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Review

Gossiping about death: Apoptosis-induced ERK waves as coordinators of multicellular fate decisions

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ABSTRACT

Apoptosis is now recognized as a highly dynamic process that involves the release of a large set of signaling molecules that convey information to cells neighboring an apoptotic site. Recent studies in epithelial systems have discovered that apoptotic cells trigger waves of pulses of mitogen-activated protein kinase (MAPK) / extracellular signal-regulated kinase (ERK) pathway activity in their neighbors. At the single-cell level, the ERK pulses emerge from the MAPK pathway's excitable network properties, such as ultrasensitivity and adaptation. At the cell population level, apoptosis-induced ERK waves (AiEWs) emerge from propagation of ERK pulses across cells via a mechanism that involves mechanical inputs and paracrine signaling. AiEWs enable cell populations to dynamically coordinate fate decision signaling during tissue homeostasis and development. This spatio-temporal signaling mechanism can be hijacked by cancer cells to induce drug-tolerant persister states when apoptosis is triggered by cytotoxic or targeted therapies, undermining treatment efficacy. In this review, we summarize our current understanding of AiEWs, including their initiation, propagation, and coordination of fate decision signaling within a population. We discuss how the relatively simple properties of single cells, and their interactions within a collective coordinate these dynamic signaling patterns. We highlight their implication in resistance to cancer therapy and explore potential strategies to target these waves to re-sensitize cancer cells. Finally, we discuss emerging technologies and future directions to expand the study of this biological phenomenon.

1. Main body

Apoptosis [1] is the best characterized form of programmed cell death. It plays a crucial role in the precise removal of old, damaged or unnecessary cells throughout an animal organism's life. During development, apoptosis sculpts tissues and organs, while in adulthood, it maintains tissue homeostasis [2]. Additionally, it serves as a critical defense mechanism against cancer. Consequently, evasion of apoptosis is recognized as one of the most important hallmarks of cancer [3]. Due to its ability to remove dying cells without triggering inflammation, or recruitment of immune cells, it was once considered a "silent" form of cell death. Recently a series of discoveries, summarized in [4], has shed light on the ability of apoptotic cells to communicate to neighboring cells in a tissue, via soluble molecules, microvesicles or apoptotic bodies. In this review, we discuss apoptosis-induced ERK waves (AiEWs) as another recently discovered mechanism through which cells "gossip about death".

2. Single-cell and collective signaling dynamics by the MAPK/ERK signaling pathway

The MAPK/ERK signaling pathway plays a crucial role in transducing growth factor signaling and in the regulation of fate decisions [5]. It is activated by growth factors binding to receptor tyrosine kinases (RTKs) like the epidermal growth factor receptor (EGFR), as well as by G-protein–coupled receptors and integrins. These receptors activate small GTPases, such as Ras, that subsequently activate the kinase cascade constituted by Rapidly Accelerated Fibrosarcoma (RAF) kinase,

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mitogen-activated protein kinase (MEK) and ERK. The kinase ERK is the last effector of the signaling cascade, phosphorylating a large array of cytosolic substrates, and also translocating to the nucleus to trigger wide transcriptional programs. Mutations in this pathway are frequently observed in cancer, driving evasion of apoptosis and unrestrained cell proliferation.

While it has been clear for three decades that the MAPK signaling network can induce different ERK activity dynamics [6], the development of a large panel of fluorescent biosensors of ERK activity, summarized in [7], now enables the observation of ERK activity dynamics (hereafter referred to as ERK dynamics) in single living cells. This technology, combined with image and data analysis, has revealed that ERK activity is highly dynamic at the single-cell level. A prime example is 20–30 min-long ERK pulses of constant amplitude whose frequency corresponds to the concentration of epidermal growth factor (EGF) added to confluent epithelial cell cultures [8]. These ERK pulses arise from the intrinsic excitability of the MAPK signaling network. Nonlinear kinetic modeling using ordinary differential equations, together with measurements of MAPK activation in Xenopus oocyte extracts, has demonstrated that the sequential phosphorylations within the RAF–MEK–ERK cascade confer ultrasensitivity. This mechanism enables the MAPK pathway to convert graded stimuli into switch-like, all-or-nothing responses [9]. The inherent nonlinear dynamics of the MAPK pathway have also been captured through live fluorescent imaging, by monitoring both ERK nuclear translocation and ERK activity via biosensors [10,11]. Furthermore, self-reinforcement mediated by positive feedback loops amplifies activation and stabilizes the switch-like state, thereby enhancing ultrasensitivity and generating the bistable behavior of the MAPK network. This phenomenon has been validated through mathematical modeling [12] as well as experiments in PC-12 cells stimulated with nerve growth factor (NGF) [13]. In addition to the roles of positive feedback and bistability, studies utilizing ordinary differential equations [14], different cellular models [15], and combinations of fluorescent biosensors with optogenetic activation [16] have shown that negative feedback loops within the MAPK network drive adaptation. This adaptation is crucial for generating pulses of constant amplitude, even under continuous stimulation. It is important to note, however, that while negative feedback is essential for establishing pulsatile dynamics, factors such as baseline ERK dephosphorylation and the finite size of the ERK pool may also influence the limitation of pulse amplitude [14].

Thanks to these properties, the MAPK excitable signaling network encodes the EGF concentration into a frequency-modulated ERK pulse regime [8]. Low, intermediate, and high ERK pulse frequencies are then decoded into apoptosis, survival, and proliferation fates respectively [8,17]. Beyond mere correlations between ERK pulse frequencies and fate decisions, causal relationships between both processes can be obtained by using optogenetics to evoke synthetic frequency-modulated ERK pulse regimes to trigger specific fate decisions [18–20]. For instance, we demonstrated that triggering an ERK activity pulse every 3 hours, or at a higher frequency using an artificial optogenetic FGF receptor, induces cell survival in 2D epithelia and in 3D acini during mammary morphogenesis *in vitro* [17,21]. Two recent review articles discuss in detail how ERK dynamics are encoded and decoded [22,23].

ERK activity waves (hereafter referred to as ERK waves) emerge from the ability of adjacent cells within a confluent monolayer to sequentially transmit ERK pulses from one cell to the next, through different self-reinforcing mechanisms that depend on the cell type and model tissue/organism. ERK waves were first observed in epithelial cell cultures *in vitro* and in the epidermis *in vivo* [24,25]. Wounded monolayers of Madin-Darby canine kidney (MDCK) cells exhibit ERK waves that start at the wound edge and penetrate inwards. These ERK waves spatio-temporally control myosin contractility to coordinate collective migration towards the wounded edge, facilitating wound repair [24,26,27]. The observations of ERK waves in the mouse skin epidermis *in vivo* [25] and in osteoblasts during fish scale regeneration [28], or the ultrafast ERK waves that propagate in longitudinal muscles in Planaria and coordinate whole body regeneration [29] demonstrated that these phenomena occur in intact physiological tissues in living animals. By travelling from cell to cell, ERK waves convey information about the events that triggered the waves. Such information propagates in a tissue until the waves dissipate, enabling localized cellular responses [30]. ERK waves observed to date exhibit a wide variety of shapes, propagation distances, and speeds, playing important roles in development, regeneration, and response to bacterial infections [29,31,32]. Current knowledge about ERK waves has been reviewed here [23,33,34].

3. Apoptosis-induced ERK signaling waves

A subtype of ERK waves includes those that are induced by apoptotic cells in a tissue. These phenomena have been observed in epithelial monolayers made of the rat renal cell line NRK-52E [17,35], the dog renal cell line MDCK [17], the human mammary epithelial cell line MCF10A [17,36], the human bronchial epithelial cell line 16HBE [37], in patient-derived 2D intestinal organoids [38] and in the Drosophila pupal notum [39]. In all these cases, a single apoptotic cell can trigger an ERK activity wave that propagates radially through the surrounding healthy cells up to a finite distance (Fig. 1a). The fact that AiEWs can be observed in different *in vitro* mammalian cell cultures, as well as *in vivo* in Drosophila, indicates that these waves are evolutionarily conserved phenomena, spanning from arthropods to vertebrates [17,39]. These waves are initiated during apoptosis by the activation of matrix metalloproteinases (MMPs), which cleave membrane-bound EGFR ligands. These ligands bind to EGFR on the neighboring cells, activating not only the downstream MAPK/ERK but also the PI3K/Akt signaling cascades (Fig. 1b). Our previous work demonstrated that ERK/Akt waves are triggered when apoptosis is induced by optogenetic activation of mitochondrial outer membrane permeabilization (MOMP). In these experiments, we have observed that an AiEW is initiated concomitantly with nuclear compaction that precedes apoptosis and epithelial extrusion [17]. Thus, AiEWs are independent of the downstream caspase cascade, and occur before the execution phase responsible for apoptotic blebbing and epithelial extrusion.

Once initiated, AiEWs propagate through a variable number of neighboring cells, ranging from waves that only propagate one row of neighbors [39], to those that potentially propagate for more than 10 rows of neighbors [17,38]. Because these waves have a finite range, their propagation requires both a self-reinforcing mechanism to relay the signal from cell to cell and a dissipative mechanism to limit their spread. For example, MDCK cells, which produce long-ranging AiEWs, may utilize a mechanism similar to that observed in ERK wave propagation during wound healing. In this scenario, ERK wave propagation would depend on the crosstalk with the actomyosin cytoskeleton: ERK is activated by cell stretching induced by contractility in neighboring cells, and in turn, ERK activity triggers localized contractility that stretches the next cell, thereby coordinating collective directional migration [26]. This review article summarizes the current understanding of the mechanochemical crosstalk between ERK and actomyosin cytoskeleton during ERK wave propagation [40]. Moreover, the strict dependence of AiEWs on MMPs and EGFR activity supports the idea that EGFR ligand signaling is central to their propagation. According to this hypothesis, a cell receiving an EGFR ligand input releases additional ligands to activate an adjacent cell, establishing a sequential relay mechanism that spans multiple cells and leads to the formation of AiEWs (Fig. 1c). In other systems, such as osteoblasts during fish scale regeneration [28] or during the invagination of the Drosophila tracheal placode [41], ERK activity spreads by inducing the transcription of its activators. However, because this process relies on the time required for transcription and translation, it is slower than that observed in AiEWs.

The nature of the mechanisms of dissipation has not been identified so far. An attractive hypothesis is that a sequential ERK-triggered mechanical input wanes over time, determining the ERK wave's size. The

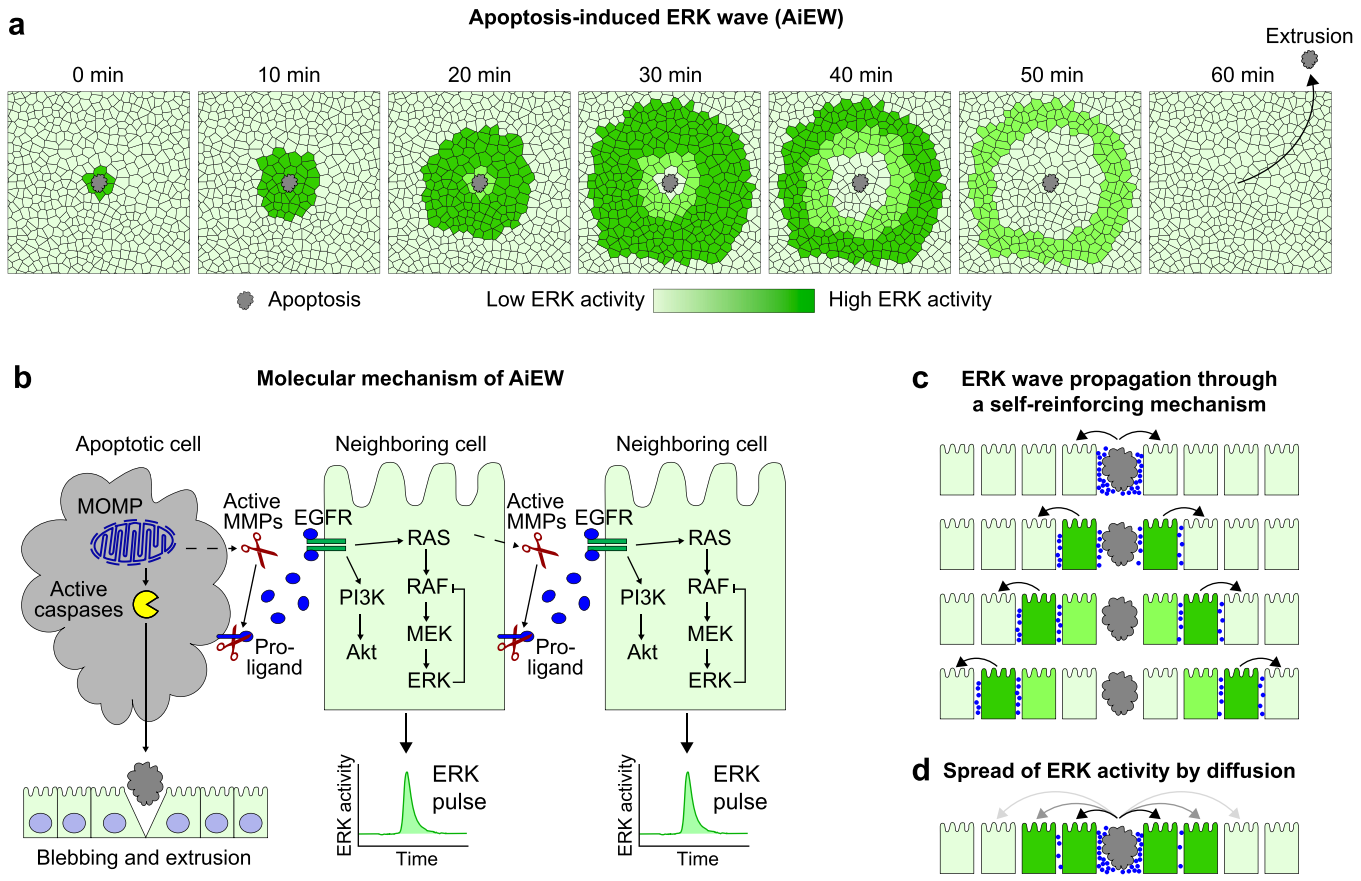

Fig. 1. Apoptosis-induced ERK waves (AiEWs). a) An AiEW is triggered by the apoptotic cell in the center, and then it propagates radially into the surrounding healthy cells until it dissipates upon reaching a certain propagation distance. Following this process, the apoptotic cell is typically extruded from the epithelial monolayer. b) Apoptotic cells trigger the ERK wave by activating MMPs, which shed EGFR ligands. These ligands bind to EGFR on neighboring cells, leading to a transient pulse of MAPK/ERK pathway activity. The activation of MMPs is dependent on MOMP but is independent of caspases, which instead regulate cellular blebbing and epithelial extrusion. The ERK wave is then propagated from cell to cell. The illustrated scheme represents the hypothesis of active propagation, where the first neighboring cell releases additional EGF, stimulating the next neighboring cell in sequence. c) AiEWs propagation may occur through a self-reinforcing mechanism, in which each neighboring cell actively produces the signal required to sustain and propagate the wave. This type of ERK wave has the potential to travel greater distances compared to those mediated solely by passive diffusion. The finite propagation distance of AiEWs also requires the existence of a dissipating mechanism, that reduces the intensity of the ERK input over the distance from the apoptotic site. d) Similar phenomena of ERK activity spreading can also be generated by simple diffusion, where the MAPK pathway transforms a decaying gradient of the ligands into an all-or-nothing response. These events should not be defined as ERK waves because they lack a self-reinforcing mechanism.

observation that MDCK cells, known for forming very strong adherent junctions, display ERK waves that propagate over long distances, while MCF10A cells, which produce less robust adherent junctions, display waves that propagate for shorter distances, may support this idea. A key question is how a diminishing input is transformed into all-or-nothing ERK activity pulses with constant amplitude, as seen in experiments. This can be explained by the ultrasensitivity of the MAPK network: cells exposed to an EGFR ligand concentration above a specific threshold produce a full-amplitude ERK pulse, while those below the threshold do not trigger a pulse.

Alternatively, short-range activation of ERK in neighboring cells could result from the simple diffusion of EGFR ligands released by the apoptotic cell (Fig. 1d). However, such diffusion events lack the self-reinforcing intercellular propagation that defines true “waves” and therefore should not be defined as such. These ligands likely diffuse basolaterally, below the tight junction barrier that insulates epithelia, with different ligands exhibiting different diffusion properties [42]. Consistently, spread of ERK activity over different distances can be generated by a synthetic *in vitro* system with inducible secretion of different EGFR ligands from a source cell [43]. Here, high affinity EGFR ligands produce ERK waves that propagate for shorter distances than low affinity ligands, suggesting that ligand identity controls wave size. Similarly to the self-reinforcing mechanism with dissipation explained above, the ultrasensitivity of the MAPK pathway would transform a decaying input into a full-amplitude ERK activation, until the input remains above a threshold.

Further research is needed to elucidate the mechanisms governing AiEW propagation and dissipation, and to determine whether different models produce true AiEWs or merely reflect the spread of EGFR ligands from apoptotic cells.

4. ERK waves as spatial organizers of ERK pulse frequency

Each ERK wave corresponds to a single ERK pulse experienced by cells within its propagation zone. In tissues, where multiple ERK waves can arise sequentially or simultaneously, a single cell can experience multiple pulses of ERK activity. The number of these pulses per unit of time determines the ERK pulse frequency perceived by individual cells. This relationship makes ERK waves key spatial and temporal organizers of ERK pulse frequency, with profound implications for cell fate decisions.

In the case of AiEWs, size and number of the ERK waves have a major role in controlling ERK pulse frequency in individual cells. Generally, a higher number of ERK waves at the population level or ERK waves that

propagate for longer distances result in an increased ERK pulse frequency. Conversely, fewer ERK waves or ERK waves that propagate for shorter distances lead to a reduced ERK pulse frequency (Fig. 2a). This establishes a direct relationship between ERK wave properties and the ERK pulse frequency observed in individual cells. The propagation distance of AiEWs controls tissue patterning in different ways, reflecting distinct biological functions. AiEWs that propagate for short distances are important when the information of an apoptotic event is required only in the closest neighbors. For example, in the Drosophila pupal notum, AiEWs affect only the cells directly adjacent to the apoptotic cell [39]. This is expected to play a role in a transient and local phenotype in only the direct neighbors, but have a modest effect on single-cell ERK pulse frequency at the population scale.

In contrast, AiEWs that propagate for longer distances tend to overlap, resulting in higher single-cell pulse frequencies and coordinating the behavior of larger groups of cells (Fig. 2a). In 2D patient-derived primary intestinal cell cultures, AiEWs are triggered by extruding cells in an area corresponding to the tips of intestinal villi [38]. These waves define a zone of high ERK activity, creating a compartment of a specific size around the apoptotic cells. Because apoptosis occurs consistently at the tip of the villi, multiple consecutive and overlapping AiEWs are expected to arise. Although the original study did not measure ERK pulse frequency directly, it is likely that consecutive AiEWs triggered from the same site result in a zone of high ERK pulse frequency. This high-frequency zone corresponds to the differentiated enterocyte zone (Fig. 2b), while cells outside this zone retain stem-cell properties. Through this mechanism, AiEWs dynamically organize the intestinal epithelium, ensuring a balance between crypt (stem cell) and villi (enterocyte) compartments. Disrupting this spatial patterning of ERK waves, and consequently ERK pulse frequency, can disturb this balance, leading to abnormal epithelial organization [38].

Another example of this spatial encoding of ERK pulse frequency is the self-organization of 3D mammary acini morphogenesis (Fig. 2c). Here, proliferating mammary epithelial cells initially grow into a sphere, transition to a quiescent state upon reaching a critical size, and ultimately undergo cavitation through apoptosis of the inner cells, resulting in the formation of the lumen [44]. During the cavitation stage,

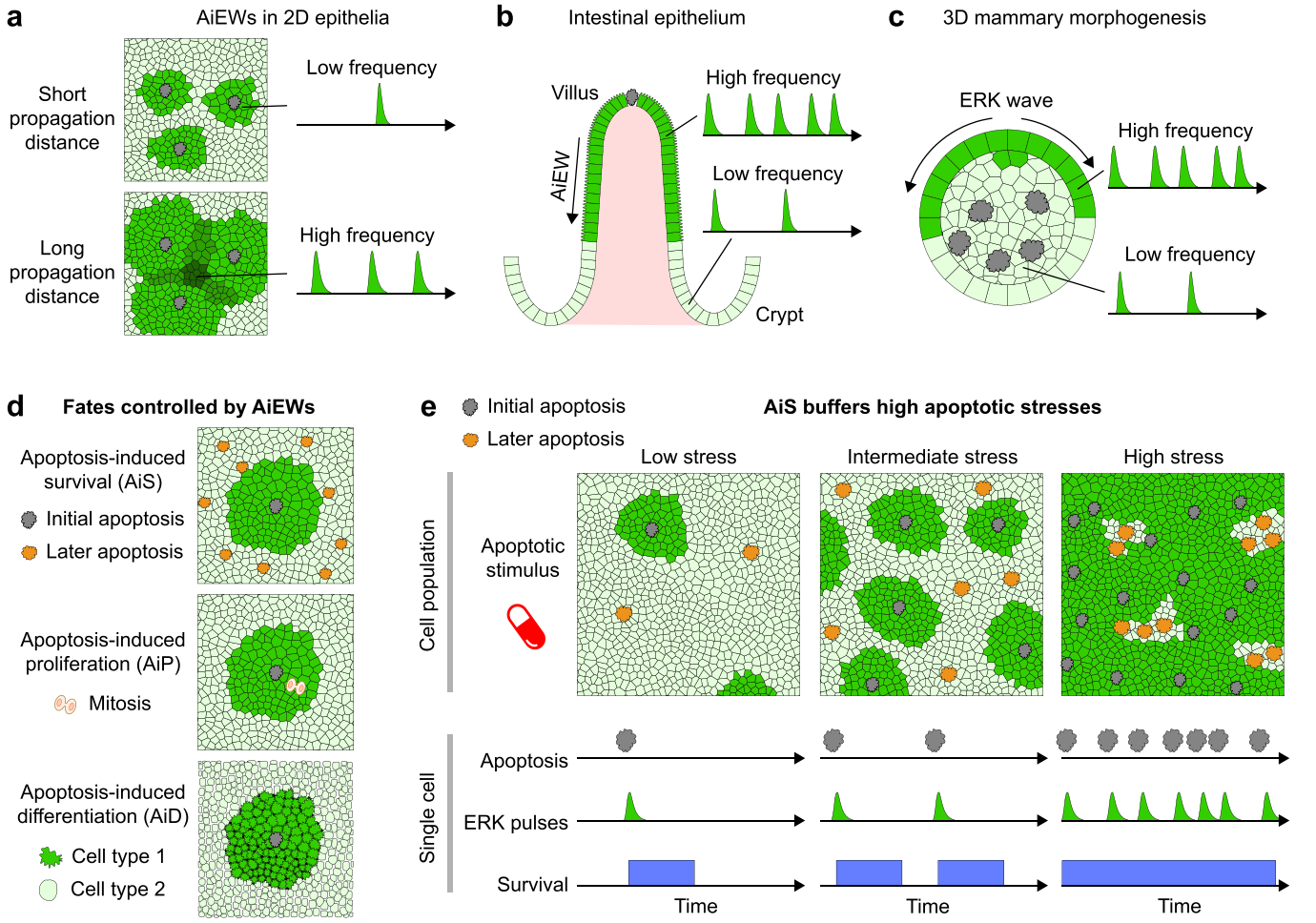

Fig. 2. ERK waves as spatial organizers of ERK pulse frequency and cell fate decisions. a) ERK waves play a critical role in the spatiotemporal regulation of ERK pulse frequency within tissues. AiEWs that propagate for short distances result in low ERK pulse frequencies, while AiEWs that propagate for longer distances generate higher frequencies. Notably, the cartoon illustrates three distinct waves occurring at different times. b) In 2D primary intestinal cultures, apoptosis consistently occurs in regions corresponding to the tips of the villi, triggering AiEWs that propagate over long distances. These waves establish distinct zones: a high-ERK-frequency zone associated with differentiated enterocytes and a low-ERK-frequency zone encompassing the stem cell compartment. c) During 3D mammary acini morphogenesis, non-apoptotic ERK waves are primarily triggered by the outer cells and rarely propagate inward. This creates two distinct zones of ERK pulse frequency: a high-frequency zone in the outer cells, which exhibit increased survival, and a low-frequency zone in the inner cells, which undergo apoptosis. d) AiEWs can regulate diverse cellular fates in the affected cells, including survival, cell cycle progression, and differentiation. e) Apoptosis-induced survival (AiS), mediated by AiEWs, serves as a buffering mechanism against high apoptotic stress at the tissue level. As the apoptotic rate rises, a larger portion of the tissue is influenced by AiEWs, resulting in more cells becoming resistant to subsequent apoptosis. At the single-cell level, multiple ERK waves triggered by distinct apoptotic events in the surrounding area increase the frequency of ERK pulses, thereby prolonging cell survival.

non-apoptotic ERK waves originate and mostly propagate in the outer cells of a sphere, and only rarely propagate to the cells in the center of acini. Consequently, ERK waves dynamically pattern outer and inner zones with respectively high and low ERK pulse frequency. These different ERK pulse frequencies are then decoded in quiescence/survival (outer cells, high ERK frequency) and apoptosis fates (inner cells, low ERK frequency), dynamically and spatially regulating lumen formation. In this system, AiEWs might instead control mammary acini homeostasis, once their morphogenesis is completed [21].

These examples illustrate that AiEWs, and in general ERK waves, organize fate decisions by encoding spatiotemporal patterns of ERK activity into frequency-modulated ERK pulse regimes that are sensed and decoded in specific fates at the single-cell level. By modulating ERK wave size, frequency, and shape, tissues dynamically adjust ERK pulse frequencies to respond to specific triggers to maintain tissue architecture and function. Disruption of ERK wave patterns, and consequently ERK pulse frequency, leads to aberrant fate decisions during tissue self-organization. Future studies should explore how tissues fine-tune ERK wave properties to control pulse frequency and how this impacts development, homeostasis, and disease progression.

5. Decoding of AiEWs into cell fate decisions

As mentioned above, AiEWs spatially contribute to ERK pulse frequency in the vicinity of apoptotic lesions in epithelia. Different ERK pulse frequencies in turn can be decoded into different fates such as apoptosis, survival, or proliferation. This establishes a relationship between AiEWs and decoding of cell fate decisions.

In epithelia, the most immediate effect of AiEWs is a transient reduction in the probability of apoptosis in cells neighboring an apoptotic lesion. We termed this process apoptosis-induced survival (AiS) [17,39] (Fig. 2d), but a similar process was already described in other biological contexts, and named apoptosis-induced death resistance [45]. Current evidence suggests that this reduction in apoptosis is only transient, and after its expiration, the basal probability of apoptosis is restored. Decreased probability of apoptosis might occur via different regulatory mechanisms. In the case of the Drosophila pupal notum, the neighboring cells that experience a pulse of ERK activity display a reduction in caspase activity, leading to an 1-hour AiS episode [39]. In mammalian cells, the longer duration of the ERK pulse triggered AiS episode (3–4 hours) [17] might be consistent with the kinetics of transcription/translation of immediate early genes that are transcribed downstream of ERK [46]. Immediate early genes encode mRNA and proteins that are highly unstable [47], and thus a rapidly turning over transcriptional program might mediate the AiS response.

Beyond this local effect in the vicinity of the apoptotic lesion, AiS also allows tissues to tune survival fates to environmental stresses of different intensities at the whole cell population level. This emergent property results from the cooperation of multiple ERK waves that are triggered by different apoptotic events. When environmental stress is low, few apoptotic events occur, leading to a limited number of AiEWs. This results in a low single-cell ERK pulse frequency that falls below the critical threshold to induce survival. For instance, in the mammary epithelial cell line MCF10A, at least three pulses per hour are required to shift fate decision to survival [17]. In contrast, stronger environmental stress increases the frequency of apoptotic events, which in turn produces more AiEWs. In the case of MCF10A cells, this elevated number of waves raises the ERK pulse frequency in single-cells above the three-pulse threshold, triggering a full survival response (Fig. 2e). This cooperative effect of multiple ERK waves arises from this non-linear relationship between ERK pulse frequency and cell survival. Because of the steep curve, even minor changes in the number of AiEWs can shift cells from non-survival to survival. This mechanism is essential for maintaining epithelial integrity, as it dynamically scales the AiS response in proportion to the intensity of environmental stress. We demonstrated that, in the presence of high doses of a chemotherapeutic agent, this mechanism maintains epithelial integrity for several hours despite the presence of high apoptotic stress. However, when the pro-survival ERK waves are abrogated, the epithelial integrity is rapidly compromised by the formation of holes in the epithelial monolayer structure [17].

Tissue homeostasis might also require that cells neighboring an apoptotic lesion proliferate to compensate for cell loss (Fig. 2d). This mechanism, known as apoptosis-induced proliferation (AiP), has been found to be critical for tissue integrity and regeneration, and to contribute to cancer progression [48,49]. However, a direct implication of AiEWs in AiP remains unclear. While we have not tested this experimentally, we propose that in the presence of a large amount of environmental stress leading to many AiEWs, the induction of a very high ERK pulse frequency might also induce proliferation rather than only survival. This is supported by the finding that cell cycle progression in healthy, non-mutated mammary epithelial cells can be promoted by repetitive non-apoptotic ERK waves, triggered by clusters of cells harboring the oncogenic BRAF V600E mutation [36]. In this scenario, the oncogenic MAPK network within source cells hijacks wave generation, driving ERK waves independently of apoptosis. Additionally, a study on AiP regulation identified an interplay with a mechanotransduction signaling pathway. Kawaue et al. demonstrated that the decision of a cell neighboring an apoptotic lesion to proliferate depends on inhomogeneities in Yes-associated protein (YAP)-mediated mechanotransduction [50]. In this context, ERK waves could act synergistically with YAP signaling to determine the spatial range of AiP. Moreover, ERK activity has been shown to be essential for compensatory proliferation in response to macrophage recruitment by vesicles released from apoptotic cells [51]. These findings suggest that the cells neighboring apoptotic lesions might integrate multiple signals, including AiEWs and mechanical cues, to induce proliferation. Future studies are needed to elucidate the precise contribution of AiEWs, and YAP-mediated mechanotransduction, as well as possible other signaling networks in the regulation of AiP.

Besides survival and proliferation, AiEWs can serve as spatial organizers of cell differentiation (Fig. 2d), as in the abovementioned case of intestinal cultures *in vitro* [38]. The ability of AiEWs to regulate distinct fates in different cell systems/tissues underscores their significance in development and homeostasis of tissues. We propose that AiEWs are a general mechanism to spatially encode different ERK pulse frequencies that can then be decoded into fate decisions. The mechanism of ERK pulse frequency decoding might then be different depending on a specific cell system or tissue.

6. Role of ERK signaling waves in cancer and therapy

While AiEWs act as a physiological mechanism to ensure tissue development and homeostasis, its detrimental side might be the potential contribution to cancer growth and resistance to therapies. ERK waves, triggered by apoptotic tumor cells in response to drug regimes, can lead to mitogenic stimulation and drug resistance in the neighboring cancer cells (Fig. 3a).

Already in 1956, a seminal paper from László Révész showed that irradiated dying tumor cells mixed with untreated tumor cells make the latter more aggressive, causing shorter life expectancy in mice models [52], providing the first example of the paradoxical role of apoptosis in cancer. While evasion of apoptosis is considered a universal hallmark of cancer, low levels of apoptosis coincide with aggressive phenotypes in a wide range of cancer types [53]. It is worth considering whether the role of apoptosis to promote tumor aggressiveness is driven by non-autonomous activation of the MAPK/ERK pathway in cancer cells mediated by AiEWs.

Recently, Gonzalez-Martinez et al. studied ERK waves in cells harboring the EML4-ALK (echinoderm microtubule associated protein-like 4-anaplastic lymphoma kinase) fusion oncogene. This oncogene drives the formation of cytoplasmic condensates containing EML4-ALK

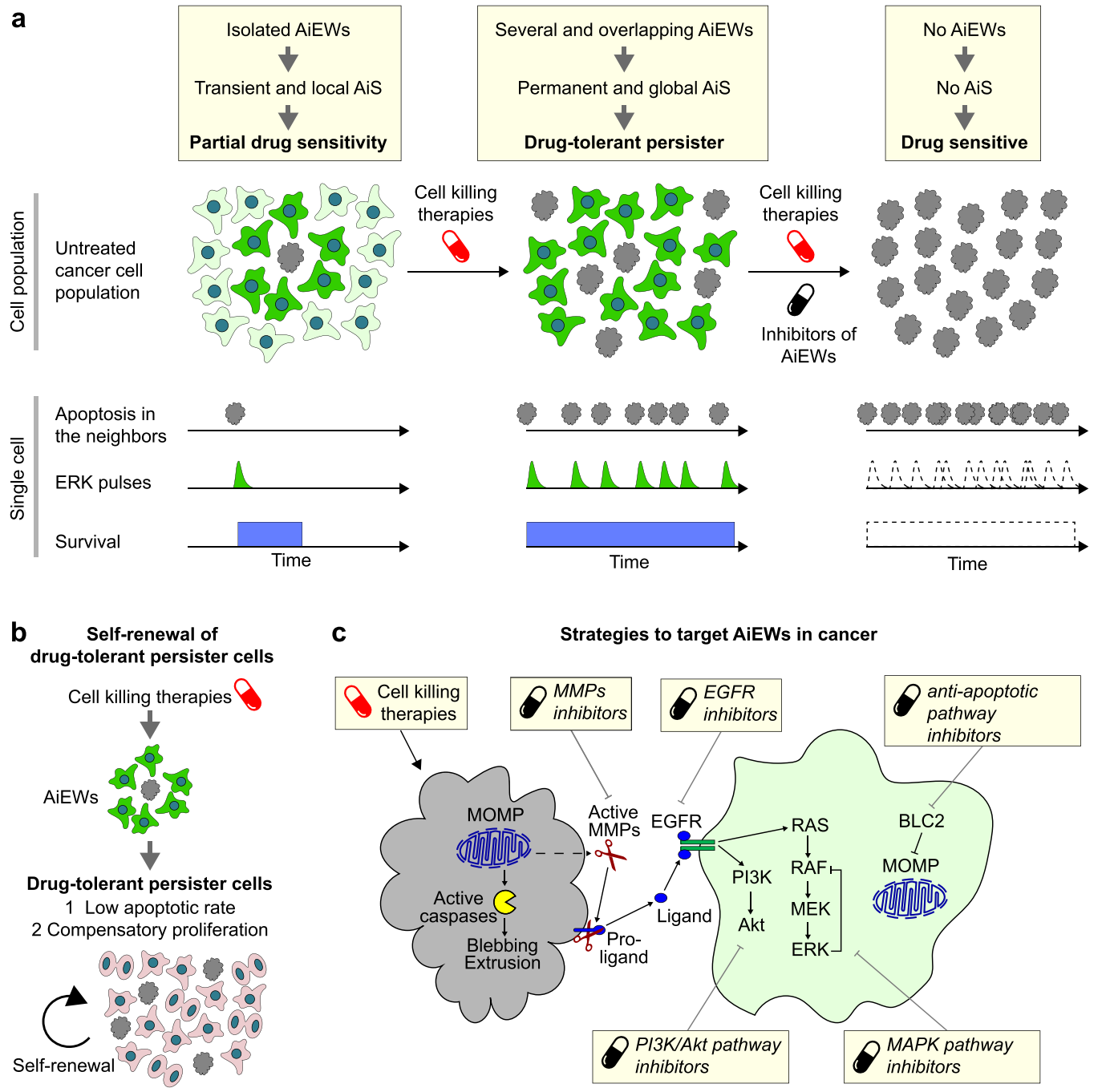

Fig. 3. AiEWs as a promising target in cancer therapy. a) Survival driven by AiEWs can compromise the efficacy of cancer therapies. In untreated cancers, rare apoptotic events have minimal impact on tumor sensitivity to drugs. However, when a therapeutic agent induces widespread apoptosis, the resulting surge in ERK waves can lock surviving cells into a state of drug resistance, often referred to as the drug-tolerant persister state. Inhibiting ERK wave propagation is anticipated to restore drug sensitivity, overcoming this resistance mechanism. b) ERK waves might play a pivotal role in the maintenance of the drug-tolerant persister state, by keeping a low apoptotic rate and promoting compensatory proliferation. c) There are different possibilities to block AiEWs in cancer cells, including inhibition of MMPs, inhibition of the receptor on the neighboring cells or direct targeting of the MAPK, PI3K/Akt and anti-apoptotic pathways.

itself and adaptor proteins like GRB2 and SOS1, both crucial components of the MAPK pathway. These condensates constitutively activate the MAPK pathway, bypassing the need for external stimuli. Treatment with an ALK inhibitor dissolves these condensates, sensitizing cells to external signals and allowing the transmission of ERK waves. Under ALK inhibition, apoptotic cells trigger AiEWs, which promote drug resistance in neighboring cancer cells. However, this resistance mechanism can be mitigated by inhibiting EGFR or MMPs, which enhances cell killing and reduces survival signaling [54]. Another example of this effect has been reported in apoptotic HeLa cervical cancer cells that make the cells neighboring a lesion more resistant to apoptosis via secretion of fibroblast growth factor (FGF) [55]. This again involves activation of the MAPK pathway in cells neighboring the apoptotic lesion to transcriptionally upregulate the pro-survival BCL-2 protein. This might be consistent with the observation that in certain cancers, FGF-signalling and BCL-2 expression correlates with worse prognosis. In this case, the involvement of FGF suggests that RTKs other than EGFR can mediate the pro-survival effect of ERK waves. Two other oncogenes, KRAS G12V and PIK3CA H1047R, cause ERK waves that propagate for longer distances, possibly due to alteration of cell-cell transmission of ERK pulses [56].

This suggests that mutations in the MAPK, or in the PI3K/Akt network (that crosstalks with the latter) additionally feed in the regulation of ERK wave propagation.

One of the major problems in modern oncology is that, even with the most advanced therapies, some cells persist and eventually cause tumor relapse when therapy is suspended. There is an emerging awareness that these drug-tolerant persister cells acquire a transient state through which they can survive in the presence of the drug [57]. The results with EML4-ALK fusion oncogene report, for the first time, an example of how AiEWs are involved in the drug persister state of cancer cells. It is possible to imagine a scenario in which chronic cell death of some cancer cells induces survival of other cancer cells in the same cell population (Fig. 3b). This scenario might involve compensatory proliferation through AiEWs. Experiments with the chemotherapeutic agent doxorubicin demonstrate that AiEWs can buffer an apoptotic rate to 3 % of apoptosis per hour (Gagliardi et al., 2021), that is compatible, in principle, with a cell cycle duration of ~24 hours, to maintain a stable pool of drug-tolerant persister cells.

Our current knowledge on the molecular mechanism responsible for AiEWs propagation might already be ready to be translated in cancer therapy (Fig. 3c). Inhibitors are available for targeting MMPs, EGFR ligands, EGFR, and the MAPK pathway that regulate AiEW propagation. Cleavage of EGFR ligands from the transmembrane to the soluble extracellular form is mediated by MMPs, and can be inhibited by pan-MMP or MMP-specific (ADAM17) inhibitors. EGFR ligands and receptors can be inhibited by antibodies and small molecules. Direct targeting of EGFR ligands might however be problematic, as ERK waves are only partially inhibited after genetic ablation of four of the seven known EGFR ligands [58]. On the contrary, blocking the EGFR receptor with the antibody Cetuximab, commonly used for colorectal cancer treatment, efficiently blocks ERK waves [17]. The same result can also be achieved by small molecule inhibitors of EGFR kinase activity and of the downstream MAPK pathway [17]. Another strategy might be to target the anti-apoptotic protein Bcl2, responsible for AiS mediated by non-autonomous activation of MAPK pathway in cells neighboring a lesion [55]. All the abovementioned inhibitors are already used in the treatment of cancer or are under clinical development, facilitating a possible clinical translation of targeting AiEWs. However, further research is required to identify novel mediators of AiEWs with high specificity to cancer cells to reduce potential toxicity to healthy cells. Future studies should also characterize the best ratio between the drugs that induce apoptosis and those that suppress AiEWs. It is possible that inhibiting AiEWs might have an optimal therapeutic effect at an intermediate apoptotic rate, at which a sufficient cell number undergoes apoptosis to trigger a global survival effect, without however inducing a cell population collapse.

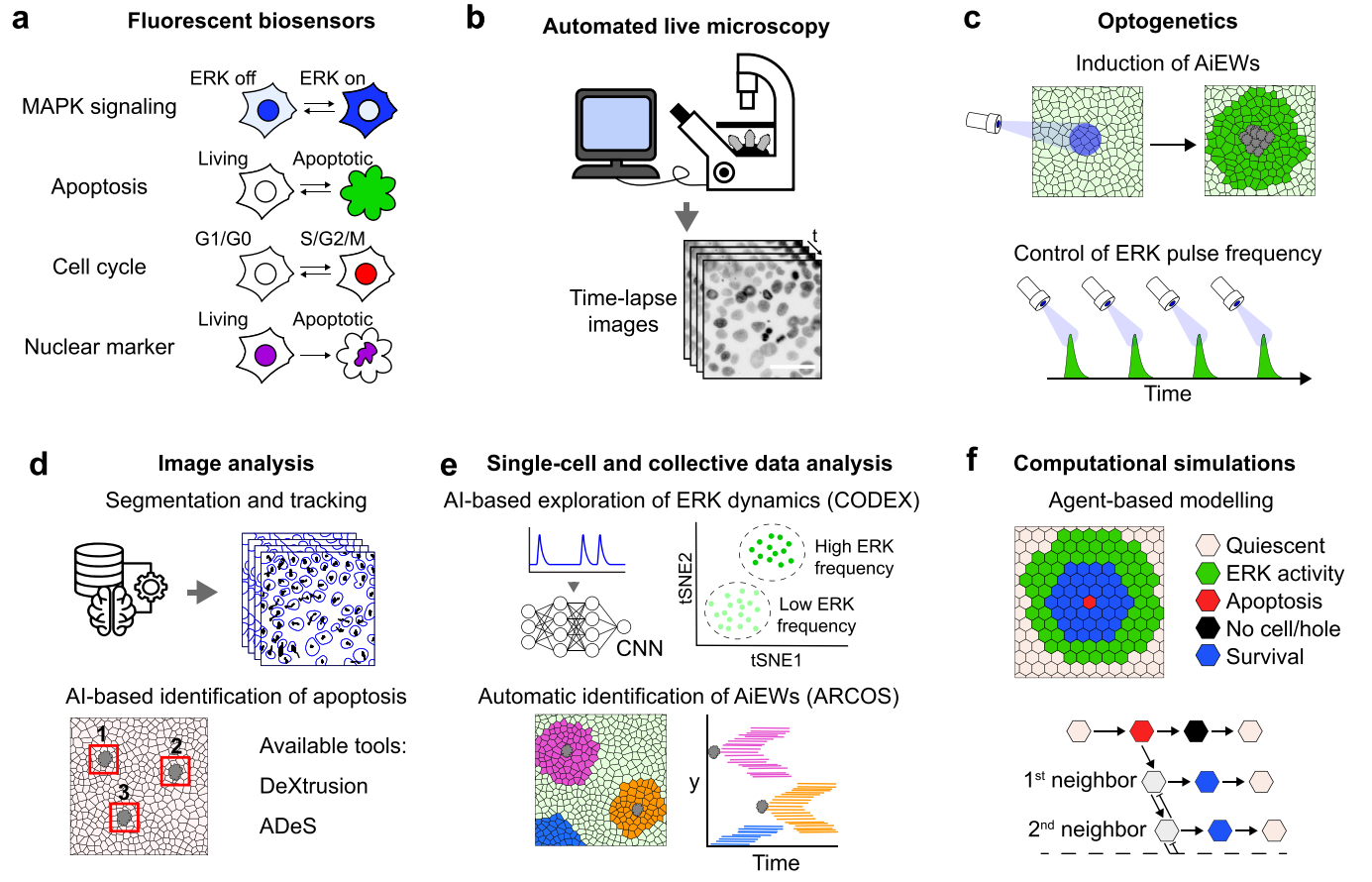

Fig. 4. State-of-the-art technologies to study AiEWs. a) Fluorescent biosensors of ERK activity, apoptosis, and cell cycle, and a stable nuclear marker are required to observe AiEWs and their consequences for cell fate decisions. b) Live fluorescence microscopy with a time resolution of 1–5 minutes over several hours is crucial for capturing highly dynamic events, such as AiEWs. c) Optogenetics is a powerful tool for manipulating ERK dynamics and establishing causal relationships between ERK activity and its downstream effects. It enables precise control over cellular processes, such as inducing apoptosis in individual cells or groups of cells, generating synthetic ERK waves, and orchestrating various temporal patterns of ERK activity. d) Image analysis enables segmentation and tracking of cells, the extraction of single-cell ERK dynamics, and the automatic identification of apoptotic events. e) Data-driven computational tools are essential for unraveling the complexity of single-cell ERK dynamics and enabling the automated detection of collective ERK waves. These tools provide the analytical power needed to explore complex temporal patterns and spatial propagation within cellular populations. f) Agent-based simulations offer a powerful approach to replicating AiEWs in silico, enabling the exploration of ERK wave parameter spaces that are challenging or impossible to investigate experimentally. These simulations provide important insights into the dynamics and regulation of ERK waves under various conditions.

7. Technologies to quantitatively study these emergent properties

The observation and characterization of AiEWs has only been possible by the emergence of technologies that resolve the dynamics of this process at the single-cell level, and from computational methods that can extract relevant information from single-cell high dimensional datasets. This includes live cell imaging of signaling dynamics using fluorescent biosensors, optogenetics, image analysis, statistical analysis of single-cell biosensor trajectories, detection of collective signaling events, and computational modelling. Below, we describe these technologies, and how they can be used to characterize AiEWs.

7.1. Live microscopy with fluorescent biosensors

Because of AiEWs's dynamic nature, it is not possible to analyze them at steady state using tissue immunostaining for phosphorylated, active ERK. This explains why AiEWs remained elusive before the use of fluorescent biosensors of ERK activity (Fig. 4a). Two main categories of biosensors exist: FRET-based biosensors [59] and nuclear translocation reporters [60]. Both biosensor types have been iteratively improved over the years to increase their dynamic range or to eliminate the spurious phosphorylation by cyclin-dependent kinase 1 (CDK1) [61,62]. In addition, the availability of multiple fluorescent proteins with different spectral properties, now allows one to perform multiplexed measurements using different biosensors. For example, one can engineer cell lines expressing an ERK biosensor, a caspase biosensor (that becomes fluorescent when cleaved by caspases [63]), a cell cycle reporter and a stable nuclear marker for segmentation and tracking. Live imaging then enables the measurement of ERK activity, apoptosis, and cell cycle fates at single-cell resolution in over 1000 cells (Fig. 4b). This provides the opportunity to better link ERK signaling dynamics with their downstream fates. Such experiments require 3–5 min-scale time resolution over several hours to capture asynchronous, sometimes rare signaling phenomena as AiEWs.

7.2. Optogenetics

Causal understanding of how AiEWs are triggered and propagated, or how ERK pulse frequency regimes are decoded into cell fates, is facilitated by the ability to precisely control apoptosis or ERK activity dynamics in time and space, with single-cell resolution. This can be achieved using optogenetics that uses light-triggerable protein switches, combined with optical setups to shine light only in desired cells (Fig. 4c). We used optogenetics to induce MOMP in target cells by recruiting a pro-apoptotic optogenetic Bax domain to the outer membrane of mitochondria [17]. This tool, called OptoBax [64], and other optogenetic tools, that allow for direct activation of the caspase cascade [65], enable the precise induction of apoptosis at different points in the apoptotic signaling cascade with single-cell resolution. In another experimental scenario, optogenetic tools that control MAPK signaling dynamics, as the optogenetic actuator of FGFR [66] or RAF [35], can be used to create synthetic ERK waves to study their propagation [56]. This approach can also be used to control the ERK pulse frequency [17,67] to establish a causal relation between ERK dynamics and cell fate decisions.

7.3. Image analysis

AiEWs can be visualized using ERK activity biosensors. Image analysis methods are then required for the extraction of single-cell time-series of ERK activity of each cell in a population (Fig. 4d). Modern image analysis pipelines now combine artificial intelligence-based segmentation and tracking of nuclei. A typical image analysis pipeline to perform these operations is discussed here [68]. In addition to measuring ERK activity dynamics, it is also crucial to identify apoptotic events. This allows one to build a spatio-temporal map that associates each apoptotic event to a specific AiEW. This can be achieved by automatically identifying the apoptotic events through a fluorescent apoptosis reporter, or through morphological features of apoptotic cells. DeXtrusion and ADeS are neural network-based algorithms designed to automatically detect apoptosis and extrusion events by analyzing morphological changes in the cell body or nucleus [69,70]. Thus, these methods circumvent the requirement of an apoptosis reporter.

7.4. Statistical methods for analysis of time-series and collective behaviors

High-dimensional datasets, extracted through image analysis and including single-cell timeseries of ERK activity and apoptotic events, must be statistically analyzed to extract spatio-temporal dependencies between apoptotic events, ERK activity timeseries, and collective ERK activity behaviors (Fig. 4e). Using the spatiotemporal coordinates of apoptotic events, one can generate density maps of subsequent apoptotic occurrences relative to initial events or perform a nearest-neighbor analysis [39]. These approaches can reveal the survival effect of AiEWs even in the absence of direct ERK activity measurements. When ERK activity dynamics is included in the analysis, the heterogeneity of ERK time series makes it difficult to identify temporal patterns across thousands of single cells through visual inspection. In cells synchronized by acute growth factor or drug stimulation, single-cell ERK timeseries can be hierarchically clustered to identify cell subpopulations with specific ERK dynamics [71]. However, AiEWs, and their resulting single-cell ERK dynamics are asynchronous within a population, requiring a different approach. CODEX (COnvolutional neural networks for Dynamics EXploration), solves this issue by taking advantage of convolutional neural networks that are trained to identify specific patterns in the timeseries (e.g. ERK pulse frequencies, shape of ERK pulses, ….) relevant to specific classes (e.g. cell subpopulations of cells that exhibit a different fate, or that experience different perturbations) (Jacques et al., 2021). This approach revealed in a completely data-driven manner that MCF10A cells within a monolayer that survive or die display different ERK pulse frequencies (Gagliardi et al., 2021). These signaling timeseries analysis techniques do not take into account any spatial information. To analyze how single-cell ERK dynamics are coordinated across AiEWs within a population, we developed Automatic Recognition of COllective Signaling (ARCOS) [56,68]. This method identifies and tracks clusters of cells that display coordinated ERK dynamics within a cell population, and provides statistics about the collective events (size, duration, speed of propagation). ARCOS is available in R- and Python, as well as Napari [73] plugin that facilitates analysis for users without programming expertise.

7.5. Mathematical modelling

A correct understanding of AiEWs requires analyzing signaling dynamics at both the single-cell scale and the population level, a task that often surpasses the limits of human intuition. Computational simulations are useful to explore such multiscale phenomena. Collective ERK waves can be simulated with continuum computational models, where a tissue is simulated as a field of ERK activity and other properties, such as mechanical forces. With these models, ERK signaling can be coupled with a delay to tissue mechanical relaxation, predicting oscillatory instabilities consistent with experimental wave patterns [74]. Similarly, it is possible to use a reaction-diffusion framework to capture how ERK activity waves propagate and coordinate tissue regeneration [75].

As an alternative approach, agent-based computational simulations offer a number of advantages compared to continuum models, including capturing cell-cell variability and observing emergent collective properties. Here, individual cells are modeled as agents that can assume different states (e.g., different ERK dynamics or specific fates), and interact with each other within a population using defined rules (Fig. 4f). These virtual cell communities can be simulated as simple hexagonal meshes or triangular lattices, allowing for instance to study

how cellular interactions and noise determine optimal long-range information transmission [30]. More complex models that integrate cell geometries and mechanics allow a more comprehensive understanding of how ERK waves propagate. Cells can be simulated with a cellular Potts model, where each cell is represented as a cluster of lattice sites with energy terms for adhesion, elasticity, and ERK-dependent, anisotropic contraction. Using this model, it is possible to simulate that cell stretching activates ERK, which in turn induces anisotropic cell contraction, establishing a feedback loop that drives long-range ERK waves and coordinated migration [26]. Further, simulations combining a self-propelled vertex model with mechanochemical feedback and glassy dynamics, demonstrate how the interplay between active migration forces and ERK–density couplings can control transitions between uniform and periodic wave states that modulate tissue fluidity and collective migration [76].

With these tools, one can simulate AiEWs and the fates of the neighbors of an apoptotic lesion, while varying parameters such as apoptotic rate, wave size, duration, and strength of the survival effect that are not accessible to experimentation. Altogether, these tools enable one to evaluate the global output of different AiEW spatiotemporal dynamics on the cell population, and provide intuition about the emergent properties that govern collective behavior. This approach could for example be used to simulate the effect of cell killing therapies, therapies targeting the ERK waves and their combination, providing new insight about optimal therapy not directly accessible to human intuition.

8. Future directions and conclusive remarks

AiEWs provide a simple mechanism for “gossiping about death”, enabling neighboring cells to sense and respond to an apoptotic event. This process is key for coordinating survival, compensatory proliferation, and differentiation fates in cells neighboring apoptotic lesions. By facilitating communication between apoptotic and bystander healthy cells, AiEWs allow tissues to organize and achieve coordinated collective behaviors, such as epithelial homeostasis and tissue development. Usurpation of this mechanism by cancer cells can, however, hamper the response to targeted therapies and lead to drug tolerance.

Beyond AiEWs, additional spatiotemporal signaling networks are likely involved in regulating collective fate decision signaling. Supporting this idea, recent multiplexed measurements of signaling activity in steady-state conditions have shown that the heterogeneity of multiple signaling networks can drive context-dependent fate decisions within a cell population. This context dependency is determined by a combination of the internal cell state and local environmental signals [77]. This suggests that a fully deterministic understanding of fate decision signaling at the single-cell level is possible. An arising question is, therefore, how the heterogeneity of multiple signaling networks is dynamically and spatially regulated to balance apoptosis with other fates during epithelial homeostasis. For example, as discussed above, an additional mechanism driving compensatory proliferation in response to apoptosis in epithelia involves localized YAP activity patches, triggered by the loss of mechanical homogeneity at the site of apoptotic cell extrusion [50]. Thus, distinct signaling processes with different spatio-temporal logics balance cell death with compensatory proliferation during epithelial homeostasis. A key future goal is to comprehensively map these processes, unravel their spatio-temporal logic, and explore how individual cells integrate multiple signaling inputs to ultimately determine a specific fate. Recent technology allows one to measure the dynamics of up to 7 biosensors in single living cells [78]. This has the potential for example to measure simultaneously ERK, AKT, YAP, JNK, calcium, caspase activity, cell cycle fate, and to understand how these pathways collectively regulate fate decisions. This could in principle resolve both stress pathways (JNK and caspase activity, that lead to apoptosis), as well as survival and proliferation pathways (ERK, AKT, YAP). Such an approach might elucidate how these pathways feed on each other, and how they correlate with apoptosis, survival, and proliferation fates. The bioinformatic approaches to analyze such multidimensional datasets remain to be set up, but now seems to be in reach thanks to machine learning techniques such as CODEX [72], that we described in the previous section.

Finally, our understanding of spatio-temporal signaling mechanisms that collectively balance apoptosis with other fates mostly come from simple *in vitro* models such as 2D epithelial cell cultures, even if *in vivo* data has already corroborated the existence of AiEWs in living systems [25,39]. Since tissue mechanics and geometry feed in the spatial regulation of fate decisions, it will be instrumental to study such signaling responses in realistic contexts. Imaging of signaling activities *in vivo* is the gold standard. However, biosensor imaging will be greatly facilitated in organoids and organs on a chip [79] that recapitulate such geometries/mechanical properties, and also allow for a higher throughput in system perturbations. A combination of all these technologies has the potential for a deterministic understanding of collective apoptosis, survival and proliferative fate decision signaling in homeostasis, development, and cancer.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used ChatGPT/OpenAI in order to improve the readability and language of some sections of the manuscript. This AI tool was never used to generate new content. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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