Calcium as a signal integrator in developing epithelial tissues

Pavel A. Brodskiy[1] and **Jeremiah J. Zartman**[1,#]

[1]Department of Chemical and Biomolecular Engineering, University of Notre Dame, 205 McCourtney Hall, Notre Dame, IN 46556, USA

Abstract

Decoding how tissue properties emerge across multiple spatial and temporal scales from the integration of local signals is a grand challenge in quantitative biology. For example, the collective behavior of epithelial cells is critical for shaping developing embryos. Understanding how epithelial cells interpret a diverse range of local signals to coordinate tissue-level processes requires a systems-level understanding of development. Integration of multiple signaling pathways that specify cell signaling information requires second messengers such as calcium ions. Increasingly, specific roles have been uncovered for calcium signaling throughout development. Calcium signaling regulates many processes including division, migration, death, and differentiation. However, the pleiotropic and ubiquitous nature of calcium signaling implies that many additional functions remain to be discovered. Here we review a selection of recent studies to highlight important insights into how multiple signals are transduced by calcium transients in developing epithelial tissues. Quantitative imaging and computational modeling have provided important insights into how calcium signaling integration occurs. Reverse-engineering the conserved features of signal integration mediated by calcium signaling will enable novel approaches in regenerative medicine and synthetic control of morphogenesis.

Keywords

developmental biology; morphogenesis; bow-tie network; cell signaling; information processing; communication; collective behavior; computational modeling

1. Introduction

Organ development requires extensive coordination among and within heterogeneous cell populations to generate complex organ morphologies [1–3]. During morphogenesis, epithelial cells must process a large range of environmental signals to implement the genetic program that drives development. To do so, a sophisticated cell communication network processes a broad range of inputs to compute output responses [3,4]. As a second messenger, the calcium ion (Ca^{2+}) is a central integrator embedded in this network (Figure 1A, [5,6]). However, the central role and pleiotropic functions of Ca^{2+} signaling make it difficult to assign mechanistic roles to specific Ca^{2+} signaling dynamics. The recent development of

#jzartman@nd.edu, Phone number for correspondence: 574-631-0455.

Conflict of Interest

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genetically encoded Ca^{2+} indicators such as the fluorogenic GCaMP sensors have begun to reveal new roles for Ca^{2+} signaling in epithelial development [7,8]. Due to nonlinear and stochastic dynamics, computational modeling is essential for decoding Ca^{2+} signals in developing tissues as reviewed in [9–11].

This review is structured as follows. First, the Ca^{2+} signaling network is described, with emphasis on how the core Ca^{2+} toolkit encodes dynamic Ca^{2+} signals, how downstream transduction machinery decodes these signals, and general principles of the network structure of Ca^{2+} signaling. Second, several biological examples of decoding and encoding of Ca^{2+} dynamics in epithelial development are described. Finally, the review closes with a summary and an overview of unanswered questions for Ca^{2+} signaling in epithelia. Many of these questions are applicable to other second messengers and for other tissue types.

2. Ca²⁺ signaling: ubiquitous, pleiotropic, yet specific

The molecular mechanisms defining the core Ca^{2+} signaling toolkit in cells are well established and ubiquitous across cell types (a few representative reviews include [12–16]). Yet, cellular behaviors in response to Ca^{2+} signaling are often cell type specific. This is in part because the encoding of information through Ca^{2+} dynamics depends on the signal transduction genes expressed (Figure 1A–B). Ca^{2+} is downstream of many chemical, mechanical, and electrical inputs, and transduces these inputs into a dynamic signal: the concentration of cytosolic Ca^{2+}. This dynamic signal encodes more information than a static signal, and a large network of genes interprets this information, allowing Ca^{2+} to affect many diverse processes (Figure 1B–C).

Transmission of Ca^{2+} signaling information between compartments in the cell or between cells occurs through the control of fluxes by Ca^{2+} channels. Several organelles serve as Ca^{2+} ion reservoirs that can be regulated to amplify signals (Figure 1B). Decoding Ca^{2+} information requires downstream proteins with protein domains that reversibly bind to Ca^{2+} as well as proteins that are regulated by Ca^{2+}-binding proteins (Figure 1C). Cellular processes that are influenced by Ca^{2+} signaling are diverse and include cell growth, division, death, differentiation, migration, cytoskeletal mechanics, and protein secretion (reviewed in, among others, [17–19]). Here we briefly discuss these components and their functions.

2.1. Molecular components of the Ca²⁺ signaling toolkit

The cytosol must maintain low concentrations of Ca^{2+} (~0.1 μM [12,13]) to avoid toxicity. The mitochondrial matrix is maintained at slightly higher levels (~0.1–2.0 μM [20]). Conversely, the extracellular fluid, other organelles, and the space between the inner and outer mitochondrial membranes are generally maintained at high Ca^{2+} concentrations (~50–1000 μM [12,13]). Channels permit diffusion of Ca^{2+} ions down a concentration gradient, and can be opened to induce a rapid and efficient Ca^{2+} response [13,21–23]. Mechanically-gated [24,25], electrically-gated [26], and receptor-operated channels [14,27–30] all contribute to the wide range of stimuli that can regulate Ca^{2+} concentrations in the cell. For example, extrinsic tensile forces on epithelia causes the stretch-activated Ca^{2+} channel Piezo to induce a Ca^{2+} response, causing extracellular signal-regulated kinase (ERK) phosphorylation, which leads to cell division [31]. Two additional classes of membrane-

bound ligand receptors can activate Ca^{2+} signaling: G-protein-coupled receptors (GPCRs) and receptor tyrosine kinase (RTKs), which stimulate release of Ca^{2+} from the ER stores through phospholipase C (PLC) activation of inositol trisphosphate receptors (IP_3Rs).

The cell actively sequesters Ca^{2+} into organelles and across the plasma membrane to maintain a low Ca^{2+} concentration in the cytosol (Figure 1B). This role is fulfilled by various Ca^{2+}-ATPases, which are high-affinity, low-capacity pumps. The high affinity of Ca^{2+}-ATPases ensures the pumps are constitutively active. A low capacity results in a long time-scale required for significantly changing cytosolic Ca^{2+} concentrations. Sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA, [32]), plasma membrane Ca^{2+} ATPase (PMCA, [33]), and secretory pathway Ca^{2+} ATPase (SpcA, [34]) maintain a gradient of Ca^{2+} across the ER, plasma membrane, and Golgi apparatus membranes respectively. When the concentration of Ca^{2+} in the cytosol spikes beyond the limits of what the Ca^{2+}-ATPase pumps can efficiently remove, the dormant capacity of low-affinity, high-capacity pumps such as the Na^+/Ca^{2+} exchanger (NCX, [33]) jump into action to rapidly return the cytosol to basal levels. The mitochondria has an analogous mitochondrial Na^+/Ca^{2+} exchanger (mNCX, [35]) as its primary Ca^{2+}-extrusion pump. Additionally, stress can cause the permeability transition pore (PTP) complex to assemble, leading to a rapid equilibration of cytosolic and mitochondrial Ca^{2+} concentration, frequently leading to cell death [36]. Together, these molecular components maintain a low Ca^{2+} concentration in the cytosol and mitochondrial matrix, and a high concentration in other organelles.

A recent quantitative study of organelle distribution in fibroblast-like cells has revealed the extent to which the ER colocalizes with other organelles [37]. In particular, 25% the volume of mitochondria was in contact with ER volume [37]. These connections are stabilized by the mitofusin proteins (MFN, [38]) and facilitate Ca^{2+} transfer from the ER to the mitochondria. Ca^{2+} released at these sites enters the mitochondrial lumen through the voltage-dependent anion channel (VDAC, [39]) and the low-affinity mitochondrial uniporter complex (MCU, [40]).

When the nuclear pore complex (NPC) is open, Ca^{2+} freely diffuses between the cytosol and the nucleus. The degree to which cells regulate the concentration of Ca^{2+} in the nucleus is debated; however nuclear Ca^{2+} has been shown to affect transcription [41–43], and nuclear Ca^{2+} oscillations have also been implicated in cell division [44]. The NPC can undergo conformational changes in response to Ca^{2+} concentration [45], and can close, which can result in differential Ca^{2+} concentration in the nucleus and the cytosol.

Many proteins contain a Ca^{2+}-binding domain. Ca^{2+} binding to an EF-hand or C2 domain causes proteins to alter their structure and therefore function [46,47]. Calmodulin (CaM [48]) is a key protein that transduces changes in Ca^{2+} concentration to downstream target proteins (Figure 1C). Additionally, proteins such as calbindin (Calb) buffer Ca^{2+} concentration. Ca^{2+} buffering proteins alter the dynamics of free cytosolic Ca^{2+} and slow down Ca^{2+} diffusion. CaM and similar effectors can result in specific and rapid activation of kinases and phosphatases including CaM kinase (CaMK [49,50]), myosin light chain kinase (MLCK [51]), and calcineurin (CalN [50,52]), and transcription factors such as nuclear factor of activated T-cells (NFAT [53]), serum response factor (SRF [54,55]), and cAMP

response element-binding protein (CREB [56,57]). These kinases and transcription factors decode Ca^{2+} signals and enact the relevant outcomes including cell death [58], proliferation [43,59–61], migration [62,63], secretion [64,65], and differentiation [66–68].

2.2. Spatial and dynamical properties of Ca^{2+} signaling

The buffering of Ca^{2+} leads to subcellular compartmentalization of Ca^{2+} responses [69,70]. Ca^{2+} dynamics can occur on the order of less than a micron (reviewed in [13,71,72]). The two smallest scales of Ca^{2+} signaling that have been described are blips (also referred to as quarks), and puffs (also referred to as sparks) (Figure 2A). Blips are caused by the opening of an individual membrane-bound channel, while puffs are caused by multiple adjacent channels opening in concert. Generation of Ca^{2+} signals can be subcellularly-localized due to sparsity of ligand-gated Ca^{2+} channels such as IP_3R and RyR [13,72,73], especially near the nuclear pore complex [74]. One functional implication of subcellular compartmentalization of Ca^{2+} signals is that transcriptional regulation by Ca^{2+} may be localized to the nucleus and may be independent from signaling events at the cell membrane [71,75]. Another scale of Ca^{2+} signaling are spikes, which are large enough to excite most of the channels in the cell [66,76–79]. Ca^{2+} signals of sufficiently-high magnitude can propagate to neighboring cells through gap junctional diffusion or paracrine signaling, resulting in intercellular Ca^{2+} transients or waves. This wide range of spatial scales greatly increases the design-space for Ca^{2+} to impact the behavior of the whole system.

Ca^{2+} oscillations contain far more information than the static concentrations of non-oscillatory signals through the amplitude, frequency, shape, and basal concentration, which can all be decoded by independent signaling pathways (Figure 2B). Ca^{2+} response times range from the order of nanoseconds or microseconds during membrane damage [80], seconds in response to mechanical or wounding stimuli [81–85], and on longer time-scales in response to changes in basal Ca^{2+} concentrations [62].

Oscillations in the cellular concentration of a species occur when the rate of production and decay rapidly shift to adapt to changes in inputs to restore homeostasis [4,86]. The requirement for every cell to control cytosolic Ca^{2+} leads to an increased tendency for oscillations to develop [86]. Ca^{2+} oscillations emerge as temporary increases in cytosolic Ca^{2+} inevitably lead to a strong push towards homeostasis, a feature common to other oscillatory phenomena [86]. Ca^{2+} channels are often inactivated by the resulting Ca^{2+} response, or close naturally over time allowing the cell to return to a basal Ca^{2+} level. The dynamic nature of these oscillations allows cytosolic Ca^{2+} to encode multiple signals.

One mechanism for this is demonstrated in Figure 2C, which shows how "ratcheting" and "non-ratcheting" mechanisms can be used to discriminate between high basal-concentration and high frequency signals [62]. Slow responses to Ca^{2+} signaling will integrate the signal over time, converting stationary but highly-dynamic Ca^{2+} signals into a first-order signal with a static set-point (Figure 2C). If the response is fast and insensitive to small perturbations in the signal, it will convert the signal into a spike train, which is a rapid series of discrete spikes similar to a neuronal action potential ([87], Figure 2C). The downstream transcription factor NFAT integrates the frequency of oscillatory Ca^{2+} signals, but not the amplitude [88]. $NF\kappa B$ is sensitive to infrequent Ca^{2+} oscillations that NFAT cannot detect

[88,89]. A field of cells that would oscillate with different frequencies when cultured independently can be induced to undergo coordinated oscillations through the oscillation of a small population of "pacemaker cells" [90,91]. This is similar to applying a MAX operation to a pool of Ca^{2+} oscillation frequencies, in which the fastest-oscillating cells tend to set the pace for oscillations within the tissue, but also tend to slow down slightly. This adds an additional layer of spatial and population-level complexity to signal integration.

Ca^{2+} oscillations have been modeled extensively, both in excitable [92,93] and non-excitable [94,95] systems. Modeling has uncovered universal principles and archetypical motifs of Ca^{2+} signaling. One such work is the distinction between Class I and Class II models of IP_3R-mediated Ca^{2+} oscillations, whereby IP_3 is stable in the former and IP_3 oscillates in the latter [9]. Additionally, extensive work has been done on the encoding and decoding of the frequency of Ca^{2+} oscillations [96–98]. These models have been reviewed ([10,11,99–101]) and are briefly summarized here.

Models for single-cell cytosolic Ca^{2+} concentration generally rely on three classes of fluxes: channel, pump, and leak (Figure 3A, [94]). These fluxes can occur across the plasma membrane or the membranes of organelles such as the ER. The molecular components involved in each of these fluxes are described in section 2.1. Release of Ca^{2+} into the cytosol through Ca^{2+} channels incorporates case-specific flux equations, and is often regulated by signaling from IP_3, ATP, or Ca^{2+} (Figure 3A). Ca^{2+} pumping out of the cytosol is assumed to occur in a concentration-dependent manner. Another common assumption is that Ca^{2+} leaks from high concentration compartments into the cytosol at a constant rate. Tissue-scale models generally couple individual cells through extracellular diffusion across cells, jump boundary conditions between juxtaposed cell membranes, or homogenization of the tissue domain to model "effective" diffusion (Figure 3B). Common mechanisms include flux of Ca^{2+} or IP_3 through gap junctions [102–104] or release of ligands such as ATP into the extracellular space [29,103,105]. Recent models coupling Ca^{2+} signaling and stress fiber formation can also lead to models for propagation of mechanical waves [106]. These modeling efforts explain how differential expression of Ca^{2+} toolkit components can result in different Ca^{2+} dynamics from the same input signal. The delicate balance of the three cellular fluxes and cell-cell communication can be summarized in bifurcation diagrams (Figure 2C). Bifurcation diagrams offers insight into movement of the system equilibrium to and from states of dynamic instability (Figure 2C).

Recently, stochastic models have been used to explore the kinetics of IP_3R [107,108]. IP_3R shifts stochastically between a highly-active "drive" and mainly inactive "park" mode. This model involves 10 rate constants, of which 2 depend on Ca^{2+}, IP_3, and ATP concentration [107]. Because ligand concentration is only important for switching between drive and park mode, this model updates the prior hypothesis that Ca^{2+} inhibits IP_3R slowly over time. However, these mechanistic differences do not affect previous models as the overall temporal kinetics of the two cases are similar [9]. Other epithelial systems with recent advances in modeling Ca^{2+} waves include retinal pigment epithelium [109], urothelial cells [110], and the developing cochlea [111]. Additionally, experimentally-developed models have been proposed for coupled transport of Ca^{2+} and actomyosin after wounding that will require the development of computational models to test [84].

2.3. Information flow through the Ca²⁺ signaling network

Information in biological systems is frequently processed through signal transduction networks that include bow-tie motifs. In a bow-tie motif, many inputs are integrated into a specialized processing system with very few components, forming a knot, and regulate many targets (Figure 1A, [6,112–115]). Examples of bow-tie networks in biology include metabolism of key nutrients [112,116], and the immune response [117]. By relying on few components to perform many functions, the system becomes vulnerable to perturbations to those key components. Despite this fragility, bow-tie networks are able to react faster and more efficiently than networks with more central nodes [113].

Second messengers like Ca^{2+} are suited for a role as nodes within the central knot as their homeostasis is critical for cell survival. Therefore a great energetic investment is already made to regulate their concentrations [12,13,32,118]. This property improves the speed and efficiency by which a decision can be reached, and "consensus forming," in which multiple competing inputs are combined into a single central message [6,113–115], (Figure 1A). Characterizing bow-tie networks that rely on second messengers offers valuable insight into how cells perform complex collective behaviors despite limited and local information. The problem of collective cell behavior mirrors the problem solved by "swarm intelligence," wherein a system has many individuals interacting by simple rules, to self-organize into complex higher-order behaviors [119]. Ca^{2+} signaling contributes to the regulation of collective behaviors such as morphogenesis, regeneration, and wound healing by regulating cellular processes like division, death, migration, secretion, and differentiation. Compared with the great wealth of studies modeling the encoding of Ca^{2+} oscillations, there has been considerably less effort on the mechanisms behind the decoding of Ca^{2+} oscillations. Recent modeling efforts have yielded insight on how specific transcription factors decode frequency or amplitude of Ca^{2+} oscillations [96,120,121]. For example, shorter trains of oscillatory signals may target proteins more specifically than longer trains [122]. Much integrative modeling work remains to be done to connect both the coding and decoding modules of Ca^{2+} bow-tie network within the context of collective cell decision making that occurs during epithelial morphogenesis and homeostasis.

3. Ca²⁺ signaling during epithelial development

Development occurs over multiple spatial and temporal scales. Embryonic morphogenesis in many animal systems takes place over the course of many hours, and yet critical steps in gastrulation occur on the order of minutes. Precise coordination of adjacent cells is required for tissue-scale morphogenesis, despite individual cells only interacting with their immediate neighbors. Morphogen signaling through diffusible ligands is the primary mechanism by which cells coordinate throughout development [1–3,79,123]. Morphogen-based receptors can stimulate Ca^{2+} fluxes into cells (Figure 1B). The presence or absence of Ca^{2+} toolkit components and Ca^{2+} signal transducers allows for high specificity in the Ca^{2+} response to morphogen signals [13]. Several specific roles for Ca^{2+} regulation of morphogenesis have been proposed and have been reviewed in depth [17,123]. Here we review a selection of recent discoveries in which Ca^{2+} encodes diverse inputs to guide epithelial development.

3.1. Ca²⁺ dynamics encode growth factor concentration to coordinate tubulogenesis

Endothelia are specialized epithelial tissues that develop into vessels or tubes. Endothelial cells must reliably and irreversibly commit to specific cell behaviors that are needed to form a tube [124–126]. The growing tube is led by a tip cell. Adjacent cells referred to as stalk cells divide to increase the length of the tube (Figure 4A, [62,127,128]). A central regulator of tube formation is vascular endothelial growth factor (VEGF), which can stimulate cells to divide through NFAT signaling, or to migrate through MLCK, but both behaviors are mutually exclusive [62,124]. Ca^{2+} is the second messenger mediating these responses [62]. To determine how endothelial cells stimulated with VEGF choose between these two fates, Noren et. al. treated porcine aortic and human umbilical vein endothelial cells with VEGF and observed the resulting Ca^{2+} dynamics (Figure 4A, [62]). VEGF is required to stimulate a Ca^{2+} response. However, addition of VEGF at low concentrations led to populations of cells exhibiting rapid Ca^{2+} oscillations, and at higher concentrations led to cells exhibiting one large peak, followed by a return to basal Ca^{2+} concentration. These findings were confirmed in vivo by showing that high-frequency oscillations in zebrafish tip cells reduced tip migration, and was correlated with division in stalk cells [62].

The authors propose that Ca^{2+} signaling functions to specify which of these fates is selected (Figure 4A, [62]). The two downstream pathways described in this work can be classified as "ratchet-like" and "non-ratcheting," as described in Figure 2C. Ratchet-like mechanisms integrate many peaks because the signal transducer has a low decay rate and can accumulate over time as additional oscillations occur. Transducers in non-ratcheting mechanisms have a high decay rate, and reproduce the input signal with high fidelity, but are unable to maintain a "chemical memory" like ratchet-like pathways can. Many peaks of Ca^{2+} signaling are required to accumulate sufficient levels of activated NFAT to trigger downstream targets, making such a mechanism effective for filtering high-frequency oscillations. A non-ratcheting mechanism is unable to reach its activation threshold without a large increase in basal concentration as its downstream products rapidly decay. This makes non-ratcheting mechanisms effective in filtering short, high-magnitude signals. The cell utilizes the Ca^{2+} toolkit to robustly determine local VEGF concentration, specifying to the cell whether to express NFAT, activate MLCK, or exhibit no response in a mutually-exclusive manner [62]. These findings demonstrate that Ca^{2+} encodes VEGF concentration and is decoded to regulate both tip cell and stalk cell behavior during tube growth.

3.2. Ca²⁺ dynamics encode morphogen concentration to specify cell fate

The neural crest is the tissue from which neurons, glia, and other nervous system cell types are derived [129]. It is derived from the same embryonic tissue as epithelia—the ectoderm—and is initially comprised of tightly-adherent cells [129,130]. A gradient of Sonic Hedgehog (Shh) signaling directs the patterning of neuronal differentiation in the neural crest [77]. The development of the spinal cord in frogs is correlated with Ca^{2+} spike activity [66].

Recently, Belgacem and Borodinsky implicated a Ca^{2+}-dependent form of non-canonical Type II Hh signaling in differentiation of neural crest cells (Figure 4B, [77,131]). The authors dissected the developing spinal cord and found a positive relationship between activation of Hh signaling through Shh ligand or Smoothened Agonist, and Ca^{2+} spike

activity. The authors also demonstrated a decrease in GABAergic neuronal cell fates when decreasing Ca^{2+} signaling through voltage-gated channel blockers. Effectively, these spikes convert a noisy, continuous signal (Hh concentration) into a binary signal that specifies cell fates. Overall, these findings suggest that Ca^{2+} signaling encodes Shh concentration and is later decoded to differentiate neuronal crest cells into a GABAergic neuronal cell fate. A recent study has since found that Ca^{2+} signaling mediates the rearrangement of neural stem cells into rosettes in the neural tube, which is critical for neural development [132].

3.3. Ca^{2+} dynamics encode multiple inputs to coordinate epithelial stem cell division

Intestinal epithelial stem cells (ISCs) must continuously divide to maintain homeostasis in the intestine in response to metabolic conditions, stress, and Wnt signaling [133–136]. The rate of ISC proliferation is a critical factor in controlling the size and structure of the gut, as insufficient proliferation may cause a reduction in integrity and an increase in proliferation may cause hyperplasia or lead to tumorigenesis [133,136,137]. There are many inputs that an intestinal stem cell considers when deciding whether to divide including morphogenetic signals [138], tissue stress [139,140], and the nutritional state of the surrounding tissues [141,142]. For example, intestinal stem cells obtain metabolic information by binding of L-Glutamate to the metabotropic glutamate receptor (mGluR), a GPCR [134].

Deng et. al. recently showed Ca^{2+} functions as a key signal integrator to regulate proliferation in intestinal stem cells (Figure 4C [134]). They identified three "states" of Ca^{2+} oscillations that lead to different cell fates, including: 1. fast, low-amplitude oscillations ("resting", 1–2 Hz), 2. slow, high amplitude oscillations ("activated", 0.2–0.5 Hz), and 3. low amplitude, low frequency oscillations that were generally not observed in healthy tissues ("Ca^{2+} deficient", less than 0.1 Hz). The authors found that the "activated" states led to proliferation specifically through activation of CREB by CalN and speculate that the role of the "resting" state is for cells to be able to rapidly adapt to environmental changes. They observed that glutamate stimulated cells to transition from a resting Ca^{2+} state to an activated state. Stimulation of the immune response with ecc15 bacterial infection also induced an "activated" Ca^{2+} waveform. This is consistent with evidence that bacterial infection increases division in intestinal stem cells [140]. A specific cell must make a consensus decision regarding proliferation despite receiving many potentially contradicting inputs. Ca^{2+} signaling provides a way for cells to encode these inputs into a single consensus signal that can then be decoded to regulate ICS homeostasis.

3.4. Ca^{2+} dynamics encode morphogen signal in multiple dynamic features in epithelia

Ca^{2+} signaling dynamics can also be spatially patterned in tissues, mirroring the patterns of morphogen signaling pathways. Spontaneous intercellular Ca^{2+} transients have recently been discovered in the developing butterfly and fly wing in vivo [78,143,144], and ex vivo in the presence of fly extract, a growth serum that contains a large range of active proteins [76,78,81,144,145]. Perturbation of Ca^{2+} signaling in the wing leads to a range of adult wing defects [78,144,146]. In the developing wing, the spatial range and frequency of these signaling events is decreased as the larvae ages. Ca^{2+} signaling dynamics can be investigated ex vivo by subjecting wing discs to different concentrations of fly extract. These yield qualitatively different types of Ca^{2+} signals in the wing disc, ranging from spikes, to long-

range transients, to oscillatory waves, while absence of fly extract led to no Ca^{2+} oscillations, or rare, single-cell spikes [78]. The amplitudes of the Ca^{2+} oscillations ex vivo are higher in the posterior compartment of the pouch. Hh is secreted from the posterior compartment, but Hh signaling is not active in the posterior compartment (Figure 4D, [145,147]). Inhibition of the primary transducer of Hh signaling, Smoothened, in the pouch not only leads to an increase in oscillation frequency in the whole tissue, but also led to an increase in amplitude in the anterior compartment, leading to a uniform amplitude across the entire pouch [145]. These findings indicate that Ca^{2+} signaling exhibits spatial asymmetries downstream of morphogen signaling. Network inference methodologies may enable systems-level investigation of crosstalk between morphogen signaling and Ca^{2+} signaling dynamics in this tissue [148,149].

3.5. Modeling reveals the mechanism behind ICWs in the developing cochlea

The cochlea is the epithelial inner-ear organ responsible for converting mechanical signals caused by sound waves into electrical signals that can be transmitted to the brain [150]. The developing cochlea exhibits Ca^{2+} waves that are critical to development. In the cochlea, binding of ATP to purinergic (P2Y) receptors results in IP_3R-mediated Ca^{2+} oscillations [151]. However, it is unknown whether the propagation of the resulting ICWs is due to diffusion of the resulting IP_3 through gap junctions, or paracrine feedback through secretion of additional ATP by activated cells to facilitate activation of P2Y receptors in neighboring cells. This leads to two questions: 1. what is the mechanism by which the Ca^{2+} signal propagates in the cochlea, and 2. are IP_3 oscillations required for the corresponding Ca^{2+} oscillations [111], (Figure 4E)?

Ceriani et. al. recently approached these questions using a mechanistic model incorporating generation of IP_3 through P2Y signaling, IP_3R-induced Ca^{2+} release, diffusion of ATP and IP_3 [111]. The model predicted a Hopf bifurcation depending on the concentration of ATP outside of the stimulated cell (Figure 3C, 4E). This predicts that stable oscillations are obtained if and only if ATP is within a range of concentrations. This result was independent of IP_3 oscillations, indicating that IP_3 oscillations are not required for Ca^{2+} oscillations in the developing cochlea. This identifies the developing cochlea as a Class I system [9]. Additionally, the speed and extent of ICWs predicted by the model were consistent with those observed experimentally. The model showed that the wave propagation speed was bistable with respect to maximum rate of ATP release, either exhibiting a constant speed of around 10 μm/s, or no propagation at all. Conversely, IP_3 diffusion through gap junctions did not affect wave propagation within physiological limits. This implicates ATP diffusion as the primary mechanism for ICW propagation in the developing cochlea over IP_3 diffusion through gap junctions. Here, computational models played a critical role in understanding ICW formation in epithelial development.

4. Summary

Quantitative studies that map the encoding and decoding of Ca^{2+} signals during morphogenesis are beginning to crack the Ca^{2+} signaling code in epithelia. These encoding and decoding modules of Ca^{2+} signal transduction form a bow-tie network, which is critical

for the role of Ca^{2+} as a signal integrator in epithelial development. In effect, Ca^{2+} signaling acts as an "embedded consultant" that helps inform cellular decisions. Differential expression of channels, pumps, and buffering proteins provide cell-type specificity in signals that are encoded by Ca^{2+} dynamics. The versatility of the Ca^{2+} toolkit allows many inputs to be encoded into a single dynamic signal (Figure 1A, [13]). Decoding Ca^{2+} dynamics through the bow-tie networks structure allows one signal to affect many downstream targets. There are many downstream proteins that decode Ca^{2+} signaling, including enzymes with fast dynamics on the scale of seconds. In this case, these proteins quickly respond to changes in Ca^{2+} binding kinetics. Transcription factors that act on the time scale of hours can integrate multiple peaks of Ca^{2+} oscillations through ratchet-like effects. Regardless of the variety of up-stream inputs, cytosolic Ca^{2+} encodes a single, albeit non-binary signal, allowing redundant or contradictory information to be removed in favor of consensus-building, which increases both the speed and efficiency of signal processing.

As demonstrated in several biological examples (Figure 4), Ca^{2+} signaling integrates information from multiple sources and can lead to different cell behaviors in developing epithelia. Recent mapping studies suggest that the developmental program generates spatiotemporal patterns of Ca^{2+} signal signatures, which include Ca^{2+} oscillation amplitude, frequency, and basal Ca^{2+} concentration. The broader implications of these signals remain to be discovered. However, like a consultant, Ca^{2+} is not always essential in the processes that it modulates. Depending on the context, Ca^{2+} oscillations could largely be a epiphenomenon or by-product of morphogenesis, provide primary features required for robust and efficient morphogenesis, or hint at the generation of a physiological-based memory system of morphogenetic patterns, necessary for wound healing and regeneration [152–154].

Many questions remain to be answered about the roles of Ca^{2+} as a signal integrator in developing epithelia and other tissue types. Ca^{2+} is necessary for morphogenesis, as no embryonic system has been found that can complete gastrulation in media devoid of Ca^{2+} [17]. Discerning the specific roles of Ca^{2+} in collective behavior within developing systems will require computational studies of tissue morphogenesis that couples chemical signaling with multi-scale descriptions of tissue morphogenesis [155–158]. Further, the generality of encoding/decoding modules across developmental systems needs to be characterized. The switch between division and migration in developing tubes has been observed in human and pig cells, as well as in zebrafish. Is this signaling motif universal, and if so, what other universal motifs rely on Ca^{2+} as a signal integrator? What specific morphogenetic unit operations rely on Ca^{2+} as a central integrator [159–161]? Part of the challenge in answering these questions is controlling the Ca^{2+} concentration of specific cells in developing epithelia. Optogenetic tools have recently been developed that will allow specific tissue regions, cells, or organelles to be targeted for Ca^{2+} release through optogenetic control of Ca^{2+} channels [121,162,163]. Further, control of downstream signal transducers including kinases, phosphatases, and transcription factors will allow a better understanding of the mechanisms involved in decoding Ca^{2+} signals. A better understanding of the role that Ca^{2+} plays in the integration of signals and the regulation of cellular processes will reveal systems-level insights into collective behavior in morphogenesis, wound healing, and regeneration. Future studies that create synthetic computational modules of Ca^{2+} signaling could be employed to control tissue mechanics synthetically or to embed

sensors within developing tissues [164]. Finally, chemical-mechanical coupled models are needed to test develop theory linking Ca^{2+} signaling and actomyosin flow to cell mechanics.

Acknowledgments

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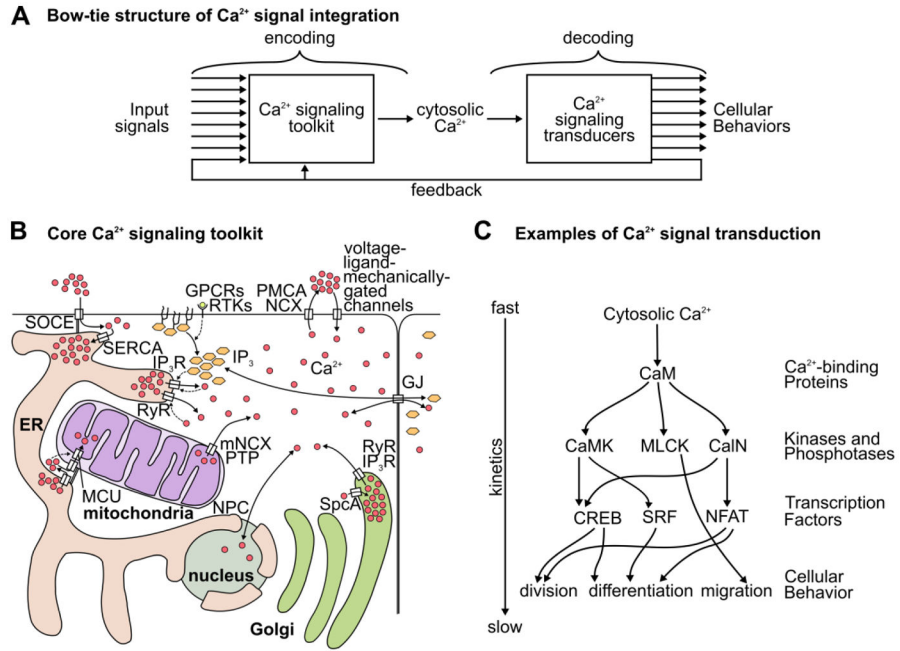
Figure 1. The transport and activity of Ca^{2+} in signal integration

(A) General network structure of Ca^{2+} signaling. (B) Molecular diagram of cellular Ca^{2+} transport. Solid arrows represent transport. Dashed arrows represent regulatory interactions. (C) Ca^{2+} binds to Ca^{2+}-binding proteins, which can buffer Ca^{2+} concentrations and activate enzymatic targets and transcription factors. Activation of kinases and phosphatases can affect the cell rapidly. Altering cell fate through transcription factors takes longer because the timescale of transcriptional machinery is slower. Ca^{2+} impacts many cellular functions including division, growth, death, differentiation, and migration. Arrows represent activating or inhibitory regulations. Specific components of the calcium signaling machinery are described in the text.
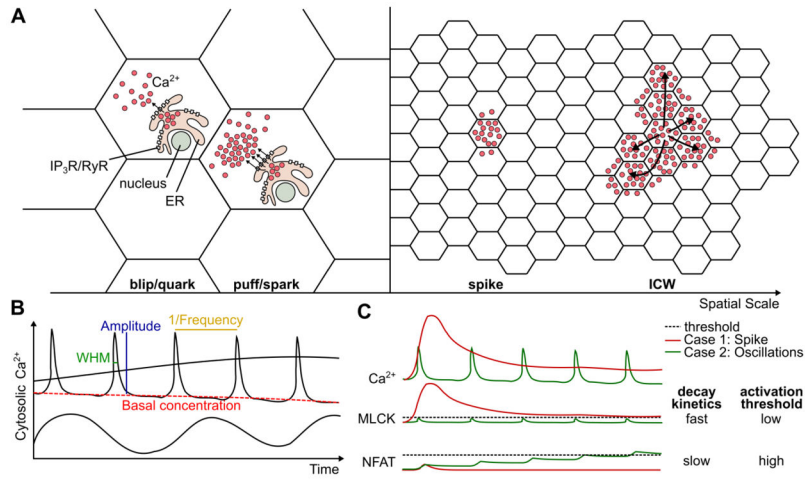
Figure 2. Spatial and temporal properties of Ca²⁺ dynamics

(A) Cartoon of four spatial scales of Ca^{2+} dynamics. Blips involve only one receptor activated channel. Puffs involve more than one receptor-activated channel (IP_3R or RyR). In a spike, the entire cell is active, and Ca^{2+} may spread to adjacent cells. An intercellular Ca^{2+} wave (ICW) involves multiple full-cell responses that are chained together, resulting in a self-sustaining transient of Ca^{2+} signaling across an entire tissue. Arrows represent the diffusion of the Ca^{2+} signal dynamics. (B) Several examples of Ca^{2+} signals. Shown is a slow change in Ca^{2+} concentration, a sinusoidal Ca^{2+} oscillation, and Ca^{2+} oscillation spikes. An oscillatory signal can contain more information than a static signal including oscillation Frequency, Amplitude, Basal concentration, and Width at Half Max (WHM). (C) Example of how a dynamic signal can be decoded to activate one of two different targets. In this case, the red signal is a non-oscillatory sustained increase in basal Ca^{2+} concentration, and the green signal is an oscillatory signal with high frequency and low amplitude and WHM. MLCK has a low activation threshold and can detect the change in basal concentration caused by the red signal. NFAT has a slow decay rate and can accumulate after many individual Ca^{2+} oscillations.
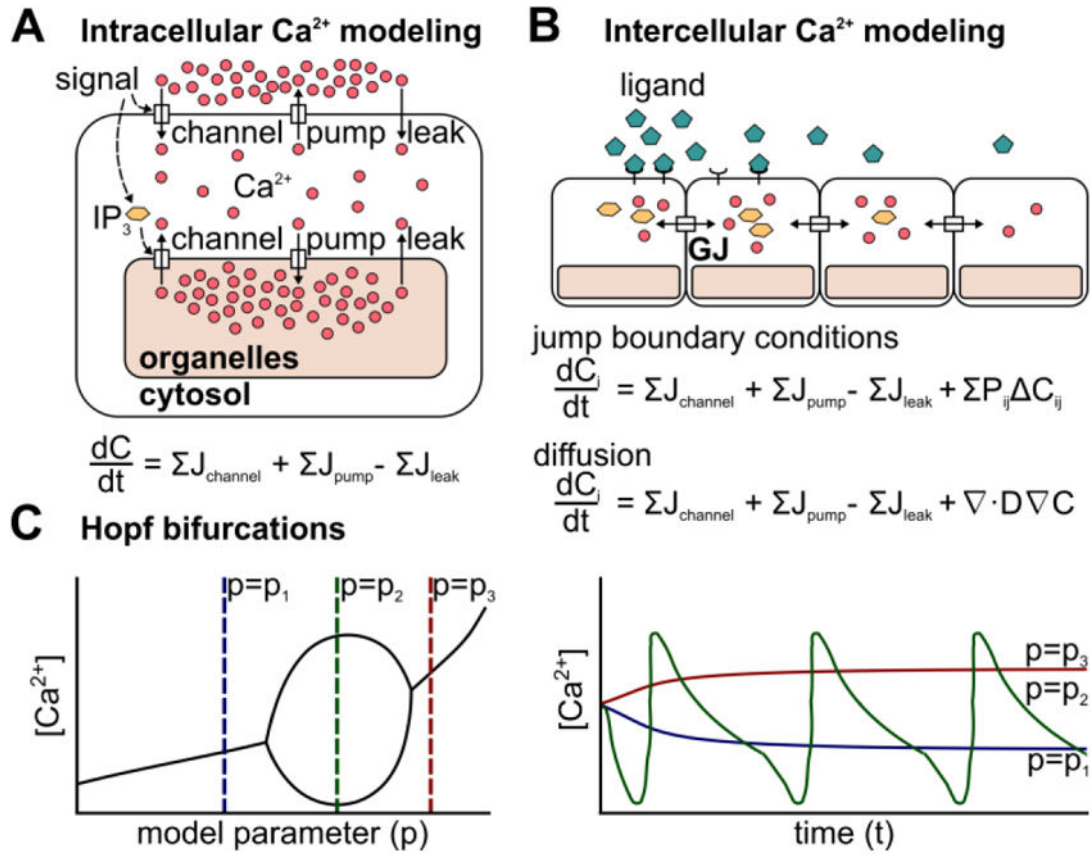
Figure 3. Modeling Ca^{2+} dynamics in non-excitable epithelial tissues

(A) Cartoon representation of molecular sources of various flux terms. General equation for chemical balance arising from molecular components of Ca^{2+} signaling toolkit. (B) Cartoon representation of Ca^{2+} flux through an epithelial sheet. General equation for chemical balance arising from cell-cell interactions. P_{ij} represents the relative permeability of cells i and j, and is 0 when cells are not in contact. (C) Cartoon representation of Hopf-bifurcation in the parameter domain (p) and in the time domain (t). Oscillations arise for a specific range of model parameters.
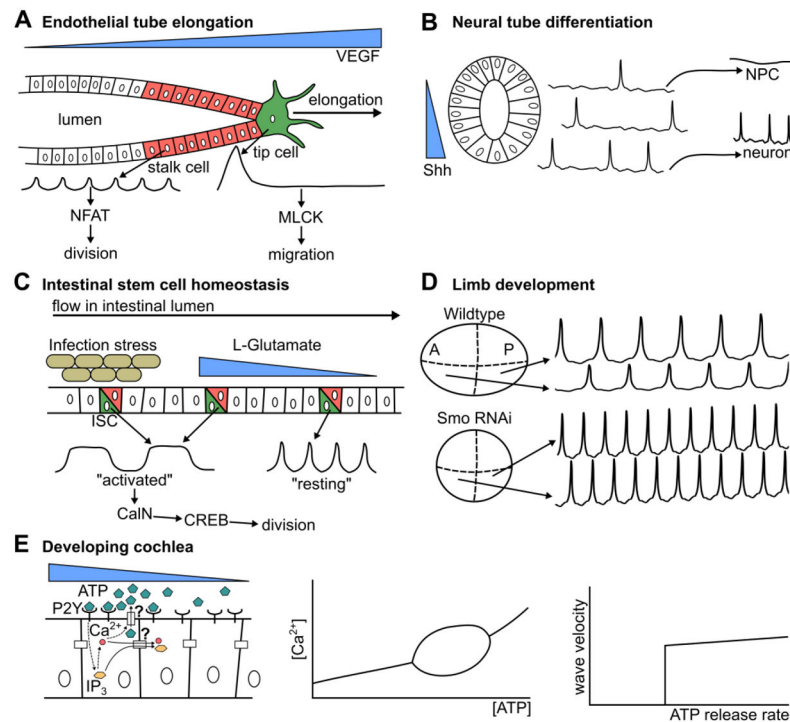
Figure 4. Ca^{2+} multiplexes morphogen and other ligand signals

(A–E) Several examples where Ca^{2+} signaling interprets ligand concentrations and encodes information in dynamic features. (A) Ca^{2+} interprets VEGF signaling in porcine aortic endothelial cells. Cells closest to VEGF undergo a large, singular Ca^{2+} spike, which activates MLCK and induces migration. Cells further away undergo many smaller spikes, over time inducing NFAT activation and inducing proliferation. (B) In the *Xenopus* neural tube, Ca^{2+} spike frequency encodes Shh concentration and is correlated with neuronal fate specification. (C) High L-Glutamate or bacterial infection lead to longer, higher magnitude Ca^{2+} oscillations that can be interpreted by CREB through CalN and result in intestinal stem cell renewal. Low levels cause smaller spikes that do not induce cell division. ISC indicates intestinal stem cells. (D) Cells that secrete Hh exhibit higher oscillatory amplitudes in the developing wing disc. When Hh signaling is suppressed, the frequency and amplitude of Ca^{2+} oscillations are uniform and elevated in the entire disc. This indicates that multiple features of Ca^{2+} oscillations reflect the morphogen state of cells. A represents anterior, and P represents posterior compartment. (E) ATP induces Ca^{2+} waves across the developing cochlea. Mathematical modeling indicates that these oscillations primarily require ATP concentration to be within a specific range. The mechanism of propagation of the waves in the cochlea is paracrine signaling and not gap-junction mediated diffusion.