Supplementary Information

Supplementary Materials and Methods

In vitro transcription and translation
TNT High-Yield Wheat Germ In vitro system (Promega) was used to produce protein from HA-Krz, HA-Krz-R209E, ERK-Flag, Cactus and GFP constructs cloned into the pSP73 vector. Reaction was brought up to 250 µl with PBS and incubated with 20 µl anti-HA beads with rotation at 4°C for 2 hours. Samples were washed and then treated for western blotting as described in the main text.

Ubiquitination Assay
S2 cells were transfected with HS-HA-Ubiquitin (a gift from R. Fehon), ERK-Flag, and/or GFP-Krz, as indicated in Supplemental Figure 5. ERK-Flag and GFP-Krz were expressed under a copper inducible promoter overnight. HA-ubiquitin was expressed from a heat shock promoter by incubation at 37°C for 30 minutes. After heat shock cells were allowed to recover at 25°C for 2 hours. Protein extraction and co-immunoprecipitation was performed as described in the main text, except 10 mM NEM (Sigma, St.Louis, MO) was added to the default lysis buffer. Samples were analyzed by western blotting.

ERK degradation Assay
S2 cells were transfected with ERK-Flag alone, or together with either HA-Krz or HA-Krz-R209E. After an overnight induction with 0.35 mM CuSO₄ cells were treated with MG132 at a final concentration of 50 µM and incubated for 4 hours at 25°C. An initial 0 time-point sample was taken before addition of cycloheximide (CHX, Sigma, St Louis, MO) to a final concentration of 100 µg/ml. Once CHX was added samples were taken every hour for 5 hours.
Cells were transferred to conical tubes and gently rocked during the incubation period to avoid cell settling. As each time-point collected samples were spun down at 500 g for 5 min at 4°C. These samples were then lysed with default lysis buffer, cleared at 15,000 g for 15 min at 4°C, and boiled for 6 min in SDS buffer. Samples were analyzed by western blotting.

**Expression constructs**

Full-length *Drosophila* MEK (Dsor1) and human β-arrestin2 open reading frames were amplified by PCR using epitope tag-containing primers and cloned into the pMT/V5-His series vectors (Invitrogen, Eugene, OR) to generate carboxy-terminally tagged MEK-V5 and amino-terminally tagged HA-β-arrestin2. *Drosophila* activated mutant MEK-S237E-S241E (MEK-SE-SE) and a β-arrestin2 “pre-activated” mutant R170E were generated with GeneTailor site-directed mutagenesis system (Invitrogen, Eugene, OR).

**Supplementary Figure legends**

**Supplementary Figure S1. Analysis of Krz expression in *krz* maternal mutants and rescue of *krz* function by the *krz 5.7* transgene.**

(A, B) Visualization of Krz with anti-Krz antibody. (A) A stage 4 FRT control embryo. Krz protein is distributed throughout the embryo and is primarily localized in the cytoplasm. (B) A stage 4 *krz* maternal mutant embryo. Krz protein is undetectable. (C) General embryo morphology visualized using anti-Elav antibody which stains all neurons in stage 15 *krz* maternal mutant embryo obtained from females carrying one copy of the genomic rescue construct, *krz 5.7*. Wild type morphology suggests full rescue of *krz* maternal mutant phenotype. (D) A western blot on extracts from 0-4 hour FRT control embryos, *krz* maternal mutant embryos, and *krz* maternal mutant embryos obtained from females carrying one copy of the
genomic rescue construct, _krz 5.7_. Krz protein is lost in the mutants but is present in _krz 5.7_ rescued embryos. IB: immunoblot. HSP70 antibody was used as a loading control. (E) Cuticular preparation of a _krz<sup>1</sup>_ maternal mutant embryo obtained from females carrying one copy of the genomic rescue construct, _krz 5.7_. Rescued embryos were indistinguishable from wild type controls.

**Supplementary Figure S2. An algorithm to quantify dpERK levels in embryos and a comparison of dpERK expression in FRT control and Hist-GFP embryos.**

Quantification of dpERK patterns was performed as in {Coppey, 2008 #372}. (A) Quantification begins at the mid-dorsal point of the embryo and continues toward the anterior, ventral, posterior and finally ending back at the mid-dorsal point of the embryo. At each point along the embryo an intensity measurement is taken. (B) The data are plotted starting from the center of the dorsal side of the embryo. At approximately 2.5 hours of embryonic development dpERK is expressed at the embryo poles and thus is seen as peaks in intensity with the dorsal and ventral expression as troughs on the graph. (D, dorsal; A, anterior; V, ventral; P, posterior). (C, D) Expression of doubly phosphorylated activated ERK (dpERK) detected using anti-dpERK antibody in a stage 4 _Histone-GFP_ embryo (C) and an FRT control embryo (D). (E, F) Quantification of dpERK in 28 _FRT_ and 28 _Hist-GFP_ embryos. (E) Average dpERK intensity at the anterior and posterior termini (au, arbitrary signal intensity units). (F) Average width of dpERK expression extending from anterior and posterior termini. (G) Average intensity of dpERK expression plotted around entire embryos on one graph to show a complete representation of the data. Red line represents _FRT_ control embryos. Blue line represents _Hist-GFP_ embryos. Note that the two datasets are indistinguishable.

**Supplementary Figure S3. Interactions between _Drosophila_ ERK and human β-arrestin2.**
S2 cells were transfected with human HA-Arrb2 or HA-Arrb2-R170E together with the indicated *Drosophila* ERK-Flag versions. Samples were immunoprecipitated with anti-HA beads and analyzed by western blotting. IP: immunoprecipitated samples. IB: immunoblots.

**Supplementary Figure S4. Interactions between Krz and ERK using in vitro translated proteins.**

Using in vitro translation, GFP (A) and *Drosophila* ERK-Flag (B) were translated either alone or together with HA-Krz-R209E. Samples were immunoprecipitated with anti-HA beads and analyzed by western blotting. IP: immunoprecipitated samples. IB: immunoblots.

**Supplementary Figure S5. Effects of co-expression of Krz and ERK on ERK ubiquitination and turnover.**

(A) S2 cells were transfected with various combinations of *Drosophila* ERK-Flag, HA-Ubiquitin, Krz-GFP, and/or Krz-R209E-GFP as indicated. Samples were immunoprecipitated with anti-Flag beads. Lysates and immunoprecipitates were analyzed by western blot with anti-Flag, anti-HA, and anti-GFP antibodies. (B) S2 cells were transfected with *Drosophila* ERK-Flag alone or in combination with HA-Krz or HA-Krz-R209E. Cells were treated with cycloheximide (CHX) and collected at one hour intervals. Lysates were analyzed by western blot with anti-Flag antibody. Protein levels were measured by densitometry and plotted as a fraction of ERK remaining at each time-point. IP: immunoprecipitated samples. IB: immunoblots.

**Supplementary Figure S6. Krz interacts with *Drosophila* Raf and MEK and can compete with MEK for binding to ERK.**

(A) S2 cells were transfected with GFP-Krz or GFP-Krz-R209E alone or together with *Drosophila* HA-Raf (phl). Samples were immunoprecipitated with anti-HA beads and analyzed by western blotting. (B) S2 cells were transfected with *Drosophila* MEK-V5 (Dsor1) or an
activated mutant Drosophila MEK-SE-SE-V5 alone or together with indicated HA-Krz versions. Drosophila MEK-S237E-S241E construct corresponds to activated mammalian MEK2-S218E-S222E, which was reported to phosphorylate ERK independent of pathway activation. Samples were immunoprecipitated with anti-HA beads and analyzed by western blotting. (C) S2 cells were transfected with ERK-Flag in combination with HA-Krz and/or MEK-V5. Samples were immunoprecipitated with anti-V5 beads and analyzed by western blotting. (D) S2 cells were transfected with Myc-Krz alone or in combination with ERK-Flag or ERK-Flag and MEK-V5. Samples were immunoprecipitated with anti-Flag beads and analyzed by western blotting. IP: immunoprecipitated samples. IB: immunoblots.

**Supplementary Figure S7. Interaction between Krz and Cactus using in vitro translated proteins.**

Cactus was translated either alone or together with HA-Krz-R209E, or HA-Krz. Samples were immunoprecipitated with anti-HA beads and analyzed by western blotting. Anti-Cactus antibody was a gift from Steven Wasserman. IP: immunoprecipitated samples. IB: immunoblots.
**Average dpERK intensity**

<table>
<thead>
<tr>
<th></th>
<th>Anterior (au)</th>
<th>Posterior (au)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hist-GFP</strong></td>
<td>61.0 ± 9.8</td>
<td>43.3 ± 9.7</td>
</tr>
<tr>
<td><strong>FRT</strong></td>
<td>62.2 ± 11.2</td>
<td>41.9 ± 12.0</td>
</tr>
<tr>
<td>t-test</td>
<td>0.66</td>
<td>0.62</td>
</tr>
</tbody>
</table>

**Width of dpERK expression**

<table>
<thead>
<tr>
<th></th>
<th>Anterior (au)</th>
<th>Posterior (au)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hist-GFP</strong></td>
<td>13.3 ± 2.1</td>
<td>15.7 ± 2.2</td>
</tr>
<tr>
<td><strong>FRT</strong></td>
<td>12.6 ± 1.9</td>
<td>16.7 ± 2.1</td>
</tr>
<tr>
<td>t-test</td>
<td>0.19</td>
<td>0.07</td>
</tr>
</tbody>
</table>
Supplementary Figure S3
Supplementary Figure S4
Supplementary Figure S5

A

<table>
<thead>
<tr>
<th></th>
<th>ERK-Flag</th>
<th>HA-Ubiquitin</th>
<th>Krz-GFP</th>
<th>Krz-R209E-GFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysates:</td>
<td>anti-Flag</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Lysates:</td>
<td>anti-HA</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Lysates:</td>
<td>anti-GFP</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

B

- **A**
  - Time, hrs: 0 1 2 3 4 5
  - Fraction of ERK-Flag
- **B**
  - Time, hrs: 0 1 2 3 4 5
  - Fraction of ERK-Flag
Supplementary Figure S7