Activation-induced substrate engagement in ERK signaling

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ABSTRACT The extracellular signal-regulated kinase (ERK) pathway is an essential component of developmental signaling in metazoans. Previous models of pathway activation suggested that dissociation of activated dually phosphorylated ERK (dpERK) from MAPK/ERK kinase (MEK), a kinase that phosphorylates ERK, and other cytoplasmic anchors, is sufficient for allowing ERK interactions with its substrates. Here, we provide evidence for an additional step controlling ERK’s access to substrates. Specifically, we demonstrate that interaction of ERK with its substrate Capicua (Cic) is controlled at the level of ERK phosphorylation, whereby Cic binds to dpERK much stronger than to unphosphorylated ERK, both in vitro and in vivo. Mathematical modeling suggests that the differential affinity of Cic for dpERK versus ERK is required for both down-regulation of Cic and stabilizing phosphorylated ERK. Preferential association of Cic with dpERK serves two functions: it prevents unproductive competition of Cic with unphosphorylated ERK and contributes to efficient signal propagation. We propose that high-affinity substrate binding increases the specificity and efficiency of signal transduction through the ERK pathway.

INTRODUCTION

The extracellular signal-regulated kinase (ERK) is the final component of the Raf-MAPK/ERK kinase-ERK (Raf-MEK-ERK) signaling module, which functions downstream of receptor tyrosine kinases (RTKs) and controls multiple cellular processes, including cell proliferation and differentiation (Lemmon and Schlessinger, 2010; Futran et al., 2013). Activated dually phosphorylated ERK (dpERK) relays pathway activation to the cell via phosphorylation of multiple substrates (Futran et al., 2013). ERK interactions with substrates are typically mediated by two docking domains, the D-site recruitment site (DRS, also known as the common docking, or CD domain), which binds the conserved D-site motif in the substrates, and the F-site recruitment site (FRS), which binds to the F XF motif (also known as the DEF motif) (Jacobs et al., 1999; Sharrocks et al., 2000; Tanoue et al., 2000; Futran et al., 2013).

To understand how ERK carries out its multiple cellular functions, it is critical to establish how pathway activation affects the ability of ERK to recognize its substrates. It is thought that unphosphorylated ERK is sequestered in the cytoplasm by MEK and other cytosolic scaffold and anchor proteins (Roskoski, 2012). Phosphorylation of ERK by MEK results in the release of dpERK from complexes with scaffold and anchor proteins (Roskoski, 2012). Whether ERK activation contributes to substrate selection is not well understood. It has been proposed that formation of dpERK results in a conformational change that allows binding of the FRS motif in ERK to the FXF motif in substrate proteins such as ELK1 (Lee et al., 2004). However, a subsequent study found that ERK phosphorylation results in reduced binding to ELK1 (Burkhard et al., 2011).
One of the key ERK targets in Drosophila and mammals is the high mobility group–box transcriptional repressor Capicua (Cic), which controls tissue patterning and organ growth (Jimenez et al., 2000; Astigarraga et al., 2007; Tseng et al., 2007; Ajuria et al., 2011; Grimm et al., 2012; Lim et al., 2013; Jin et al., 2015; Yang and Veraksa, 2017). In Drosophila, Cic phosphorylation and downstream regulation is involved in most developmental contexts that are under ERK control (Jimenez et al., 2012). In humans, mutations in Cic have been implicated in the neurodegenerative disease spino-cerebellar ataxia type 1 (Lam et al., 2006; Fyrer et al., 2011) and in the majority of oligodendroglioma cases, as well as other cancers (Simon-Carrasco et al., 2017; Tanaka et al., 2017; Tan et al., 2018). In all contexts studied so far in Drosophila, Cic functions as a transcriptional repressor whose activity is inhibited in response to ERK activation (Jimenez et al., 2012). Recent evidence suggests that Cic phosphorylation by ERK leads to rapid relief of repression through interference with DNA binding or interactions with corepressors, followed by slower export from the nucleus and eventual proteolytic degradation (Astigarraga et al., 2007; Grimm et al., 2012; Lim et al., 2013, 2015). Because all of the proposed modes of Cic inactivation are dependent on the ERK-mediated phosphorylation of Cic, it is essential to determine how ERK associates with Cic. Previous studies identified a region in Drosophila Cic (the C2 motif) that mediates its binding to ERK (Astigarraga et al., 2007; Futran et al., 2015); however, these studies used the inactive (unphosphorylated) form of ERK.

Here, we report that Cic interacts with activated, dually phosphorylated ERK with a much higher affinity compared with unphosphorylated ERK. Our data suggest that preferential Cic-dpERK interaction prevents unproductive competition of Cic with unphosphorylated ERK, when both Cic and ERK are localized in the same cellular compartment. Furthermore, higher affinity of Cic for dpERK may be required for efficient signal propagation, as it contributes to pathway output (down-regulation of Cic) and increases the steady-state level of dpERK. Based on this, we propose that activation-dependent association with Cic is an integral part of ERK signaling dynamics that serves as an additional checkpoint to regulate the specificity and efficiency of signal propagation.

RESULTS AND DISCUSSION

ERK activation is required and sufficient for high-affinity interaction with Cic

Previous studies of interactions between ERK and Cic used unphosphorylated forms of ERK in binding experiments (Astigarraga et al., 2007; Futran et al., 2015). For testing whether activation of ERK affects its interaction with Cic, V5-tagged Cic (Cic-V5) expressed in Drosophila S2 cells was immobilized on streptavidin beads and incubated with bacterially expressed, purified rat ERK2, which was converted into the active form by coexpression with active MEK (Figure 1A). ERK2 phosphorylation resulted in a much more robust interaction with Cic (Figure 1A). We observed a similar result with the fly ERK protein using extracts from S2 cells expressing either Drosophila ERK alone or ERK in combination with MEK and Raf, which is sufficient to induce dpERK formation (Tipping et al., 2010), and streptavidin binding peptide (SBP)-tagged Cic (Figure 1B). Activation of ERK by MEK by dual phosphorylation is therefore sufficient to convert it into a form that has a higher affinity for Cic compared with unphosphorylated ERK.

To determine whether full activation of ERK is required for its increased affinity for Cic, we studied ERK/Cic binding using the T198A and Y200F ERK mutants that disrupt the TEY phosphorylation motif targeted by MEK (Canagarajah et al., 1997). As shown in Figure 1C, mutation of either residue impaired the binding between Cic and Drosophila ERK-Flag when both were coexpressed in S2 cells, relative to the wild-type enzyme, suggesting that full ERK activation and formation of dpERK is required for its highest affinity for Cic. We note that in this experiment coexpression of ERK with Cic resulted in the stabilization of the activated form of ERK, likely due to the effect of shielding of dpERK from the action of phosphatases in the Cic-dpERK complex (Kim et al., 2011). Collectively, these studies reveal that ERK phosphorylation is required and sufficient to induce a high-affinity interaction with Cic (Figure 1D).

Preferential binding of Cic to dpERK in vivo

To determine whether Cic preferentially associates with dpERK in vivo, we studied the binding between Cic-Venus (Grimm et al., 2012), which is expressed from genomic regulatory sequences at the endogenous level, and the endogenous ERK protein in 0- to 4-h Drosophila embryos. At this stage, dpERK is formed specifically at the embryonic termini downstream of RTK Torso activation (Gabay et al., 1997). Immunoprecipitation of Cic-Venus resulted in copurification of endogenous ERK, which also gave a strong dpERK signal (Figure 2A). To test whether dpERK is limiting in this assay, we generated additional dpERK via maternal expression of a dominantly active form of MEK, MEK[K203] (Goyal et al., 2017a). In these embryos, dpERK was produced throughout the embryo, including the middle region, where dpERK is normally absent (Figure 2, B and C), while the total ERK level was unchanged (Figure 2A and Supplementary Figure S1). Remarkably, under these conditions, a higher amount of total ERK was associated with Cic-Venus, consistent with a higher overall level of dpERK in these embryos (Figure 2A). We note that the level of Cic-Venus was lower in MEK[K203]-overexpressing embryos (Figure 2A, GFP signal), likely because of excessive degradation due to ERK hyperactivity (Goyal et al., 2017b). However, we still observed an increase in the amount of total ERK in our communoprecipitation experiment, despite it being pulled down by this lower amount of Cic-Venus. In summary, Cic has a higher affinity for dpERK in vivo, and the amount of ERK bound to Cic is limited by the level of dpERK produced downstream of RTK activation.

Preferential association of Cic with dpERK prevents competition with ERK and contributes to efficient signal propagation

Previous quantitative models of dpERK interactions with substrates assumed that dpERK is largely nuclear (Kim et al., 2011). However, several studies showed that both Cic and dpERK can localize in the cytoplasm in developing tissues (Roch et al., 2002; Astigarraga et al., 2007; Coppey et al., 2008; Grimm et al., 2012). We studied ERK and Cic localization in Drosophila cultured S2 cells under the basal conditions of limited ERK phosphorylation or in cotransfection with Raf and MEK, which generates large amounts of dpERK (Tipping et al., 2010). Transfection of Cic alone resulted in a predominantly cytoplasmic localization, with some nuclear signal (Figure 3, A–A″). Cotransfection of ERK with Raf and MEK led to the formation of dpERK, which was also localized mostly in the cytoplasm (Figure 3, B–B″). Upon cotransfection of Cic with ERK, Raf, and MEK, the Cic and dpERK signals were still primarily cytoplasmic, with detectable but low nuclear staining (Figure 3, C–C″). These data showed that Cic, ERK, and dpERK can coexist in the cytoplasm in S2 cells, which agrees with previously reported localization of Cic and dpERK in both the nucleus and the cytoplasm in vivo (Roch et al., 2002; Astigarraga et al., 2007; Coppey et al., 2008; Grimm et al., 2012). Subcellular localization of the two major ERK phosphatases in

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FIGURE 1: ERK phosphorylation in the activation loop is required and sufficient to induce strong binding to Cic. (A) An in vitro binding assay in which bacterially expressed purified rat ERK2 and dpERK2 were incubated with beads bound with Cic-V5 purified from S2 cells, analyzed by Western blotting. Cic-V5 strongly prefers dpERK2 over unphosphorylated ERK2. (B) An in vitro binding assay in which protein lysates from S2 cells cotransfected with Drosophila ERK-Flag, Raf, and MEK (dpERK-Flag) or transfected only with ERK-Flag were incubated with beads bound with separately expressed Cic-SBP, analyzed by Western blotting. Cic-SBP strongly prefers dpERK over unphosphorylated ERK. (C) Coimmunoprecipitation between Cic and ERK mutants in Drosophila S2 cells, analyzed by Western blotting with the indicated antibodies. Blocking the formation of dually phosphorylated ERK (dpERK) results in a lower affinity for Cic. Asterisks in B and C indicate endogenous (untagged) dpERK present in S2 cells. (D) Summary of Cic interactions with ERK and dpERK. Cic preferentially associates with dpERK.

FIGURE 2: Cic preferentially associates with dpERK in vivo. (A) Extracts from embryos expressing Cic-Venus alone or Cic-Venus with activated MEK (MEK\textsuperscript{E203K}) were immunoprecipitated with anti-green fluorescent protein (GFP) beads and analyzed by Western blotting. Up-regulation of ERK signaling is sufficient to increase ERK association with Cic in vivo. (B, C) Overexpression of activated MEK (MEK\textsuperscript{E203K}) results in an increase of dpERK formation in the middle of the embryo. (B) Schematic diagram of the embryo, indicating the location of the images shown in C.

Drosophila is consistent with the importance of cytoplasmic regulation of dpERK; both Mkp3 and PTP-ER are cytoplasmic proteins (Karim and Rubin, 1999; Molnar and de Celis, 2013).

We hypothesized that preferential association of Cic with dpERK may be important for preventing competition between ERK and dpERK for binding to Cic, when both ERK forms and Cic are localized in the same cellular compartment (cytoplasm). Such competition would occur if ERK was still present in significant amount after pathway activation. To determine whether the production of dpERK in the cells that are cotransfected with ERK, Raf, and MEK is limited, we analyzed extracts from S2 cells expressing ERK alone or ERK together with MEK and RAF by Western blotting with an antibody specific for unphosphorylated ERK (unphospho-ERK). We did not observe a significant down-regulation of the unphospho-ERK signal upon cotransfection with Raf and MEK, despite detecting robust formation of dpERK (Figure 3, D and E), suggesting that dpERK is present in limited amounts in these cells, and unphosphorylated ERK is still the predominant form. We also compared the normalized ratios of unphospho-ERK to total ERK by immunofluorescence of transfected S2 cells. These measurements showed that, upon cotransfection with Raf and MEK, the amount of unphosphorylated ERK was reduced from 1 to
0.75 (a 25% decrease), again indicating only limited dpERK formation in cells (Supplemental Figure S2).

To explore the consequences of colocalization of Cic, ERK, and dpERK, we formulated a mathematical model in which Cic and both the unphosphorylated and phosphorylated forms of ERK are localized in the same compartment (Figure 4A; see Supplemental Materials and Methods for model details). The model describes the conversion of an inactive form of ERK (E) to its active dually phosphorylated form, dpERK (E*), in the presence of active enzyme (MEK) that phosphorylates ERK and phosphatases that

FIGURE 3: Localization studies of Cic, ERK, and dpERK in S2 cells, and ERK phosphorylation analysis. In A–C‴, transfected expression constructs are shown on the left, and staining signals are shown on the individual panels. In A and C, dashed lines indicate nuclear boundaries. Scale bars, 5 µm. (A–A‴) Cic-V5 was predominantly cytoplasmic, with some nuclear distribution. (B–B‴) When cotransfected with MEK and Raf, both ERK-Flag and dpERK signals were mostly cytoplasmic but also showed nuclear localization. (C–C‴) When Cic-V5 and ERK-Flag were cotransfected with Raf and MEK, the Cic-V5 and dpERK signals remained predominantly cytoplasmic, with some nuclear distribution. (D) Extracts from S2 cells transfected with vector control, ERK alone, or ERK together with MEK and RAF were analyzed by Western blotting. A representative blot of three independent experiments is shown. (E) Quantification of results in D. No significant down-regulation of unphospho-ERK signal was observed with cotransfection of Raf and MEK, despite a detectable up-regulation of dpERK. n = 3; n.s., not significant (p > 0.05).
dephosphorylate ERK. Active enzyme E* binds to and promotes the degradation of its substrate (S), which is continuously synthesized and also undergoes intrinsic degradation when free or in any complex. Inactive enzyme E also binds S, but does not cause its degradation. In this model, an outcome of successful signal propagation is a reduction in the level of S (Figure 4A).

We investigated the effects of the relative strengths of binding of substrate (S) to the inactive (E) and active (E*) forms of enzyme, which are controlled by the binding parameters $\beta_3$ and $\beta_4$, respectively. The $\beta$ parameters in this model are Michaelis–Menten constants rescaled by the total ERK concentration and indicate the concentrations of unmodified ($\beta_2$) or modified ($\beta_4$) enzyme at which unbound substrate concentration falls due to binding or degradation. Therefore, smaller values of $\beta_3$ and $\beta_4$ indicate stronger interactions of Cic with the corresponding form of ERK; that is, smaller $\beta_3$ indicates stronger binding of S to E, whereas smaller $\beta_4$ indicates stronger binding of S to E*. At steady state, varying the ratio of $\beta_3/\beta_4$ revealed that strong preferential association of E* with S (large values of $\beta_3/\beta_4$) is required for efficient substrate down-regulation (Figure 4B, blue curve). In principle, inactive E could interact with S and compete with active E*. However, under this condition, the pathway cannot function efficiently, as shown by an inability of S to be degraded under small values of $\beta_3/\beta_4$.
either the single H138A mutation, the activating mutation in the residue in vivo, we generated SBP-tagged variants of ERK that carry its interactions with the C2 domain of mammalian ERK2 reduced the binding affinity to the C2 domain (Futran et al., 2015). One such residue is H123, whose mutation to alanine in Drosophila Cic (Futran et al., 2011). Overexpression of wild-type ERK-SBP did not alter the normal venation pattern (Figure 5A), while overexpression of ERK<sup>Sen</sup>-SBP promoted formation of ectopic veins (Figure 5B). This phenotype is likely caused by ectopic inhibition of Cic function by overactive ERK, outside the normal areas of vein formation (Roch et al., 2002; Ajuria et al., 2011). Interestingly, while overexpression of ERK<sup>H138A-SBP</sup> alone did not alter the normal venation pattern (Figure 5C), this mutation in combination with Sevenmaker within the same polypeptide (ERK<sup>Sen/H138A-SBP</sup>) strongly suppressed the ability of the double-mutant protein to induce ectopic veins (Figure 5D). Therefore, the H138A mutation is completely dominant over the activating mutation ERK<sup>Sen</sup>, when tested in cis within the same protein.

The inability of ERK<sup>Sen/H138A-SBP</sup> to induce ectopic veins might be interpreted solely through loss of binding to Cic, which is expected for the H138A mutation (Futran et al., 2015). However, it is possible that a reduction of binding to Cic would also lead to a reduction in steady-state level of dpERK due to the action of phosphatases (Kim et al., 2011), which would contribute to the inability of the double-mutant protein to induce ectopic veins. To distinguish between these possibilities, we compared steady-state phosphorylation levels of various SBP-tagged ERK mutants. Protein extracts from dissected larval wing disks did not provide sufficient dpERK for analysis on Western blots. We therefore analyzed dpERK levels of the ERK-SBP variants in Drosophila embryos using the da-GAL4 driver, which is expressed ubiquitously. Because all ERK-SBP variants were injected as a matching set into the same genomic location and provided equivalent levels of total ERK (Figure 5E), expression in the embryo can be used as a valid method of comparison of dpERK levels. The level of phosphorylation (dpERK signal) of the ERK<sup>Sen</sup>-SBP protein was much higher than that of the wild-type ERK-SBP or ERK<sup>H138A-SBP</sup> (Figure 5E). Interestingly, the level of phosphorylation of the double-mutant ERK<sup>Sen/H138A-SBP</sup> protein was much lower than that of ERK<sup>Sen-SBP</sup> (Figure 5E). This result reveals two important properties of the mutant ERK proteins. First, it shows that the ERK<sup>Sen</sup> protein is still susceptible to inactivation by phosphatases, because a reduction in Cic binding due to the H138A mutation resulted in a reduction in steady-state phosphorylation of ERK<sup>Sen/H138A-SBP</sup>. Second, it suggests that the inability of the ERK<sup>Sen/H138A-SBP</sup> protein to induce the ectopic vein phenotype is due to a combination of both reduced binding to Cic and a strong reduction in phosphorylation. These results underscore the importance of Cic-ERK interactions in vivo.
and highlight their importance for maintaining high levels of dpERK, which is required for pathway activity (seen here as formation of ectopic wing veins).

Conclusion

Previous studies have shown that substrates of ERK may have preferences for binding to either the active or the inactive form of ERK (Lee et al., 2004; Burkhard et al., 2011) and have mapped the dynamic ERK interactome (von Kriegsheim et al., 2009). Here, we established that phosphorylated ERK (dpERK) interacts with its substrate Cic more strongly than unphosphorylated ERK. Our data suggest that the preferred binding of Cic to dpERK is functionally important at two levels: first, it prevents a possible competition between ERK and dpERK for binding to Cic, when these proteins are localized in the same cellular compartment; second, it may allow for efficient signal propagation when only a small proportion of ERK is converted to dpERK. In support of the latter, we have shown that only a small fraction of ERK gets phosphorylated upon activation with Raf and MEK in cultured S2 cells (Figure 3 and Supplemental Figure S2), consistent with our previous results in vivo (Johnson et al., 2017).

In Drosophila, ERK-mediated Cic phosphorylation and down-regulation is involved in most developmental contexts that are under ERK control. Drosophila Cic interacts with dpERK through the C2 docking site, which is not well conserved in other species (Astigarraga et al., 2007; Futran et al., 2015). C2 domain-mediated down-regulation is therefore a unique property of fly Cic; however, the exact mechanism of Cic down-regulation upon dpERK binding is not fully understood. It likely involves phosphorylation of multiple sites in Cic upon binding of dpERK through the C2 domain (S.P., unpublished data). One of the potential mechanisms of Cic down-regulation is proteolytic degradation (Roch et al., 2002; Astigarraga et al., 2007; Suisse et al., 2017). A stronger binding of Cic to dpERK may thus represent a mechanism to ensure efficient phosphorylation and subsequent degradation of Cic, possibly still in the dpERK-bound state. This would ensure that dpERK is available for phosphorylating other substrates like Bicoid and Hunchback during embryonic patterning, after Cic protein levels are reduced (Ronchi et al., 1993; Löhrr et al., 2009; Kim et al., 2010). We have shown by mathematical modeling that preferential association of Cic with dpERK also contributes to the maintenance of a steady-state level of dpERK, which in turn is required for pathway output (i.e., Cic degradation), which is critical for the patterning of the embryonic termini (Astigarraga et al., 2007). Together, our results suggest that activation-induced high-affinity binding of dpERK to Cic is an important part of ERK signaling dynamics that can increase both the specificity and efficiency of signaling.

MATERIALS AND METHODS

Expression constructs, cell culture, and immunoprecipitations

Construction of C-terminally tagged full-length pMT-Cic-V5 and pMK33-Cic-SBP was described in Yang et al. (2016). Construction of C-terminally Flag-tagged Drosophila ERK(Rolled)-SBP was described in Yang et al. (2017). Site-directed mutagenesis was performed using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs) on pMT-ERK-Flag following the manufacturer’s protocol to generate ERK<sub>Gan</sub>, ERK<sub>Gan/H138A</sub>, and ERK<sub>Gan/H138A</sub>, which were then subcloned into pUAST-attB-SBP. Drosophila S2 cells were used for all cell-based assays. Cells were cultured at 25°C in standard Schneider’s S2 medium with 10% fetal bovine serum (Life Technologies) and 5% Pen/Strep (Invitrogen). For stable expression in S2 cells, a pMK33-Cic-SBP construct was transfected by using Effectene transfection reagent (Qiagen), and stable cell lines were selected in the presence of 300 μg/ml hygromycin (Sigma). Transient DNA transfections were performed using Effectene transfection reagent (Qiagen). At 24 h after transfection with indicated plasmids, cells were induced with 0.35 mM CuSO<sub>4</sub> and incubated overnight to allow expression of the protein. Cells were harvested and then lysed with default lysis buffer (50 mM Tris, pH 7.5, 125 mM NaCl, 5% glycerol, 0.2% IGEPAL CA-630, 1.5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 25 mM NaF, 1 mM Na<sub>2</sub>VO<sub>3</sub>, 1 mM EDTA) containing 2X Complete protease inhibitor (Roche). Cleared cell lysates were incubated with anti-V5 beads (Sigma) or Streptavidin beads (Pierce) at 4°C for 2 h. Beads were washed three times with default lysis buffer, and the protein complexes were eluted with SDS sample buffer. Immunoprecipitations using Cic-Venus–expressing embryos followed a similar protocol using GFP-Trap beads (Chromotek). All experiments were carried out at least twice, and representative results are shown.

Immunoblotting and immunostaining

Protein complexes were resolved on 8% SDS protein gels and transferred onto Millipore Immobilon-FL PVDF Transfer Membranes with 0.45-μm pores. Primary antibodies used for Western blots were as follows: mouse anti-ERK 1:1000 (Sigma), rabbit anti-total ERK 1:1000 (Cell Signaling Technology), mouse anti-V5 1:1000 (Sigma), rabbit anti-Flag 1:1000 (Sigma), and mouse anti-SBP 1:1000 (Santa Cruz Biotechnology). Secondary antibodies used were as follows: IRDye 800CW donkey anti-rabbit immunoglobulin G (IgG) 1:20,000 (LI-COR) and IRDye 680CW donkey anti-mouse IgG, 1:20,000 (LI-COR). Primary antibodies used for S2 cell staining were as follows: mouse anti-dpERK 1:500 (Sigma), rabbit anti-VP 1:500 (Sigma), rabbit anti-Flag 1:500 (Sigma), and mouse anti–nonphosphorylated ERK 1:500 (Sigma). Secondary antibodies used were as follows: donkey anti-rabbit Alexa Fluor 488 1:500 (Invitrogen) and goat anti-mouse Alexa Fluor 555 1:500 (Invitrogen). Stained cells were mounted with Prolong Gold anti-fade mounting reagent (Life Technologies) and 5% Pen/Strep (Invitrogen). For stable expression in S2 cells, overnight cultures were subcultured into 1 l LB (Luria–Bertani) medium supplemented with 100 μg/ml ampicillin to a starting OD<sub>600</sub> of 0.02, and cultures were grown at 37°C with agitation at 250 rpm until they reached an OD<sub>600</sub> of 1.0. Protein expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and cultures were grown at 22°C for 6 h with agitation at 250 rpm. Bacterial cell pellets were harvested by centrifugation and stored at −20°C. For the expression of phosphorylated ERK2 (dpERK2), the plasmids encoding tagged rat ERK2 in pQE80 and tagged constitutively active (CA)-MEK1 (MEK1-G7B; Mansour et al., 1996) in

Purification of unphosphorylated and phosphorylated ERK from bacteria

For the expression of ERK2, the plasmid encoding tagged rat ERK2 in pQE80 was transformed into Escherichia coli BL21(DE3)-competent cells. Overnight cultures were subcultured into 1 l LB (Luria–Bertani) medium supplemented with 100 μg/ml ampicillin to a starting OD<sub>600</sub> of 0.02, and cultures were grown at 37°C with agitation at 250 rpm until they reached an OD<sub>600</sub> of 1.0. Protein expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and cultures were grown at 22°C for 6 h with agitation at 250 rpm. Bacterial cell pellets were harvested by centrifugation and stored at −20°C. For the expression of phosphorylated ERK2 (dpERK2), the plasmids encoding tagged rat ERK2 in pQE80 and tagged constitutively active (CA)-MEK1 (MEK1-G7B; Mansour et al., 1996) in...
pBAD33 were transformed into E. coli BL21(DE3)-competent cells. Overnight cultures were subcultured into 1 L LB medium supplemented with 100 μg/ml ampicillin and 25 μg/ml chloramphenicol to a starting OD600 of 0.02, and cultures were grown at 37°C with agitation at 250 rpm. At an OD600 of 0.8, 0.1% l-arabinose was added, and the temperature was shifted to 22°C. At OD600 1.0, ERK2 protein expression was induced with 1 mM IPTG, and cultures were grown at 22°C for 6 h with agitation at 250 rpm. Bacterial cell pellets were harvested by centrifugation and stored at −20°C. For all protein purifications, cell pellets were resuspended in 40 ml of 10 mM imidazole, 300 mM NaCl, and 50 mM Na2HPO4 (pH 8) and lysed by treatment with lysozyme and sonication on ice. Cell debris was removed by centrifugation, and the supernatant was sterile filtered. All proteins were purified from clarified lysate using Ni-NTA agarose resin (Qiagen) following the manufacturer’s recommendations. ERK2 was buffer exchanged into 50 mM HEPES, 100 mM NaCl, 20 mM MgCl2, and 10% glycerol (pH 7.4) using PD-10 desalting columns (Bio-Rad). Aliquots of 5–50 μl were snap frozen in liquid nitrogen and stored at −80°C.

In vitro binding assays
S2 cells were transfected with pMT-Cic-V5 or empty vector. Transfected cells were preincubated with 2 μM PD0325901, an MEK inhibitor (Biotang) with dimethyl sulfoxide as vehicle, for 3 h before induction. Cells were induced with 0.35 mM CuSO4 and incubated overnight. Extracts prepared as described earlier (except additional IGEPAL was added to default lysis buffer for a final concentration of 0.4%), and were incubated with anti-V5 beads for 2 h at 4°C. After three washes, the Cic-V5-bound beads were incubated with 500 ng of purified ERK2 or dPERK2 in default lysis buffer for 2 h at 4°C. Bovine serum albumin was added in binding solution to the final concentration of 0.05%, to reduce nonspecific binding to the beads.

**Drosophila melanogaster stocks**
Fly stocks and crosses were maintained on standard yeast–cornmeal–agar medium at 25°C or 18°C. MS1096-GAL4 was from the Bloomington Drosophila Stock Center. UAS-MEKK203K, Histone-GFP, and P(α4-GAL-VP16)67, used as a maternal driver, were described in Goyal et al. (2017a). Wild-type Drosophila ERK (rolled) as well as ERK mutants, C-terminally tagged with GFP, were subcloned into pUAST-attB, and transgenic lines were generated by inserting the constructs into the attP40 genomic site by using epC31-based integration system (Venken et al., 2006; Bischof et al., 2007). All constructs were sent for injection together and therefore provide a matching set for comparisons of expression levels. Cic-Venus uses genomic regulatory sequences for expression and was described in Grimm et al. (2012).

**Wing phenotypes**
Transgenic male flies were crossed with M51096-GAL4 virgins, and the wings of the resulting female progeny were imaged with Olympus BX60 compound microscope using bright-field illumination and a 4× objective.

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