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REPORT

Substrate-dependent control of MAPK phosphorylation *in vivo*

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Phosphorylation of the mitogen-activated protein kinase (MAPK) is essential for its enzymatic activity and ability to control multiple substrates inside a cell. According to the current models, control of MAPK phosphorylation is independent of its substrates, which are viewed as mere sensors of MAPK activity. Contrary to this modular view of MAPK signaling, our studies in the *Drosophila* embryo demonstrate that substrates can regulate the level of MAPK phosphorylation *in vivo*. We demonstrate that a twofold change in the gene dosage of a single substrate can induce a significant change in the phosphorylation level of MAPK and in the conversion of other substrates. Our results support a model where substrates of MAPK counteract its dephosphorylation by phosphatases. Substrate-dependent control of MAPK phosphorylation is a manifestation of a more general retroactive effect that should be intrinsic to all networks with covalent modification cycles.
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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 (Chen *et al*, 2001; Shaul and Seger, 2007). 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 (Huang and Ferrell, 1996; Fujioka *et al*, 2006; Qiao *et al*, 2007; Nakakuki *et al*, 2010). 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 (Lenormand *et al*, 1998; Tanoue *et al*, 2002; Bardwell *et al*, 2003; Grewal *et al*, 2006; Blackwell *et al*, 2007).

Substrate-dependent control of MAPK phosphorylation has been demonstrated in studies with heterologous expression systems (Bardwell *et al*, 2003), 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.

Results and discussion

Removal of endogenous MAPK substrates reduces MAPK phosphorylation *in vivo*

Drosophila uses its ERK/MAPK pathway throughout embryonic development (Gabay *et al*, 1997b). 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 (Figure 1A and B; Furriols and Casanova, 2003; Li, 2005). In the absence of MAPK signaling, *tll* and *hkb* are repressed by the ubiquitously expressed transcriptional repressors Capicua (Cic) and Groucho (Gro; Paroush *et al*, 1997; Jimenez *et al*, 2000). At the termini, their action is counteracted by MAPK, which phosphorylates both Cic and Gro and thus derepresses *tll* and *hkb* (Cinnamon *et al*, 2008). MAPK also phosphorylates Bicoid (Bcd) and Hunchback (Hb), two other transcription factors (Ronchi *et al*, 1993; Kim *et al*, 2010). In contrast to Cic and Gro, Bcd and Hb are localized to the anterior of the embryo (Figure 1C).

We have previously shown that removal of a single MAPK substrate, Bcd, leads to a significant reduction of MAPK phosphorylation in the embryo (Kim *et al*, 2010). 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 ERK/MAPK (dpERK) (Gabay *et al*, 1997a; Coppey *et al*, 2008). In all cases, the level of dpERK was significantly reduced (Figure 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 (Figure 1H–K), whereas removal of either Cic or Gro influenced both the anterior and posterior levels of dpERK (Figure 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 (Supplementary Figure S1). Thus, the level of MAPK phosphorylation is reduced by removing any one of the four known MAPK substrates in the early embryo.

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 (Lenormand *et al*, 1998; Tanoue *et al*, 2002; Bardwell *et al*, 2003; Grewal *et al*, 2006; Blackwell *et al*, 2007; Liu *et al*, 2010). 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 (Kim *et al*, 2002, 2004; Gomez *et al*, 2005). 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′UTR of the *mkp3* gene (Rintelen *et al*, 2003). 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 (Rintelen *et al*, 2003). 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 (Figure 2A and B). 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 (Figure 2I and J, data not shown). 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 (see Materials and methods). This led to a significant reduction of MAPK phosphorylation, with greater effect in the posterior region (Figure 2C and D). On the basis of these results, we conclude that MKP3 negatively regulates MAPK phosphorylation in the early embryo.

A model for substrate-dependent control of MAPK phosphorylation

Thus, the phosphorylation level of MAPK is negatively regulated by MKP3, a cytoplasmic phosphatase (Dowd *et al*, 1998; Kim *et al*, 2002), 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 (Astigarraga *et al*, 2007). This protein retains a MAPK-docking site, which is essential for its interaction with and down-regulation by MAPK. Expression of this protein led to a marginal increase of MAPK phosphorylation (Supplementary Figure S2). 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 3A and B). On the basis of these observations, we favor a model where substrates control MAPK phosphorylation by affecting its subcellular localization.

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 phosphorylated/active form of the enzyme (E*) shuttles in and out of the nucleus, where it interacts with multiple substrates. Steady state of this model reveals that

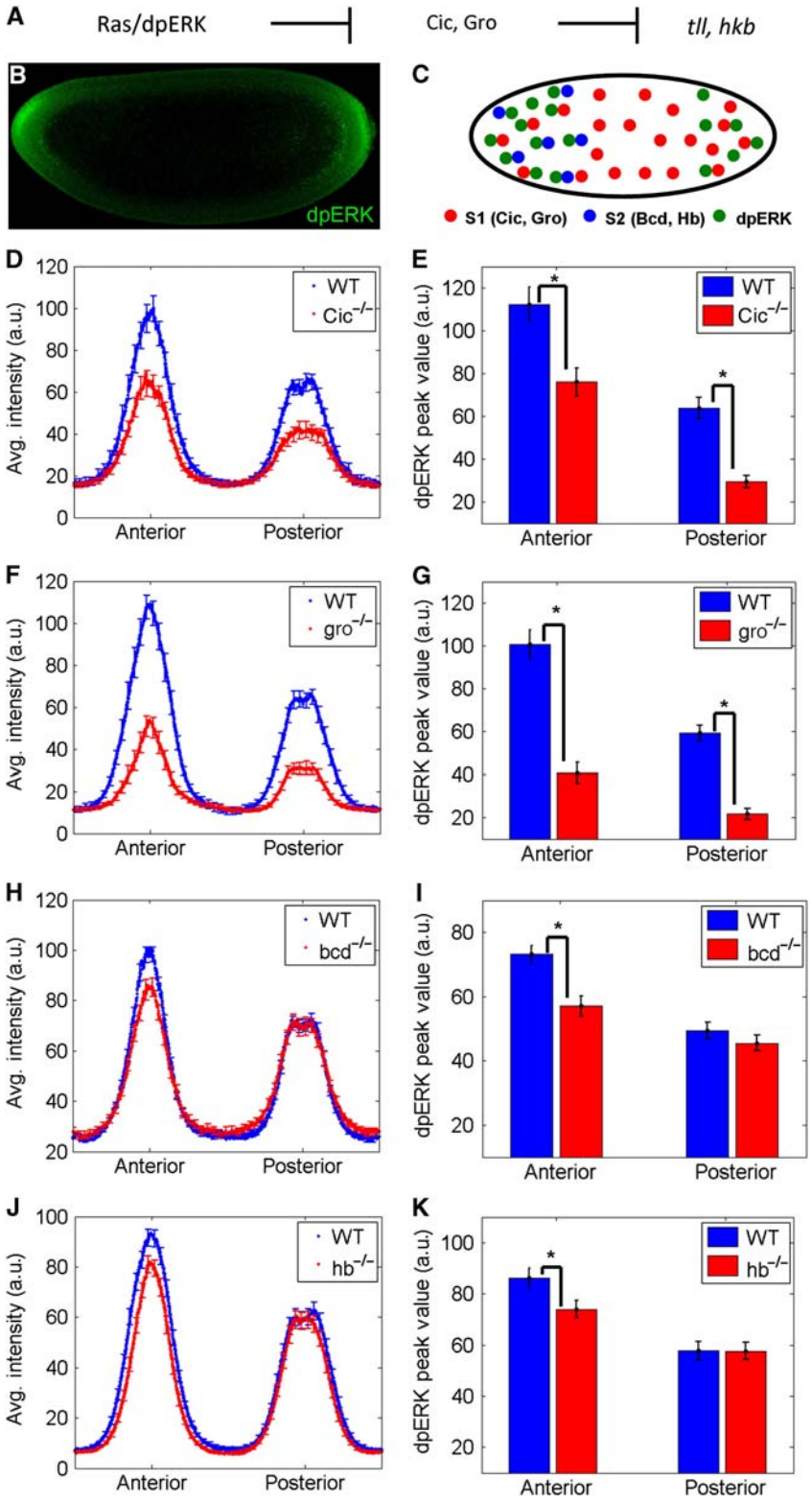


Figure 1 Removal of MAPK substrates reduces the level of MAPK phosphorylation in the embryo. (**A**) Schematic of signal transduction in the terminal patterning system of the *Drosophila* embryo. Activated MAPK controls expression of the terminal gap genes *tll* and *hkb* by downregulating transcriptional repressors Cic and Gro. (**B**) MAPK phosphorylation detected with an antibody that recognizes the double phosphorylated form of ERK/MAPK (dpERK). (**C**) MAPK substrates can be categorized into either uniformly distributed (S1, such as Cic and Gro, red) or localized to the anterior region of the embryo (S2, such as Bcd and Hb, blue). (**D**–**G**) Quantified average dpERK gradients and peak levels in wild-type embryos (blue) and mutant embryos lacking *cic* (D, E), *gro* (F, G), *bcd* (**H**, **I**) or *hb* (**J**, **K**). Error bars are standard error of the mean, and numbers of embryos used in the analysis are N_{WT}=17, N_{cic}=17 for (D, E), N_{WT}=21, N_{gro}=27 for (F, G), N_{WT}=18, N_{bcd}=28 for (H, I) and N_{WT}=32, N_{hb}=32 for (J, K). *Indicates $P<0.01$. Source data is available for this figure at http://www.nature.com/msb.

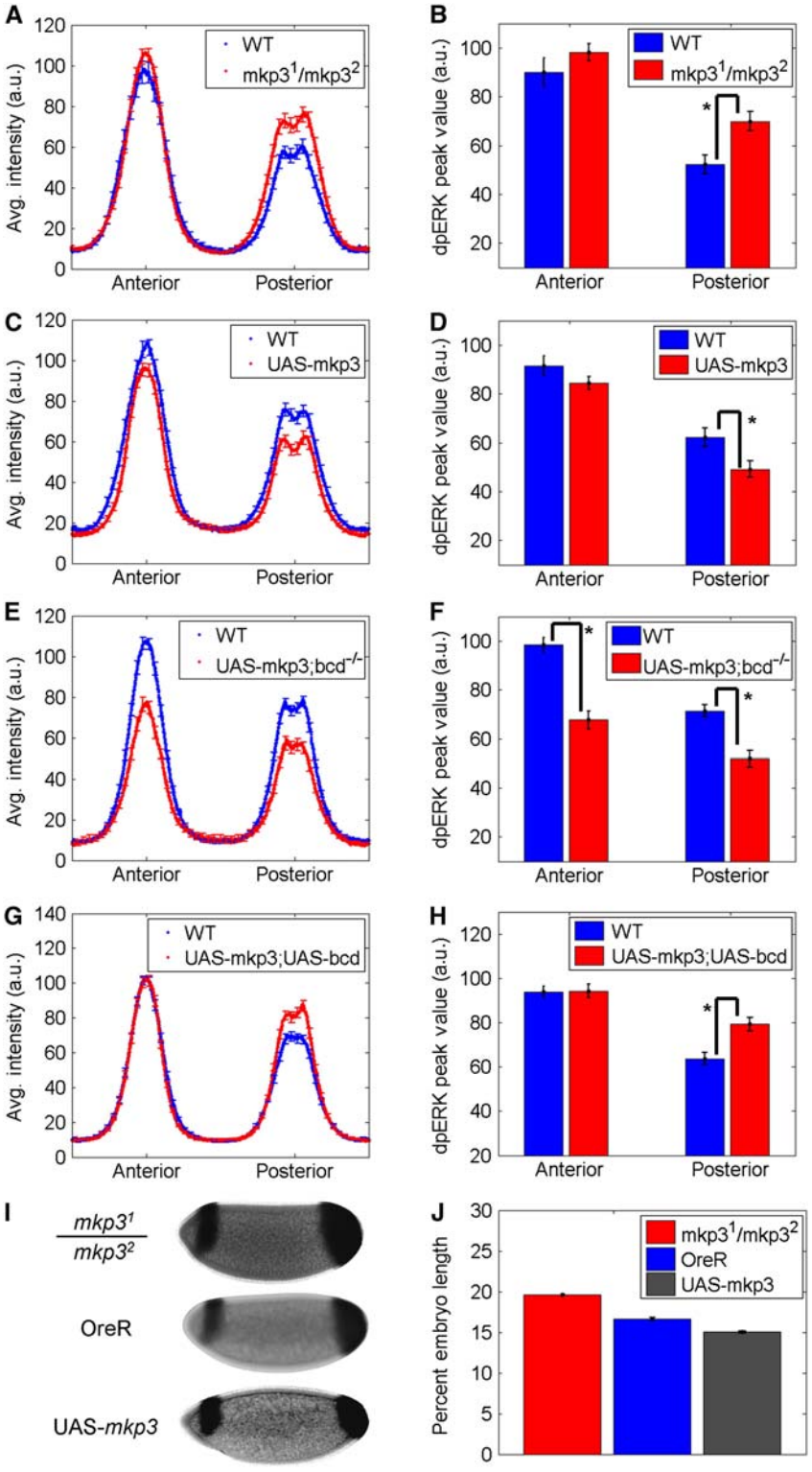


Figure 2 MKP3 negatively regulates MAPK phosphorylation and signaling in the terminal system. (**A–H**) Quantified average dpERK gradients and peak levels in wild-type embryos (blue) and mutant embryos with lower level of MKP3 (*mkp3*^{1}/*mkp3*^{2}) (A, B), ectopic overexpression of MKP3 (C, D), ectopic overexpression of MKP3 in *bcd* null background (E, F) and ectopic overexpression of both MKP3 and Bcd (G, H). Error bars are standard error of the mean, and numbers of embryos used in this analysis are N_{WT}=23, N_{mutant}=34 for (A, B), N_{WT}=28, N_{mutant}=31 for (C, D), N_{WT}=23, N_{mutant}=24 for (E, F) and N_{WT}=38, N_{mutant}=36 for (G, H). (**I**, **J**) Altering the level of MKP3 is accompanied by changes in the expression of the downstream genes *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). The numbers of embryos used in this analysis are 71 for wild type, 104 for *mkp3* transheterozygous and 86 for UAS-*mkp3* embryos. *Indicates $P<0.01$. Source data is available for this figure at http://www.nature.com/msb.

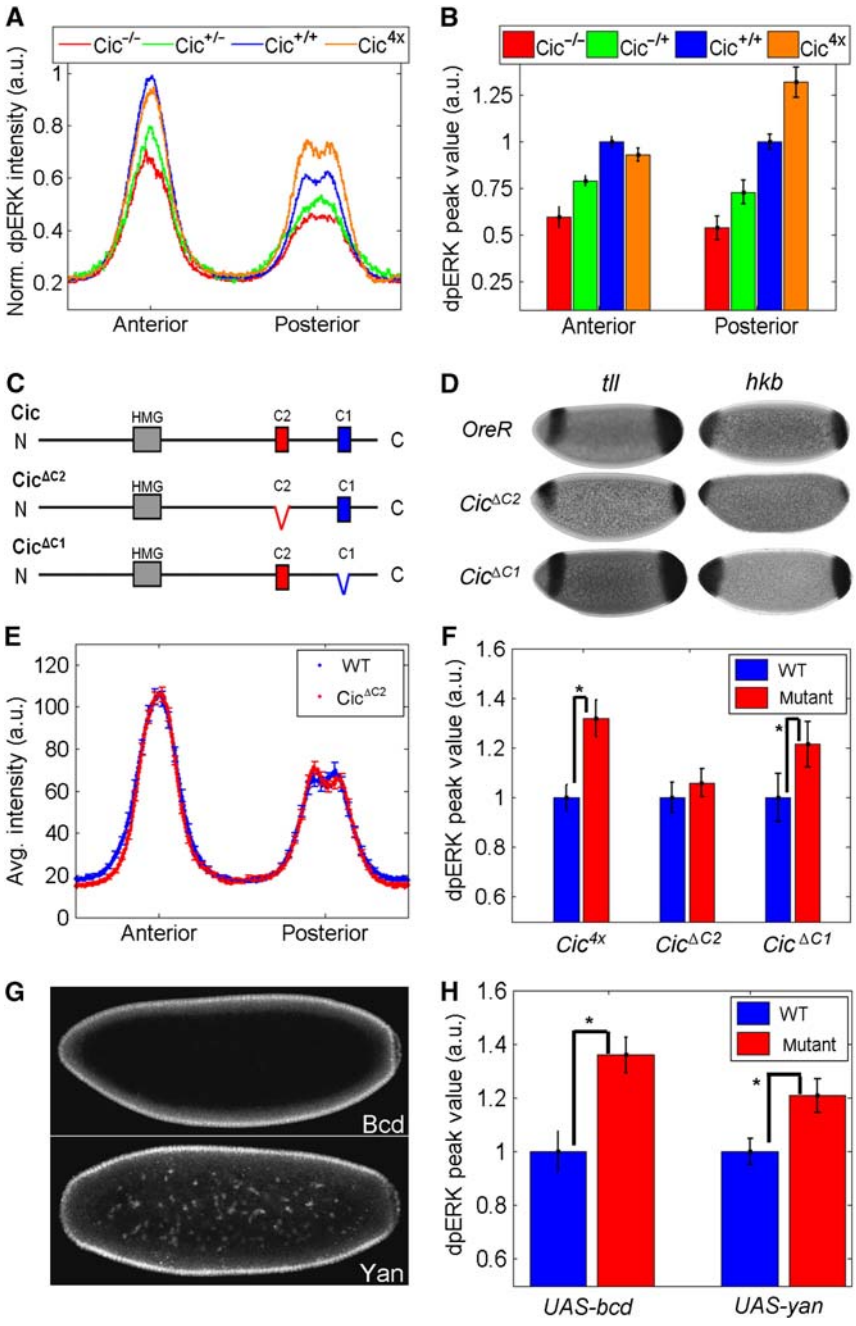
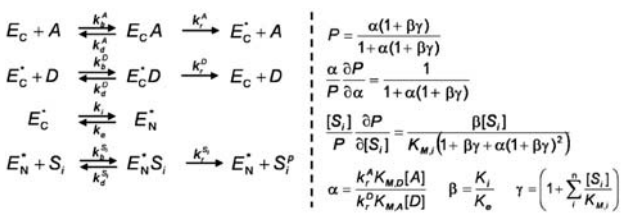


Figure 3 Cic-dependent control of MAPK phosphorylation. (**A**) dpERK gradients in embryos with different *cic* copy number; each gradient is an average of ~20 embryos of the same genotype. (**B**) The anterior and posterior peak levels of dpERK in embryos with different *cic* copy number. Each bar indicates 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 $Cic^{\Delta C2}$ in wild-type embryos decreases the expression domains of *tll* and *hkb*. In contrast, addition of $Cic^{\Delta C1}$ does not affect the gene expression. (**E**) Averaged dpERK gradients in wild-type embryos (blue, N=28) and embryos expressing $Cic^{\Delta C2}$ (red, N=31). (**F**) Quantitative comparison of posterior dpERK levels in the wild-type embryos (blue) and embryos overexpressing wild-type Cic (cic^{4x}), $Cic^{\Delta C2}$ or $Cic^{\Delta C1}$. The numbers of embryos used in the analysis are N_{WT}=22, N_{mutant}=22 for cic^{4x}, N_{WT}=28, N_{mutant}=31 for $Cic^{\Delta C2}$ and N_{WT}=26, N_{mutant}=29 for $Cic^{\Delta C1}$. (**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). Each bar indicates averaged peak dpERK values of N_{WT}=23, N_{mutant}=23 for UAS-*bcd* and N_{WT}=25, N_{mutant}=24 for UAS-*yan*. *Indicates $P<0.01$. Source data is available for this figure at http://www.nature.com/msb.

decreasing the level of a nuclear substrate always leads to decrease in the total fraction of enzyme in phosphorylated state (P). 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 (Supplementary Figure S3).

Box 1 Kinetic model of substrate-dependent control of MAPK phosphorylation



An enzyme is converted between active (E*) and inactive (E) forms in cytoplasm by a kinase (A) and phosphatase (D). E* shuttles between cytoplasm and nucleus, where it phosphorylates multiple substrates (S_i). Under a number of assumptions made to simplify the algebra (see the Supplementary information), we derived the expression for the total fraction of enzyme in the phosphorylated state (P), this includes free active enzyme (E*) and its complexes. P depends on three dimensionless parameters that characterize the input to the phosphorylation–dephosphorylation cycle (α), the ratio of import and export rate of the phosphorylated enzyme (β) and the collective effect of the substrates (γ). $K_{M,i}$ is the Michaelis constant of the i-th reaction, and $K_{M,A}/K_{M,D}$ are the Michaelis constants for the activation/deactivation reactions, respectively. Upon differentiating the expression for P, we find that it is an increasing function of both α and S_i, but the magnitudes of both effects are attenuated by the total amount of substrates (γ).

Following the same reasoning, the effects of spatially uniform changes in the level of any given MAPK substrate should be always smaller at the anterior pole (Supplementary Figure S3). 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 3A, B and H). 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 (Rebay and Rubin, 1995; Gabay *et al*, 1996), significantly increased the dpERK level at the posterior pole, whereas the anterior level was indistinguishable from that of the wild type (Figure 3G and H).

Thus, a model in which substrates positively regulate the level of MAPK phosphorylation successfully predicts the effects 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 2E and F).

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 3H), but reduced by ectopic expression of MKP3 (Figure 2D). When Bcd and MKP3 were overexpressed together, the effect was intermediate (Figure 2G and H). 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.

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, Cic acts as a repressor of *argos*, an inhibitor of the epidermal growth factor receptor that signals through the MAPK pathway (Roch *et al*, 2002). In the early embryo, we found that increasing the gene copy number of Cic leads to a clear increase of MAPK phosphorylation (Figure 3A and B). 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 ($Cic^{\Delta C2}$) and is therefore insensitive to MAPK-dependent downregulation, but is otherwise active as a repressor (Astigarraga *et al*, 2007). As expected, adding the MAPK-insensitive mutant Cic has a strong effect on the expression of *tll* and *hkb* (Figure 3D). If the effect of Cic overexpression is mainly transcriptional, then expression of the $Cic^{\Delta C2}$ 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 (Figure 3E and F). Thus, even though $Cic^{\Delta C2}$ 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 ($Cic^{\Delta C1}$). The mutant protein still interacts with and is downregulated by MAPK, but it is functionally weaker (Astigarraga *et al*, 2007); for instance, its expression does not alter the expression boundaries of *tll* and *hkb* (Figure 3D). At the same time, the level of MAPK phosphorylation was significantly increased upon expressing the $Cic^{\Delta C1}$ derivative (Figure 3F). Thus, despite the weaker activity as a transcriptional repressor, $Cic^{\Delta C1}$ 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 (Kim *et al*, 2010). 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).

Conclusions

MAPK phosphorylation is controlled by multiple enzymes, adaptors and scaffolds that regulate its catalytic activity, interaction partners and subcellular localization (Kolch, 2005; Karlsson *et al*, 2006; Shaul and Seger, 2007; Kiel and Serrano, 2009; von Kriegsheim *et al*, 2009). Our study demonstrates that MAPK substrates can be equally important in controlling the level of MAPK phosphorylation *in vivo*. Similarly, the level of MAPK activity toward any one of its substrates can be significantly affected by other co-expressed substrates (Kim *et al*, 2010). As shown in Supplementary Figure S4, this effect is not limited to Bcd: Cic downregulation is increased upon removal of Gro, another substrate of MAPK.

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 (Del Vecchio *et al*, 2008; Saez-Rodriguez *et al*, 2008). 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 (Qiao *et al*, 2007; Ventura *et al*, 2008, 2010; Kiel and Serrano, 2009; Cirit *et al*, 2010). In the case of retroactivity, feedback cannot be removed genetically or pharmacologically without affecting the input-to-output connection.

In some systems, retroactive effects may be small (Del Vecchio *et al*, 2008). 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.

Materials and methods

Drosophila strains and germline clones

The following *Drosophila* strains and germline clones were used: Histone-GFP, OreR, cic^{1}, FRT82B-gro^{MB36}, bcd^{E1}, FRT82B-bcd^{E1}-hb^{FB}-nos^{BN} (a gift from E Wieschaus), FRT82B-hb^{FB}-spz^{4} (a gift from E Wieschaus), w; cic-HA (cic^{4x}), w; $cic^{\Delta C2}$, w; $cic^{\Delta C1}$, yw; $MKP3^{1}$, yw; $MKP3^{2}$. yw; UAS-BcdGFP (a gift from E Wieschaus), yw; UAS-mkp3, matα4-GAL-VP16 (a gift from E Wieschaus), and yw; 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 (Hacker and Perrimon, 1998). 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^{D} were crossed with females carrying the mutant of interest. The resulting progenies were heat-shocked at 37.5°C for 1 h for 2 consecutive days at 3rd instar larva stage.

Immunostaining and *in situ* hybridization

Primary antibodies used in this study were as follows: 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) were used as secondary antibodies. Embryos were mounted in Aqua PolyMount and kept in 4 °C. To detect *tll* and *hkb*, embryos were hybridized overnight at 60°C with DIG-labeled anti-sense probes. Embryos were then incubated with alkaline phosphatase-conjugated anti-DIG (Roche, 1:2000) antibody for 1 h at room temperature and developed in NBT/BCIP solution for 20 min.

Imaging was carried out on a Zeiss LSM510 confocal microscope, with a Zeiss 20× (NA 0.6) A-plan objective. Images of individual embryos were automatically extracted from raw confocal images and quantified as described elsewhere (Coppey *et al*, 2008).

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 *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 dorsoventral axis. This was performed for 1000 points uniformly spaced along the anterior–posterior axis, generating an anterior–posterior expression profile of the gene. The locations of the half maximum level were used as boundaries of the expression domains.

Supplementary information

Supplementary information is available at the *Molecular Systems Biology* website (http://www.nature.com/msb).

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Author contributions: YK, ZP, GJ and SYS designed the experiments. YK performed the experiments. KN and EF provided phosphatase flies. YK and SYS wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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