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REVIEW

Shedding light on developmental ERK signaling with genetically encoded biosensors

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ABSTRACT

The extracellular signal-regulated kinase (ERK) pathway governs cell proliferation, differentiation and migration, and therefore plays key roles in various developmental and regenerative processes. Recent advances in genetically encoded fluorescent biosensors have unveiled hitherto unrecognized ERK activation dynamics in space and time and their functional importance mainly in cultured cells. However, ERK dynamics during embryonic development have still only been visualized in limited numbers of model organisms, and we are far from a sufficient understanding of the roles played by developmental ERK dynamics. In this Review, we first provide an overview of the biosensors used for visualization of ERK activity in live cells. Second, we highlight the applications of the biosensors to developmental studies of model organisms and discuss the current understanding of how ERK dynamics are encoded and decoded for cell fate decision-making.

KEY WORDS: Biosensors, ERK, FRET, KTR, Optogenetics

Introduction

Animal embryonic development is a complex process, in which mitotic division, differentiation, and tissue remodeling take place coordinately (Wolpert et al., 2015; Barresi and Gilbert, 2020). So far, researchers have extensively studied the roles played by intracellular signal transduction systems in embryonic development, mainly using model organisms such as mammalian cultured cells, *Caenorhabditis elegans*, *Drosophila*, zebrafish and mice. The cell signaling machinery in embryonic development is known to be repurposed in physiological and pathological processes including wound healing, regeneration, cancer cell invasion and metastasis. Interestingly, a small set of core cell signaling systems is used to drive these processes in a time- and site-specific manner (Arias et al., 2002).

The RAS-extracellular signal-regulated kinase (ERK) mitogen-activated protein (MAP) kinase signaling pathway is an important core signaling pathway (Nishida and Gotoh, 1993) (Fig. 1). Activation of receptors such as receptor tyrosine kinases (RTKs) triggers the initiation of the RAS-ERK signaling. Upon receptor activation, cytoplasmic guanine exchange factors (GEFs) translocate to the plasma membrane, and activate a low molecular weight G protein, RAS. GTP-bound active RAS at the plasma membrane recruits and activates RAF, a MAP kinase kinase kinase (MAPKKK). The activated RAF then phosphorylates and activates MEK, a MAP kinase kinase (MAPKK), leading to dual phosphorylation in the activation loop of ERK. Consequently, ERK enhances kinase activity and catalyzes the phosphorylation of various substrates. Transcription factors phosphorylated by ERK induce gene expression/repression, whereas other proteins such as cytoskeletal regulators function directly upon phosphorylation, causing a wide range of cellular phenotypic changes, such as cell proliferation, differentiation, cell motility and tumorigenesis (Lavoie et al., 2020). Furthermore, germline mutations in the RAS-ERK signaling genes are associated with developmental disorders in humans called RASopathies (Rauen, 2013).

RAS-ERK signaling has been extensively studied in diverse research fields, including developmental biology (Patel and Shvartsman, 2018). Many of the findings have been clarified using immunostaining, inhibitors, and gain- and loss-of-function experiments, representing the multifunctional aspect of ERK. However, we still do not fully understand why and how RAS-ERK signaling participates in such diverse functions during development and regeneration processes. An emerging area of research is spatial and temporal coding of the ERK activation dynamics originally described in cultured cells; ERK activation dynamics are associated with distinct cellular phenotypes (Marshall, 1995) much like the cell signaling dynamics of p53, NF-κB and Ca^{2+} (Purvis and Lahav, 2013). The recent advance of genetically encoded fluorescent biosensors, which allow visualization of signal transduction systems at the single-cell level, has contributed to the verification of this concept. In this Review, we first outline genetically encoded biosensors that monitor ERK activity in living cells. Secondly, we discuss the spatiotemporal ERK dynamics and their roles in developmental contexts such as embryogenesis, regeneration, differentiation and collective migration in model organisms.

Genetically encoded ERK biosensors

Fluorescent proteins and biosensors have enabled visualization of various biological molecules (proteins, lipids, nucleotides, ions, etc.), biochemical reactions (association, dissociation, enzymatic reactions, etc.) and biophysical properties (diffusivity, temperature, force, etc.) taking place within a cell with high temporal and spatial resolutions (Zhang et al., 2002; Miyawaki, 2003). Here, we focus on genetically encoded fluorescent biosensors for monitoring ERK activation and their mode of action. The current biosensors for ERK are mainly classified into two types: Förster (or fluorescence) resonance energy transfer (FRET)-based biosensors and single fluorophore-based biosensors. The former rely on the principle of FRET to detect ERK activity, whereas the latter monitor ERK activity as a change in fluorescence intensity. First, we discuss the

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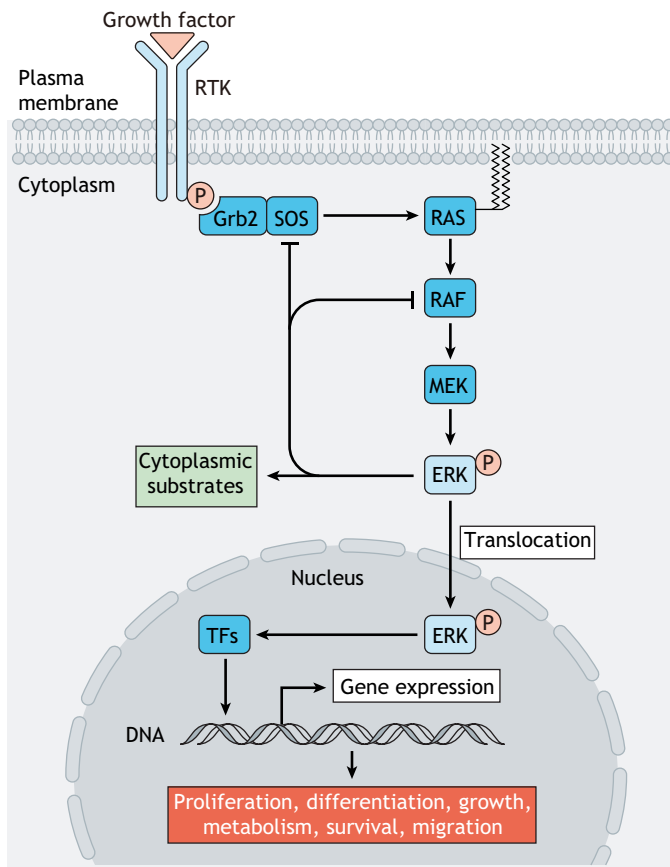A.N., 0000-0002-5717-4912; Y.G., 0000-0002-5597-158X; Y.K., 0000-0002-2801-4522; K.A., 0000-0001-7263-1555

DEVELOPMENT

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Fig. 1. The RAS-ERK signaling pathway. The RAS-ERK mitogen-activated protein (MAP) kinase pathway is known to be activated mainly through receptor tyrosine kinase (RTK) activation. Upon growth factor stimulation, RAS is activated, leading to the subsequent RAF and mitogen-activated protein kinase kinase (MEK) activation. Eventually, MEK phosphorylates and activates ERK, which phosphorylates cytoplasmic and nuclear substrates including transcription factors. These phosphorylated substrates induce a wide variety of cellular functions such as proliferation, differentiation, growth, metabolism, survival and migration.

basic principle of FRET and FRET-based biosensors for ERK. Many excellent reviews have provided details on the aspects of fluorescent proteins and genetically encoded biosensors, and we encourage the reader to refer to them (Zhang et al., 2002; Miyawaki, 2003; Greenwald et al., 2018; Lambert, 2019).

FRET-based biosensors
FRET is a non-radiative energy transfer process, in which the excitation energy of a donor fluorophore is transferred to an acceptor fluorophore (Foster, 1948; Lakowicz, 1999). Although FRET efficiency is affected by several factors, the relative distance and angle between the donor and acceptor fluorophores make the main contributions to most FRET biosensors. Taking advantage of these properties, FRET-based ERK biosensors have been designed to estimate ERK activity as a change in FRET signals. Several pairs of fluorescent proteins suitable for FRET have been reported, with the most widely used pair being a yellow fluorescent protein (YFP) as acceptor and cyan fluorescent protein (CFP) as donor (Greenwald et al., 2018). The wide use of this particular pair could be due to the large overlap between the emission spectrum of CFP and the absorption spectrum of YFP and the convenience of estimating the FRET efficiency with acceptor photobleaching. In recent years, pairs of longer-wavelength fluorescent proteins have also been

developed for FRET-based biosensors (Watabe et al., 2020; Mo et al., 2020).

The first reported FRET biosensor for ERK was Miu2 (Fujioka et al., 2006), which consists simply of ERK2 sandwiched between YFP and CFP. The conformational change mediated by MEK binding to ERK2 in Miu2 increases the FRET efficiency (Fig. 2A). In the inactivated state, ERK is constitutively associated with MEK and localized in the cytoplasm, but upon phosphorylation and activation by MEK, ERK is dissociated from MEK and translocated into the nucleus (Adachi et al., 1999). Miu2 monitors both the dissociation from MEK and nuclear translocation of ERK2 upon epidermal growth factor (EGF) stimulation (Fujioka et al., 2006). Almost all ERK FRET biosensors reported after Miu2 have been of the substrate type. For example, the biosensor per se is phosphorylated by the endogenous ERK and the change in phosphorylation is detected by FRET.

The advent of the extracellular signal-regulated kinase activity reporter (EKAR) has had a particularly major impact on the subsequent ERK FRET biosensors (Harvey et al., 2008). EKAR is composed of a YFP, WW domain, Gly linker, ERK phosphorylated peptide containing ERK docking motif, and CFP, and exhibits an increase in FRET by the phosphorylation (Fig. 2B). Following the initial report of EKAR, several researchers have reported improvements of this biosensor (Fig. 2C), either by using a long flexible linker and dimerization-prone fluorescent protein pair (EKAREV) (Komatsu et al., 2011), or optimization of fluorescent proteins and the order of domains (EKAR2G, EKAR-TVV, EKAR3, EKAR4) (Fritz et al., 2013; Vandame et al., 2014; Sparta et al., 2015; Keyes et al., 2020). These improvements have significantly enhanced the gain of the FRET signal, allowing more researchers to easily employ the FRET biosensors. One drawback of the EKAR-based FRET biosensor is that the phosphorylation peptide originally derived from Cdc25 is non-specifically phosphorylated by cyclin-dependent kinase 1 (CDK1), thereby resulting in an artificial increase in the FRET signal immediately before the onset of M-phase and a decrease in the FRET signal at the end of M-phase even under an MEK-inhibition condition (Aoki et al., 2013). Recently, however, two new ERK FRET biosensors, EKAREN4 and EKAREN5 (Fig. 2C), have been reported that resolve the undesired CDK1-sensitivity by introducing two amino acid mutations in the phosphorylation peptide (Ponsioen et al., 2021). These mutations almost completely suppress the steep increase in the FRET level at the onset of the M-phase and the rising slope during the G2 phase. Intriguingly, the linker length, which is the difference between EKAREN4 and EKAREN5, fine-tunes the sensitivity and dynamic range of each biosensor to ERK activity, as previously suggested (Komatsu et al., 2011). In addition to these single-chain FRET biosensors, ERK biosensors using an intermolecular FRET have been reported (Depry et al., 2015), but they are rarely used in the field of cell and developmental biology, possibly due to the difficulty of optimizing the acceptor and donor expression levels.

Single fluorophore-based biosensors
In contrast to FRET-based biosensors, single fluorophore-based biosensors enable the measurement of ERK activity by changes in the subcellular localization (relocation-type) or changes in the brightness of fluorescent reporters. Nuclear accumulation of fluorescent protein-fused ERK is the most classical way to monitor the ERK activation in living cells (Fig. 2D) (Lenormand et al., 1993; Adachi et al., 1999). This relocation-type ERK biosensor is still widely used, but as the use of the wild-type ERK induces excessive
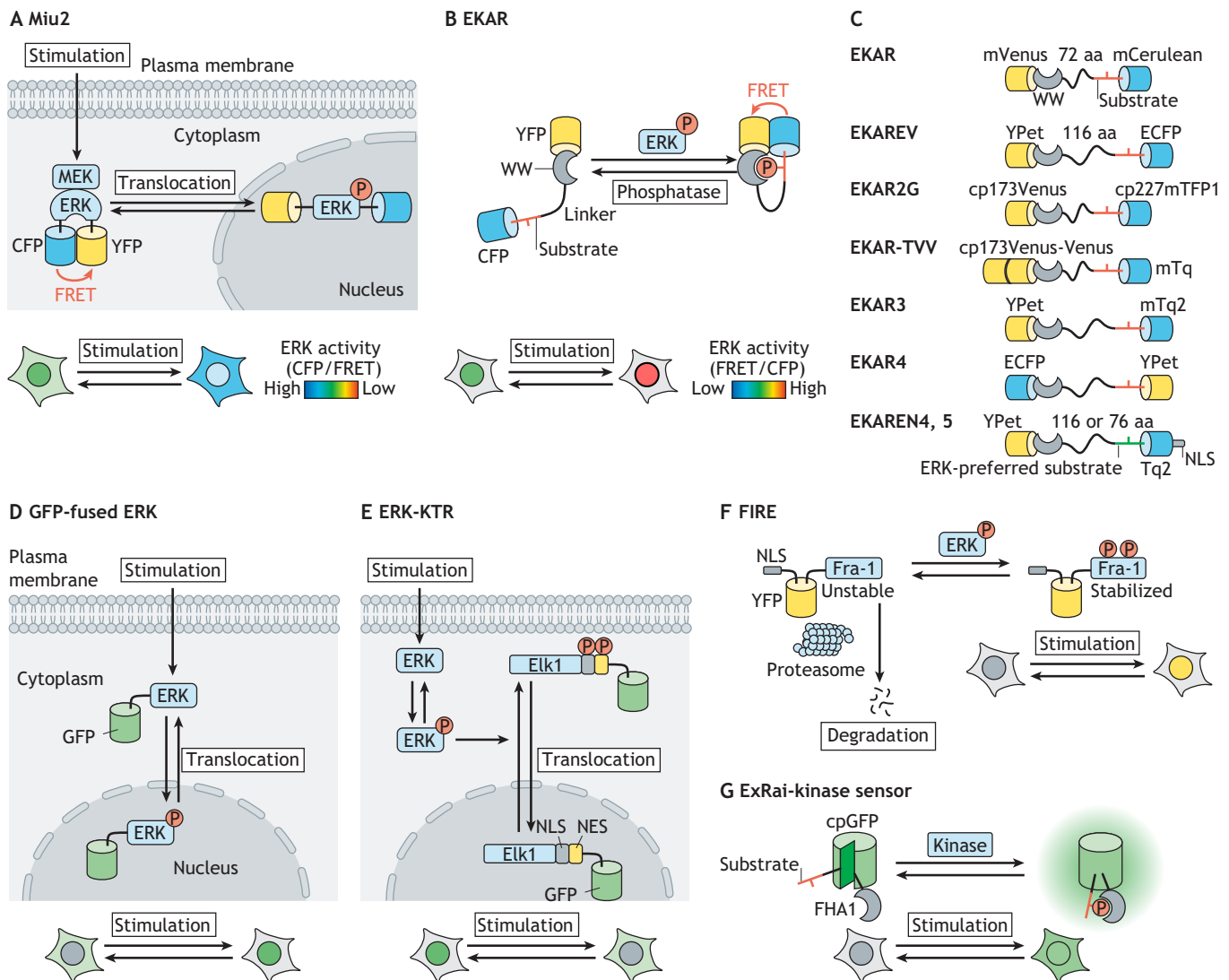
Fig. 2. Genetically encoded fluorescent biosensors for ERK. (A) Miu2 binding to mitogen-activated protein kinase kinase (MEK) shows closed conformation with high Förster (or fluorescence) resonance energy transfer (FRET) level. Upon stimulation, Miu2 is dissociated from MEK, leading to a decrease in FRET level and nuclear translocation of Miu2. (B) Original extracellular signal-regulated kinase activity reporter (EKAR) consists of a YFP, WW domain, linker, substrate peptide and CFP. The endogenous ERK phosphorylates its substrate peptide, which is derived from Cdc25, increasing FRET efficiency. (C) Schematic of the structures of EKAR and its improved versions. (D) Translocation of GFP-fused ERK from cytoplasm to the nucleus in response to the stimuli. (E) ERK-KTR demonstrates ERK-dependent nucleocytoplasmic shuttling, allowing estimation of ERK activity from the subcellular localization. (F) FIRE, degradation-based ERK activity reporter, is stabilized by ERK phosphorylation. Therefore, the brightness of FIRE reflects the time-integrated value of ERK activity. (G) ExRai kinase sensor includes a circularly permuted GFP, FHA1 domain and substrate peptide. Once the peptide is phosphorylated by kinase, the intramolecular binding between the peptide and FHA1 domain undergoes a change in the chromophore of cpGFP, increasing the fluorescence.

negative feedback through gene expression, the use of kinase-dead ERK as an ERK activity reporter is strongly recommended. A new framework, called kinase translocation reporters (KTRs) system, is composed of a kinase substrate fused to a bipartite nuclear localization signal (bNLS), a nuclear export signal (NES) and a fluorescent protein (Fig. 2E) (Regot et al., 2014; Kudo et al., 2017). The introduction of the negative charge by phosphorylation of the substrate modulates nucleocytoplasmic shuttling, thereby regulating the subcellular distribution of the reporter as a proxy for the kinase activity. Single fluorophore-based biosensors, such as KTRs, enable multiplexed imaging of kinase activity at the single-cell level (Regot et al., 2014; Maryu et al., 2016; Jacques et al., 2021) and combination with optogenetics (Zhou et al., 2017; Goglia et al., 2020; Gagliardi et al., 2021). The sensitivity of ERK-KTR is

comparable with that of FRET-based ERK biosensors (Sparta et al., 2015; Yang et al., 2018). In addition, a liquid-liquid phase separation-based ERK biosensor has been reported, called ERK-SPARK (separation of phases-based activity reporter of kinase), in which multivalent protein-protein interactions upon ERK activation are induced to form fluorescent droplets (Zhang et al., 2018).

In addition to the aforementioned relocation-type biosensors, there have been biosensors that monitor changes in fluorescence intensity as an ERK activity. An ERK biosensor called FIRE (Fra-1-based integrative reporter of ERK) is normally degraded, but when phosphorylated by ERK it escapes degradation and stabilizes, increasing its fluorescence intensity (Fig. 2F) (Albeck et al., 2013). FIRE is incapable of measuring the fast dynamics often observed in ERK (e.g. pulsatile dynamics), but would provide an index

corresponding to the time-integrated ERK activity. A different type of kinase biosensor has been demonstrated, in which the circularly permuted green fluorescent protein (cpGFP) is employed to detect kinase activity (Kawai et al., 2004; Mehta et al., 2018). In particular, the ExRai (excitation ratiometric) biosensor shows a higher dynamic range than that of the conventional FRET-based biosensor (Fig. 2G) (Mehta et al., 2018; Zhang et al., 2021), but no ExRai-ERK has yet been reported. Finally, a dimerization-dependent fluorescent protein (ddFP)-based single fluorophore ERK biosensor (RAB-EKARev) has been reported (Mehta et al., 2018). RAB-EKARev contains a fluorogenic ddRFP-A and a non-fluorescent ddRFP-B instead of YPet and ECFP in EKAREV, and ERK-dependent dimerization results in an increase in ddRFP-A fluorescence intensity.

The number of developmental biology studies using these genetically encoded ERK biosensors is still limited. There are several possible reasons: the development of the ERK biosensor is relatively new, the development of *in vivo* imaging methods and the image analysis are still challenging, and sensitive biosensors are needed to visualize subtle changes in ERK activation *in vivo*. At present, EKAREV and ERK-KTR have been almost exclusively used in developmental biology research. The roles of ERK dynamics in invertebrate and vertebrate development studies with model organisms are introduced below.

Temporal dynamics of ERK activation within a cell: insights from *C. elegans* development

We first introduce investigations of temporal dynamics of ERK activation at the single-cell level. Seminal studies in PC12 cells have shown that EGF-induced cell proliferation relies on a transient ERK activation, whereas nerve growth factor (NGF)-induced cell cycle arrest and neuronal differentiation require a sustained ERK activation (Marshall, 1995). The question is how temporal dynamics

of ERK activation within one cell are impacted by changing the nature of a stimulus *in vivo*. The invertebrate model organism, *C. elegans*, has an advantage for addressing this question, because of the invariant cell lineage, which allows examination of the same cell in different individuals.

Cell fate patterning of vulval precursor cells (VPCs) in *C. elegans* is the most prominent example relevant to RAS-ERK signaling. Intensive studies of this system have revealed many fundamental mechanisms underlying the regulation of VPC differentiation by canonical EGF-EGFR-RAS-ERK signaling (Patel and Shvartsman, 2018). VPCs are made up of six epithelial cells in line, which are named P3.p to P8.p. The VPCs are undifferentiated in the L2 stage and potent to generate vulval cells. The anchor cell (AC) in the gonad secretes EGF toward the neighboring VPCs, which evokes RAS-ERK signaling activation in VPCs, finally resulting in distance-dependent graded ERK activity and different cell fates of VPCs at the L3 stage (Fig. 3A). ERK activity is highest in P6.p, which is the center cell in VPCs and also the cell closest to the AC, lower in neighboring P5.p and P7.p, and not detectable in peripheral cells (Yoo et al., 2004; Burdine et al., 1998). In these earlier studies, graded ERK activity has been visualized by the expression of the *egl-17* transcriptional reporter, although the time-lag from ERK activation to transcriptional activation constituted a limitation in the measurement of real-time ERK dynamics.

There exist only a few studies using genetically encoded fluorescent biosensors to visualize ERK dynamics in living worms. A substrate-type FRET-based biosensor, ERKy, is the first biosensor showing ERK dynamics in *C. elegans* (Tomida et al., 2012). The basic design of ERKy is similar to that of EKAR, but modified with a dimerization-prone acceptor fluorophore (YPet), a distinct substrate peptide and an optimized linker length. It has been shown that the temporal pattern of stimulation with Ca^{2+} imaging determines the intensity and duration of ERK activity in a sensory
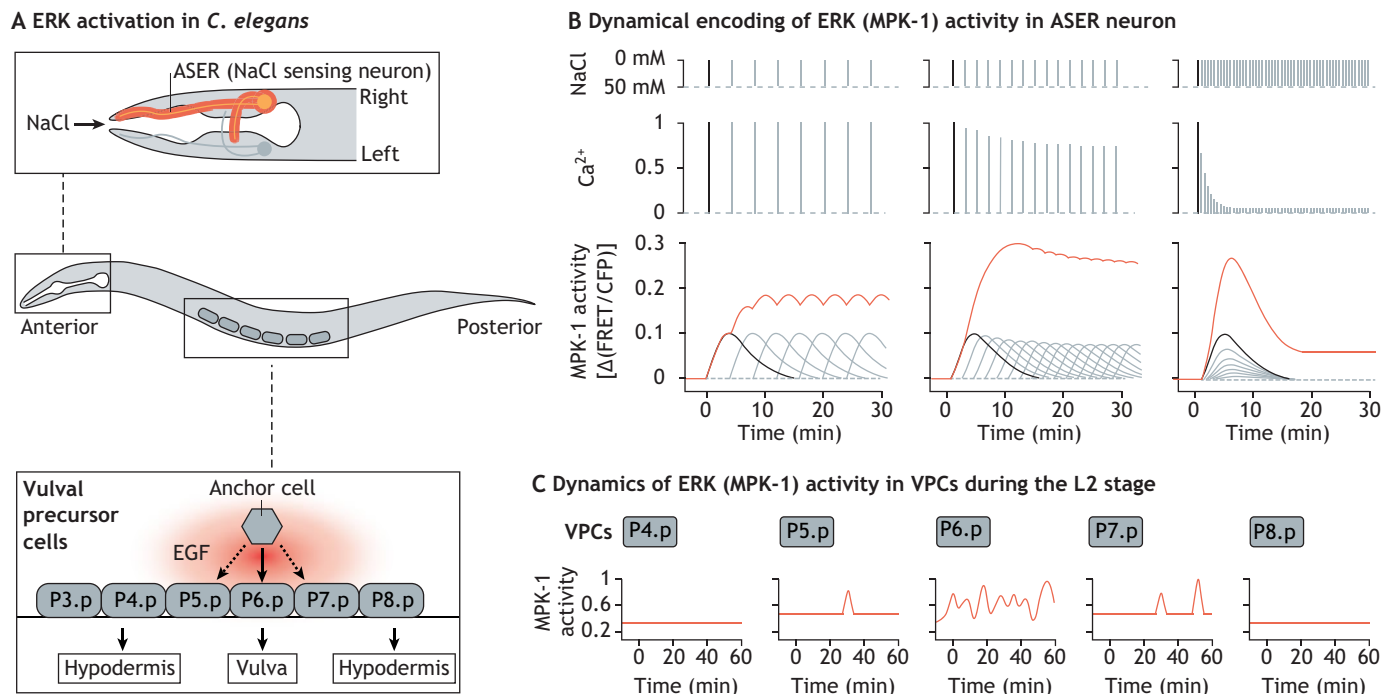

Fig. 3. ERK dynamics in *Caenorhabditis elegans*. (A) Schematic of ERK activation in *C. elegans*. (B) Dynamical encoding of ERK (MPK-1) activity in ASER neuron. NaCl-induced ERK activity in ASER neuron was measured by ERKy. The ERK activation dynamics in ASER neuron non-linearly encodes the information of NaCl input through Ca^{2+} dynamics. (C) ERK (MPK-1) activation dynamics in vulval precursor cells (VPCs) at the L2 stage as visualized by ERK-nKTR.

neuron, ASER (Fig. 3A,B). A second report of ERK dynamics in *C. elegans* has employed a relocation-type single-fluorophore biosensor, ERK-KTR (de la Cova et al., 2017). As most cells have complex cytoplasmic shapes or form syncytia, even while the nucleus remains discrete and stable (except during cell division), a robust system known as ERK-nKTR (where 'n' is the nuclear ratio) has been developed that allows estimation of ERK activity by only nuclear fluorescence intensity of ERK-nKTR, instead of the cytoplasm/nucleus ratio. To normalize the expression level, ERK-nKTR-mClover and the nuclear marker mCherry-Histone H2B are expressed from a 2A peptide-linked multicistronic vector, and the ratio of ERK-nKTR-mClover to mCherry-Histone H2B at the nucleus is used as a proxy for ERK activity. ERK-nKTR allows *in vivo* visualization of ERK activity in various tissues and cell types of *C. elegans*, including the sex myoblast, sensory neurons and germline cells.

ERK dynamics have been examined with ERK-nKTR throughout the differentiation process of VPCs from the L2 to L3 stage (Fig. 3C) (de la Cova et al., 2017, 2020). ERK-nKTR detects spatial gradients of ERK activity in VPCs from cells close to the AC, suggesting the concentration gradient of EGF *in vivo*. Further, even in the early L2 stage, in which the AC is not yet specified, EGF-dependent ERK activation begins to be observable by ERK-nKTR in the P6.p cell. Interestingly, ERK dynamics in the P6.p cell are pulsatile rather than sustained throughout the L2 and L3 stages. Consistent with the response in mammalian cells treated with different concentrations of EGF (Albeck et al., 2013), pulsatile ERK dynamics can also be seen in the nascent VPCs, especially in P7.p, with less frequency but with comparable amplitude. It remains unclear how the pulsatile dynamics of ERK activation contribute to the cell fate determination of VPCs. The relationship between ERK dynamics and cell fate determination is highly dependent on cell type and context, and therefore it would be necessary to more

directly validate the causal relationship through an approach such as optogenetics (Aoki et al., 2013; Toettcher et al., 2013; Johnson and Toettcher, 2019).

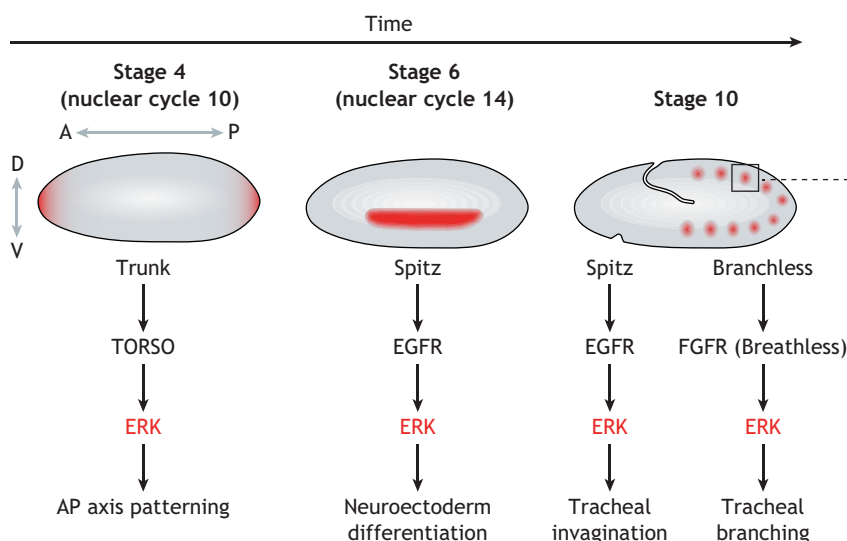## Temporal and spatial dynamics of ERK activation within a tissue

In addition to the ERK activation dynamics at the single-cell level, it has been observed that the ERK activation dynamics are temporally and spatially coordinated at the tissue level during the development and regeneration processes. We next introduce what types of ERK dynamics occur at the tissue level and how these ERK dynamics generate multi-cellular responses in model organisms from *Drosophila*, zebrafish and mouse.

ERK dynamics in *Drosophila* embryogenesis
In *Drosophila* embryogenesis, ERK is known to be activated downstream of RTKs, playing important roles in dorso-ventral (DV) and anterior-posterior (AP) axis patterning, cell differentiation and morphogenesis (Gabay et al., 1997; Furriols and Casanova, 2003; Schweitzer et al., 1995; Johnson and Toettcher, 2019). In general, RTKs activate the PLCγ and PI3K pathways in parallel with the RAS-ERK pathway. Interestingly, however, the RTKs in *Drosophila* embryogenesis give rise to their phenotypes largely via the ERK pathway, based on the phenotypes observed in a series of genetic experiments (Shilo, 2014). In many cases, the RTKs are ubiquitously expressed during embryogenesis, whereas the ligands are processed and activated in a spatially and temporally restricted manner, resulting in the specific ERK activation dynamic.

Here, we outline three events related to ERK activation across the developmental stage of *Drosophila* embryogenesis. First, in stage 4 early syncytial embryos (nuclear cycle 10, 80-130 min after fertilization), the RTK Torso is activated by its ligand Trunk in
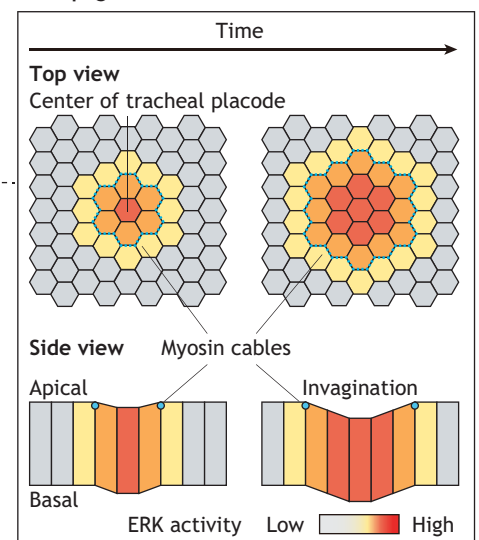

Fig. 4. ERK dynamics in *Drosophila* embryogenesis. (A) Schematic of stage-specific ERK activation in *Drosophila* embryos and their signaling pathways. In stage 4 (nuclear cycle 10), anterior-posterior (AP) axis patterning is achieved by localized activation of ERK through the TORSO receptor tyrosine kinase and the ligand Trunk. In stage 6 (nuclear cycle 14), activated Spitz triggers epidermal growth factor receptor (EGFR) activation, resulting in ERK activation on the ventrolateral stripes along the AP axis and neuroectoderm differentiation. At stage 10, in the initial process of the trachea formation, Spitz-EGFR-ERK signaling is activated within the tracheal placode, followed by the trachea branching through Branchless [a fibroblast growth receptor (FGFR) ligand]-Breathless (FGFR)-ERK signaling. (B) Propagation of ERK activation from the center of tracheal placodes accounts for the coordinated apical constriction and cell intercalation. The circular pattern of myosin cables (blue lines) form at the interface of cells with different ERK activities and expand radially in a manner dependent on wave front of ERK activation.

both the anterior and the posterior poles, subsequently delineating a steep gradient of ERK activation from the poles (Fig. 4A). This Trunk-mediated Torso and ERK activation lasts for 60-90 min and specifies the DV axis. Second, between stages 5 and 6 (nuclear cycle 14, 130-180 min after fertilization), when cellularization and gastrulation take place, an intracellular protease called Rhomboid is expressed on the ventrolateral stripes along the AP axis, cleaving and activating Spitz, a ligand for EGFR. The secretion of Spitz accomplishes the ERK activation in that region and promotes differentiation into neuroectoderm (Fig. 4A). Furthermore, from stage 10 (260-320 min after fertilization), tracheal placodes appear and begin to invaginate, followed by branching of the ectodermal epithelium (Samakovlis et al., 1996) (Fig. 4A). During the initial process of the trachea formation, Spitz-EGFR-ERK signaling is activated within the tracheal placode, accounting for the coordinated apical constriction and cell intercalation observed in the trachea formation (Gabay et al., 1997). Later, Branchless (FGFR ligand)-Breathless (FGFR)-ERK signaling regulates trachea branching (Sato and Kornberg, 2002) (Fig. 4A).

A few examples of ERK dynamics visualized by ERK biosensors and their roles have been demonstrated in the embryogenesis of *Drosophila*. First, the ERK activity dynamics in the early phase of trachea development have been visualized using an ERK FRET biosensor, EKAREV, showing that ERK activation propagated concentrically from the center of the placode (Fig. 4B) (Ogura et al., 2018; Hayashi and Ogura, 2020). It has been shown that actomyosin contractility is elevated in ERK wavefront cells and is required for invagination. Interestingly, mathematical model analysis has shown that the propagation of ERK activation requires a switch-like response for sufficient induction of invagination. Control of tissue morphogenesis by ERK waves has also been observed in collective migration in cultured mammalian cells, which is consistent with the use of a molecular mechanism by which actomyosin contractility is increased through the ERK pathway (Aoki et al., 2017; Hino et al., 2020). In the late phase of trachea development, ERK activity is visualized during the formation of the air sac primordium (ASP) (Zhang et al., 2018). By combining a phase separation-based ERK sensor, SPARK, with various genetic mutants, ERK has been shown to be activated by FGF signals in ASP (Zhang et al., 2018), consistent with previous studies (Sato and Kornberg, 2002). More recently, an ERK-KTR for *Drosophila*, miniCic, has been developed, demonstrating the role of ERK in the cell extrusion and cell death of epithelial cells of the pupal notum (Moreno et al., 2019). It has been shown by live-cell imaging that the epithelial cells of the pupal notum undergo inactivation of ERK activity by compaction, which causes cell death and extrusion. Moreover, it has been reported that when cell extrusion takes place, cells surrounding the extruded cells extend the cell area and demonstrate propagation of ERK activation, rendering the cells resistant to cell death for a short period of time (Valon et al., 2021). The propagation of ERK activation has been observed in cultured cells surrounding oncogene-expressing cells or apoptotic cells (Aikin et al., 2020; Gagliardi et al., 2021). These results indicate that mechano-chemical feedback exists between cell morphology and ERK activity in line with similar mechanisms proposed in cultured cells (Hino et al., 2020). It is fascinating that the mechano-chemical feedback between ERK and actin cytoskeleton is evolutionarily conserved, although the molecular mechanisms underlying the signal transduction pathways still remain unknown, particularly with respect to how ERK regulates actin cytoskeleton and actomyosin contractility, and which factors link cell morphology to ERK activity.

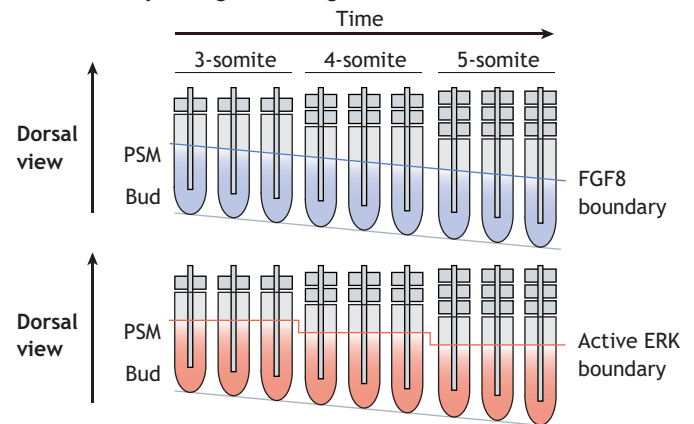## ERK dynamics in the development and regeneration of zebrafish

Zebrafish has been widely used as a model organism for various developmental processes, such as epiboly, bone generation and pigment patterning – just to name a few (Bruce and Heisenberg, 2020; Lepage and Bruce, 2010; Marques et al., 2019; Patterson and Parichy, 2019). In zebrafish development, ERK is also activated downstream of RTKs, especially FGF receptors. Sixteen FGF family genes are encoded in the zebrafish genome (Itoh, 2007). Owing to overlapping functions of FGF family genes, phosphorylated ERK detected by whole-mount immunostaining has been used as a readout for FGF signals (Shinya et al., 2001; Akiyama et al., 2014). ERK activation is detected in the marginal mesoderm at the 75% epiboly stage, and the forebrain, hindbrain, somites, and tailbud at the six somite stage (Fig. 5A).

A FRET biosensor, EKAREV, was first introduced into zebrafish for the visualization of ERK activity in a variety of embryonic

A ERK activation in zebrafish embryo

Lateral view
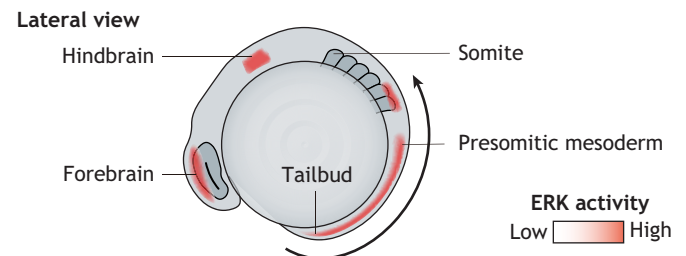
B ERK activity during somite segmentation

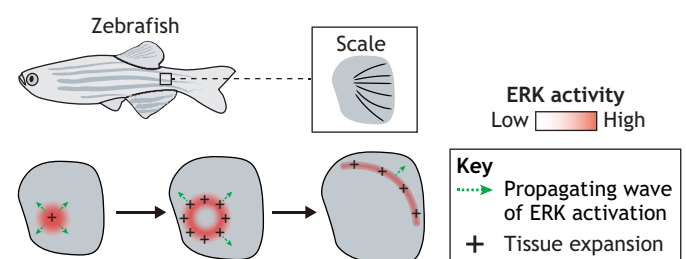**C Propagating wave of ERK activation in scale regeneration**

Fig. 5. ERK dynamics in the development and regeneration of zebrafish. (A) Schematic of ERK activation in zebrafish embryo at the six somite stage. (B) Representative patterns of FGF8 expression (purple) and ERK activity (red) during the three- to five-somite stages of zebrafish development. (C) Osteoblast ERK activity waves during scale regeneration. ERK activation propagates as excitable propagating waves that are able to traverse the entire scale.

Box 1. Mechanisms of ERK activation propagation in different organisms
The propagation wave of ERK activation has been widely observed in cultured cells, *Drosophila*, zebrafish and mouse. These studies have revealed both common and unique features of the mechanisms underlying propagating waves of ERK activation in different biological systems. A common feature is that intercellular propagation of ERK activation is achieved through the activation of ligands for the growth factor receptor. In cultured cells, ERK activates ADAM17 (TACE), a membrane-bound metalloprotease, leading to solubilization of pro-EGFR ligands on the plasma membrane and activation of ERK in adjacent cells (Aoki et al., 2013, 2017). A recent study has reported the redundant roles of pro-EGFR ligands in the propagation of ERK activation (Lin et al., 2021 preprint). There are some notable differences of the propagating waves of ERK activation in different biological contexts, including the traveling velocity [from 2-3 µm/min in mammalian cells (Aoki et al., 2017) to 10 µm/hour in zebrafish scale regeneration (De Simone et al., 2021)], traveling distance [from only one cell in *Drosophila* epithelial cells of the pupal notum (Moreno et al., 2019), to several cells in mammalian cells and mouse, to more than 10 cells in zebrafish scale regeneration (De Simone et al., 2021)] and duration of ERK activation [transient (Aoki et al., 2017; De Simone et al., 2021; Valon et al., 2021) versus sustained (Ogura et al., 2018)]. In addition, cell functions caused by ERK activity propagation range from morphogenesis to cell proliferation to regeneration. Future studies are needed to clarify how these phenotypic differences are generated.

processes (Sari et al., 2018; Ishimatsu et al., 2018; Wong et al., 2018). The results obtained with EKAREV are in very good agreement with those of immunostaining with phospho-ERK antibodies. ERK activation dynamics have further been analyzed in the somite segmentation, where the spatially regular pattern of cell differentiation is thought to be controlled by combining oscillatory gene expression of Hes family transcription factors, Her1 and Her7, with a spatial gradient of FGF/ERK activity from the end of the posterior presomitic mesoderm (PSM). According to this clock and wavefront model (Cooke and Zeeman, 1976), the oscillation of Her1 and Her7 determines the regular size of somites (Bessho and Kageyama, 2003), whereas an anterior limit of ERK activation marks the earliest future somite boundary (Akiyama et al., 2014). Time-lapse imaging of ERK activity by EKAREV has revealed stepwise regression of ERK activity (Sari et al., 2018) in spite of the gentle FGF8 gradient (Akiyama et al., 2014), suggesting that positive feedback mechanisms underlie the steep gradient and stepwise regression of ERK activity (Fig. 5B). This observation, combined with mathematical modeling of the signaling circuit, has resolved how mutants without the oscillatory gene expression still produce irregularly-sized somites (Naoki et al., 2019), which had puzzled researchers in the field.

A few studies with transgenic zebrafish expressing ERK-KTR have been reported. They have revealed ERK dynamics during the wound response of muscle cells (Mayr et al., 2018), angiogenesis (Okuda et al., 2021) and scale regeneration (De Simone et al., 2021). In particular, De Simone and colleagues have nicely demonstrated periodic concentric propagating waves of ERK activation (Box 1) among regenerating osteoblasts after a scale was plucked, and the radial waves were shown to be necessary for proper tissue expansion (Fig. 5C). The propagating waves of ERK activation were blocked by the treatment with FGFR inhibitor, suggesting the involvement of FGF in the propagating waves. Live imaging has revealed dynamic properties; for example, how the wave propagation is perturbed by laser ablation of the wavefront. The data support the idea that the ERK propagation is a reaction-diffusion trigger wave,

in which the long-range ERK propagation is mediated by excitability of the signaling circuit that includes positive and negative feedback, as observed for action potentials in neurons (Gelens et al., 2014). These results, when combined with previous observations on cultured mammalian cells (Matsubayashi et al., 2004; Aoki et al., 2017; Hino et al., 2020), indicate the surprisingly persistent and broad importance of ERK waves in tissue regeneration.

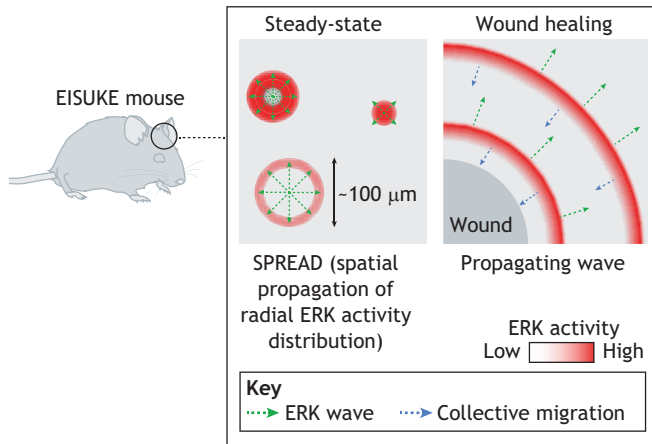## ERK dynamics in development and regeneration of mouse

ERK signaling is important in many aspects of mammalian development (Lavoie et al., 2020). Many gain- and loss-of-function studies have shown the involvement of RTK/ERK signaling in various developmental processes, including gastrulation, vasculogenesis, limb development, neural patterning and placentation (Deng et al., 1994; Srinivasan et al., 2009; Min et al., 1998; Hatano et al., 2003; Newbern et al., 2008). Spatial and temporal patterns of ERK activation during mouse embryogenesis have been examined with anti-phospho-ERK antibodies (Corson et al., 2003). However, the live imaging of ERK activation dynamics is still in the early stages of investigation.

The pioneering work for visualization of the ERK activity in mice was the establishment of a transgenic mouse line, EISUKE, which expresses EKAREV (Kamioka et al., 2012). EISUKE mice allow the visualization of *in vivo* ERK dynamics in many developmental and regeneration processes. For example, the epidermis occasionally exhibits bursts and radial propagations of ERK activation, designated as the spatial propagation of radial ERK activity distribution (SPREAD), and the frequency of SPREAD correlates with the rate of epidermal cell division (Fig. 6A) (Hiratsuka et al., 2015). Meanwhile, in wounded skin, ERK activation has been shown to proceed as a propagating wave in parallel to the wound edge (Fig. 6A) (Hiratsuka et al., 2015). More recently, ERK activation waves have been found to control collective cell migration during the cochlear duct development of mice (Ishii et al., 2021). As described above, intercellular propagating waves of ERK activation are widely observed in cultured cells, flies, zebrafish and mice, and may constitute an evolutionarily conserved mechanism.
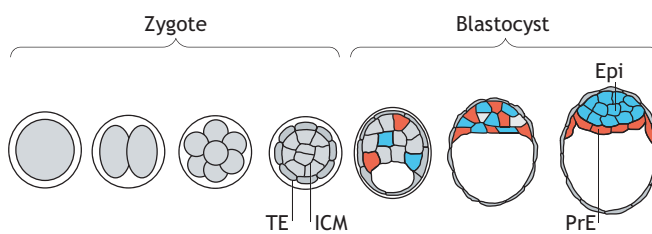
Here, we focus on cell-fate decisions during early mouse development, which are exemplified by stem cell self-renewal. The mouse blastocyst is an excellent model to understand ERK signaling and cell-fate determination (Simon et al., 2018). The preimplantation mouse blastocyst undergoes two consecutive differentiation events and produces three types of cell lineage (Fig. 6B). The first differentiation event is governed by polarity-mediated Hippo signaling (Nishioka et al., 2009; Hirate et al., 2013; Wicklow et al., 2014; Frum et al., 2018), which segregates the trophectoderm (TE) and inner cell mass (ICM). Next, the epiblast (Epi) and primitive endoderm (PrE) cells appear within an ICM marked by the mutually exclusive expression patterns of NANOG and GATA6, respectively (Guo et al., 2010; Plusa et al., 2008). FGFR/ERK signaling plays the pivotal roles in the differentiation of Epi and PrE cells; inhibition of FGFR/ERK signaling either by chemical inhibitors or genetic ablations prevents all ICM cells from selecting PrE, resulting in the Epi cell lineage of all ICM cells, whereas treatment with the high dose of FGF causes all ICM cells to select PrE (Yamanaka et al., 2010; Saiz et al., 2016; Kang et al., 2017, 2013; Molotkov et al., 2017; Chazaud et al., 2006).

Although the importance of FGFR/ERK signaling for the segregation of Epi and PrE has been proved by genetic and pharmacological analyses over the past decade, ERK activity in the

DEVELOPMENT

A ERK activation in basal epidermis of mouse ear

EISUKE mouse

Steady-state

Wound healing

~100 μm

SPREAD (spatial propagation of radial ERK activity distribution)

Propagating wave

Wound

ERK activity
Low — High

Key
····▸ ERK wave   ····▸ Collective migration

B Mouse early embryogenesis

Zygote

Blastocyst

Epi

TE ICM

PrE

C Spatial pattern of ERK activity in TE

Polar TE

FGF gradient

Mural TE

ERK activity
Low — High

Cell fate determination by bifurcation of ERK activity dynamics

G2 M G1

Epi
(NANOG+)

ERK activity

G2 M G1
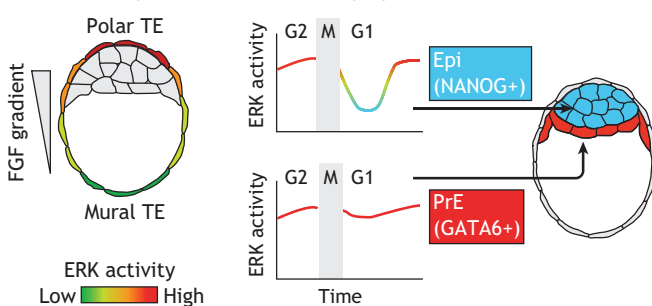
PrE
(GATA6+)

ERK activity

Time

Fig. 6. ERK dynamics in mouse development and regeneration. (A) ERK activation in the basal epidermis of the mouse ear. In steady-state, bursts of ERK activation occur where ERK activity radially propagates from cell to cell, called SPREAD. Meanwhile, in wound healing, ERK activation propagates as a propagating wave in parallel to the wound edge. (B) Schematic of early mouse embryogenesis. Red and blue cells correspond to cells specified to primitive endoderm (PrE) and epiblast (Epi), respectively. ICM, inner cell mass; TE, trophectoderm. (C) Spatial ERK activity gradient of TE in blastocyst (left). ERK activity dynamics during G1 phase determines cell fate specification to either Epi or PrE (right).

ICM has recently been described by the immunofluorescence of phosphorylated active ERK (Azami et al., 2019). Two recent cooperative works have established an ERK-KTR-expressing mouse line and observed time-resolved ERK dynamics during mouse blastocyst differentiation (Pokrass et al., 2020; Simon et al., 2020). With respect to the ERK activity pattern in TE, the polar TE juxtaposing the ICM retains high ERK activity induced by Epi-produced FGF4 (Guo et al., 2010; Nowotschin et al., 2019), whereas mural TE shows much lower ERK activity in a manner dependent on the distance from the nearest ICM (Simon et al., 2020). These distinct ERK signaling profiles between polar and

mural TE emerge at the early blastocyst (Fig. 6C), suggesting that ERK-KTR is more sensitive than immunofluorescence with phospho-ERK antibodies, which detects such differences at later stages (Azami et al., 2019; Christodoulou et al., 2019). Time-lapse imaging of ERK-KTR followed by immunostaining for cell fate markers, such as NANOG and GATA6, has enabled investigators to connect temporal ERK activity patterns to the terminal cell fates. ICM blastomeres are initially both NANOG- and GATA6-positive, but the PrE begins to exhibit increased expression of GATA6, whereas the Epi shows higher NANOG expression, in a mutually exclusive manner (Guo et al., 2010; Plusa et al., 2008). Interestingly, ERK activity in ICM blastomeres bifurcates at the end of mitosis; some ICM cells undergo the transient ERK inactivation after the cell division whereas others retain high ERK activity (Pokrass et al., 2020). Moreover, this bifurcation of ERK activity at mitotic exit governs the commitment of the specification to Epi or PrE; NANOG and GATA6 expression observed by the immunofluorescence after time-lapse imaging are negatively and positively correlated with the ERK activity at the end of mitotic exit, respectively (Fig. 6C). As ERK activity at the end of time-lapse imaging shows no correlation with the expression level of cell fate markers, the temporal pattern of ERK activity – but not the endpoint activity – plays a pivotal role in the cell fate determination, which highlights the importance of live-cell imaging using biosensors.

Conclusion

We have provided an overview of genetically encoded fluorescent biosensors for monitoring ERK activity, and introduced how these live-imaging technologies provided insights into spatial and temporal ERK dynamics in developmental contexts. Here, we briefly discuss a few research directions relevant to these issues.

With respect to the biosensors, further improvement in terms of brightness, dynamic range and specificity will be reasonably expected by using novel fluorescent proteins and the design of biosensors. The typical time constant of ERK dynamics ranging from several minutes to 20 min requires the acquisition of fluorescence images with a higher sampling rate than half the time constant of ERK dynamics (that is, the Nyquist frequency) to sufficiently capture ERK dynamics. Taking fluorescence images of long-term biological events such as embryogenesis with a high sampling rate is quite challenging, even at present, because of the phototoxicity. To mitigate this problem, a hopeful research direction is to design synthetic gene/protein circuits to detect specific signaling dynamics (Lormeau et al., 2021; Ravindran et al., 2021 preprint). An example is READer (recorder of ERK activity dynamics), which is composed of ERK-KTR and an incoherent feedforward circuit so that pulsatile, but not sustained, ERK activation can be recorded (Ravindran et al., 2021 preprint). The output of the protein circuit is GFP expression, and thus observation at a single time point allows estimation of ERK pulses. The design principle of READer is generalizable to a variety of cell signals other than ERK.

ERK activation dynamics in space and time accomplish the induction of distinct cellular and tissue phenotypes. We still do not fully understand what kind of input information ERK activation dynamics encodes *in vivo*, or how ERK activation dynamics are decoded to lead to different cellular behaviors. To address the first issue, we need the spatial and temporal information of ligands such as growth factors. Again, the development of genetically encoded biosensors might open the door to visualizing ligands themselves, such as G-protein coupled receptor (GPCR) ligands (Sun et al., 2018; Patriarchi et al., 2018). Regarding the second issue, it is

necessary not only to quantitatively understand the gene expression patterns and function of downstream molecules of ERK, but also to develop tools for controlling ERK activity *in vivo* to verify the causality between ERK activation dynamics and cellular phenotype.

Although the current applications of genetically encoded ERK biosensors have been limited to popular model systems, a growing body of evidence implies that ERK plays key roles in a much wider variety of developmental processes and organisms. For example, previous studies on planarians indicated that opposing gradients of ERK and β-catenin signals provide a positional cue throughout the adult body, which enables the strong regeneration capability from any part of the body (Gurley et al., 2008; Petersen and Reddien, 2008; Umesono et al., 2013). Moreover, it has been reported that transient inhibition of ERK after amputation permanently blocks regeneration, further supporting the importance of dynamics (Owlarn et al., 2017). We are certain that the characterization of dynamical aspects of ERK signaling in underrepresented systems would not only demonstrate novel biological phenomena, but also shed light on how developmental processes have evolved.

We believe that, although the previous applications of live-imaging techniques have already been fruitful, they are only the tip of the iceberg. In order to draw the entire picture of the RAS-ERK pathway, we need to explore the full potential of the rapidly evolving technologies and apply them to a wider variety of systems.

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