

Developmental Cell

A Real-Time Biosensor for ERK Activity Reveals Signaling Dynamics during *C. elegans* Cell Fate Specification

Highlights

- ERK-nKTR is a genetically encoded biosensor for extracellular signal-regulated kinase
- Qualitative or quantitative assessment is possible by analyzing individual nuclei
- ERK-nKTR is a faithful reporter for ERK activity in several different cell types
- In the vulval precursor cells, there is pulsatile, frequency-modulated signaling

Authors

Claire de la Cova, Robert Townley,
Sergi Regot, Iva Greenwald

Correspondence

sregot@jhmi.edu (S.R.),
isg4@columbia.edu (I.G.)

In Brief

A genetically encoded biosensor for ERK activity allows qualitative or quantitative assessment by analysis of individual nuclei during development and neural function in diverse cell contexts in *C. elegans*. In-depth analysis of ERK activity over time in the vulval precursor cells has identified dynamic features including pulsatile frequency-modulated signaling.

A Real-Time Biosensor for ERK Activity Reveals Signaling Dynamics during *C. elegans* Cell Fate Specification

Claire de la Cova,^{1,2} Robert Townley,¹ Sergi Regot,^{3,4,*} and Iva Greenwald^{1,2,4,5,*}

¹Department of Biological Sciences, Columbia University, New York, NY, USA

²Department of Biochemistry & Molecular Biophysics, Columbia University Medical Center, New York, NY, USA

³Department of Molecular Biology & Genetics, The Johns Hopkins University School of Medicine, Baltimore, MD, USA

⁴Senior author

⁵Lead Contact

*Correspondence: sregot@jhmi.edu (S.R.), isg4@columbia.edu (I.G.)

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SUMMARY

Kinase translocation reporters (KTRs) are genetically encoded fluorescent activity sensors that convert kinase activity into a nucleocytoplasmic shuttling equilibrium for visualizing single-cell signaling dynamics. Here, we adapt the first-generation KTR for extracellular signal-regulated kinase (ERK) to allow easy implementation *in vivo*. This sensor, “ERK-nKTR,” allows quantitative and qualitative assessment of ERK activity by analysis of individual nuclei and faithfully reports ERK activity during development and neural function in diverse cell contexts in *Caenorhabditis elegans*. Analysis of ERK activity over time in the vulval precursor cells, a well-characterized paradigm of epidermal growth factor receptor (EGFR)-Ras-ERK signaling, has identified dynamic features not evident from analysis of developmental endpoints alone, including pulsatile frequency-modulated signaling associated with proximity to the EGF source. The toolkit described here will facilitate studies of ERK signaling in other *C. elegans* contexts, and the design features will enable implementation of this technology in other multicellular organisms.

INTRODUCTION

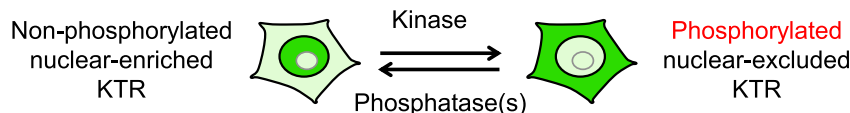
Studies of extracellular signal-regulated kinase (ERK) function have revealed its profound and pervasive roles in development, physiology, and disease (Arur et al., 2009; Kolch et al., 2015). ERK is the most downstream protein kinase of the canonical Ras-Raf-MEK-ERK cascade that is activated by a diverse set of stimuli and transduced by growth factor or G-protein-coupled receptors. Upon stimulation, positive and negative feedback mechanisms act on multiple levels of the pathway, operating in a stimulus-specific and cell type-specific manner. As a consequence, a variety of ERK activity kinetics are observed in a range of cell types or in response to different stimuli; these include

transient, sustained, and pulsatile ERK responses (Albeck et al., 2013; Aoki et al., 2013; Hiratsuka et al., 2015; Lim et al., 2015; Mattingly et al., 2015; Santos et al., 2007; Shankaran et al., 2009). It has been proposed that such dynamic characteristics of signaling may in themselves account for the diversity of ERK-dependent outcomes, such as its ability to trigger graded or switch-like events at the molecular level, and its potential to drive a number of different biological processes at the cellular level (Avraham and Yarden, 2011). In a classic example, rat PC12 cells employ different positive or negative feedback mechanisms to regulate the nerve growth factor receptor (TrkA) and epidermal growth factor receptor (EGFR), resulting in either sustained ERK activity and differentiation in response to TrkA activation, or transient ERK activity and proliferation in response to EGFR activation (Marshall, 1995; Ryu et al., 2015; Santos et al., 2007).

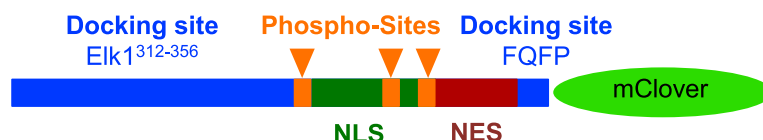
Our understanding of how protein kinases transform stimuli into cellular responses has been enhanced by the development of genetically encoded fluorescent activity sensors, the first generation of which were based on Förster resonance energy transfer (FRET) (Harvey et al., 2008; Ting et al., 2001). Assayed in living cells, genetically encoded sensors allow monitoring of ERK activity in a longitudinal fashion, in the same cells over time, and reveal additional levels of ERK signaling complexity. For example, in steady-state conditions in cultured mammalian cells, ERK activity oscillates with a frequency modulated by epidermal growth factor (EGF) dose as a result of mechanisms acting at the level of the receptor (Albeck et al., 2013; Aoki et al., 2013; Sparta et al., 2015). Intriguingly, within intact living mouse skin, pulses of ERK activity spontaneously arise and spread to neighboring cells (Hiratsuka et al., 2015). Together, these observations highlight the relevance of visualizing ERK signaling activity in real time in a variety of cellular contexts *in vitro* and *in vivo*.

Kinase translocation reporters (KTRs) offer a powerful approach to studying kinase activity and dynamics *in vivo* that is not based on FRET technology (Regot et al., 2014). KTRs are genetically encoded fluorescent sensors that convert phosphorylation state into a nucleocytoplasmic localization within the cell. KTRs are constructed by juxtaposing a docking site for a kinase to a module containing a phospho-inhibited nuclear localization signal (NLS), a phospho-enhanced nuclear export signal (NES), and a fluorescent protein. When the cognate kinase

A KTR method



B ERK-KTR protein



C Bi-cistronic reporter transgene (nKTR)



D Ratio Red/Green used to quantify ERK-nKTR localization

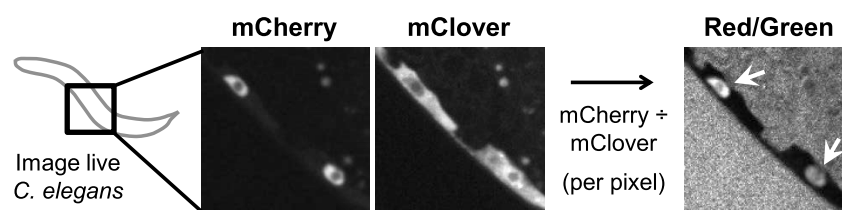


Figure 1. The Nuclear Ratio ERK-nKTR Design

(A) The KTR method (Regot et al., 2014). When kinase activity is low, the KTR sensor protein is not phosphorylated and is nuclear enriched. When kinase activity is high, the KTR sensor is phosphorylated and excluded from the nucleus. (The KTR is always largely excluded from the nucleolus.) (B) The ERK-KTR sensor is composed of peptides from Elk1 (Elk1³¹²⁻³⁵⁶ and FQFP), which promote ERK docking to substrates, followed by a module containing NLS and NES sequences, S/T-P phospho-acceptor residues for phosphorylation by ERK (Phospho-Sites), and the GFP mClover. An annotated protein sequence of ERK-nKTR-mClover is shown in Table S2.

(C) Bicistronic transgene design of ERK-nKTR: the coding region for a viral 2A peptide (T2A) is inserted between the coding regions for ERK-KTR-mClover and mCherry-H2B. The T2A peptide triggers “ribosomal pausing” efficiently in *C. elegans* (Ahier and Jarriault, 2014), such that ERK-nKTR-mClover and mCherry-H2B are encoded by a single transcript but produced as two separate, functional proteins in an equimolar ratio. Tissue-specific expression of the bicistronic transcript is achieved using appropriate promoter (prom) and 3' UTR sequences.

(D) After imaging of live *C. elegans*, the ratio of mCherry to mClover intensity is calculated per pixel, producing a new image (Red/Green), a pictorial representation of the red/green ratio within nuclei (arrows), as quantified in Figures 2 and 3.

is inactive, the KTR is unphosphorylated and mostly nuclear; when the cognate kinase is active, phosphorylation of the KTR results in cytoplasmic translocation (Figure 1A). The modularity of KTRs enabled the development of ERK-KTR, a sensor for ERK kinase activity, utilizing conserved ERK docking sites from the ERK substrate Elk1 (Figure 1B) (Regot et al., 2014). Furthermore, this sensor was shown to be a rapid, specific, and quantitative reporter for ERK kinase activity in human cultured cells, revealing fast pulsatile dynamics in individual cells in response to growth factors; the kinetics are similar to an FRET-based reporter but has the advantages of a higher dynamic range and a more rapid response to downregulation of kinase activity (Regot et al., 2014; Sparta et al., 2015). The success of this approach in cultured cells suggested that, for developmental biology questions, the versatility of the ERK-KTR method would be especially valuable compared with other methods, as it can be observed by eye without the need for image processing, making it appropriate for rapid visual screens or, alternatively, used for image capture and quantification of ERK activity dynamics during development.

Here, we report that we have successfully adapted this technology for visualizing ERK activity *in vivo*, and show that it has the anticipated versatility for both visual inspection and quantitative analysis. We term the adapted design a “nuclear KTR” (nKTR) because it allows for quantitation of kinase activity based solely on the level of the sensor in nuclei. This adaptation facilitates the use of the KTR approach when cells are closely packed

together, a situation often encountered *in vivo*. We then showed that the ERK-nKTR sensor faithfully reports activity of the ERK ortholog MPK-1 in several cell types and different developmental processes in *Caenorhabditis elegans*: vulval precursor cell (VPC) fate specification, sex myoblast (SM) migration, AWC and ASE sensory neurons, and germline development. Finally, we analyzed ERK activity dynamics in VPCs, where the role of EGFR-Ras-ERK signaling promotes VPC “competence” and vulval fate induction. Our observations indicate that sensors have the potential to reveal insights into spatial, temporal, and dynamic aspects of signaling *in vivo*.

Design

A Bicistronic Transgene Strategy for Quantifiable KTR Activity in Nuclei: “nKTR”

An ERK-KTR sensor developed in mammalian cells utilized a conserved ERK docking site from the mammalian ERK substrate Elk1, and kinase activity was quantified by measuring the ratio of cytoplasmic to nuclear fluorescent signal (Figures 1A and 1B) (Regot et al., 2014). Within whole organisms, however, most cells do not reside in flat monolayers; instead, they may be packed tightly against other cells in three dimensions, have complex cytoplasmic shapes, and change in size, shape, or form syncytia even while the nucleus remains discrete and stable (except during cell division). These situations can make quantitation of the cytoplasm unreliable (Figure S1A); we therefore sought to develop a method that would allow us to quantify KTR

localization based solely on fluorescence level in the nucleus, and furthermore would not rely on absolute values of fluorescence signal and thus would be relatively insensitive to cell size and transgene vagaries such as transcriptional noise and promoter strength.

Our solution was to create reporters in which equimolar expression of a KTR-mClover biosensor and the nuclear marker mCherry-H2B is achieved from a single transcript using the 2A peptide. This virus-derived structural motif causes “ribosomal pausing,” leading to the production of distinct proteins during translation, and works efficiently in *C. elegans* (Ahier and Jarriault, 2014) (Figure 1C). We designate such reporters “nKTRs,” for nuclear ratio Kinase Translocation Reporters. To quantify kinase activity using the nKTR form of the ERK-KTR, we (1) acquire z stacks of nKTR-mClover and mCherry-H2B fluorescence, (2) generate a ratiometric image by dividing the mCherry (“red”) by the mClover (“green”) intensities, pixel by pixel, resulting in a new image called “red/green” (Figure 1D) (STAR Methods), and (3) use the mCherry-H2B image to segment and track the nuclei of all cells over the entire z stack. This design innovation will facilitate analysis in any other context, including cultured cells, and should be able to be incorporated into any KTR-type reporter, because the docking site is what governs kinase specificity (Regot et al., 2014).

We also incorporated other features and tests into the reporter design, as will be described further below. First, to simplify image collection and cell identification as we developed and tested the design, we used tissue-specific regulatory sequences to drive ERK-nKTR expression in defined cells. Second, to avoid expression-level vagaries typical of multicopy transgenes in *C. elegans* and to minimize protein overexpression that might overwhelm the equilibrium of nuclear import and export, we made single-copy integrated transgenes for all tissue-specific ERK-nKTR reporters. Third, we made variant ERK-nKTRs in an attempt to improve the dynamic range of the reporter. Finally, we performed genetic tests to validate ERK-nKTR as a faithful reporter of ERK activity in different cell contexts.

RESULTS

Vulval Precursor Cells: Optimizing and Validating the ERK-nKTR Reporter

Studies of VPC fate specification have illuminated many fundamental and conserved features of canonical EGFR-Ras-ERK signaling, including the initial discovery of how ERK docks with its substrates (Jacobs et al., 1998, 1999). The tools and knowledge accumulated through these studies have made it an attractive paradigm for developing and validating the ERK-nKTR approach.

The VPCs are six polarized epithelial cells, numbered P3.p to P8.p, that in the L2 stage are multipotent and have the potential to generate vulval cells. In the L3 stage, the VPCs adopt different fates in a defined pattern via cell-cell interactions (Figure 2A; reviewed in Sternberg, 2005). The pattern is established by an EGF-like signal produced by the anchor cell of the gonad, which activates a canonical EGFR-Ras-Raf-MEK-ERK cascade in VPCs. There are different roles for EGFR-Ras-ERK signaling over the course of the lifetime of the VPCs, and different responses to the EGF-like signal. We

will consider these properties in more detail below, but in this section we focus our analysis on the mid-L2 stage. At this time, the LIN-3/EGF signal evokes a graded ERK response whereby activity is highest in P6.p, lower in the neighboring VPCs, P5.p and P7.p, and not detectable in other VPCs, based on a transcriptional reporter (Yoo et al., 2004). We first demonstrate that the pattern of nuclear accumulation of the nKTR form of the mammalian ERK-KTR reflects the inferred pattern of MPK-1/ERK activity. Then, to validate the new nKTR and nuclear quantitation method, we show that the observed pattern of ERK-nKTR localization requires phosphorylation of the reporter protein and that its localization is correlated with MPK-1/ERK activity levels. Later, we examine ERK-nKTR in a broader range of times to examine signaling dynamics.

The Mammalian ERK-KTR Sensor Is Excluded from the Nucleus Specifically in P6.p

We fused the original ERK-KTR-mClover sensor sequence characterized in mammalian cells (Regot et al., 2014) to T2A-mCherry-H2B and expressed it specifically in VPCs. All details about this transgene and further variants are described in STAR Methods and Table S1. We refer to this reporter as ERK-nKTR(NLS0) to denote the original NLS, as subsequently we mutated the NLS in attempts to achieve a greater dynamic range. We found that localization of the ERK-nKTR(NLS0) reporter was indeed correlated with the inferred pattern of MPK-1 activity in VPCs: it was visibly excluded from the nucleus of P6.p, consistent with high activity of MPK-1, whereas it was present in both the cytoplasm and nucleus of all other VPCs, where MPK-1 is less active (Figure S1B).

Analysis of Variants to Improve the Dynamic Range

Our initial results with the original ERK-nKTR(NLS0) were promising, because quantification indicated a significant red/green signal in P6.p (Figure S1C). To see whether it would be possible to achieve a greater dynamic range, we tested if substituting known *C. elegans* bipartite NLS sequences for the original NLS would improve its nuclear localization and dynamic range (Figures S2A–S2C). We found that, although the NLS sequence from EGL-13 or OCR-2 is sufficient to produce nuclear localization of GFP (Ezak and Ferkey, 2011; Lyssenko et al., 2007), neither NLS was as effective as the original NLS0 when introduced into the sensor (Figure S2D). Most of the smaller engineered changes to NLS0, to alter the number or spacing of basic residues known to be important for NLS function (Conti et al., 1998; Fontes et al., 2000), also did not result in a significant improvement (Figure S2D). However, one variant, ERK-nKTR(NLS3), seemed to be as effective as NLS0, as it was visibly excluded from the nucleus of P6.p where MPK-1 activity is high, and present in the nucleus of other VPCs where MPK-1 activity is low (Figure 2B). When quantified, ERK-nKTR(NLS3) allowed detection of significant differences not only between P6.p and other VPCs but also between the VPCs flanking P6.p, which also receive the EGF-like signal (P5.p and P7.p) and the outermost VPCs (P4.p and P8.p) which do not (Figure 2F). These data suggest that the dynamic range of the ERK-nKTR(NLS3) has an improved linear correlation with the levels of ERK activity found in VPCs. Therefore, we have used the NLS3 variant as the canonical reporter for all subsequent analysis, and designate it ERK-nKTR for simplicity.

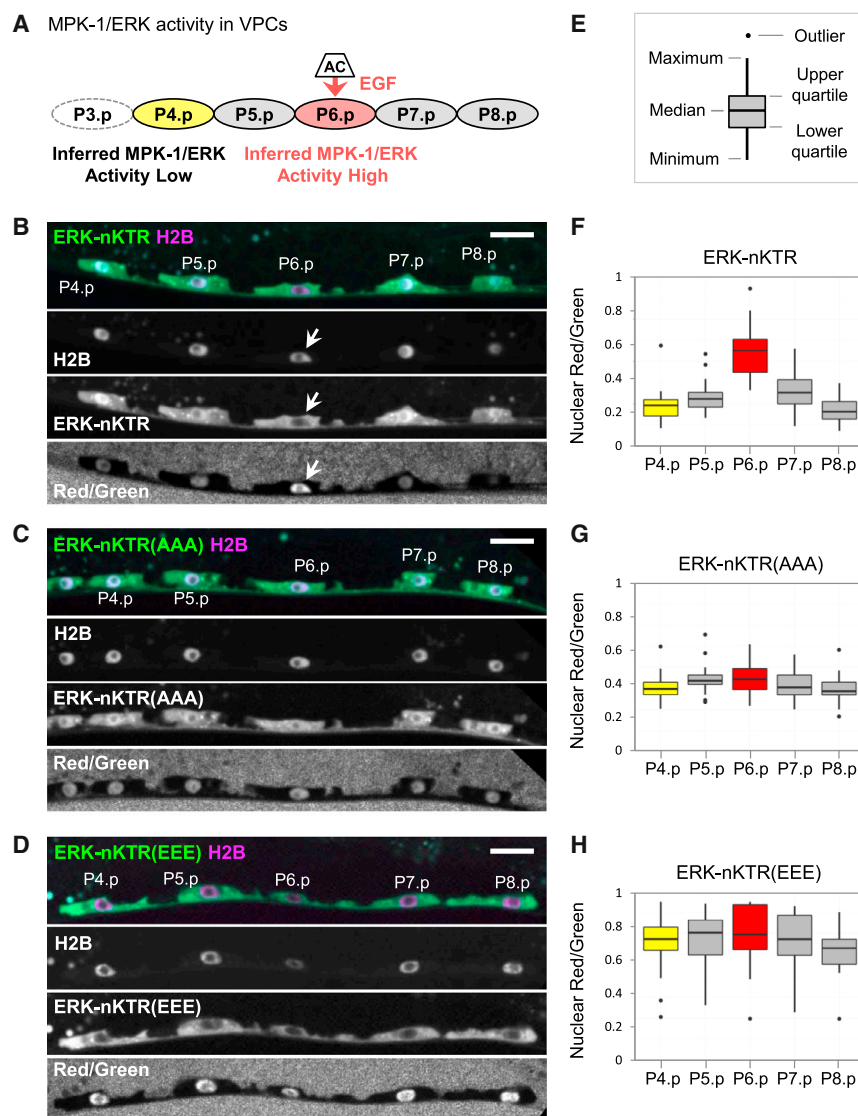


Figure 2. The ERK-nKTR Reports MPK-1/ERK Activity in a Phosphorylation-Dependent Manner

(A) The six VPCs, P3.p to P8.p, receive an EGF signal from the anchor cell (AC). Genetic and transcriptional reporter analyses indicate that the EGF signal results in high MPK-1/ERK activity in P6.p (red), the VPC closest to the AC. In the L2 stage, there is low activation of the EGFR-Ras-ERK pathway in P5.p and P7.p (Yoo et al., 2004); VPCs further from the AC, such as P4.p (yellow), have no MPK-1 activity. This drawing and all images of VPCs are oriented with anterior at the left and dorsal at the top. In half of wild-type hermaphrodites, P3.p often fuses with the hypodermal syncytium in the L2 stage instead of remaining as a VPC, so it is omitted from our analysis.

(B–D) Here, and in all following images of ERK-nKTR expression (Figures 3, 4, and 5), the ERK-nKTR-mClover reporter (ERK-nKTR) is shown in green and mCherry-H2B (H2B) is in magenta. ERK-nKTR, H2B, and the calculated red/green ratio (Red/Green) are also shown separately in grayscale. Scale bars, 10 μ m. (B) The transgene *arT185* expresses ERK-KTR(NLS3), which we call “ERK-nKTR,” in VPCs. ERK-nKTR protein is excluded from the nucleus of P6.p (arrows). (C) Mutation of ERK-nKTR phospho-sites to alanines in the ERK-nKTR(AAA) transgene *arT101* prevents key phosphorylations that occur upon ERK activation; thus, this mutant form is not responsive to ERK activity and accumulates in the nucleus of P6.p. (D) Mutation of the phospho-sites to glutamic acids, mimicking constitutive phosphorylation, in the ERK-nKTR(EEE) transgene *arT100* also makes the mutant form independent of ERK activity, and is excluded from nuclei in all the VPCs.

(E) Explanation of subsequent box plots. Each box-plot graph indicates the median, upper quartile, lower quartile, maximum and minimum values, and outliers (values more than 1.5-fold the interquartile range from either the upper or lower quartile).

(F–H) Red/green ratios for VPC nuclei expressing (F) ERK-nKTR (n = 23), (G) ERK-nKTR(AAA) (n = 19), and (H) ERK-nKTR(EEE) (n = 19).

To assess whether the localization of each reporter is patterned in VPCs, a one-way ANOVA was performed to compare the red/green ratios in all VPCs. For ERK-nKTR, there is a highly significant effect on red/green ratios when different VPCs are compared (at the $p < 0.01$ level, $F(4,109) = 32.1$, $p = 1.16 \times 10^{-17}$). Post hoc pairwise comparisons find significant differences between red/green ratios for the following: P6.p versus P4.p, P5.p, P7.p, or P8.p (all $p < 0.01$), P5.p versus P4.p or P8.p ($p < 0.01$), and P7.p versus P8.p ($p < 0.05$) (see statistical tests in Supplemental Information). In contrast to ERK-nKTR, neither phospho-site mutant is patterned; there are not significant differences in red/green ratios between VPCs expressing ERK-nKTR(AAA) (at the $p < 0.01$ level, $F(4,90) = 2.36$, $p = 0.060$) or ERK-nKTR(EEE) (at the $p < 0.01$ level, $F(4,89) = 0.661$, $p = 0.621$).

Mutation of ERK-nKTR Indicates that the Sensor Subcellular Localization Is Phosphorylation Dependent

The translocation of KTRs upon phosphorylation depends on the negative charge introduced into the NLS segment by kinase-specific phosphorylation. Thus, non-phosphorylatable and phospho-mimetic mutants should represent the full dynamic range of the sensor, and would be expected to have a similar red/green ratio in all VPCs even though ERK activity state differs among them. We therefore created variants in which three phospho-acceptor sites (see ERK-nKTR protein sequence in Table S2) were mutated to either alanine (AAA) or glutamic acid (EEE). Localization of ERK-nKTR(AAA) should be the most nuclear possible for ERK-nKTR in VPCs; conversely, localization

of ERK-nKTR(EEE) should be the most cytoplasmic possible. Indeed, unlike wild-type ERK-nKTR, the non-phosphorylatable ERK-nKTR(AAA) form was not excluded from the nucleus in P6.p (Figure 2C), whereas the phospho-mimetic ERK-nKTR(EEE) form was excluded from the nucleus in all VPCs (Figure 2D).

We quantified the intensity of the red/green ratio image within VPC nuclei in worms expressing ERK-nKTR, ERK-nKTR(AAA), and ERK-nKTR(EEE) reporters (Figures 2F–2H). Our analysis of ERK-nKTR indicates that red/green in P6.p is on average 2-fold higher than the other VPCs. Interestingly, the phospho-mimetic ERK-nKTR(EEE) mutant also shows a 2-fold increase in all VPCs with respect to the non-phosphorylatable

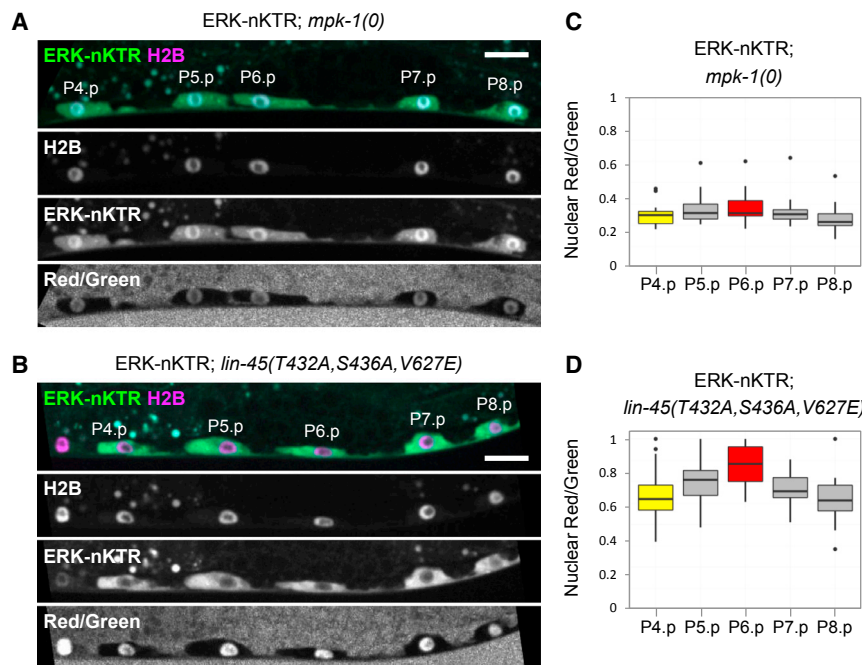


Figure 3. The ERK-nKTR Is Sensitive to Levels of MPK-1/ERK Activity

Images of ERK-nKTR transgene *arTi85* in genetic backgrounds with altered MPK-1/ERK activity show ERK-nKTR-mClover (ERK-nKTR), mCherry-H2B (H2B), and the calculated red/green ratio (Red/Green). All scale bars are 10 μ m.

(A) Loss of MPK-1/ERK activity in *mpk-1(0)* causes nuclear accumulation of ERK-nKTR in P6.p, similar to the pattern seen with the non-phosphorylatable ERK-nKTR(AAA) (see Figure 2D).

(B) Ectopic activation of MPK-1 in all VPCs, achieved by expression of a constitutively active and stable form of LIN-45/Raf (see STAR Methods), results in ectopic exclusion of ERK-nKTR from the nuclei of P4.p, P5.p, P7.p, and P8.p, similar to the result seen with the phospho-mimetic ERK-nKTR(EEE) (see Figure 2E).

(C and D) Red/green ratios for VPC nuclei expressing ERK-nKTR in (C) *mpk-1(0)* ($n = 19$) or (D) the presence of hyperactive LIN-45/Raf ($n = 16$). Each boxplot graph indicates the median, upper, and lower quartiles, maximum and minimum values, and outliers as described in Figure 2E.

To assess whether the localization of ERK-nKTR is patterned in VPCs of each genotype, one-way ANOVAs were performed to compare the red/green

ratios in all VPCs. ERK-nKTR is not patterned in *mpk-1(0)*; there are not significant differences in red/green ratios between VPCs (at the $p < 0.01$ level, $F(4,90) = 1.66$, $p = 0.165$). However, in the presence of hyperactive LIN-45/Raf, there is a significant effect on red/green ratios when different VPCs are compared (at the $p < 0.01$ level, $F(4,79) = 4.52$, $p = 0.002$). In this case, post hoc pairwise comparisons indicate that the red/green ratio of P6.p is significantly different from those of P8.p ($p < 0.01$) and P4.p ($p < 0.05$) (see statistical tests in Supplemental Information).

ERK-nKTR(AAA) (compare Figures 2G and 2H), similar to what is observed in P6.p for the wild-type sensor. These data indicate that the dynamic range of the ERK-nKTR sensor captures the MPK-1 activity level in P6.p.

The Subcellular Localization of ERK-nKTR in VPCs in Mutants Correlates with ERK Activity

To assess whether ERK-nKTR localization reflects EGFR-Ras-ERK signaling, we examined it in genetic backgrounds where ERK activity is lost or constitutively active. We found that ERK-nKTR accumulates uniformly in all VPC nuclei in an *mpk-1* null mutant background (hereafter *mpk-1(0)*) (Figures 3A and 3C), similar to that of the non-phosphorylatable ERK-nKTR(AAA) in a wild-type background (Figures 2C and 2G). Conversely, ERK-nKTR is excluded from the nuclei of all VPCs when MPK-1/ERK is activated by uniform expression of a highly active and stable form of LIN-45/Braf in all VPCs (transgene *arTi31*, see STAR Methods) (Figures 3B and 3D), similar to the phospho-mimetic ERK-nKTR(EEE) (Figures 2D and 2H). Together, the results of the *cis* mutations in ERK-nKTR and manipulation of MPK-1/ERK activity in *trans* indicate that ERK-nKTR functions as a faithful reporter for ERK activity.

ERK-nKTR Accurately Reflects MPK-1/ERK Activity in Different Cell Contexts

To establish the generality of the ERK-nKTR approach, we examined three additional cell contexts. These contexts differ from VPCs in the upstream receptors that lead to activation, and that *mpk-1*/ERK activity performs a biological function other than cell fate specification (Sundaram, 2013). In all three cases nuclear exclusion of the sensor is seen, and such exclusion

depends on *mpk-1*/ERK activity, indicating that ERK-nKTR is an accurate reporter of ERK activity in diverse cell types (Figure 4). All details about the transgenes for these additional contexts are described in STAR Methods and Table S1.

Migrating Sex Myoblasts

The sex muscles that control egg-laying are derived from two precursors, the SMs, which are born near the posterior of the larva at the first larval (L1) stage and migrate toward the mid-body of the larva in the L2 stage, attracted by a fibroblast growth factor (FGF)-like signal that emanates from the somatic gonad (Burdine et al., 1998). The precise positioning of the SM at the proper location is mediated by the EGL-15/FGF receptor via the canonical Ras-Raf-MEK-ERK cascade, implying that MPK-1 should be active in migrating SMs (DeVore et al., 1995; Sundaram et al., 1996). Indeed, ERK-nKTR is visibly excluded from nuclei in migrating SMs in a wild-type background, demonstrating that MPK-1/ERK is active. Importantly, ERK-nKTR is no longer excluded from the nucleus in *mpk-1(0)*, indicating that ERK-nKTR is indeed reporting *mpk-1* activity in SMs (Figure 4A).

Sensory Neurons

The two AWC neurons mediate chemotaxis to volatile odorants, and the two ASE neurons mediate chemotaxis to water-soluble molecules (Bargmann, 2006). G-protein-coupled receptor stimulation of MPK-1/ERK activity has been implicated in the function of these sensory neurons (Chen et al., 2011; Hirotsu and Iino, 2005; Tomida et al., 2012). We examined ERK-nKTR in well-fed worms in which sensory neurons should be well stimulated by compounds produced by bacteria and chemicals in the growth media, and thus where ERK should be active. Indeed, we find that ERK-nKTR is excluded from nuclei of AWC and ASE,

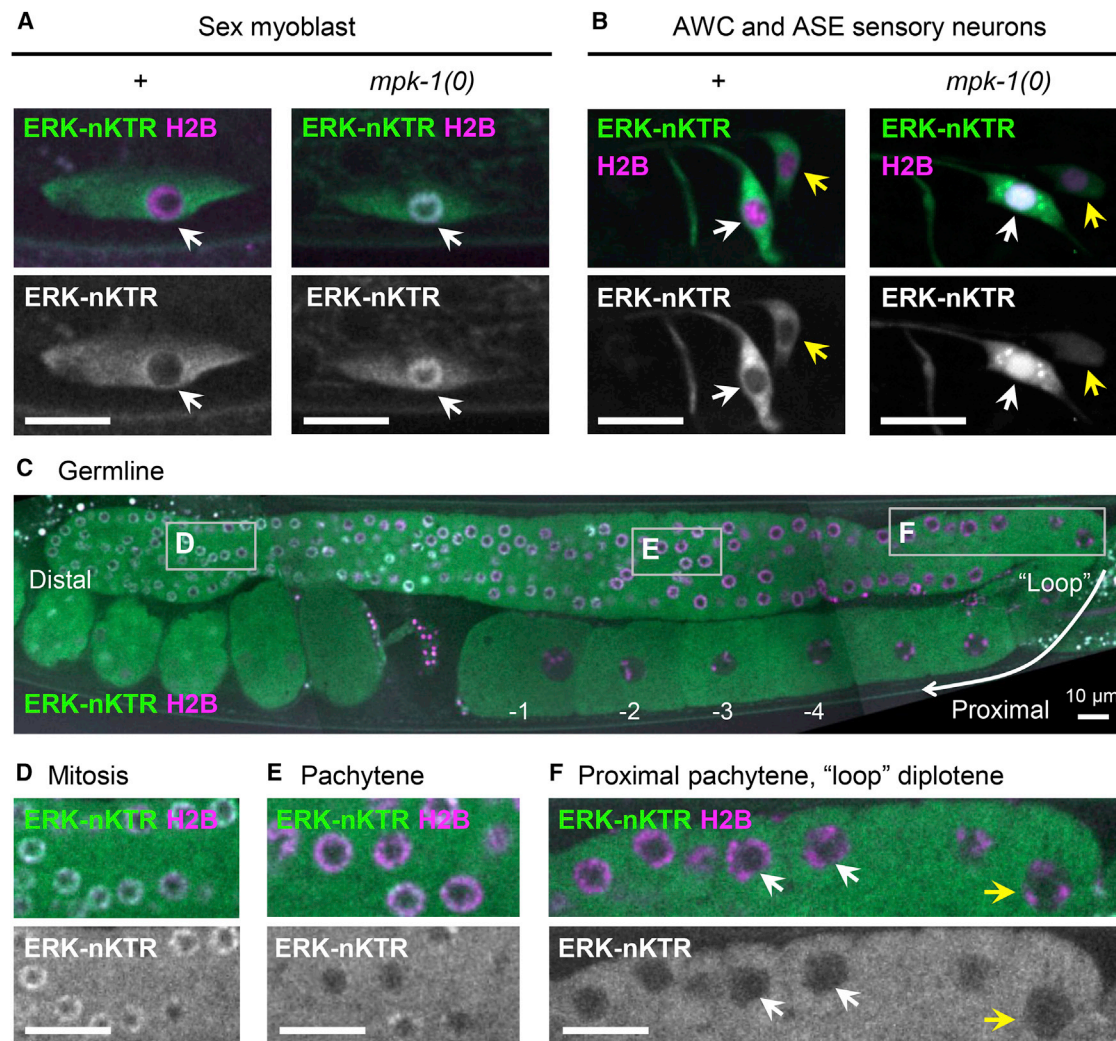


Figure 4. The ERK-nKTR Reports MPK-1/ERK Activity in Multiple Cell Contexts

(A) ERK-nKTR is excluded from nuclei (white arrows) of migrating sex myoblast (SM) cells in wild-type, but not in *mpk-1(0)*. SM-specific ERK-nKTR transgene *arTi133*, showing ERK-nKTR-mClover (ERK-nKTR) and mCherry-H2B (H2B) in wild-type (+) and *mpk-1(0)*.

(B) ERK-nKTR is excluded from the nuclei of AWC and ASE sensory neurons in wild-type, but not in *mpk-1(0)*. AWC- and ASE-specific ERK-nKTR transgene *arTi137*, showing ERK-nKTR-mClover (ERK-nKTR) and mCherry-H2B (H2B) in wild-type (+) and *mpk-1(0)* AWC (white arrows) and ASE (yellow arrows) neurons.

(C) ERK-nKTR is localized in a pattern correlated with MPK-1 activity in the hermaphrodite germline. Germline-expressed ERK-nKTR transgene *arSi4*, showing ERK-nKTR-mClover (ERK-nKTR) and mCherry-H2B (H2B). The most distal germline nuclei (oriented to left in this micrograph) are in mitosis. More proximally, nuclei enter meiosis and most of the length of germline remains in pachytene of meiosis I. Proceeding proximally, the germline “loops,” and in this region nuclei enter diplotene, which is followed by diakinesis and oocyte maturation.

(D–F) Images from insets indicated by boxes in (C). (D) ERK-nKTR accumulates in nuclei within the distal mitotic region, consistent with low MPK-1 activity detected in fixed samples (Lee et al., 2007). (E and F) ERK-nKTR levels are low in some nuclei in pachytene (E), but becomes highly excluded from nuclei in the proximal pachytene (white arrows) and loop region (yellow arrows) (F). In the most proximal region, ERK-nKTR is excluded from the nuclei undergoing diakinesis and oocyte maturation (C, labeled –1, –2, –3, –4). Nuclear exclusion of ERK-nKTR in proximal pachytene, diplotene, and diakinesis regions is consistent with regions where high levels of phosphorylated substrates are detected in fixed samples (Arur et al., 2011; Drake et al., 2014).

Scale bars in all images represent 10 μ m.

and that nuclear exclusion is abrogated in *mpk-1(0)*, indicating that ERK-nKTR is reporting MPK-1/ERK activity in these sensory neurons (Figure 4B).

Germline

The developing germline displays a spatial pattern along a proximal-distal axis: at the distal end, germline progenitors proliferate mitotically; as they move more proximally, away from the proliferative signal, they enter meiosis. Continuing proximally, germ

cells progress through meiosis and become oocytes, undergoing maturation during ovulation. *mpk-1* acts autonomously in the germline for multiple, genetically separable roles including meiotic progression and oocyte maturation (Arur et al., 2009; Church et al., 1995; Kim et al., 2012; Lee et al., 2007; Miller et al., 2001; Sundaram, 2013). Staining of fixed worms with an anti-phospho-ERK antibody revealed activity of this pathway in a corresponding spatial pattern: while MPK-1/ERK protein is

present throughout the germline, phospho-ERK immunostaining is highest in two zones: the proximal half of the pachytene zone and in the oocytes undergoing diakinesis (Lee et al., 2007). Phosphorylation of MPK-1 substrate proteins is also patterned, with high levels of phospho-substrates in the proximal pachytene, loop, and diakinesis (Arur et al., 2011; Drake et al., 2014). The germline therefore provides an opportunity for assessing the performance of the nKTR in a syncytium with spatially graded ERK activity.

Using a germline-specific promoter, we expressed ERK-nKTR throughout the germline and found that ERK-nKTR localization *in vivo* correlates with independent, direct measurements of active MPK-1/ERK and its phosphorylated substrates seen with immunostaining in extruded and fixed germlines described above. ERK-nKTR is enriched in nuclei in the mitotic zone where genetic analysis and phospho-ERK staining has indicated that MPK-1/ERK activity is low (Figures 4C and 4D). ERK-nKTR is excluded from the nuclei of germ cells in the proximal pachytene region, the loop, and in diakinesis (Figures 4C, 4E, and 4F), a pattern that closely resembles that of phosphorylated MPK-1 substrates.

Quantitative Imaging of ERK-nKTR in VPCs Over Time

The VPCs are born in the mid-L1 stage. During the L2 stage, Wnt produced from different cellular sources and EGF from the gonad maintains VPC competence for later vulval induction (Myers and Greenwald, 2007; Sternberg, 2005). At that time, graded expression of the *egl-17* transcriptional reporter suggests graded activation of EGFR-Ras-ERK signal transduction in VPCs, with a peak in P6.p and lower activity in two neighboring VPCs, P5.p and P7.p (Burdine et al., 1998; Yoo et al., 2004). As development proceeds the role of EGF changes, specifying P6.p to adopt the 1° fate. The fate specification process appears to begin in the L2 molt, based on the temperature-sensitive period of the *let-23/EGFR* gene (Ferguson et al., 1987) and the activation of *lag-2*, a second ERK-dependent transcriptional reporter that is directly regulated by the ERK substrate LIN-1/Elk1 (Zhang and Greenwald, 2011). P6.p becomes committed to the 1° fate early in the L3 phase, prior to entry into S phase of the cell cycle (Ambros, 1999). Fate commitment of P6.p is associated with positive and negative feedback mechanisms on the EGFR-Ras-ERK pathway (Berset et al., 2005; de la Cova and Greenwald, 2012). The flanking VPCs, P5.p and P7.p, are specified to adopt the 2° fate by activation of LIN-12/Notch, which negatively regulates EGFR-Ras-ERK signaling in the prospective 2° fate cells (Berset et al., 2001; Yoo et al., 2004).

Although useful, reliance on transcriptional reporters for precise inferences about ERK activation status and its quantitation is problematic because: (1) there may be a significant lag between the time transcription is activated and fluorescence is visualized; (2) the available evidence suggests that *egl-17* is not a direct transcriptional target of the EGFR-Ras-ERK pathway and responds to other inputs (Cui and Han, 2003; Tiensu et al., 2005); and (3) the restricted regulatory regions, multicopy nature, and random insertion sites of “classic” reporter transgenes may affect their expression dynamics. The ERK-nKTR sensor we developed here can overcome these problems as it more directly reports ERK activation status.

We examined ERK activity in VPCs during the course of development by generating time-lapse movies of individual larvae. We used two different imaging protocols. In one protocol, we imaged at 2-min intervals within a 1-hr time period in the late L2 stage, so as to capture potential ERK activation dynamics and resolve the frequency and amplitude of pulsatile activity in wild-type (Figures 5A–5D). In the second protocol, we imaged at 10-min intervals within 3.5-hr windows, limited by the efficacy of the anesthetic and the inability of larvae to survive the molting period on a slide. This protocol allowed us to span the entire L2 phase and early L3 stage, exclusive of the molt, and revealed lower-resolution information on ERK activity dynamics, including the earliest time of ERK activation in P6.p (Figure 5E).

Frequency-Modulated Signaling in P6.p

In P6.p, ERK activity is consistently elevated compared with other VPCs, and displays pulse-like changes over time when sampled at high resolution (Figures 5A and 5C); in addition, we observed infrequent activity pulses in other VPCs (Figure 5A). The ERK activity dynamics we observe are the result of meaningful biochemical activity and not an artifact of our experimental system because ERK activity pulses are absent in the ERK-nKTR(AAA) reporter in an otherwise wild-type background, and when the wild-type ERK-nKTR reporter is examined in the *mpk-1(0)* mutant background (Figure 5B).

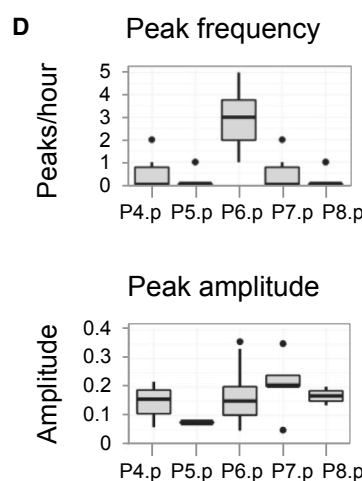
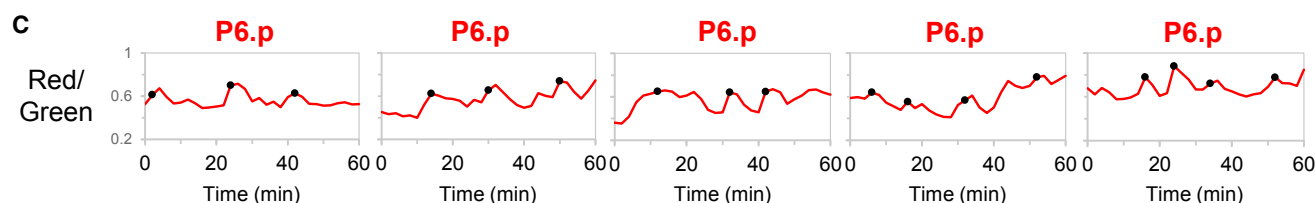
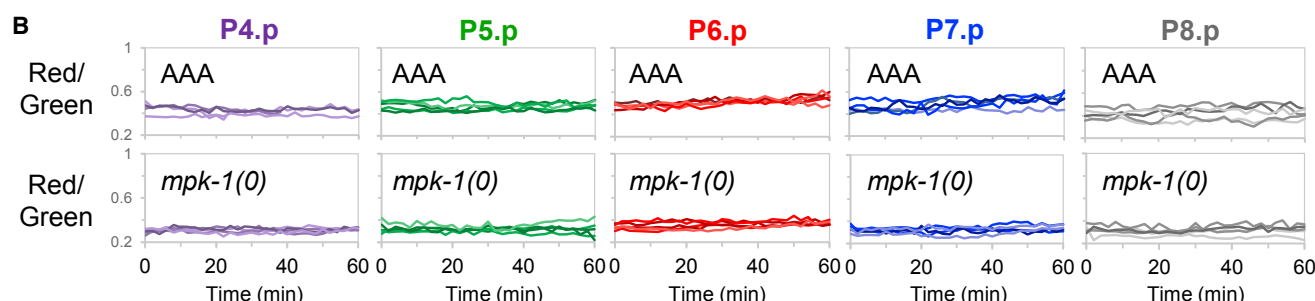
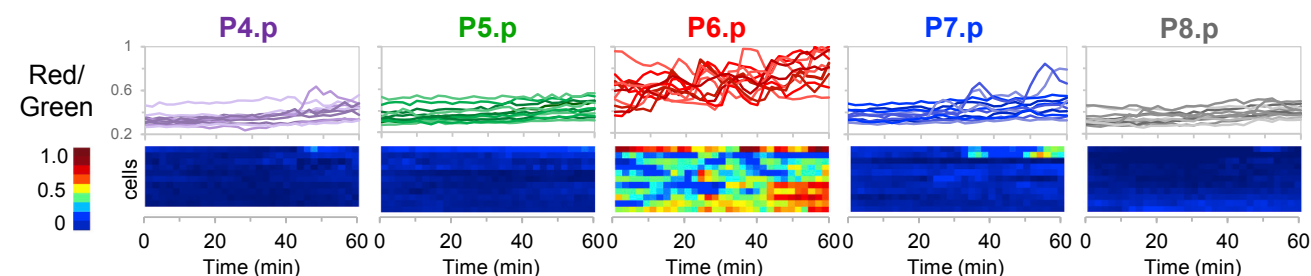
Analysis of the frequency and amplitude of ERK activity pulses in VPCs shows that P6.p sustains significantly more peaks over time, but the relative peak amplitude does not differ from that of other VPCs (Figure 5D). These and similar observations in cultured cells have been modeled as a frequency-modulated response whereby frequency of ERK activity pulses is proportional to EGF concentration, and whereby very high EGF concentration produces highly frequent pulses manifested as a steady, elevated response. In sum, our observations of signaling dynamics during VPC development show frequency-modulated signaling in a developmental context, underscoring the utility of the ERK-nKTR as a reliable reporter for single-cell and real-time characterization of ERK signaling *in vivo*.

LIN-3/EGF-Dependent ERK Activity in Early L2 Stage

In movies covering 3.5-hr intervals across the L2 and L3 stages, we were surprised to find that the earliest ERK activity begins in P6.p 1 hr after the L1–L2 molt (Figure 5E), before the anchor cell has been specified and prior to expression of the *egl-17* transcriptional reporter used to assess EGFR-Ras-ERK activation (Yoo et al., 2004). At this time, however, the four somatic gonad cells with anchor cell potential transcribe the *lin-3/EGF* gene (Hwang and Sternberg, 2004), suggesting that ERK-nKTR activity may still be responding to gonadal *lin-3/EGF*. Indeed, ERK activity is absent in a mutant that lacks *lin-3/EGF* expression in the anchor cell (Figure S3C), indicating that EGF, and not just the transcript, is produced at this early stage and is responsible for ERK activation.

During early and mid L2, the amplitude of ERK activity in P6.p steadily rises and exhibits fluctuations (Figure 5E). By late L2, activity in P6.p continues to fluctuate and appears to reach a plateau in amplitude. We also detect less frequent ERK activity pulses in other VPCs, notably P7.p, in mid and late L2 stages. In the early L3 stage, ERK remains active in P6.p, in the same time frame that it becomes committed to the 1° fate (Ambros, 1999). The infrequent ERK activity pulses observed in P7.p

A Dynamic ERK activity in VPCs during the L2 stage



E Time course of ERK activity in VPC development

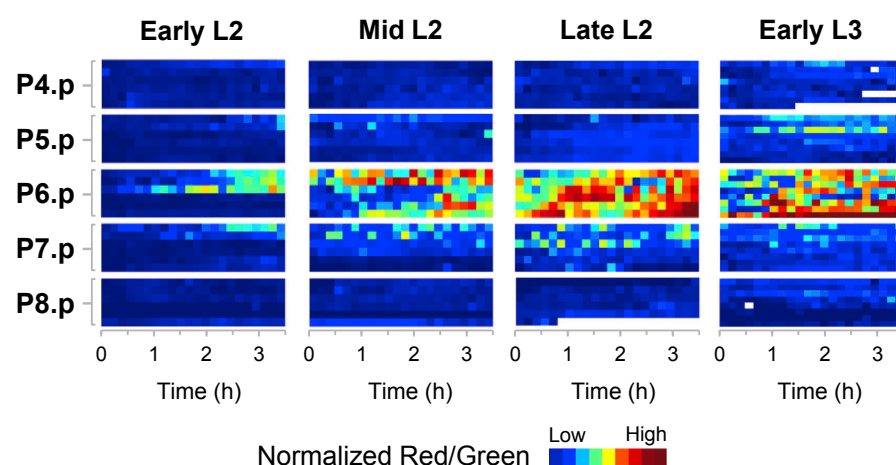


Figure 5. Live Imaging of ERK-nKTR during VPC Development

Unless otherwise indicated, movies were made of strains containing the transgene *arT185*, which expresses ERK-nKTR in VPCs.

(A) To examine ERK activity dynamics in wild-type late L2 larvae, time-lapse movies were captured by imaging at 2-min intervals over a 1-hr time period (see [STAR Methods](#)). The red/green ratio of individual VPC nuclei over time is shown in two ways: all individual traces overlaid for each VPC (top), and a heatmap (bottom) where each row represents a cell and the red/green ratio is represented by color ($n = 11$ larvae).

(B) Time-lapse movies were captured as described in (A) to examine *arT101* larvae that express non-phosphorylatable ERK-nKTR(AAA) in an otherwise wild-type background (top, $n = 5$ larvae), and *mpk-1(0)* larvae that express ERK-nKTR (bottom, $n = 5$ larvae). Shown are all individual traces overlaid for each VPC.

(legend continued on next page)

in the L2 stage appear to be diminished in early L3. We verified that the ERK activity we detect at all stages is absent in the ERK-nKTR(AAA) reporter and in the wild-type ERK-nKTR reporter in the *mpk-1(0)* background (Figures S3A and S3B).

Finally, we note that we used 2-min interval movies to assess the premise that the red/green nuclear ratio is an accurate method. First, we examined the intensity of ERK-nKTR and H2B over time and found that the observed pulsatile changes in red/green are due to fluctuations in nuclear ERK-KTR, while nuclear H2B is constant (Figure S1D). Second, we compared red/green with the ratio of cytoplasmic/nuclear green as used for the original KTR (Regot et al., 2014), and 1/green, using the nucleus only as a mask (Figure S1E). The dynamics look similar regardless of the ratiometric paradigm used. However, the red/green nucleus-only design facilitates segmentation and hence remains our preferred method of data analysis.

DISCUSSION

We have established that the ERK-nKTR biosensor technology is readily implemented through standard transgenesis and can be used for monitoring ERK activity in individual cells in *C. elegans*, at single time points or over prolonged periods of time. We validated the sensor as responsive to ERK in developing polarized epithelial cells (VPCs), migrating muscle cell precursors (SMs), sensory neurons (AWC and ASE), and in the germline. Each of these cases has been paradigmatic for developmental or neural processes in mammals. Furthermore, the bicistronic sensor design described here and the approach to image analysis, and the various considerations we describe in developing it, will be useful in extending ERK-nKTR to other experimental systems, as well as for adapting KTR sensors for other kinases (Regot et al., 2014) for use *in vivo*.

Limitations of Fluorescent Sensor Technology and Potential Solutions *In Vivo*

A limitation of KTR technology (and other fluorescent sensors of kinase activity) is that it is necessary to correct for kinase-independent parameters such as expression level, protein stability, and nuclear import/export rates that might otherwise affect the reporter baseline. Reporter baseline must be established by including controls to measure reporter activity in the absence of phosphorylation and kinase activity.

In our experiments, we determined the baseline red/green value in two ways. First, we used expression of a non-phosphorylatable derivative, ERK-nKTR(AAA), to measure basal variation in each VPC in the absence of phosphorylation. Similarly, we

used *mpk-1(0)* to assess basal variation in each VPC in the absence of ERK activity. As a general approach for normalization in long-term imaging in other cellular paradigms, we suggest that ERK-nKTR(AAA) could be co-expressed with the native ERK-nKTR using the T2A linker; this strategy would be similar to that used for the “in-cell” control in the study of JNK-KTR (Regot et al., 2014). For *in vivo* analysis, co-expression of the two forms may best be done by incorporating the non-phosphorylatable KTR form into a bicistronic transcript with a native KTR; an additional nuclear marker would be necessary for segmentation.

We note also that the KTR approach measures the phosphorylation state of the ERK substrate, which reflects both the forward phosphorylation of the substrate and dephosphorylation by unknown phosphatases. This same limitation applies to other approaches to measuring ERK activity in live individuals, such as FRET sensors, as well as genetic assays such as suppression or enhancement of phenotypes or expression of transcriptional reporters, which are furthermore likely to have additional inputs other than ERK activity per se that affect the output. Of these various options, we think the ERK-nKTR approach is the most generally useful and easily implemented technology.

Signaling Dynamics in VPCs in Wild-Type Hermaphrodites

ERK-nKTR has allowed us to quantify and compare ERK signaling activity and signaling dynamics in VPCs in wild-type hermaphrodites, yielding a more complete and finer-grained picture of ERK activity in this cell fate specification paradigm. Our analysis revealed frequency-modulated dynamics in VPCs in response to EGF. Previously, ERK activity sensors revealed frequency modulation in human cells provided different concentrations of EGF (Albeck et al., 2013), similar to what we have observed in VPCs. In contrast, amplitude modulation was observed in intact mouse skin epithelia mediated by EGFR (Hiratsuka et al., 2015). These observations suggest that there are different modes of regulating ERK activity dynamics.

Data collected from time-lapse movies indicate that ERK activity in VPCs is dynamic and pulsatile, and P6.p is distinguished from all other VPCs in sustaining more frequent ERK activity peaks. At this time, all VPCs have the potential to adopt the 1° fate (Greenwald et al., 1983; Sternberg and Horvitz, 1986; Sulston and White, 1980); however, P6.p is closest to the EGF-producing anchor cell. Thus, we infer EGF level differences found in physiological gradients *in vivo* are capable of producing different frequencies of pulsatile ERK activity. Interestingly, while the frequency of ERK activity pulses differed in P6.p compared with other VPCs, the relative amplitude of pulses did not, a behavior

(C) Time-lapse red/green data for all individual nuclei were analyzed for peaks (STAR Methods). Shown are five representative traces for P6.p cells, where red/green of individual nuclei is shown over time and the position of activity peaks are indicated by black circles.

(D) Peak frequency (peaks/hr) is shown for each VPC (top). A two-sample k test was performed to make pairwise comparisons of peak number (per hour) between VPCs. The peak frequency found in P6.p differed significantly from peak frequency in all other VPCs ($p < 1 \times 10^{-6}$). The relative peak amplitude (difference between red/green ratio at peak and nearest trough) is shown for each VPC (bottom). No significant differences in relative peak amplitude were found in pairwise comparisons of different VPCs using a two-sample k test. Each boxplot graph indicates the median, upper, and lower quartiles, maximum and minimum values, and outliers as described in Figure 2E.

(E) Time-lapse movies were captured by imaging at 10-min intervals over four overlapping 3.5-hr time periods after egg lay to examine ERK activity in wild-type VPC development: 20–23.5 hr (Early L2), 23–26.5 hr (Mid L2), 26–29.5 hr (Late L2), and 30–33.5 hr (Early L3) (see also STAR Methods and Figure S3A). In the heatmaps each row represents a cell and the red/green ratio of nuclei is represented by color. To correct for variation between animals, the average red/green value for the P4.p nucleus of a given larva was used as an internal baseline for normalization of other VPCs in that individual, since P4.p experiences minimal EGF signal (see Discussion and STAR Methods). Occasional missing values in heatmaps (white) result when the nucleus is out of focus for the time point.

comparable with the response of human cells provided different concentrations of EGF (Albeck et al., 2013). Although our short-time-period movies did not capture a significant difference in the ERK pulse frequency of P5.p and P7.p compared with more distant VPCs, we were able to observe a graded pattern of ERK activity when quantified in a large number of animals at a single time point, consistent with graded expression of the *egl-17* transcriptional reporter at this stage (Yoo et al., 2004). Future genetic manipulations of LIN-3/EGF and LET-23/EGFR signaling will help illuminate whether different frequencies of pulsatile ERK activity relate to different ERK-triggered outcomes.

In our survey of ERK activity across VPC developmental stages (Figure 5E), we find that the earliest detectable ERK activity in VPCs is evident in P6.p in the first hour of the L2 stage, an earlier time than activation of known ERK-dependent transcriptional outcomes. This activity is LIN-3/EGF dependent, reflecting the immediacy of KTR technology for assessing signaling compared with transcriptional reporters, which require transcription, translation, and maturation of the fluorescent protein. This early time frame is before the anchor cell has been specified, and suggests that LIN-3/EGF is produced, not just transcribed, in the four somatic gonad cells with anchor cell potential (Hwang and Sternberg, 2004; Sallee and Greenwald, 2015; Seydoux and Greenwald, 1989). This early L2-stage ERK activity is interesting in view of the redundant role of EGF with Wnt signaling to maintain P6.p in an uncommitted, competent state (Myers and Greenwald, 2007). Interestingly, the number of cells with vulval competence is a variable feature in nematode evolution and there has been divergence in the quantitative contribution of EGF-like signal to vulval induction (Félix, 2007; Kiontke et al., 2007); furthermore, the robustness of VPC fate is influenced by signal strength (Barkoulas et al., 2013; Braendle and Félix, 2008).

The analysis described here for VPCs in wild-type development provides a road map for future examination of such dynamics in different cell types *in vivo*. This description of *in vivo* ERK signaling dynamics will also provide the baseline for interpreting experimental perturbations of Ras-Raf-MEK-ERK regulation mechanistically, and for understanding the contributions of individual conserved regulators such as SEL-10/Fbw7 (de la Cova and Greenwald, 2012), GAP-1/RasGTPase-activating protein (Hajnal et al., 1997), and others to signaling dynamics in development.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
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- EXPERIMENTAL MODEL AND SUBJECT DETAILS
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- DATA AND SOFTWARE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and six tables and can be found with this article online at <http://dx.doi.org/10.1016/j.devcel.2017.07.014>.

AUTHOR CONTRIBUTIONS

C.d.I.C., R.T., S.R., and I.G. designed the experiments. C.d.I.C. and R.T. conducted the experiments. S.R. designed and supervised the image quantification methods. C.d.I.C., R.T., S.R., and I.G. wrote the paper.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental Models: Organisms/Strains		
<i>C. elegans</i> : N2: <i>C. elegans</i> , Bristol isolate	Caenorhabditis Genetics Center	WB strain: N2
<i>C. elegans</i> : SD378: <i>mpk-1(ga117)/dpy-17(e164) unc-79(e1069)</i>	Caenorhabditis Genetics Center	WB strain: SD378
<i>C. elegans</i> : EG7893: <i>oxTi615 unc-119(ed3)</i>	Caenorhabditis Genetics Center	WB strain: EG7893
<i>C. elegans</i> : CB1417: <i>lin-3(e1417)</i>	Caenorhabditis Genetics Center	WB strain: CB1417
<i>C. elegans</i> : JCC888: <i>jccTi1 [lin-31p::mCherry-H2B::unc-54 3'UTR]</i>	Julie Canman	N/A
<i>C. elegans</i> : GS7845: <i>arTi22 [hlh-2prox::GFP::H2B::unc-54 3'UTR]</i>	Michelle Attner	N/A
<i>C. elegans</i> : GS7890: <i>arTi31 [lin-31p::lin-45(T432A,S436A,V627E)::unc-54 3'UTR]</i>	This paper	N/A
<i>C. elegans</i> : GS8213: <i>arTi92 [lin-31p::ERK-KTR(NLS0)-mClover-T2A-mCherry-H2B::unc-54 3'UTR]</i>	This paper	N/A
<i>C. elegans</i> : GS8207: <i>arTi60 [lin-31p::ERK-KTR(NLS2)-mClover::unc-54 3'UTR]; jccTi1</i>	This paper	N/A
<i>C. elegans</i> : GS8190: <i>arTi85 [lin-31p::ERK-KTR(NLS3)-mClover-T2A-mCherry-H2B::unc-54 3'UTR]</i>	This paper	N/A
<i>C. elegans</i> : GS8188: <i>arTi83 [lin-31p::ERK-KTR(NLS4)-mClover-T2A-mCherry-H2B::unc-54 3'UTR]</i>	This paper	N/A
<i>C. elegans</i> : GS8221: <i>arTi95 [lin-31p::ERK-KTR(egl-13 NLS1)-mClover-T2A-mCherry-H2B::unc-54 3'UTR]</i>	This paper	N/A
<i>C. elegans</i> : GS8206: <i>arTi89 [lin-31p::ERK-KTR(egl-13 NLS2)-mClover-T2A-mCherry-H2B::unc-54 3'UTR]</i>	This paper	N/A
<i>C. elegans</i> : GS8210: <i>arTi90 [lin-31p::ERK-KTR(ocr-2 NLS1)-mClover-T2A-mCherry-his-11::unc-54 3'UTR]</i>	This paper	N/A
<i>C. elegans</i> : GS8187: <i>arTi82 [lin-31p::ERK-KTR(ocr-2 NLS2)-mClover-T2A-mCherry-H2B::unc-54 3'UTR]</i>	This paper	N/A
<i>C. elegans</i> : GS8255: <i>arTi101 [lin-31p::ERK-KTR(NLS3, S43A,T55A,T62A)-mClover-T2A-mCherry-H2B::unc-54 3'UTR]</i>	This paper	N/A
<i>C. elegans</i> : GS8254: <i>arTi100 [lin-31p::ERK-KTR(NLS3, S43E,T55E,T62E)-mClover-T2A-mCherry-H2B::unc-54 3'UTR]</i>	This paper	N/A
<i>C. elegans</i> : GS8232: <i>arTi85 [lin-31p::ERK-KTR(NLS3)-mClover-T2A-mCherry-H2B::unc-54 3'UTR]; mpk-1(ga117)/oxTi615 unc-119(ed3)</i>	This paper	N/A
<i>C. elegans</i> : GS8222: <i>arTi85 [lin-31p::ERK-KTR(NLS3)-mClover-T2A-mCherry-H2B::unc-54 3'UTR]; arTi31</i>	This paper	N/A
<i>C. elegans</i> : GS8458: <i>arTi133 [hlh-8p::ERK-KTR(NLS3)-mClover-T2A-mCherry-H2B::unc-54 3'UTR]</i>	This paper	N/A
<i>C. elegans</i> : GS8484: <i>arTi133 [hlh-8p::ERK-KTR(NLS3)-mClover-T2A-mCherry-H2B::unc-54 3'UTR]; mpk-1(ga117)/oxTi615 unc-119(ed3)</i>	This paper	N/A
<i>C. elegans</i> : GS8462: <i>arTi137 [ceh-36p::ERK-KTR(NLS3)-mClover-T2A-mCherry-H2B::unc-54 3'UTR]</i>	This paper	N/A
<i>C. elegans</i> : GS8485: <i>arTi137 [ceh-36p::ERK-KTR(NLS3)-mClover-T2A-mCherry-H2B::unc-54 3'UTR]; mpk-1(ga117)/oxTi615 unc-119(ed3)</i>	This paper	N/A
<i>C. elegans</i> : GS8601: <i>arSi4 [mex-5p::ERK-KTR(NLS3)-mClover-T2A-mCherry-H2B::tbb-2 3'UTR]</i>	This paper	N/A
<i>C. elegans</i> : GS8472: <i>arTi85 [lin-31p::ERK-KTR(NLS3)-mClover-T2A-mCherry-H2B::unc-54 3'UTR]; arTi22</i>	This paper	N/A
<i>C. elegans</i> : GS8713: <i>arTi85 [lin-31p::ERK-KTR(NLS3)-mClover-T2A-mCherry-H2B::unc-54 3'UTR]; lin-3(e1417); arTi22</i>	This paper	N/A

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant DNA		
Plasmid pENTR-ERKKTRClover	Addgene	#59138
Plasmid pCFJ90	Addgene	#19327
Plasmid pGH8	Addgene	#19359
Plasmid pMA122	Addgene	#34873
Plasmid pCFJ601	Addgene	#34874
Plasmid pCFJ910	Addgene	#44481
Plasmid pWZ111	Bob Goldstein	N/A
Plasmid pAP082	Bob Goldstein	N/A
Plasmid pCC318 <i>lin-31p::ERK-KTR(NLS0)-mClover-T2A-mCherry-his-11::unc-54 3'UTR</i>	This paper	N/A
Plasmid pCC306 <i>lin-31p::ERK-KTR(NLS2)-mClover::unc-54 3'UTR</i>	This paper	N/A
Plasmid pCC324 <i>lin-31p::ERK-KTR(NLS3)-mClover-T2A-mCherry-his-11::unc-54 3'UTR</i>	This paper	N/A
Plasmid pCC326 <i>lin-31p::ERK-KTR(NLS4)-mClover-T2A-mCherry-his-11::unc-54 3'UTR</i>	This paper	N/A
Plasmid pCC331 <i>lin-31p::ERK-KTR(egl-13 NLS1)-mClover-T2A-mCherry-his-11::unc-54 3'UTR</i>	This paper	N/A
Plasmid pCC322 <i>lin-31p::ERK-KTR(egl-13 NLS2)-mClover-T2A-mCherry-his-11::unc-54 3'UTR</i>	This paper	N/A
Plasmid pCC329 <i>lin-31p::ERK-KTR(ocr-2 NLS1)-mClover-T2A-mCherry-his-11::unc-54 3'UTR</i>	This paper	N/A
Plasmid pCC320 <i>lin-31p::ERK-KTR(ocr-2 NLS2)-mClover-T2A-mCherry-his-11::unc-54 3'UTR</i>	This paper	N/A
Plasmid pCC335 <i>lin-31p::ERK-KTR(NLS3, S43A, T55A, T62A)-mClover-T2A-mCherry-his-11::unc-54 3'UTR</i>	This paper	N/A
Plasmid pCC336 <i>lin-31p::ERK-KTR(NLS3, S43E, T55E, T62E)-mClover-T2A-mCherry-his-11::unc-54 3'UTR</i>	This paper	N/A
Plasmid pCC344 <i>ceh-36p::ERK-KTR(NLS3)-mClover-T2A-mCherry-his-11::unc-54 3'UTR</i>	This paper	N/A
Plasmid pCC346 <i>hlh-8p::ERK-KTR(NLS3)-mClover-T2A-mCherry-his-11::unc-54 3'UTR</i>	This paper	N/A
Plasmid pCC357 <i>mex-5p::ERK-KTR(NLS3)-mClover-T2A-mCherry-his-11::tbb-2 3'UTR</i>	This paper	N/A
Software and Algorithms		
Zen	Zeiss.com	N/A
ImageJ	Imagej.nih.gov	N/A
Matlab	Mathworks.com	N/A
CellProfiler	Cellprofiler.org	N/A
Matlab and Cellprofiler scripts used in this study	github.com/GreenwaldLab	de-la-Cova-2017

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Iva Greenwald (isg4@columbia.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

C. elegans Strains

Strain names and full genotypes are listed in the [Key Resources Table](#). *Caenorhabditis elegans* strain N2 was the parent strain in which transgenes were generated. The following alleles were obtained from the Caenorhabditis Genetics Center: LGIII: *mpk-1(ga117)*, *oxTi615[eft-3::tdTomato-H2B::unc-54 3'UTR]*, LGIV: *lin-3(e1417)*.

Because *mpk-1(ga117)* (null) adults are sterile, strains carrying *mpk-1(ga117)* were maintained as heterozygotes over the visible marker *oxTi615*. Homozygous *mpk-1(ga117)* segregants—designated *mpk-1(0)* in text—were picked for analysis. We note that in all cases where we analyzed ERK-nKTR in *mpk-1(0)*, ERK-nKTR was not excluded from the nucleus, indicating that potential maternal contribution was negligible and consistent with the completely penetrant Vulvaless phenotype of such hermaphrodites.

jccTi1, which marks all VPCs, is a single-copy insertion containing *lin-31p::mCherry-his-58::unc-54 3'UTR*, and was a gift of Julie Canman.

arTi22, which marks the four proximal somatic gonad cells with anchor cell (AC) potential in the L2 stage, is a single-copy transgene containing *hlh-2prox::gfp-H2B::unc-54 3'UTR* made by Michelle Attner, based on the promoter *hlh-2prox* (Sallee and Greenwald, 2015).

The transgene *arTi31* encodes a mutant LIN-45 protein that is constitutively active and stable. It is a single-copy insertion containing *lin-31p::lin-45(T432A,S436A,V627E)::unc-54 3'UTR* and was modeled after transgenes described in de la Cova and Greenwald (2012).

METHOD DETAILS

Plasmids and Transgenes Used for Expression in *C. elegans*

All plasmids used are listed in the Key Resources Table and new plasmids and transgenes generated in this work are described in Table S1. The ERK-nKTR cDNA was derived from pENTR-ERKKTRClover (Addgene, #59138) and encodes ERK-KTR fused to mClover (Regot et al., 2014) followed by a T2A peptide (Ahier and Jarriault, 2014) fused to mCherry and HIS-11. Established regulatory sequences were used for tissue-specific expression.

VPCs

The VPC-specific transgene *lin-31p::ERK-KTR-mClover-T2A-mCherry-his-11::unc-54 3'UTR* contains 5' and intronic regulatory sequences derived from the *lin-31* gene (Tan et al., 1998), designated “*lin-31p*.” VPC-specific transgenes that express “AAA” and “EEE” mutant derivatives were identical other than the phosphorylation site mutations.

SM

The SM-specific transgene *hlh-8p::ERK-KTR-mClover-T2A-mCherry-his-11::unc-54 3'UTR* contains regulatory sequences from -1791 to +1 upstream of the *hlh-8* gene (Harfe et al., 1998), designated “*hlh-8p*.”

AWC and ASE

The sensory neuron-specific transgene *ceh-36p::ERK-KTR-mClover-T2A-mCherry-his-11::unc-54 3'UTR* contains regulatory sequences named “*prom2*” derived from the *ceh-36* gene (Etchberger et al., 2007).

Germline

The germline-specific transgene *mex-5p::ERK-KTR-mClover-T2A-mCherry-his-11::tbb-2 3'UTR* contains regulatory sequences from the *mex-5* and *tbb-2* genes that allow uniform germline expression throughout larval and adult development (Merritt et al., 2008).

All somatically-expressed transgenes (driven by regulatory sequences from the *lin-31*, *hlh-8*, or *ceh-36* genes) contain the *unc-54 3'UTR*, and were cloned in the miniMos transgenesis vector pCFJ910 (Frøkjær-Jensen et al., 2014) (Addgene, #44481). Random, single-copy miniMos transgenic strains were generated in the N2 strain by germline injection of hermaphrodites with 10 ng/μL transgene plasmid with a co-injection mixture of pCFJ601[*eft-3::mos1 transposase::tbb-2 3'UTR*], pCFJ90[*myo-3::mCherry::unc-54 3'UTR*], pGH8[*rab-3::mCherry::unc-54 3'UTR*], and pMA122[*hsp-16.41::peel-1::tbb-2 3'UTR*]. Strains carrying integrated transgenes were isolated as described by (Frøkjær-Jensen et al., 2014).

The germline-expressed *mex-5*-derived transgene contains the *tbb-2 3'UTR*, and was cloned in pWZ111, a vector designed for Caspr/Cas9-mediated single-copy insertion at the germline-permissive *ttTi4348* locus (Frøkjær-Jensen et al., 2012). Caspr/Cas9-mediated single-copy transgenic strains were generated in N2 by germline injection of 10 ng/μL transgene plasmid with a co-injection mixture of pAP082[*eft-3::Cas9::tbb-2 3'UTR*, *U6::sgRNA targeting the ttTi4348 locus*], pCFJ90[*myo-3::mCherry::unc-54 3'UTR*] and pGH8[*rab-3::mCherry::unc-54 3'UTR*]. Strains carrying integrated transgenes were isolated as described (Dickinson and Goldstein, 2016; Dickinson et al., 2015). We gratefully acknowledge that the reagents used for insertion at this locus were made by Ari Pani and Bob Goldstein, and gifted to us by David Matus.

Image Acquisition

To image ERK-KTR in VPCs and SMs in the L2 stage, larvae were grown at 25°C, mounted on an agarose pad at 24 hours after an egg lay, and anesthetized using 0.5 μM Levamisole in M9. Similarly, the ERK-KTR was imaged in sensory neurons and germline of young adults mounted on agarose pads at 48 hours after an egg lay. To image development in real-time, L2 larvae were anesthetized for 20 minutes using a mixture of 0.1% Tricaine/0.01% Tetramisole, then mounted on an agarose pad in M9 without anesthetic as described in (McCarter et al., 1999). We used different imaging protocols to generate 1) short, high-resolution data, and 2) longer, low-resolution data. To capture ERK dynamics with high resolution in time, we imaged at two-minute intervals within a 1 hour time period beginning at 26 hours after an egg lay. To image ERK activity over longer developmental periods, we imaged at ten-minute intervals over four developmental periods (time in hours after egg lay): early L2 (20–23.5 hr), mid L2 (23–26.6 h), late L2 (26–29.5 hr), early L3 (30–33.5 hr). The duration times of our movies were limited to 3–4 hours because the anesthetic typically became ineffective after 4 hours. Additionally, developing larvae did not survive the molting period on slides even in the absence of anesthetic.

Z-stacks of mClover and mCherry fluorescence were captured simultaneously using a spinning disk confocal, dual camera system (Carl Zeiss). For all images, exposure times used for mClover and mCherry were equal: 500 ms was used for “still” images of a single time point, and 150 ms was used for movie images. For all experiments, blank images were collected using the same parameters.

Image Quantification

A workflow of the image quantitation methods is provided in Table S4. Matlab and CellProfiler scripts used in this study are provided in <https://github.com/GreenwaldLab/de-la-Cova-2017>. Illumination correction was done using blank images obtained for every experiment. A ratiometric image was calculated by dividing mCherry intensity values by mClover intensity values for each pixel. The resulting image (Red/Green) was normalized between the percentiles 0.1 and 99.9% to avoid artifacts from hot and cold pixels in the camera. Segmentation and object quantification was performed with CellProfiler (Kamentsky et al., 2011), treating each Z stack image independently. Nuclei were segmented using the mCherry-H2B signal and the upper quartile intensity of the ratiometric Red/Green image was obtained. Tracking of cells within the Z-stack and manual annotation of the VPC identities were performed using custom software. For each VPC nucleus, the mean Red/Green of the five most equatorial Z sections is presented as the measurement “Red/Green”.

To compare Red/Green values acquired at different developmental stages, data from VPCs at each time interval was normalized using the mean Red/Green value of P4.p in the same larva. P4.p was utilized as an internal control because Red/Green values in this cell were well correlated with Red/Green values of ERK-nKTR in *mpk-1(0)* (Figures 2F and 3C).

To detect pulsatile peaks in ERK activity, time-lapse ERK-nKTR Red/Green data was analyzed using a custom peak finding algorithm in Matlab. Specifically, peaks were identified using slope and relative amplitude thresholds that were manually set to maximize agreement with visually distinct peaks. The relative amplitude threshold (0.05 in Red/Green units) was greater than the average difference between time points observed in ERK-nKTR(AAA) at each developmental stage (mean difference $\pm$ 95% confidence interval was 0.015 ± 0.024 in early L2; 0.020 ± 0.029 in mid-L2; 0.019 ± 0.016 in late L2; 0.027 ± 0.022 in early L3). To measure the relative amplitude of individual ERK activity peaks, the difference between Red/Green ratio at peak and nearest trough was calculated.

QUANTIFICATION AND STATISTICAL ANALYSIS

When comparing all VPCs for differences in mean Red/Green ratio (Figures 2 and 3, Tables S5–S6), we performed one-way ANOVA tests followed by pairwise comparisons using the Tukey HSD test. In pairwise comparisons of VPCs for differences in peak number and relative peak amplitude (Figure 5D), we performed two-sample k-tests. For all significant test results, p-values are provided within Figure legends.

DATA AND SOFTWARE AVAILABILITY

Software and algorithms used in this work are listed in the Key Resources Table and Table S3.