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Bioelectric regulation of intestinal stem cells

Afroditi Petsakou[1], **Norbert Perrimon**[1,2]

[1]Department of Genetics, Harvard Medical School, Boston, USA

[2]Howard Hughes Medical Institute, Boston, USA

Abstract

Proper regulation of ion balance across the intestinal epithelium is essential for physiological functions, while ion imbalance causes intestinal disorders with dire health consequences. Ion channels, pumps and exchangers are vital in regulating ion movements-i.e., bioelectric currents-that control epithelial absorption and secretion. Recent *in vivo* studies used the *Drosophila* gut to identify conserved pathways that link regulators of Ca^{2+}, Na^{+} and Cl^{-} with intestinal stem cell (ISC) proliferation. These studies laid a foundation for using the *Drosophila* gut to identify conserved proliferative responses triggered by bioelectric regulators. We review these studies, discuss their significance as well as the advantages of using *Drosophila* to unravel conserved bioelectrically induced molecular pathways in the intestinal epithelium under physiological, pathophysiological and regenerative conditions.

Keywords

Intestinal stem cells; gut; ion channels; bioelectric signaling; *Drosophila*

Ion regulation in the gut

The gut is an organ dedicated to digestion and nutrient absorption. Under physiological conditions, the **gut epithelium** (see Glossary), which consists of polarized epithelial cells, secretes digestive enzymes and regulates the passage of a variety of nutrients, electrolytes and water from the lumen to the body, and *vice versa*. The ability of intestinal epithelial cells to absorb nutrients and fluids is essential to body hydration and by extension the animal lifespan. **Ion channels**, **pumps** and **exchangers** are instrumental in electrolyte absorption and secretion and thereby regulate movement of water in the gut. These epithelial ion channels, pumps and exchangers include: i) Na^{+} and Cl^{-} channels that regulate water absorption and secretion; ii) K^{+} channels that are responsible for the negative membrane

*Corresponding authors Afroditi Petsakou: Afroditi_Petsakou@hms.harvard.edu; Norbert Perrimon: perrimon@genetics.med.harvard.edu.

Competing interests

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Resources

i: https://www.flyrnai.org/scRNA/gut/

ii: http://flygutseq.buchonlab.com/

iii: https://flygut.epfl.ch/

potential that is necessary for ion secretion; and iii) ion pumps and exchangers that help maintain the ionic gradient and osmotic equilibrium [1,2]. The combined functions of these ion channels, pumps, exchangers move charged ions in and out of the epithelium giving rise to the membrane potential. In addition, some receptors, such as **cholinergic receptors**, regulate Ca^{2+} signaling, which in turn stimulates ion transport [3,4] . We refer to them as **bioelectric regulators** due to their ability to regulate epithelial ion dynamics.

Bioelectricity - the flow of electric currents carried by charged ions such as Na^+, Ca^{2+}, K^+ and Cl^- - influences diverse processes including tissue development and patterning [5-7]. In particular, voltage changes in the membrane potential affect biological functions such as cell cycle regulation (reviewed by [8]), tissue patterning [9] and cytoskeletal organization in *Drosophila* [10]. In addition, endogenous ion currents triggered by ion channels and pumps influence tissue development [11,12], zebrafish fin growth [13] and wound healing [14,15]. Thereby, bioelectricity is considered an instrumental component of morphogenesis, growth and even tissue remodeling and regeneration [7]. How bioelectric currents are transduced in molecular pathways capable of modulating with precision cellular functions so that organs develop to their “correct” form and size is not clear [7]. Still, Ca^{2+}-mediated signal transduction [16] often links bioelectric changes to canonical regulatory networks [5,17]. For example, downstream Ca^{2+}-dependent pathways triggered by K^+ channels regulate human T-cell activation and proliferation [18] while promoting morphogenesis in the *Drosophila* wing [19]. In addition, membrane voltage changes act through Ca^{2+} signaling to drive anterior gene expression in planaria [20].

During the last decade the *Drosophila* midgut, which we will refer to as the gut for simplicity, has emerged at the forefront of stem cell studies [21-29]. The *Drosophila* gut is a single layered epithelium consisting of intestinal stem cells (ISCs) that give rise to daughter cells, the enteroblasts (EBs), which in turn differentiate into two distinct epithelial cell populations, enterocytes (ECs) and enteroendocrine cells (EEs) [28,29]. ECs, which make ~80% of the epithelium, are large polyploid cells specialized in absorption, and EEs are small secretory cells [30]. The simple architecture and cellular composition of the *Drosophila* gut, combined with the state-of the art genetic toolset available in *Drosophila* [31,32], facilitated several breakthrough studies that revealed conserved mechanisms underlying the regulation of ISC proliferation [21-29]. Specifically, ISC proliferation is triggered by the activity of several conserved pathways that include the JAK-STAT, epidermal growth factor (EGF), bone morphogenetic protein (BMP) and Wingless pathways [21-27]. During gut regeneration, these pathways are activated by autonomous and non-autonomous release of **cytokines** from various sources, including the EBs, ECs, ISCs and hemocytes (*Drosophila* immune cells), as well as the surrounding visceral muscle. The regenerative signaling pathways prompting ISC proliferation have been extensively reviewed elsewhere [33,34].

Even though bioelectrically-controlled regeneration in the gut is not yet established, ion channel dysregulation in the intestine is associated with several diseases including Inflammatory Bowel Diseases (IBDs) and cancer [1,35]. In this review, we highlight our current knowledge relating to the control of ISC proliferation in the *Drosophila* gut by

bioelectric regulators and examine how studies in flies could further our understanding of the role of bioelectric dynamics in gut homeostasis, regeneration and human diseases.

Conserved regulators of ion dynamics in the *Drosophila* gut

Recent studies in the *Drosophila* gut have generated single cell RNA sequencing datasets$_{i}$ and bulk RNA-seq datasets for specific cell populations$_{ii}$ or regions$_{iii}$, either under physiological conditions or following bacterial infection [36-38]. A cell atlas for the whole fly based on single nuclei sequencing has also been generated [39]. These invaluable datasets revealed that conserved ion channels, pumps and exchangers are expressed in the *Drosophila* gut, offering the opportunity to explore the physiological roles of these bioelectric regulators in the fly gut. In addition, current studies (Table 1) have identified how ISC proliferation is controlled by ion channels, pumps as well as a Ca^{2+} -regulating receptor [40-44]. Below, we review the significance of these studies and, using the available datasets, provide examples of potential regulators of K^{+} , Cl^{-}, Na^{+} and Ca^{2+} dynamics in the fly gut that appear to be worthy of further exploration (Figure) based on their conservation, downstream molecular pathways (Table 1) and association with human pathologies.

Ca^{2+} regulation

mGluR: Recent work in the *Drosophila* gut identified that Ca^{2+} oscillations in ISCs increase in response to mitogenic and dietary signals [40]. This study revealed that administration of dietary Glutamate (L-Glu) increases cytosolic Ca^{2+} concentrations in ISCs by activating the metabotropic Glutamate G-protein-coupled receptor (mGluR) and promoting proliferation through the conserved Calcineurin/ CREB-regulated transcriptional co-activator (CRTC) pathway [40]. Thus, mGluR-induced Ca^{2+} dynamics are proposed to link gut proliferation to the nutrient needs of the animal [40]. The association of Calcineurin with Ca^{2+} dynamics is conserved across animals, but the cellular function can vary for different tissues. For example, in skeletal myoblasts Ca^{2+} dependent Calcineurin activity promotes differentiation [45].

Ca^{2+} is a particularly significant and pleiotropic downstream component that impacts almost every aspect of cellular life [16]. Ca^{2+} signaling in intestinal epithelial cells is a major regulator of ion secretion [3,4]. In addition, Ca^{2+} carries out significant non-autonomous functions, for example elevated cytosolic Ca^{2+} triggers bioelectric communication of adjacent cells, electrically coupling them such that they function as a unit [46,47]. Thus, it is not surprising that the gut uses Ca^{2+} dynamics in ISCs to adapt intestinal epithelial integrity to varying amount of nutrients [40]. Future studies should examine whether mGluR interacts with other ion channels not only in ISCs but also in the adjacent EBs, ECs or EEs via bioelectric or chemical signaling. Such studies could unravel the underlying mechanisms that allow the gut to precisely adjust its physiological functions to match nutritional needs.

Piezo: The conserved mechanosensitive channel Piezo is a clear example of how Ca^{2+} dynamics maintain intestinal homeostasis in response to mechanical stress, regularly occurring during digestion [41,48]. Piezo ion channels are shaped as a propeller [49-51]. When closed, the center of the propeller has three blades that curve the membrane, generating a dome-like structure. It has been proposed that as tension on the membrane

increases due to mechanical pressure, the dome flattens, the blades straighten and the channel opens [49-51]. Opening of the Piezo channel in the plasma membrane mediates extracellular Ca^{2+} influx, whereas opening of the Piezo channel in subcellular organelles such as the endoplasmic reticulum/sarcoplasmic reticulum, mitochondria, or nucleus elevates Ca^{2+} levels by release from internal Ca^{2+} stores [52].

Two recent studies, one in mammalian cells and another in the *Drosophila* gut, highlighted how Ca^{2+} signaling through the Piezo channel triggers cell population adjustments in the intestinal epithelium so that normal gut function is maintained despite the mechanical stress that occurs during digestion [41,48]. Both studies found that Piezo activation increases ISC proliferation by stimulating Ca^{2+}-dependent phosphorylation of ERK [41,48], indicating that the mechanism controlling proliferation during mechanical stimulation in the gut is conserved. Moreover, a unique *Piezo*-expressing subpopulation of EBs was identified in the *Drosophila* gut that upon mechanical stimulation proliferates and differentiates into secretory EEs [41]. Albeit Piezo-induced proliferation is ERK-dependent in the *Drosophila* gut, Piezo-induced EE differentiation is ERK-independent and possibly regulated through Notch signaling [41].

Even though most studies focus on Piezo-induced Ca^{2+} currents [41,48], opening of Piezo channels generate cationic non-selective currents, such that Piezo can also result in flux of Na^{+} or K^{+} [53]. Therefore Piezo-induced non-selective currents in the epithelium can affect regulation of ion transport and water movement. Perhaps not surprisingly, abnormalities in gut mechano-sensation are associated with defecation disorders, obesity and colon cancer [54-56]. Further work in the *Drosophila* gut could help identify interactions of Piezo with other bioelectric regulators and unravel how ion and osmotic balance across the intestinal epithelium is preserved despite mechanical pressure.

TrpA1: TrpA1 (also known as the wasabi receptor) belongs to the highly conserved transient receptor potential (TRP) multigene superfamily and is found in various tissues including the gut. Similar to most TRPs, TrpA1 is a Ca^{2+} permeable nonselective cation homo-tetramer channel that localizes to the plasma membrane [57]. Each TrpA1 subunit is composed of six transmembrane regions flanked by amino-terminal ankyrin repeats in the N- terminal tails [57]. TrpA1 has been extensively studied in nociceptor neurons as a detector of irritants or tissue injury and can be activated by a range of chemical, thermal and mechanical stimuli [58]. It can also be activated by pro-inflammatory mediators including oxidative stress byproducts which directly modify cysteine residues in TrpA1 [59,60]. Upon inflammation, TrpA1 is reported to be upregulated, extensively trafficked and translocated to the membrane [58,61]. In the mammalian intestinal epithelium, TrpA1 is found in ECs, enterochromaffin cells and EEs [58]. Although the function of TrpA1 in mammalian gut cells is not as well understood as its function in neurons, TrpA1 has been proposed to regulate intestinal motility, aid in the digestion of specific foods and regulate mechano-sensation [62-66].

The expression of TrpA1 is conserved in the *Drosophila* gut with potential roles in EEs and ISC/EBs [42,67]. TrpA1 is proposed to sense microbicidal reactive chlorine and trigger bacterial expulsion as a protective response following the activation of the uracil/Duox

pathway by pathobionts [67]. In addition, transcriptome analysis of the *Drosophila* gut$_{ii}$ revealed that *TrpA1* is mainly expressed in EEs in steady-state conditions but this changes after bacterial infection, with *TrpA1* upregulated also in EBs[37]. This resembles the upregulation of TrpA1 after inflammation in mammals [58,61] and suggests that in the *Drosophila* gut, TrpA1 might sense noxious substances and promote bacteria expulsion from EEs and EBs. Moreover, TrpA1 is proposed to be one of the Ca^{2+} channels that drive ISC proliferation during *Drosophila* gut injury [42]. Specifically, TrpA1, together with the ryanodine receptor (RyR), increases intracellular Ca^{2+} in response to oxidative stress. This, in turn, activates the Ras/ERK MAPK pathway and induces secreted EGF cytokine to promote ISC proliferation [42]. Thus, Ca^{2+} influx from TrpA1 is associated with release of mitogenic signals and proliferation [42]. Future studies should explore if the same Ca^{2+}-induced pathway triggered by TrpA1 in response of mitogenic signals is concurrently promoting bacterial expulsion during infection or, alternatively, if TrpA1 regulates different Ca^{2+}-induced pathways. A comprehensive understanding of the molecular and bioelectric roles of TrpA1 in the *Drosophila* gut could be relevant to gastritis, for which TrpA1 has been proposed as a potential therapeutic target [68].

PMCA and Calx: The plasma membrane Ca^{2+} ATPase (PMCA) is an ATP-driven pump that removes cytosolic Ca^{2+} from cells. This function is essential in all eukaryotic cells for the maintenance of a low resting Ca^{2+} concentration [69]. In the small intestine, PMCA1 is considered the principal driver of Ca^{2+} extrusion and is linked with Ca^{2+} absorption deficiency, reduced bone mineralization and hyperparathyroidism [70-72]. In the *Drosophila* gut, knocking down *PMCA* in gut progenitor cells leads to Ca^{2+} oscillation impairment, cytosolic Ca^{2+} increase and ISC over-proliferation in a CREB-dependent manner [40]. In addition, *PMCA* reduction in *piezo*-deficient guts is sufficient to restore the normal number of EEs[41]. Together, these data support the idea that PMCA could be the main driver of Ca^{2+} extrusion in progenitor cells.

Another Ca^{2+} efflux pathway found in almost all cells is the Na^+/Ca^{2+} exchanger-mediated efflux pathway. In *Drosophila,* Calx is the homolog of the mammalian Na^+/Ca^{2+} exchanger (NCX) [73]. *Calx* is expressed in the *Drosophila* gut and based on profiling datasets$_{i\text{-}ii}$ *Calx* expression is as abundant as *PMCA* in EEs and ECs but less so in ISCs [36,37]. Perhaps Ca^{2+} removal is regulated by a different mechanism among gut cells, reflecting the different physiological requirements of Ca^{2+} between progenitor and epithelial cells. This idea would be worth exploring in the *Drosophila* gut, especially under pathophysiological conditions.

Na^+ regulation

ENAC: The epithelial sodium channel (ENaC) belongs to the voltage independent, Na^+ selective ENaC/DEG superfamily that regulates Na^+ and water homeostasis in the epithelium [74]. In epithelial cells, ENaC is the main route for Na^+ entry and therefore constitutes a vital regulatory mechanism for intracellular Na^+ influx [75]. ENaC is composed of heterotrimeric subunits that harbor protease-sensitive domains critical for opening and closing of the channel. When proteases cleave peptidyl tracts from the extracellular domain, ENaC opens and allows Na^+ to pass into the cell [76-79].

In *Drosophila,* the ENaC/DEG superfamily is comprised of 31 family members, the 'pickpocket' (*ppk*) genes [80], which are important regulators of *Drosophila* wing development [81]. *Ppks* are also expressed in the intestinal epithelium [43,82] and recent work revealed that *ppk4/ENaC* expression is downregulated in ECs by the small non-coding RNA *miR-263a* [43]. Depletion of *miR-263a* increases *ppk4/ENaC* mRNA levels, leading to a stress response in ECs due to dehydration and elevated Na^+ uptake [43]. This in turn triggers ISC proliferation as well as hyperplasia and promotes increased bacterial load and expression of antimicrobial peptides [43]. Thereby, elevated Na^+ uptake triggers responses that resemble the elevated ENaC activation associated with the multi-organ disease Cystic Fibrosis (CF), rendering the *Drosophila* intestinal epithelium a potential tissue to model CF [43].

ENaC has also been proposed to regulate wound healing [15,75] with *in vitro* studies reporting that ENaC increases the levels of Ca^{2+} through interactions with the Na^+/Ca^{2+} exchanger, generating a slow Ca^{2+} wave that controls the rate of wound healing [75]. Future work in the *Drosophila* gut could help further our understanding of ENaC-stimulated Ca^{2+} dynamics by studying the interactions of Calx with ppk4/ENaC during epithelial injury and by exploring *in vivo* the role of cytokines and other inflammatory factors in modulating ppk4/ENaC function.

Na^+/K^+-pump: Transcriptome analysis of the *Drosophila* $gut_{i\text{-}ii}$ revealed the conserved expression of the Na^+/K^+-pump [36,37]. This ATPase pump utilizes ATP to move Na^+ and K^+ against their concentration gradients across the plasma membrane to maintain ion balance. In detail, Atpα which is the essential membrane cation antiporter, forms heterodimers with the β subunits, which are non-catalytic components of ATPase, and, through ATP hydrolysis, regulates the number of Na^+ ions shuttled out of the cell while K^+ ions are shuttled in. Based on *Drosophila* gut $profiling_{i\text{-}ii}$ the Na^+/K^+-pump subunit α (Atpα) together with the three β subunits (Nervana 1-3, Nrv1-3) are enriched in EEs compared to ECs and ISC/EBs [36,37], indicating different Na^+ efflux and K^+ influx requirements among different gut cell types.

Na^+/K^+-pump dysregulation is associated with diseases like inflammatory bowel diseases (IBDs) and CF. It is proposed that IBD-related diarrhea is attributed to reduced Na^+/K^+-pump activity combined with downregulation of ENaC [1,83]. Together they potentially promote accumulation of intracellular Na^+, reducing Na^+ absorption thereby leading to water and ion imbalance that causes diarrhea [1,83]. Moreover, CF patients have elevated Na^+/K^+-pump activity across the airway epithelium and *in vitro* experiments find this activity to be mediated by ENaC [84,85]. Future studies in the *Drosophila* gut could help reveal conserved molecular pathways that are regulated by the Na^+/K^+-pump and explore how interactions with conserved Na^+ channels like ENaC contribute to disease development.

Cl^- regulation

Chloride channels and transporters play vital roles in water absorption and ion balance in the gut [86]. The movement of Cl^- throughout the gut has been proposed to control cell membrane potential and cell volume, to maintain the cellular pH and to regulate the

balance of electrolytes [86]. Among known Cl^- families of channels and transporters, CF transmembrane conductance regulator (CFTR) is one of the most well-studied due to its role in CF.

Mammalian CFTR is a Cl^- channel regulated by ATP-binding and protein Kinase A (PKA)-dependent phosphorylation [87]. CFTR is composed of a single polypeptide with two transmembrane domains, two nucleotide-binding motifs and a cytoplasmic regulatory domain that controls channel activity [87,88]. This regulatory domain needs to be phosphorylated by PKA for the CFTR channel to be able to open. Once phosphorylated, ATP binding opens CFTR, and ATP hydrolysis then closes the channel [88-90]. CFTR is highly expressed in the airway epithelium and the intestinal epithelium [91]. Mutations in CFTR lead to CF, which is characterized by infection and damage in the lung and is also associated with microbiota imbalance and inflammation in the gut [92-95].

A recent study identified the *Drosophila* equivalent of human CFTR (Dmel\CFTR) [44]. This Cl^- channel shares structural and functional properties with the human channel and is expressed in ECs [44]. ECs in the *Drosophila* gut are required for nutrient and water absorption and knocking down Dmel\CFTR in these cells leads to a disruption of Cl^- transport, abnormal cellular swelling, EC damage and increased expression of mucin genes [44], reminiscent of CF. These CF-related phenotypes are associated with release of cytokines and with non-autonomous activation of JAK/STAT and EGFR pathways which trigger extreme ISC proliferation and hyperplasia in the *Drosophila* gut [44].

In the mammalian epithelium, CFTR is proposed to interact with other ion channels such as **nicotinic receptors** and the epithelial Na^+ channel ENaC to maintain ion balance [3,96,97]. In support of the idea that ion balance in *Drosophila* and mammalian epithelial cells share similar mechanisms, Dmel\CFTR depletion increases ENaC-mediated Na^+ uptake and osmotic stress in ECs [44]. Future studies in the *Drosophila* gut focusing on the relationship between CFTR with other ion channels could help reveal conserved intracellular pathways associated with CF-related symptoms.

Based on *Drosophila* gut profiling datasets$_{i\text{-}ii}$ other chloride channels such as Chloride channel-a (CIC-a) and CIC-b are expressed in ECs, EEs and ISC/EBs [36,37] but their roles in the fly gut epithelium have not been established. Future studies in the *Drosophila* gut could explore whether additional Cl^- channels together with CFTR maintain ion balance and regulate ISC proliferation through JAK/STAT and EGFR pathways [44]. Having a comprehensive understanding of how Cl^- channels regulate proliferation is important since CFTR has been proposed to act as a tumor suppressor in intestinal cancer [98] and chloride channels in general are proposed to have roles beyond homeostatic ion balance [86].

K^+ regulation

K^+ channels are another type of ion channel found in the gut. These channels consist of a primary pore-forming α-subunit that controls K^+ transport and is often associated with a regulatory β-subunit that sense a variety of stimuli [99]. In general, K^+ channels maintain K^+ homeostasis by regulating K^+ influx and efflux and play significant roles in diverse cellular functions, including cell volume regulation, differentiation and apoptosis [100]. In

the gastrointestinal tract, K^+ channels are involved in the production of gastric acid and regulation of secretion [101-103], and changes in K^+ channel activity are associated with intestinal diseases such as IBDs [104,105].

There are four families of K^+ channels: the i) Ca^{2+}- and Na^+- activated K^+ channels ($K_{ca/Na}$); ii) inwardly rectifying potassium channels (K_{ir}); iii) two-pore domain K^+ channels (K_{2P}); and iv) voltage-gated potassium channels (K_v) [99,104,106]. Profiling of the *Drosophila* $gut_{i\text{-}ii}$ [36,37] identified the expression of a variety of K^+ channels and revealed that the expression of genes encoding K_v channels such as Shaker (Sh) and Shaker cognate I (ShaI) is enriched in secretory EEs compared to other gut cells. Therefore it would be worth exploring whether K_v channels regulate gut-hormone release from EEs, and consequentially affect intestinal lipid metabolism [107], feeding [108] and ISC proliferation [109].

In addition, gut profiling after $infection_{ii}$ [37] revealed that *Sh* and *ShaI* levels increase in EBs. K^+ channels have been reported to regulate cell cycle progression and proliferation (reviewed by [110]), so their upregulation in the infected gut could be suggestive of potential roles during damage-induced EB mitosis [111]. Since K^+ channels have been implicated in carcinogenesis [112,113], future studies in the *Drosophila* gut should explore the role of K^+ channels under pathophysiological conditions like damage-induced regeneration and help identify conserved signaling pathways triggered by K^+ channels that may contribute to tumor development.

Cholinergic and Serotonin receptors:

A group of major regulators for ion transport in the gut epithelium are the cholinergic receptors [4]. Not surprisingly, disruption of cholinergic signaling in the gut is associated with several intestinal diseases, including IBDs and cancers [4,114-116]. Cholinergic receptors fall into two categories: i) the G-coupled muscarinic receptors, which upon activation are proposed to raise intracellular Ca^{2+} causing Ca^{2+}-dependent K^+ outflow and subsequent Cl^- secretion, and ii) the ligand gated ion channels, nicotinic receptors that upon acetylcholine (Ach) activation are permeable to cations [4]. *Drosophila* gut $profiling_{i\text{-}iii}$ [36-38] revealed the expression of mAchR subtypes and different nAchR subunits, resembling the mammalian gut and suggesting that the role of the cholinergic pathway in regulating epithelial ion transport and water movement might be conserved. Moreover, *Drosophila* EC subpopulations like aEC1 and pEC3 express nAchR subunits α5 and β3 together with the Cl^- channel CFTR. Since mammalian nAchRs in airway epithelial cells are proposed to regulate Cl^- channels like CFTR [96], such interactions may be conserved and worth exploring for their role in promoting ion transport and for their contributions to CF-related symptoms in the *Drosophila* intestinal epithelium [44].

Single cell *Drosophila* $gut\ profiling_i$ [36] revealed that a specific subpopulations of EEs co-expresses cholinergic and **serotonin receptors**- also found in the mammalian gut [117]. Specifically, the serotonin G-coupled receptor 5-HT1A together with cholinergic receptors mAchR-A, mAchR-C, nAchRα3 and nAchRα5 are expressed in a subpopulation of EEs that secrete the peptide Allatostatin-A (Asta-A) which is reported to regulate K^+ transport [118]. In addition, serotonin and cholinergic receptors are being investigated as potential therapeutic targets for IBDs [119-121], albeit they are very rarely studied together. Future

studies in the *Drosophila* gut should explore whether the intersection of serotonergic- and cholinergic-induced pathways in Asta-A expressing EEs play any role in maintaining K^+ balance in the intestinal epithelium, especially since increased K^+ secretion is common in IBD patients [122].

Perspectives on bioelectric signaling in the *Drosophila* gut

Ion channels and pumps are vital in initiating bioelectric signaling (endogenous ion currents) which is followed by the flow of electric currents across adjacent cells through electrical synapses known as **gap junctions**. Gap junctions open and close in response to a variety of regulatory inputs, including voltage changes and shifts in intracellular pH or Ca^{2+} concentrations [47]. When open, gap junctions allow electric currents to flow between cells, causing them to electrically couple. This bioelectric coupling has been proposed to help cells cooperate towards larger scale outcomes; for instance, promoting proper growth and patterning during development [6,7].

Gap junctions are composed of two hexameric **hemichannels** that connect the cytoplasm of neighboring cells. The compositions of gap junctions vary because each connecting hemichannel can be the same (homotypic) or different (heterotypic) per cell and consists of transmembrane proteins which assemble in homomeric or heteromeric forms. In *Drosophila,* the transmembrane proteins that form gap junctions belong to the Innexin (Inx) family [123]. Roles for innexins have been reported in development [124-127], cell proliferation and differentiation during spermatogenesis[128]. Profiling data in the *Drosophila* $gut_{i\text{-}ii}$ show diverse Inx expression [36,37]. Specifically, Inx3, Inx7 and Inx2 are expressed in progenitor cells (ISC/EBs); Inx2, Inx7 and Inx1 in ECs; and only Inx2 and Inx7 are found in EEs. The functions of Innexins in the adult *Drosophila* gut remain understudied, but in other invertebrate systems, namely *C. elegans*, gap junctions between intestinal cells propagate Ca^{2+} waves to promote defecation[129]. Similar waves could exist in the adult *Drosophila* gut and their roles in stimulating physiological functions like defection as well as potential interactions with channels like TrpA1 or Piezo should be further explored.

Bioelectric signaling during regeneration and wound healing is getting a lot of attention for the exciting prospect of using electric interventions as therapeutics [6,7,130,131]. However, having a comprehensive understanding *in vivo* of the impact of ion currents in the regulatory networks of a regenerative tissue can be challenging given the pleotropic nature of these currents (e.g Ca^{2+} signaling). The extensive understanding of the molecular pathways regulating *Drosophila* gut regeneration combined with the state-of-the art genetic tools have the potential to make *Drosophila* gut an excellent bioelectric model during regeneration. For example, sophisticated tools available for *Drosophila* such as genetically encoded Calcium indicators (GECIs, e.g. GCAMP)[132-134], voltage indicators (GEVIs, e.g. ArcLight) [135], FRET-based indicators (e.g Cl^- reporter Clomeleon [136]), and activated cation channels (e.g. CsChrimson) [137], make *Drosophila* an attractive *in vivo* model for exploring ion current propagation via gap junctions as well as studying the initiation of endogenous ion flows by different bioelectric regulators. In addition, the multiple binary expression systems available for *Drosophila* (i.e., Gal4/UAS, LexA/LexAop and QF/QUAS) [32,138,139] allow specific temporal and spatial *in vivo* perturbations that could facilitate the precise dissection

of pathways induced by gap junctions and bioelectric regulators in different cells. Together, these tools could link with great specificity bioelectric networks to molecular pathways and expand our current view on gut regeneration and healing.

Concluding remarks

Studies in the *Drosophil*a gut, building on previous studies [40-44,67], provide an opportunity to further dissect the bioelectric roles of ion channels, pumps and receptors and explore *in vivo* their interactions with gap junctions during physiological, pathophysiological, and regenerative conditions (see Outstanding Questions box). For example, recent advances indicate that *Drosophila* ingestion involves the activation of mGluR- and Piezo-inducing Ca^{2+} dynamics in progenitor cells [40,41]. Dietary Glu has been reported to activate mGluR which increases cytosolic Ca^{2+} in ISCs triggering CREB-induced proliferation, while food-induced mechanical pressure elevates Ca^{2+} influx in Piezo-expressing EBs triggering proliferation through ERK signaling [40,41]. Therefore, what is perceived as steady-state gut physiological function likely involves two independent Ca^{2+} regulators in adjacent progenitor cells concurrently triggering proliferation through different Ca^{2+}-dependent molecular pathways. Further work studying the interactions of mGluR and Piezo in the *Drosophila* gut during physiological conditions will expand our understanding on how nutritional needs and mechanical pressures in the gut are decoded to regulate proliferative responses.

In addition, transcriptome analysis upon bacterial infection in the *Drosophila* $gut_{ii\text{-}ii}$ [37,38] revealed that the expression of various ion channels is altered across the epithelium. *TrpA1*, *Sh* and *ShaI* are all increased in EBs during infection indicative of elevated Ca^{2+} and K^{+} demands when rapid proliferation is triggered. Future studies in the *Drosophila* gut could help decipher which bioelectric regulators are vital in helping the intestinal epithelium meet the varying ion demands during pathophysiological conditions like regeneration, infection or CF-like development. The advanced genetic tools available in *Drosophila*, could help identify *in vivo* which bioelectric regulators and conserved molecular pathways are involved per cell type and find roles for gap junctions and bioelectric currents across the epithelium in different regenerative and disease-like states. Together this new information could ultimately have great therapeutic value for diseases including IBDs, CF and cancer.

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Glossary:

Gut epithelium	a single cell layer of intestinal epithelial cells
Ion channel	proteins forming pores that allow the flow of ions across membranes
Ion pump	active channels that use ATP hydrolysis to transfer ions from one side of the membrane to the other

Ion exchanger	bidirectional ion transporter
Nicotinic receptors	ligand gated ion channels, activated by acetylcholine
Cholinergic receptors	receptors activated by acetylcholine and include the nicotinic and muscarinic receptors
Serotonin receptors	G protein-coupled receptors and ligand-gated ion channels activated by serotonin
Bioelectric regulators	ion channels, ion pumps, ion exchangers or receptors regulating epithelial ion dynamics
Cytokines	any substances secreted by innate immune cells as well as epithelial cells and affect other cells
Gap junctions	specialized intercellular connections between adjacent cells allowing the flow of ions and electrical impulses
Hemichannel	half of a gap junction formed by the oligomerization of six subunits-innexins in invertebrates and connexins in vertebrates

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Highlights

1. Ion channels, pumps and exchangers regulate the transport of ions like Na^+, Cl^- , K^+ and Ca^{2+} in and out of cells and gap junctions allow the movement of ions across cells.
2. Ion imbalance is highly associated with intestinal disorders and even cancer, therefore a genetic model capable of decoding *in vivo* how ion regulation affects intestinal stem cell proliferation could have great therapeutic value.
3. Recent advances in the adult *Drosophila* gut, identify different molecular mechanisms by which conserved ion channels regulate intestinal stem cell proliferation.
4. The *Drosophila* gut is an attractive *in vivo* model to decipher how ion regulation and bioelectric currents affect conserved molecular pathways that drive proliferation during physiological and pathophysiological states.

Outstanding questions:

1. How do mGluR- and Piezo-induced Ca^{2+} dynamics work together to regulate proliferation during ingestion?
2. How do TrpA1-induded Ca^{2+} dynamics in the damaged epithelium promote proliferative and bacteria expulsion responses?
3. How do bioelectric interactions of ENaC with the Na^{+}/Ca^{2+} exchanger and the Na^{+}/K^{+}-pump contribute to gut regeneration?
4. What are the interactions of nAchRs with CFTR or other Cl^{-} channels during the development of CF-like symptoms?
5. What is the function of K^{+} channels in EEs and do they affect gut regeneration?
6. Which bioelectric regulators help the intestinal epithelium meet the variable ion demands during gut regeneration and disease development?
7. Do bioelectric regulators in the gut trigger endogenous ion currents during physiological and regenerative conditions? What is the nature of the molecular pathways that are affected in response to these bioelectric currents? What are the interactions between bioelectric regulators and gap junctions during these intestinal bioelectric currents?

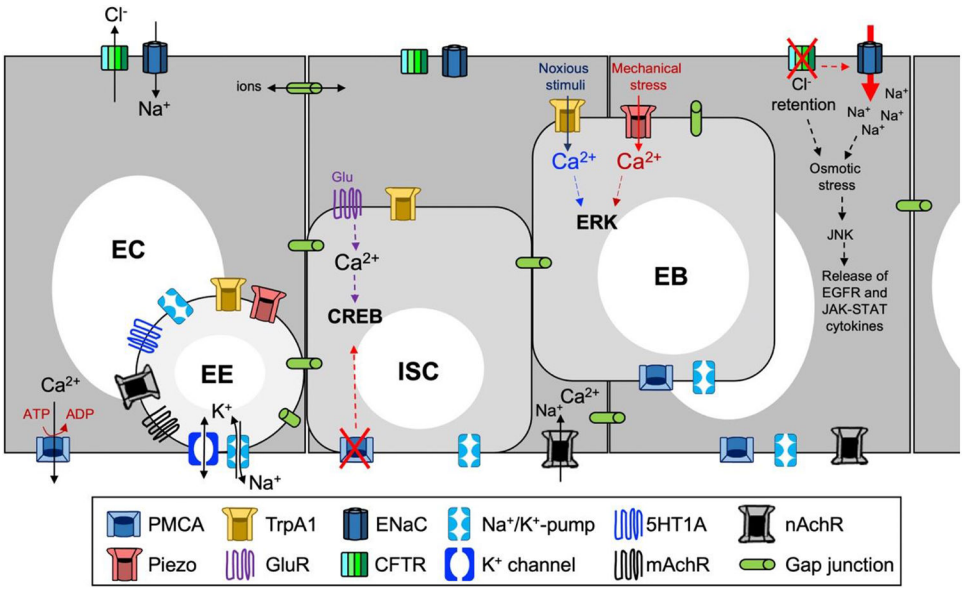

Figure: ISC proliferation under the control of bioelectric regulators in *Drosophila*.

Recent advances in the *Drosophila* gut have identified bioelectric regulators (ion channels, pumps, exchangers, receptors) which, by triggering downstream conserved molecular pathways, regulate intestinal stem cell (ISC) proliferation. Dietary Glu activates GluR in ISCs elevating cytoplasmic Ca^{2+} that promotes proliferation in a CREB-dependent manner [40]. In addition, PMCA reduction triggers proliferation in a CREB-dependent manner[40]. Mechanical pressure in a Piezo-expressing subpopulation of enteroblasts (EBs) triggers Ca^{2+} influx that promotes ERK-dependent proliferation [41]. Upon noxious stimuli TrpA1 promotes Ca^{2+} increase and ERK-dependent proliferation [42]. CFTR reduction in ECs leads to Cl^{-} retention, increased Na^{+} uptake followed by JNK induction and release of EGFR and JAK-STAT proliferative cytokines [43,44]. The schematic depicts additional conserved bioelectric regulators whose expression in the *Drosophila* gut was detected in gut profiling studies [36-38] and are worth exploring further for their roles in ion transport and gut proliferation. These include Na^{+}/K^{+} pump, K^{+} channels in EEs (enteroendocrine cells), cholinergic receptors (mAchR and nAchR), serotonin receptors (5HT1A) and gap junctions. The location of the bioelectric regulators is not representative of their subcellular localization, which in most cases has not been reported. In addition, ion exchange is not indicated in all cases for simplicity.

Table 1:

Bioelectric regulators in the *Drosophila* gut reviewed in this study

ISC/EBs	ECs	EEs	Ionic Regulation	Function	Effects on proliferation
GluR			Cytosolic Ca^{2+} increase	Regulates proliferation based on nutritional needs [40]	GluR activation increases proliferation through the CREB pathway [40]
TrpA1		TrpA1	Ca^{2+} influx	Promotes response to injury and noxious bacteria expulsion [42,67]	TrpA1 activation increases proliferation via RAS/ERK MAPK and EGFR signaling [42].
Piezo		Piezo	Ca^{2+} influx	Promotes EE differentiation in response to mechanical pressure [41].	Piezo activation increases proliferation via ERK signaling [41].
PMCA	PMCA	PMCA	Ca^{2+} efflux	Promotes Ca^{2+} balance [40].	PMCA decrease in progenitor cells triggers proliferation in a CREB-dependent manner [40]
	ppk4/ ENaC		Na^{+} influx	Regulates epithelial water movement [43].	ppk4/ENaC increase causes EC osmotic stress that triggers proliferation [43].
	CFTR		Cl^{-} transport	Regulates nutrient and water epithelial absorption [44].	CFTR reduction increases proliferation via non autonomous JAK-STAT and EGFR signaling [44].