higher than the posterior levels (Figure 1; see also Figure S1 available online). 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 [18] and/or in the intracellular localization of maternal determinants.

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 [14]. 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* (Figures 2A and 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 [19]. 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 [19–23]. Thus, the level of MAPK substrates can increase the level of MAPK phosphorylation by outcompeting the MAPK phosphatase. Correspondingly, a similar effect can be responsible for the observed dose-dependent control of MAPK phosphorylation levels 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.

The fact that the anterior level of dpERK is sensitive to the 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

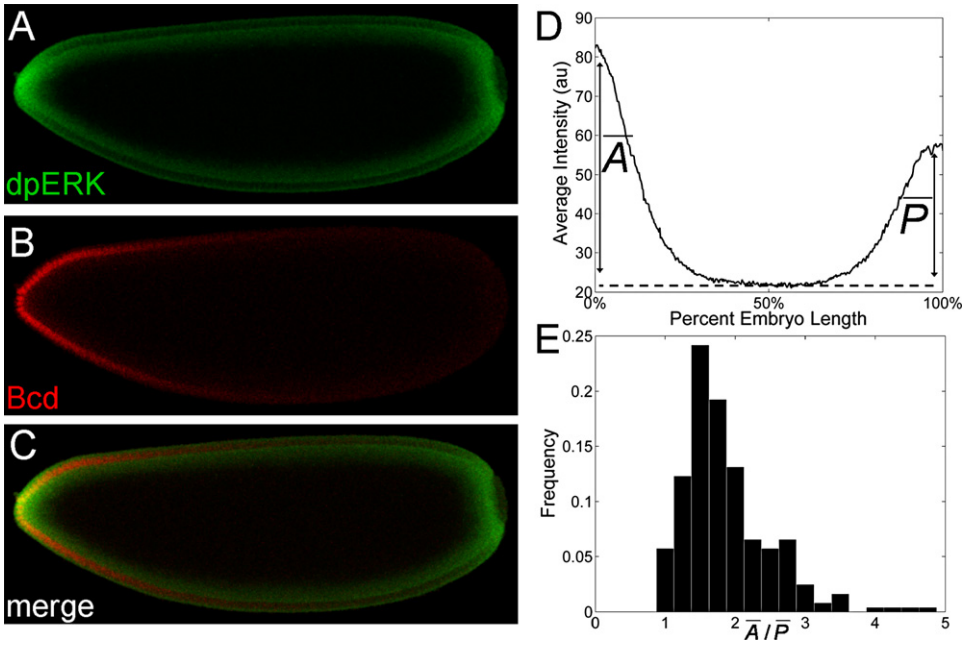

Figure 1. Spatial Asymmetries of Mitogen-Activated Protein Kinase Phosphorylation

(A–C) Coimmunostaining of the anteroposterior (AP) gradient of phosphorylated mitogen-activated protein kinase (MAPK), double phosphorylated ERK (dpERK) (green), and Bicoid (Bcd) (red). In all figures, embryos are oriented with anterior to the left and dorsal side up.

(D) An averaged AP gradient of dpERK at early cell cycle 14, obtained from 40 embryos. The dashed line indicates the reference level of dpERK. Quantification of the gradients reveals a clear AP asymmetry: the anterior levels are higher than the posterior levels.

(E) Histogram of the ratios of anterior to posterior peak levels of MAPK phosphorylation from 244 wild-type embryos. Individual peak values of dpERK at the termini are determined as described in Figure S1. In wild-type embryos, this ratio has a mean of 1.87 and a standard deviation of 0.64, which indicates that the anterior levels of dpERK are significantly higher than the posterior levels (t test, $p < 10^{-5}$).

transcriptional targets of Bcd would affect the levels of MAPK phosphorylation. To distinguish between these two alternatives, we used the *bcd* transgene (*Bcd-A*$_9$), which encodes a Bcd variant that has an impaired ability to bind its DNA recognition sequence [24] but still contains all of the sites required for binding to and phosphorylation by MAPK (Figure 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-A*$_9$ transgene (data not shown). Remarkably, the anterior level of dpERK was significantly increased in these embryos (Figure 2D). In fact, the anterior level of dpERK was indistinguishable from the anterior level of dpERK in embryos with two extra copies of wild-type *bcd* (*bcd*^{4x}). Taken together, these results imply that Bcd can affect the level of MAPK phosphorylation independently of its transcriptional activity.

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 3A–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 3D). Because 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 3E). These effects are consistent with the model, in which anteriorly localized Bcd acts as a competitive inhibitor of MAPK-mediated Cic downregulation.

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 4A and 4B). It is possible that this residual asymmetry reflects the presence of an additional MAPK substrate that is nonuniformly 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 [25, 26]. The distribution of Hb protein depends on both maternal and zygotic inputs [25, 27]. Briefly,

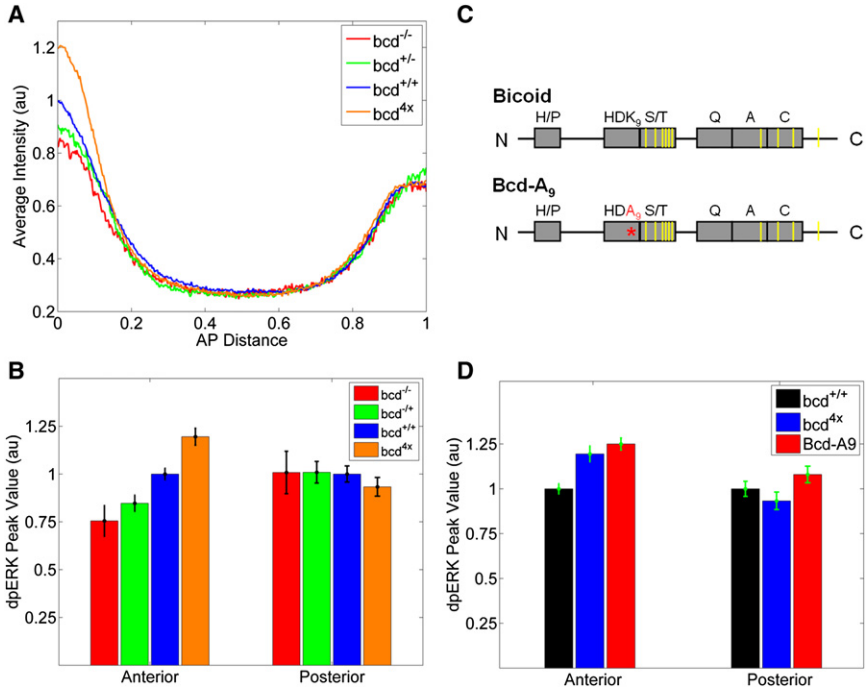

Figure 2. The Anterior Level of dpERK Responds to Changes in the Level of Bcd

(A) The AP gradient of dpERK in progeny 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-A_9) 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 [14]) are indicated by yellow bars. *Bcd-A_9* encodes a Bcd variant with a single lysine-to-alanine amino acid substitution at position 50 of the homeodomain (K_9 to A_9, shown in red).

(D) Embryos expressing two wild-type *bcd* and two copies of the *bcd-A_9* transgene show increased phosphorylation of MAPK at the anterior poles, similar to embryos with four copies of wild-type *bcd* (bcd^{4x}). Mean ± SE of 20–30 embryos for each genotype is shown; * denotes a statistically significant difference from the wild-type (t test, $p < 0.01$).

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 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)

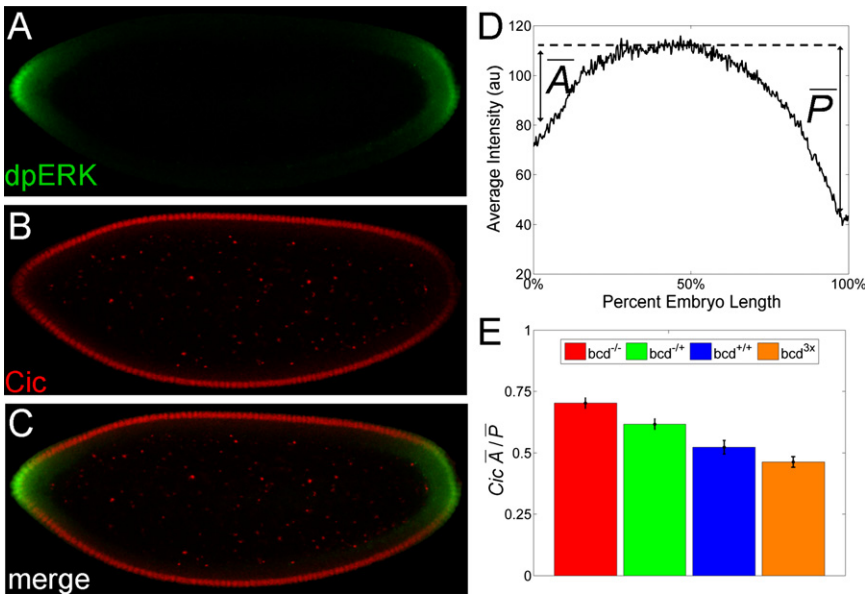

Figure 3. Asymmetry of Mitogen-Activated Protein Kinase-Mediated Downregulation of Cic

(A–C) Coimmunostaining 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: $p = 2.5 \times 10^{-12}$).

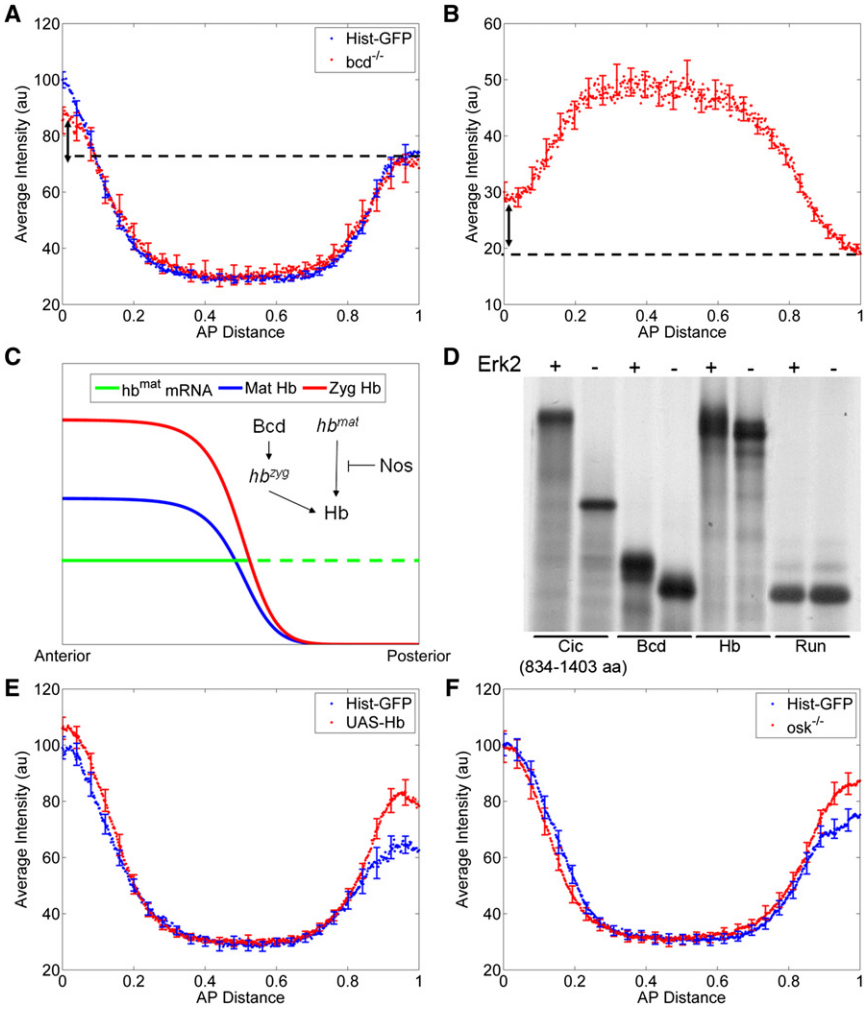

Figure 4. Hb Is a New Substrate of Mitogen-Activated Protein Kinase in the Embryo

(A) Comparison of the dpERK gradients in wild-type (histone-GFP) embryos and embryos from *bcd* null mothers. Average dpERK gradients for each genotype (n ~ 15) are plotted with SE indicated. Although the anterior levels of dpERK in *bcd* null embryos are lower than that in 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 Hunchback (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). At 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); average dpERK gradients for each genotype (n ~ 25–30) are plotted with SE indicated.

(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. Average dpERK gradients for each genotype (n ~ 25–30) are plotted with SE indicated.

(Figure 4D; data not shown). 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 4E). 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 identifies 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* [4]. In light of our results, these effects can be interpreted by the fact that, in nos^{-} or osk^{-} 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 of 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 4F). Thus, a common substrate competition mechanism 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 [5–9, 19, 28–35].

Experimental Procedures

Drosophila Strains and Whole-Mount Immunostaining

The following stocks were used in this study: histone-GFP (a gift from E. Wieschaus), bcd^{E1}, bcd^{4x}, osk^{6}, nos^{BN}, *Cic-HA; cic*^{1} [3], *Cic-HA; cic*^{1} bcd^{E1}, *UAS-hb* (a gift from N. Dostatni), and *Bcd-A*$_9$ [24]. All flies were raised and embryos were collected at 25°C. Antibody stainings were performed as described in [17]. Monoclonal mouse anti-dpERK (1:100, Sigma) and polyclonal rabbit anti-HA (1:500, Roche) were used as primary antibodies. Alexa Fluor and Oregon green (1:500, Invitrogen) were used as secondary antibodies.

Microscopy and Image Processing

Imaging was done on a Zeiss LSM510 confocal microscope, with a Zeiss 20× (NA 0.6) A-plan objective. High-resolution images (512 × 512 pixels, 12 bits depth) were obtained from the focal plane in the midhorizontal cross section of the embryo. Images of individual embryos were automatically extracted from raw confocal images and reoriented for gradient quantification, as described elsewhere [17].

In Vitro MAPK/ERK2 Phosphorylation Assays

Proteins were synthesized and labeled with 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 μg of active ERK2, in a total volume of 50 μl of kinase reaction buffer (20 mM HEPES, 0.1 mM benzamidine, 25 mM beta-glycerophosphate, 0.1 mM DTT, 1 mM Na_3VO_4, 10 mM $MgCl_2$, and 0.1 mM ATP) for 30 min at 30°C. Reactions were stopped by adding SDS sample buffer ×3 (0.25 M Tris pH 6.8, 6% SDS, 30% glycerol, B-mercapto-ethanol, and a few grains of Bromophenol-blue). The phosphorylation state of the proteins was subsequently analyzed by SDS-PAGE and autoradiography. To activate ERK2, we expressed a HIS-tagged ERK2 fusion protein in *Escherichia coli*, purified it on nickel beads (QIAGEN), and activated it with active MEK1 (Upstate).

Statistical Analysis

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 from 20–30 wild-type and mutant embryos, and the dpERK levels were determined as described in Figure S1. To examine the correlation between the amount of Bcd and the level of MAPK phosphorylation, we employed a generalized linear model (GLM) with copies of *bcd* as the independent variable and either the anterior or posterior dpERK levels as the response variable. Similarly, a 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, as described in Figure S1.

Supplemental Information

Supplemental Information includes one figure and can be found with this article online at doi:10.1016/j.cub.2010.01.019.

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