Molecular bioelectricity: how endogenous voltage potentials control cell behavior and instruct pattern regulation in vivo

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ABSTRACT **In addition to biochemical gradients and transcriptional networks, cell behavior is regulated by endogenous bioelectrical cues originating in the activity of ion channels and pumps, operating in a wide variety of cell types. Instructive signals mediated by changes in resting potential control proliferation, differentiation, cell shape, and apoptosis of stem, progenitor, and somatic cells. Of importance, however, cells are regulated not only by their own V_{mem} but also by the V_{mem} of their neighbors, forming networks via electrical synapses known as gap junctions. Spatiotemporal changes in V_{mem} distribution among nonneural somatic tissues regulate pattern formation and serve as signals that trigger limb regeneration, induce eye formation, set polarity of whole-body anatomical axes, and orchestrate craniofacial patterning. New tools for tracking and functionally altering V_{mem} gradients in vivo have identified novel roles for bioelectrical signaling and revealed the molecular pathways by which V_{mem} changes are transduced into cascades of downstream gene expression. Because channels and gap junctions are gated posttranslationally, bioelectrical networks have their own characteristic dynamics that do not reduce to molecular profiling of channel expression (although they couple functionally to transcriptional networks). The recent data provide an exciting opportunity to crack the bioelectric code, and learn to program cellular activity at the level of organs, not only cell types. The understanding of how patterning information is encoded in bioelectrical networks, which may require concepts from computational neuroscience, will have transformative implications for embryogenesis, regeneration, cancer, and synthetic bioengineering.**

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> All these facts, sufficiently numerous, ... will open a very wide field of reflection, and of view, not only curious, but particularly interesting to medicine. There will be a great deal to occupy the anatomist, the physiologist, and the practitioner.
>
> *Allesandro Volta (1800), communicating to the Royal Society his invention of the electric battery*

INTRODUCTION

Cell behavior is regulated by numerous distinct cues that impinge on them in vivo. Alongside chemical gradients (Huang *et al.*, 2005; Geard and Willadsen, 2009; Niehrs, 2010; Ben-Zvi *et al.*, 2011; Gershenson, 2012) and physical forces (Beloussov and Grabovsky, 2006; Beloussov, 2008; Nelson, 2009; von Dassow and Davidson, 2011; Davidson, 2012), cell activity is orchestrated toward the creation and repair of high-order anatomical structures by a set of bioelectrical cues (Levin, 2012a,b; Levin and Stevenson, 2012). Here *bioelectricity* refers to endogenous electrical signaling via ion channels and pumps at the plasma membrane; specifically excluded due to length constraints is the rich literature on external electromagnetic fields (Funk *et al.*, 2009; Cifra *et al.*, 2011; Hronik-Tupaj and Kaplan, 2012), ultraweak photon emission (Farhadi *et al.*, 2007; Fels, 2009; Sun *et al.*, 2010; Beloussov, 2011), and subcellular organelle potentials (Bustamante *et al.*, 1995; Mazzanti *et al.*, 2001; Yamashita, 2011).

The importance of bioelectricity for cells beyond excitable nerve and muscle was realized long ago, and solid functional data implicate steady ion currents in embryogenesis and wound healing

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Abbreviations used: dpa, days postamputation; hMSC, human mesenchymal stem cells; hpa, hours postamputation; HPLC, high-performance liquid chromatography; 5-HT, serotonin; Vmem, transmembrane voltage potential; VSP, voltage-sensitive phosphatase.

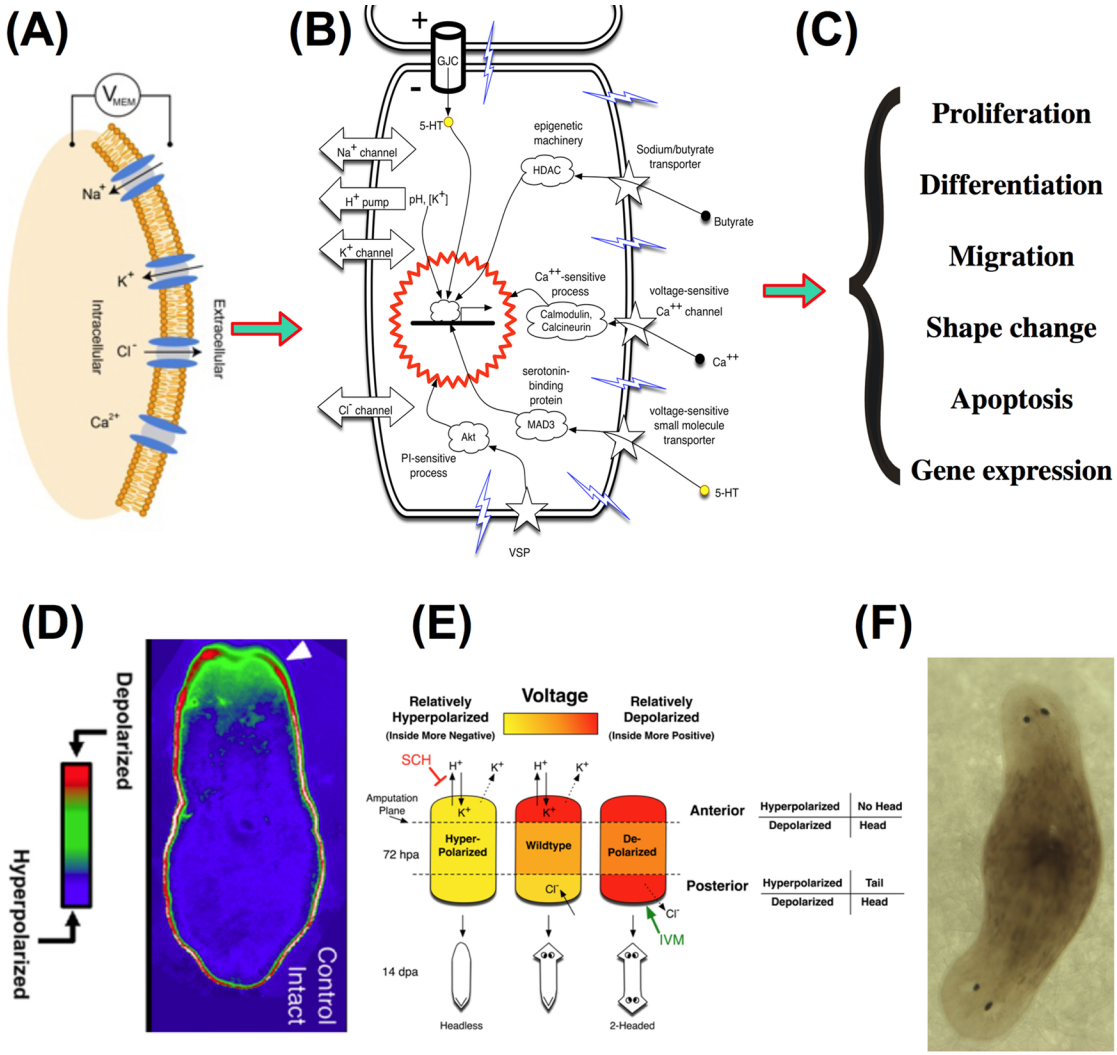

FIGURE 1: Bioelectrical signaling at the cell and organism levels, At the level of single cells, bioelectrical signals are produced by ion channel proteins, transduced into second-messenger responses, and alter key aspects of cell behavior. (A) The voltage potential (V_{mem}) at the cell membrane is produced by the movement of ions through across a cell membrane. Ions move via many different ion channels and pumps, under the control of concentration and electric gradients. (B) Change of V_{mem} is transduced into cellular effector cascades by a range of mechanisms, including voltage-sensitive phosphatases, voltage-gated calcium channels, and voltage-sensitive transporters of signaling molecules such as serotonin and butyrate. (Diagram modified, with permission, from Figure 1B of Levin, 2007.) (C) Bioelectrical signals feed into epigenetic and transcriptional cascades and thus trigger changes in cell properties such as proliferation, differentiation, migration, shape change, and programmed cell death. (D) Voltage reporter dye reveals gradients of V_{mem} across the anterior-posterior axis of planarian flatworms. (Taken, with permission, from Figure 2B of Beane *et al.*, 2013.) (E) In amputated worms, a circuit composed of proton and potassium conductances sets the voltage states at each blastema, which in turn determines the anatomical identity of each end of a regenerating fragment. (Diagram taken, with permission, from Figure 7C of Beane *et al.*, 2011.) (F) Manipulating this circuit in amputated planaria using pharmacological or genetic techniques that target ion flux allows the programming of stem cell–mediated morphogenesis to specific anatomical outcomes, such as the creation of two-head animals shown here.

(Burr and Northrop, 1935; Lund, 1947; Jaffe and Nuccitelli, 1977; Nuccitelli *et al.*, 1986; Borgens *et al.*, 1989; Hotary and Robinson, 1992). By tracking developmental currents and applying physiological-strength electric fields, it was shown that transepithelial electric fields regulate cell migration, orientation, and nerve growth (Jaffe and Poo, 1979; Patel and Poo, 1982; Borgens *et al.*, 1987; McCaig *et al.*, 2005; Nishiyama *et al.*, 2008; Cao *et al.*, 2011, 2013; Ozkucur *et al.*, 2011; Pullar, 2011; Reid *et al.*, 2011b; Vieira *et al.*, 2011; Pan and Borgens, 2012; Zhao *et al.*, 2012; Yamashita, 2013). However, recent advances and development of molecular-level techniques (Adams, 2008; Adams and Levin, 2013; Levin, 2013; Tseng and Levin, 2013) have identified a new aspect of bioelectricity that regulates individual cell function and helps coordinate the embryogenesis and regenerative repair of complex structures. This review focuses on the instructive cues mediated by spatiotemporal patterns of voltage potentials across the membranes (V_{mem}; Figure 1A) of nonneural cells and the roles these play in coordinating cell behavior during regeneration, development, and cancer.

NEW CONTROL KNOBS: RESTING POTENTIAL DETERMINES SINGLE-CELL STATE

In general, terminally differentiated, quiescent cells tend to be strongly polarized (bearing a more-negative resting potential),

Physical mechanism	References
Proliferation and cell cycle progression	Cone (1970, 1971, 1974), Cone and Tongier (1971, 1973), Cone and Cone (1976), Stillwell *et al.* (1973), Binggeli and Weinstein (1986), Arcangeli *et al.* (1993), Rouzaire-Dubois *et al.* (1993), Wonderlin and Strobl (1996), MacFarlane and Sontheimer (2000), Liebau *et al.* (2006), Morokuma *et al.* (2008a)
Apoptosis	Wang *et al.* (1999), Miki *et al.* (2001), Lauritzen *et al.* (2003), Lang *et al.* (2005), Shen *et al.* (2013)
Migration and orientation	Hyman and Bellamy (1922), Anderson (1951), Stump and Robinson (1983), Schwab *et al.* (1995), Schwab (2001), Zhao *et al.* (1997), Fraser *et al.* (2005), McCaig *et al.* (2005), Pullar and Isseroff (2005), Yan *et al.* (2009)
Differentiation	Barth and Barth (1974a,b), Konig *et al.* (2006), Hinard *et al.* (2008), Sundelacruz *et al.* (2008), Lange *et al.* (2011)
Dedifferentiation	Cone and Tongier (1971), Harrington and Becker (1973), Stillwell *et al.* (1973), Cone and Cone (1976), Sundelacruz *et al.* (2013)

TABLE 1: Cell-level properties/behaviors controlled by bioelectric events.

whereas embryonic, stem, and tumor cells tend to be depolarized (closer to zero; Binggeli and Weinstein, 1986). The picture is complicated by two still poorly understood factors: the relationship of overall V_{mem} state to the cell cycle–dependent (sinusoidally varying) changes in voltage potential (Arcangeli *et al.*, 1995; Higashimori and Sontheimer, 2007; Aprea and Calegari, 2012) and the fact that many cells in fact do not have a single V_{mem} but bear a set of distinct voltage domains over their surface (O'Connell and Tamkun, 2005; O'Connell *et al.*, 2006; Levin, 2012a).

Crucially, V_{mem} is not simply a readout but is also a functional determinant of cell behavior, such as proliferative state and plasticity (Table 1), due to a number of mechanisms that functionally couple voltage potential changes to downstream cascades (Figure 1, B and C). These data derive from genetic experiments, as well as pharmacological screens designed to identify compounds that regulate stem cell differentiation or cancer progression (Alves *et al.*, 2011; Sun *et al.*, 2013). Differentiation and proliferation are controlled by changes in V_{mem}, as shown in human mesenchymal stem cells (Sundelacruz *et al.*, 2008, 2013; You *et al.*, 2012), cardiomyocytes (Lan *et al.*, 2014), inhibitory postsynaptic currents (Jiang *et al.*, 2009), vascular muscle (Jia *et al.*, 2013), embryonic stem cells (Ng *et al.*, 2010; Du *et al.*, 2013), myoblasts (in which hyperpolarization driven by the Kir2.1 channel plays a key role; Hinard *et al.*, 2008; Li *et al.*, 2010), the specification of neurotransmitter types (Root *et al.*, 2008), and the control of precursor differentiation (van Vliet *et al.*, 2010; Yasuda and Adams, 2010; Lange *et al.*, 2011; Liebau *et al.*, 2011; Ring *et al.*, 2012; Podda *et al.*, 2013) in the developing nervous system and heart. Given the known roles of V_{mem} in regulating normal migration, differentiation, and proliferation (Aprea and Calegari, 2012; Ding *et al.*, 2012; Inaba *et al.*, 2012; Zhang *et al.*, 2012; Cao *et al.*, 2013; Yamashita, 2013), it is not surprising that control of ion flux (Park *et al.*, 2008; House *et al.*, 2010) and membrane voltage (Morokuma *et al.*, 2008a; Blackiston *et al.*, 2011; Chernet and Levin, 2013a, 2013b; Yang and Brackenbury, 2013) are also increasingly implicated in the cell dysregulation of cancer (Table 2).

Bioelectric cues also provide spatially patterned signals to cells. The differential activation of voltage-responsive transduction mechanisms on opposite sides of a cell allows bioelectric signals to regulate cell polarity. This was long ago shown in the symmetry breaking and control of outgrowth point in the algae *Fucus* (Jaffe, 1966, 1968) and has been recently shown using high-resolution imaging and genetic techniques in yeast (Minc and Chang, 2010) and pollen tubes (Certal *et al.*, 2008; Michard *et al.*, 2009). The cytoskeleton is one target of such signaling (Chifflet *et al.*, 2003; Priel *et al.*, 2006; Sekulic *et al.*, 2011; Campetelli *et al.*, 2012). Positional information can likewise be dictated by voltage properties of cells (Baglioni *et al.*, 2012) and their neighbors (Shi and Borgens, 1995). Studies of embryonic left–right patterning of the *Xenopus* embryo have revealed how bioelectrical processes link individual cell dynamics to axial patterning of the entire body plan (Levin and Palmer, 2007; Aw and Levin, 2009): cytoskeletal chirality within the fertilized egg drives

Ion translocator protein	Species	References	Function
NaV1.5 sodium channel	Human	Onkal and Djamgoz (2009), House *et al.* (2010)	Oncogene
KCNK9 potassium channel	Mouse	Pei *et al.* (2003)	Oncogene
Ductin (proton V-ATPase component)	Mouse	Saito *et al.* (1998)	Oncogene
SLC5A8 sodium/butyrate transporter	Human	Gupta *et al.* (2006)	Oncogene
KCNE2 potassium channel	Mouse	Roepke *et al.* (2010)	Oncogene
KCNQ1 potassium channel	Human, mouse	Lee *et al.* (1997), Weksberg *et al.* (2001), Than *et al.* (2013)	Oncogene
SCN5A voltage-gated sodium channel	Human	House *et al.* (2010)	Oncogene
Metabotropic glutamate receptor	Mouse, human	Song *et al.* (2012), Speyer *et al.* (2012), Martino *et al.* (2013)	Oncogene
CFTR chloride channel	Human	Xie *et al.* (2013), Zhang *et al.* (2013)	Tumor suppressor
Connexin43	Human	Sirnes *et al.* (2012)	Tumor suppressor
Acetylcholine receptor	Mouse	Felder *et al.* (1993)	Tumor suppressor

TABLE 2: Ion translocators implicated in cancer.

asymmetric distribution of ion transporter proteins in the early blastomeres, and the resulting gradient drives unidirectional (preneural) serotonin flow through cell fields, eventually triggering differential gene expression on the left versus right sides of the body (Levin, 2006; Levin *et al.*, 2006; Aw *et al.*, 2008; Lobikin *et al.*, 2012b; Vandenberg *et al.*, 2012, 2013). The dissection and synthesis of such systems at the genetic and physiological levels is beginning to reveal the properties of biophysical pathways by which individual cell polarity is integrated into large-scale patterning outcomes (Marshall, 2011).

MEASURING V_{MEM} IN VIVO

The first step in analyzing a bioelectric signal is the characterization of the spatiotemporal distributions of ionic parameters and a determination of how they correlate with patterning events. V_{mem} in cells can be quantified using several approaches; unlike mRNA and protein levels revealed by sequencing or immunohistochemistry, bioelectric properties are only ascertainable in vivo and cannot be analyzed in fixed tissue. Voltage gradients can now be visualized continuously in situ using fluorescent reporters of transmembrane potential (Adams and Levin, 2012a,b; Figure 1D) and more exotic nanoscale materials (Tyner *et al.*, 2007) suitable for use in any optically accessible tissue (Steinberg *et al.*, 2007; Yun *et al.*, 2007). These are a significant improvement on physiological impalement of single cells: far less invasive, and able to report multiple V_{mem} values across tissues and even within cell membrane subdomains (Lechleiter *et al.*, 1991; Adams and Levin, 2013). Reagents include cell-permeant dyes such as CC2-DMPE and DiSBAC$_2$(3) (Adams *et al.*, 2006; Adams and Levin, 2012b; Oviedo *et al.*, 2008; Ozkucur *et al.*, 2010) and genetically encoded protein reporters (Tsutsui *et al.*, 2008; Mutoh *et al.*, 2011; Shen *et al.*, 2011; Akemann *et al.*, 2012).

Additional tools for the characterization of bioelectrical events include highly sensitive ion-selective extracellular electrode probes (Reid *et al.*, 2007; Smith *et al.*, 2007) that reveal ion flux, microelectrode arrays (Aryasomayajula *et al.*, 2010; Schonecker *et al.*, 2014), and reporters of individual ion species such as protons (Tantama *et al.*, 2011) and sodium (Tseng *et al.*, 2010; Dubach *et al.*, 2011a,b). Significant opportunities exist for the development of specific, bright, ratiometric dyes that localize exclusively to the desired subcellular locale (e.g., plasma membrane or nucleus). Especially exciting will be the use of multiple physiological dyes in fluorescence-activated cell sorting experiments to identify subpopulations of "pure" stem and other cell types that differ in key bioelectric properties (Mello de Queiroz *et al.*, 2008), as has been observed for human endothelial cells (Yu *et al.*, 2002). Of importance, such experiments on dissociated cells will clearly highlight properties that are cell autonomous versus those physiological conditions that can only be maintained within a group context.

BIOELECTRIC SIGNALS INTERFACE WITH MOLECULAR GENETICS

The mechanistic investigation of bioelectric cues and their interactions with canonical biochemical pathways has been enriched by several new functional techniques (Adams and Levin, 2006b, 2013; Reid *et al.*, 2007; Song *et al.*, 2007). The comprehensive workflow for probing developmental bioelectricity can be illustrated by two examples. In the first, a tiered pharmacological screen (Adams and Levin, 2006a) implicated a proton pump and two channels as specifically required for tail regeneration but not for wound healing or development of the primary tail (Adams *et al.*, 2007). These loss-of-function data were confirmed using reagents with molecular specificity by misexpression of a dominant-negative form of a V-ATPase subunit protein. Marker analysis was used to show why tails failed to regenerate in V-ATPase–inhibited tails (loss of regeneration-specific gene up-regulation, lack of the obligate increase of mitosis near the wound, and abrogation of innervation into the regenerate). Fluorescent dye imaging provided physiomic profiling of the changes of V_{mem} during the stages of regeneration and confirmed that the unique voltage changes characteristic of the regenerating state were blocked by V-ATPase inhibition and were absent during stages at which tadpoles normally are not competent to regenerate their tails. On the basis of these findings, to develop a gain-of-function application, a yeast P-type proton pump was misexpressed in regeneration-incompetent animals, leading to restoration of mitosis, gene expression (MSX-1, Notch), innervation, and morphological regeneration of a complete tail. Additional rescue experiments using net-electroneutral proton exchangers allowed the independent testing of pH versus voltage signaling.

One key result was that the anatomical outcome (regeneration rescue) can be induced by a completely heterologous hyperpolarizing pump, which has no sequence or structural homology to the native *Xenopus* protein endogenously driving regeneration. This demonstrated that the necessary and sufficient trigger for regeneration is not a specific gene product (V-ATPase), but a bioelectrical state, which can be implemented using a variety of different reagents. This finding facilitated development of a purely pharmacological method of modulating ion flows in the wound to induce tail (Tseng *et al.*, 2010) and leg (Tseng and Levin, 2013) regeneration without the need for gene therapy.

The available tools enable a multistep strategy that combines pharmacological screening, physiological imaging, and molecular-genetic tools to generate loss- and gain-of-function data showing how a bioelectric pathway normally works and how it can be exploited to trigger pattern formation. A similar approach was taken with an initial gain-of-function screen, misexpressing ion channels in frog embryogenesis. One of the outcomes was the finding that a specific V_{mem} range was necessary and sufficient to trigger ectopic eye development (Pai *et al.*, 2012). Dye imaging data showed that the location of the endogenous eyes is demarcated by a prepattern of V_{mem} states in the anterior neurectoderm and that experimental alteration of this prepattern results in abnormal craniofacial gene expression and eye and facial malformations (Vandenberg, 2011; Pai *et al.*, 2012). To complement the data showing that bioelectric states are an endogenous component of eye development, it was then shown that driving eye-specific V_{mem} states in other body regions (by misexpression of ion channels) was sufficient to induce anatomically complete (well-formed) ectopic eyes (Figure 2A). Marker analysis revealed that this occurs via establishment of a positive feedback loop between hyperpolarization and Rx1/Pax6 expression, whereas a suppression screen of transduction mechanisms implicated voltage-gated calcium signaling as the transduction mechanism. However, note that, by themselves, "master" eye genes such as Pax6 do not produce eyes outside the head in vertebrates (Chow *et al.*, 1999). Moreover, as with the tail, individual cell types appropriate to the eye did not have to be specified. Together these data revealed the unique properties of bioelectric triggers to reprogram body regions at the level of organ identity and overcome lineage specification limits observed with biochemical inducers.

Of interest, many forward genetic approaches have identified ion channel genes responsible for patterning phenotypes, as have unbiased transcriptional network analyses in development (Langlois and Martyniuk, 2013) and cancer (House *et al.*, 2010). These include patterning of the face, limb, brain, and viscera in a range of model systems and a number of channelopathies that form an important

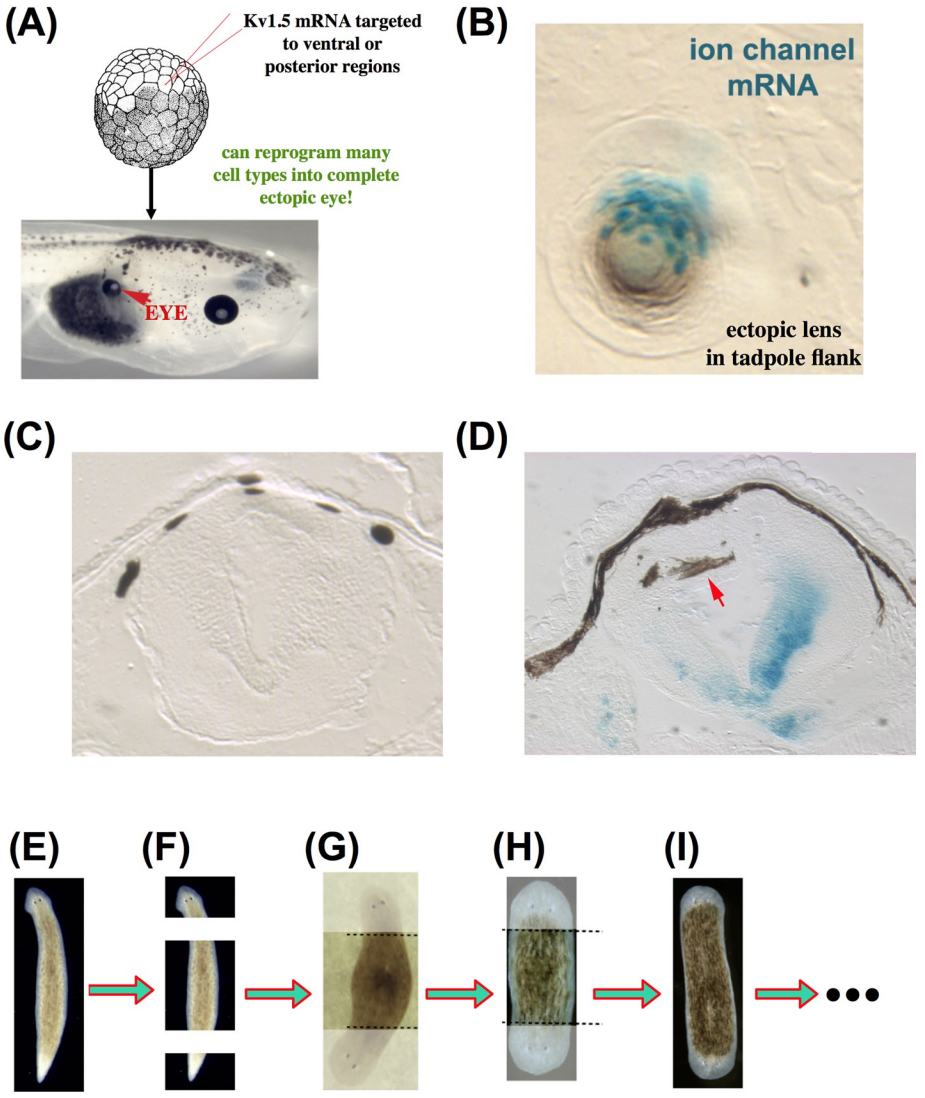

FIGURE 2: Bioelectric properties specify instructive, non–cell-autonomous patterning cues. (A) Targeted V_{mem} change, via misexpression of ion channels in the frog embryo, induces the formation of ectopic structures such as complete eyes, even in regions normally not competent to form eyes (such as on the gut). (Used, with permission, from Figure 3G of Pai *et al.*, 2012.) (B) Tracking the ion channel expression using a lineage marker reveals that the effect is not cell-autonomous: in a lens created in the tail of a tadpole by ion channel expression, only about half of the ectopic cells express the heterologous ion channel (revealed by blue lacZ staining); the other half of the induced structure consists of host cells recruited to participate in making the appropriate shape but not themselves targeted by the V_{mem}-altering reagent. (C) Melanocytes seen in a cross section of a *Xenopus* tadpole are normally few in number, round, and confined to their normal locations. (D) Depolarization induced by ion channel modulation induces these cells to overproliferate, acquire an elongated shape, and invade many organs (red arrow). Of importance, this effect is also not cell autonomous, as seen in the melanocyte phenotype, which results when cells (marked by ion channel expression construct lineage label in blue) are depolarized at a considerable distance from the melanocytes. (Taken, with permission, from Figure 6A of Chernet and Levin, 2013b.) (E) A normal planarian has a head and tail and regenerates each at the appropriate end of an amputated fragment. When it is cut into thirds and the middle fragment is briefly exposed to octanol, which temporarily blocks long-range bioelectrical signaling between the wound and mature tissues, a two-headed worm results (F). Remarkably, upon further rounds of cutting in plain water (long after the octanol has left the tissues, as confirmed by HPLC), the two-headed form results (H, I; images of two-headed worms provided by Fallon Durant, Tufts University, Medford, MA). This change in the animal's target morphology (the shape to which it regenerates upon damage) appears to be permanent and persists across the animal's normal reproductive mode (fissioning), despite the fact that the genomic sequence has not been altered. Chromatin modifications alone do not explain this, because the posterior wound cells, which could have been epigenetically reprogrammed to a head fate, are discarded at each cut: the information encoding a bipolar two-head animal is present even in the normal gut fragment—it is distributed throughout the body. We propose that this information is a kind of memory, encoded in electrical networks of somatic cells coupled by gap junctions, and is stored at the level of bioelectrical dynamics. (E–I taken, with permission, from Figure 2 of Levin, 2014; photographs of planaria taken by Taisaku Nogi, Children's Health Research Institute, Canada, and Fallon Durant.)

class of human birth defects (Table 3). Thus upstream of endogenous bioelectrical signaling lie a set of ion channel and pump proteins that establish resting potential and alter it in response to physiological, transcriptional, and mechanical signals. Such data often come from studies that, unlike the previously discussed two examples, did not set out to investigate bioelectricity, and the overall structure of developmental bioelectric signaling is starting to emerge from the synthesis of bioelectric projects investigating molecular mechanisms and molecular biology efforts that implicate ion channel activity in instructive roles.

Downstream of voltage change lie two types of endpoints—at the mRNA and chromatin modification levels. Transcriptional responses to depolarization include genes such as Notch, BMP, Sox10, Nurr1, Slug, Fos, Jun, NPY, and Wnt (Bartel *et al.*, 1989; Higuchi *et al.*, 1990; Raya *et al.*, 2004; Morokuma *et al.*, 2008a; He *et al.*, 2011; Lange *et al.*, 2011; Tseng *et al.*, 2011; Dahal *et al.*, 2012; Swapna and Borodinsky, 2012; Adams *et al.*, 2013). Epigenetic responses are triggered by movement of butyrate through an ion-dependent transporter, SLC5A8; butyrate is an HDAC1 inhibitor, and this allows voltage change to regulate chromatin acetylation (Davie, 2003; Tong *et al.*, 2004; Gupta *et al.*, 2006). This is believed to mediate control of tumorigenesis by depolarization and is also implicated in bioelectrical signaling during tail regeneration in *Xenopus* (Tseng *et al.*, 2011; Chernet and Levin, 2013a, 2014).

A set of transduction mechanisms has been identified by which changes of resting potential affect events at the nucleus (Figure 1, B and C, and Table 4). One involves voltage-gated calcium channels, which convert voltage change into signaling via this versatile second-messenger molecule (Nilius *et al.*, 1993; Dolmetsch *et al.*, 1998; Nakanishi and Okazawa, 2006; Greer and Greenberg, 2008). This mode

Protein	Morphogenetic role or loss-of-function phenotype	Species	References
TMEM16A chloride channel	Tracheal morphogenesis	Mouse	Rock *et al.* (2008)
Kir7.1 potassium channel	Melanosome development	Zebrafish	Iwashita *et al.* (2006)
Cx41.8 gap junction	Pigmentation pattern	Zebrafish	Watanabe *et al.* (2006)
Cx45 gap junction	Cardiac defects (cushion patterning)	Mouse	Kumai *et al.* (2000), Nishii *et al.* (2001)
Cx43 gap junction	Oculodentodigital dysplasia, heart defects (outflow tract and conotruncal), left–right asymmetry defects, eye defect, osteoblast differentiation in bone patterning, syndactyly, microphthalmia	Human, mouse	Britz-Cunningham *et al.* (1995), Reaume *et al.* (1995), Ewart *et al.* (1997), Pizzuti *et al.* (2004), Debeer *et al.* (2005), Civitelli (2008), Zoidl and Dermietzel (2010), Gabriel *et al.* (2011)
Kir2.1 potassium channel	Wing patterning	*Drosophila*	Dahal *et al.* (2012)
Cx43 gap junction	Fin size and pattern regulation; craniofrontonasal syndrome	Zebrafish, mouse	Iovine *et al.* (2005), Davy *et al.* (2006), Hoptak-Solga *et al.* (2008), Sims *et al.* (2009)
Kir2.1 potassium channel	Andersen–Tawil syndrome, craniofacial and limb defects	Mouse, human	Bendahhou *et al.* (2003), Dahal *et al.* (2012)
CFTR chloride channel	Bilateral absence of vas deferens	Human	Uzun *et al.* (2005), Wilschanski *et al.* (2006)
KCNK9, TASK3 potassium channels	Birk–Barel dysmorphism syndrome, craniofacial defects	Human	Barel *et al.* (2008), Veale *et al.* (2014)
Girk2 potassium channel	Cerebellar development, retina patterning	Mouse	Rakic and Sidman (1973a,b), Hatten *et al.* (1986), Patil *et al.* (1995), Tong *et al.* (1996), Savy *et al.* (1999), Liesi *et al.* (2000)
GABA-A receptor (chloride channel)	Angelman syndrome, craniofacial patterning (e.g., cleft palate) and hand defects	Mouse, human	Wee and Zimmerman (1985), Culiat *et al.* (1995), Homanics *et al.* (1997)
KCNH2 K^+ channel	Cardiac patterning	Mouse	Teng *et al.* (2008)
NHE2 Na^+/H^+ exchanger	Epithelial patterning	*Drosophila*	Simons *et al.* (2009)
V-ATPase proton pump	Wing-hair patterning, pigmentation and brain patterning, left–right asymmetry, eye development, tail regeneration, craniofacial patterning	Drosophila, medaka, human, chick, *Xenopus*, zebrafish	Hermle *et al.* (2010), Muller *et al.* (2013), Borthwick *et al.* (2003), Adams *et al.* (2006), Nuckels *et al.* (2009), Vandenberg *et al.* (2011), Monteiro *et al.* (2014)
Kv channel	Fin-size regulation	Zebrafish	Perathoner *et al.* (2014)
KCNQ1 potassium channel	Abnormalities of rectum, pancreas, and stomach, left–right patterning, Jervell and Lange-Nielsen syndrome, inner ear and limb defects	Mouse, *Xenopus*	Chouabe *et al.* (1997), Casimiro *et al.* (2004), Rivas and Francis (2005), Morokuma *et al.* (2008b), Than *et al.* (2013)
Kir6.2 potassium channel	Craniofacial defects, left–right patterning	Human, *Xenopus*	Gloyn *et al.* (2004), Aw *et al.* (2010)
NaV 1.5, Na^+/K^+-ATPase	Cardiac morphogenesis	Zebrafish	Shu *et al.* (2003), Chopra *et al.* (2010)
H^+,K^+-ATPase	Left–right patterning, polarity during regeneration	*Xenopus*, chick, sea urchin, zebrafish, planaria	Levin *et al.* (2002), Kawakami *et al.* (2005), Aw *et al.* (2008), Beane *et al.* (2011)
Innexin gap junctions	Foregut, cuticle (epithelial) patterning defects	*Drosophila*	Bauer *et al.* (2002), Bauer *et al.* (2004)
TRH1 K^+ transporter	Root-hair patterning	*Arabidopsis*	Rigas *et al.* (2001)

TABLE 3: Ion translocators implicated in patterning by genetic approaches.

Developmental role	Key biophysical event	Transduction mechanism	References
Tail regeneration in *Xenopus*: first step	Voltage change (repolarization)	Guidance of neural growth	Adams *et al.* (2007)
Tail regeneration in *Xenopus*: second step	Intracellular sodium content	SIK2 (salt-inducible kinase)	Tseng *et al.* (2010)
Neoplastic conversion of melanocytes in *Xenopus* tadpoles	Voltage change (depolarization)	Serotonin movement	Morokuma *et al.* (2008a), Blackiston *et al.* (2011)
Polarity determination in planarian regeneration, length control of zebrafish fin	Voltage change	Ca^{2+} flux through voltage-gated calcium channel	Beane *et al.* (2011), Zhang *et al.* (2011), Chan *et al.* (2014), Kujawski *et al.* (2014)
Left–right patterning in *Xenopus* embryos, melanocyte transformation toward metastatic behavior	Voltage change	Serotonin movement	Levin *et al.* (2002), Fukumoto *et al.* (2005a,b), Adams *et al.* (2006), Blackiston *et al.* (2011), Lobikin *et al.* (2012a)
Trachea size control in *Drosophila*	Ion-independent function	Planar polarity, septate junction structure	Paul *et al.* (2007)

TABLE 4: Known transduction mechanisms by which ion flows affects cell behavior.

has been implicated in control of growth-cone turning (Nishiyama *et al.*, 2008), eye patterning (Pai *et al.*, 2012), and flatworm regeneration (Nogi *et al.*, 2009; Beane *et al.*, 2011; Zhang *et al.*, 2011). Another uses the voltage gradients among cells to move small signaling molecules such as serotonin through gap junction–coupled cell fields, as occurs in left–right patterning (Fukumoto *et al.*, 2005b; Adams *et al.*, 2006) and control of neuronal pathfinding (Blackiston *et al.*, 2015). Finally, voltage-sensitive phosphatases couple V_{mem} change to the plethora of events regulated by PTEN phosphatases (Murata *et al.*, 2005; Okamura and Dixon, 2011).

Of interest, when they conflict, bioelectrical cues tend to trump chemical signals. One example is the guidance of cell motility: if a chemical gradient and an electric field are set up in opposite directions, the bioelectric vector trumps the chemical cue in directing cell movement (Zhao, 2009; Cao *et al.*, 2011). Another example is the differentiation of human mesenchymal stem cells (hMSCs), which normally hyperpolarize as they differentiate; despite the presence of potent chemical inducers, hMSCs will not differentiate if kept artificially depolarized (Sundelacruz *et al.*, 2008). Indeed, the voltage state can even partially reverse the differentiation state, inducing plasticity in differentiated hMSCs (Sundelacruz *et al.*, 2013).

By identifying the specific ion channel genes that set V_{mem} states, the transduction mechanisms that sense V_{mem} change, and the downstream transcriptional or epigenetic targets (which include ion channels themselves), recent work has established the causal chain integrating bioelectrical cues with chemical pathways (Table 5). Neither signaling mode is entirely "upstream" of the other—cellular processes are regulated by the continuous cyclical interplay between transcriptional control of ion channel profiles within cells and the regulation of transcription by voltage dynamics. Future work will identify new ion channel genes important for specific functions, additional transduction mechanisms by which cells sense their depolarization and hyperpolarization, and genome-wide (next-generation sequencing [NGS] or microarray) profiles of transcriptional programs triggered by specific V_{mem} change.

Of importance, however, V_{mem} regulation extends beyond the state of single cells. Cells can sense the voltage states of their neighbors through gap junctions (GJs)—versatile (and themselves voltage-sensitive) channels allowing the direct sharing of current and other small molecules between cells (Palacios-Prado and Bukauskas, 2009; Pereda *et al.*, 2013). The importance of GJ-mediated cues for cellular decision making has been shown, for example, in the development of the neocortex (Sutor and Hagerty, 2005) and more broadly in setting up the patterns of chemical synapses (Anava *et al.*, 2013). Cells can also read the bioelectrical state of distant regions via the chemical molecules redistributed (and transported or diffused) across long distances by bioelectric state change. This was long ago suggested by Burr, who used voltage readings at remote locations of the body to detect transplanted or induced tumors (Burr *et al.*, 1940; Burr, 1941). Recent data in the frog model implicate long-range signaling via bioelectrical control of butyrate (Chernet and Levin, 2014) and serotonin (Blackiston *et al.*, 2011; Lobikin *et al.*, 2012a) in tumorigenesis and metastatic induction. Additional modes for nonlocal bioelectrical signaling include tunneling nanotubes (Chinnery *et al.*, 2008; Wittig *et al.*, 2012) and exosomes, which contain numerous ion channels (Lotvall and Valadi, 2007; Valadi *et al.*, 2007; Wahlgren *et al.*, 2012) and could regulate bioelectric states of cells that incorporate them. Because bioelectrical gradients mediate signaling beyond the single-cell level, they form a versatile medium for carrying information.

BIOELECTRIC STATES CAN ACT AS NECESSARY, SUFFICIENT, AND INSTRUCTIVE PATTERNING SIGNALS

Spatiotemporal gradients of V_{mem} among cells in vivo are now known to regulate organ identity, positional information, size control, and polarity of anatomical axes. One mode of V_{mem} signaling is as a prepattern. Much like Hox genes, whose combinatorial patterns of gene expression encode specific body regions during development, it has recently been shown that bioelectric prepatterns in the developing face of the frog and planarian models regulate the gene expression, size, and shape of craniofacial components (Vandenberg *et al.*, 2011; Beane *et al.*, 2013). In the frog, for example, patterns of hyperpolarization in the nascent face reveal the prospective locations of the eyes and other structures; experimental perturbation of these distributions alters the boundaries of expression of face patterning genes such as *Frizzled*, with the expected effects on craniofacial anatomy. Bioelectric gradients also specify orientation of the left–right axis in frog and chick embryos (Levin *et al.*, 2002; Adams *et al.*, 2006) and set the size of regenerating structures in segmented worms and regenerating

Role	Species/ system	References
Cellular polarization (anatomical asymmetry of cell or epithelium)	Alga *Fucus*, yeast	Jaffe (1982), Minc and Chang (2010)
Migration of neurons and positional information	Chick, amphibia	Shi and Borgens (1995), Pan and Borgens (2010)
Patterning in gastrulation, neurulation, and organogenesis	Chick, axolotl, frog	Stern (1982), Hotary and Robinson (1992), Borgens and Shi (1995), Shi and Borgens (1995), Levin *et al.* (2002), Adams *et al.* (2006)
Directional transport of maternal components into the oocyte	Moth, *Drosophila*	Woodruff (2005)
Growth control and size determination	Segmented worms	Kurtz and Schrank (1955)
Neural differentiation	*Xenopus* embryo	Uzman *et al.* (1998), Lange *et al.* (2011)
Polarity during regeneration	Planaria, plants, and annelids	Marsh and Beams (1947, 1949, 1950, 1952), Marsh and Beams (1957), Bentrup *et al.* (1967), Novák and Bentrup (1972), Novak and Sirnoval (1975), Beane *et al.* (2011)
Induction of limb and spinal cord regeneration	Amphibia	Borgens (1986), Borgens *et al.* (1986, 1990)
Control of gene expression and anatomy in craniofacial patterning	*Xenopus* embryo	Vandenberg *et al.* (2011)
Induction of eye development	*Xenopus* embryo	Pai *et al.* (2012)

TABLE 5: Data on endogenous bioelectric signal roles in morphogenesis.

zebrafish tails (Kurtz and Schrank, 1955; Beane *et al.*, 2013; Perathoner *et al.*, 2014). Ion transporters, such as the V-ATPase, are required for normal left–right patterning in several vertebrate models (Adams *et al.*, 2006), zebrafish fin regeneration (Monteiro *et al.*, 2014), and zebrafish eye development (Nuckels *et al.*, 2009). These examples illustrate that bioelectric patterns can be necessary aspects of development because, when they are specifically disrupted, predictable and coherent changes in morphogenesis occur. Of importance, many of these data sets used distinct ion species (potassium, sodium, chloride, or protons) to show that the necessary parameter is indeed the voltage potential, not any one channel gene (which could have had scaffold or binding roles) or even any one ion type (which could have had chemical, not electrical, roles). As with the gain-of-function examples discussed later, the voltage is what matters for the outcome, not which ion or channel was used to set it.

In addition to specifying directly the pattern of subsequent anatomy, some bioelectric signals seem to trigger whole developmental modules. In the case of tail regeneration in *Xenopus*, genetic, optogenetic, and pharmacological experiments have been used to recapitulate a regeneration-specific bioelectric state in nonregenerative animals and induce complete regrowth of this complex neuromuscular appendage (Adams *et al.*, 2007; Tseng *et al.*, 2010). Not only could appropriate V_{mem} state overcome physiological, chemical, and age-dependent blockade of regenerative capacity, but it was seen that a very simple (low information content) stimulus, such as "pump protons," could be sufficient to trigger a complete and self-limiting cascade of events that rebuilt the appendage (Tseng and Levin, 2013), in essence providing a "build whatever normally goes here" signal. These examples reveal that bioelectric state can function as a sufficient signal or master regulator; this bodes well for the use of this approach in regenerative medicine, as we may not need to micromanage the morphogenesis of complex structures but instead rely on patterning subroutines already present in the host.

Bioelectric signals can also set the identity of whole embryonic regions to different organs. The morphogenesis of new regeneration blastemas in planaria (Figure 1, D–F) can be directed to make heads or tails by appropriate modulation of resting potential (Beane *et al.*, 2011, 2013). In vertebrates, whole-eye formation can be induced ectopically, far outside the head, even in mesoderm or endoderm (Figure 2A) by misexpression of specific ion channels in vivo (Pai *et al.*, 2012); this process is mediated by a feedback loop between hyperpolarization and expression of eye-specific genes such as Rx1 and Pax6, which in its absence cannot initiate eye formation outside of the head. It is also interesting that this signaling is not cell autonomous: cells with unique voltage characteristics serve as organizers, recruiting wild-type host tissues to participate in the ectopic morphogenesis (Figure 2B).

These examples illustrate the fact that bioelectric state provides instructive information to patterning processes and reveal that cell groups can be programmed at the level of complex organs, not only at the level of specifying individual cell types. Understanding in detail the mapping between bioelectric states and the anatomical outcomes—quantitatively cracking the bioelectric code—is a major open direction in this field. Possibilities for the parameters that functionally determine distinct organ types include spatial distribution of absolute V_{mem} values within a cell group, relative differences in V_{mem} across cell borders, and/or time-dependent changes of V_{mem} within cells. One technology that is likely to be instrumental in testing hypotheses about the bioelectric code is optogenetics (Knopfel *et al.*, 2010; Liu and Tonegawa, 2010), which will facilitate the reading and writing of bioelectric patterning information in vivo. The first steps have been taken, showing regulation of stem cells via optogenetic signaling (Stroh *et al.*, 2010; Wang *et al.*, 2014), and a recent report showed the induction of tail regeneration by optical modulation of bioelectric state after amputation (Adams *et al.*, 2013).

BIOELECTRICITY DOES NOT REDUCE TO MOLECULAR GENETICS

The information-bearing signal (the necessary and sufficient trigger) for events such as eye induction, head determination, and tail regeneration via V_{mem} change is a *physiological state*, not a gene product (Levin, 2013; Tseng and Levin, 2013). Studies reveal that the exact identity of the channel or pump used to trigger such morphological changes is often irrelevant—many sodium, potassium, chloride, or proton conductances can be used, as long as the appropriate

V_{mem} state is reached. This means that the actual cause of the given morphological change can be a bioelectrical property not necessarily in 1:1 correspondence with any genetic locus.

Because channels and pumps can open and close posttranslationally, two cells expressing precisely the same mRNA and protein can be in very different bioelectrical states. Thus rich patterns of bioelectrical gradients can exist in a transcriptionally homogeneous tissue and be completely invisible to protein and mRNA profiling until they trigger distinct downstream transcriptional targets. Conversely, cells with very different channel and pump complements may have the same V_{mem}, since resting potential is an ensemble state that is a function of many different ion flows. The implication is that mRNA and protein profiling approaches are insufficient to detect and characterize important biophysical determinants of morphogenesis, and knockout screens may completely miss bioelectric pathways, since knockouts of single ion channels will be subject to compensation and redundancy by other channels contributing to V_{mem}.

One context in which bioelectric and genetic state information can diverge is cancer (Yang and Brackenbury, 2013; Chernet and Levin, 2013b). A metastatic phenotype (overproliferation, matrix metalloprotease–dependent invasion of body tissues, and drastic arborization) can be induced in genetically normal melanocytes by depolarization of somatic cells (Blackiston *et al.*, 2011; Lobikin *et al.*, 2012a). This effect is not cell autonomous (Figure 2, C and D), showing that the bioelectric state of cells at considerable distance can trigger metastatic behavior. Conversely, the formation of tumors by human oncogenes such as p53 and KRAS mutations can be suppressed, despite the strong presence of oncogene protein within the cells, by artificially preventing the depolarization that occurs during oncogenic transformation (Chernet and Levin, 2013a). These examples reveal the potential dissociation between genetic state and disease outcome; an implication of these data is that the neoplastic state cannot always be predicted from examination of the genome, transcriptomes, or proteome, although in some cases, ion channel expression is altered (Onkal and Djamgoz, 2009; Becchetti, 2011; Lang and Stournaras, 2014). On the other hand, the functionally determinative voltage states cannot be seen in fixed tissue, stressing the importance of gathering real-time in vivo bioelectric information over and above analysis of mutations, mRNA profiles, and protein levels. Another implication for cancer biology is that although expression of some ion channel might be a useful marker (Wang, 2004; Fraser *et al.*, 2005; Stuhmer *et al.*, 2006), there will also be many cases in which the transcriptional profile reveals nothing (because of signaling via posttranslational gating of channel state), and drugs targeting one specific channel type (Arcangeli *et al.*, 2009, 2012) may have no effect (due to compensation and redundancy of channel types). If indeed cancer is augmented or induced by a depolarized bioelectric state (Binggeli and Weinstein, 1986; Olivotto *et al.*, 1996; Yang and Brackenbury, 2013), we will have to think less about individual ion channels as oncogenes (Pillozzi *et al.*, 2002; Bennett *et al.*, 2004; Lallet-Daher *et al.*, 2013; Than *et al.*, 2013) and focus instead on the way in which many channels contribute to a bioelectrical oncostate, to develop strategies for dominating the resting potential irrespective of native channel identity (Sharmeen *et al.*, 2010; Chernet and Levin, 2013a).

BIOELECTRIC GRADIENTS HAVE DISTINCT, AUTONOMOUS DYNAMICS

Bioelectric patterns are clearly important drivers of cell behavior and pattern formation, but how do these patterns originate? Diverse resting potentials across a tissue can arise from preexisting differences in ion channel transcription, but that is not the only way (Justet *et al.*, 2013). Such regionalized patterns of V_{mem} can also form de novo in transcriptionally and proteomically identical cells because cells coupled by gap junctions (electrical synapses) form a (slow) electrically excitable medium; this is a particularly interesting aspect because such media are known to have powerful computational capabilities (Fenton *et al.*, 1999; Gorgcki and Gorgcka, 2007; Adamatzky *et al.*, 2011). Positive feedback loops implemented by elements such as voltage-gated ion channels, which both set and respond to V_{mem} changes, can drive spontaneous symmetry breaking and amplification of physiological noise. Considerable self-organization dynamics can take place without a need for preexisting chemical prepattern (Toko *et al.*, 1987; Schiffmann, 1991, 1997; Palacios-Prado and Bukauskas, 2009) or transcriptional activity; for example, human red blood cells have a physiological, not genetic, circadian clock rhythm driven by a slow ionic oscillation (Chakravarty and Rizvi, 2011; O'Neill and Reddy, 2011). Such dynamics has been studied in nerve and muscle (Zykov, 1990; Chen *et al.*, 1997; Boettiger *et al.*, 2009; Boettiger and Oster, 2009), and Turing-type self-organization has long been appreciated in chemical signaling (Takagi and Kaneko, 2005; Muller *et al.*, 2012; Sheth *et al.*, 2012). However, capabilities and properties of self-organization of voltage patterns in groups of nonneural cells remain to be formally analyzed. Quantitative analysis of in silico models of bioelectric dynamics will need to be integrated with deep new data sets from appropriate physiomic technologies to fully understand and control developmental patterning in vivo.

One unexpected recent finding illustrates the storage of patterning information in physiological networks and has significant implications for evolution. Planarian flatworms have the remarkable ability to regenerate completely from partial body fragments (Reddien and Sanchez Alvarado, 2004; Salo *et al.*, 2009; Lobo *et al.*, 2012). After a surgical bisection, the cells at one edge make a tail, whereas those at the other edge make a head, revealing that the adult stem cells that implement regeneration are not locally controlled (since the cells were direct neighbors until the scalpel separated them) but must communicate with the remaining tissue to decide what anatomical structures must be formed. It was shown that this long-range communication occurs via gap junction–mediated electrical synapses (Scemes *et al.*, 2007; Marder, 2009; Pereda *et al.*, 2013), and works together with a bioelectric circuit that determines head versus tail identity in each end's blastema (Beane *et al.*, 2011, 2013). Brief inhibition of this gap junction–mediated communication results in worms developing heads at both ends (Nogi and Levin, 2005; Oviedo *et al.*, 2010).

What is remarkable (Figure 2, E–I) is that weeks later, when these two-headed animals have their heads and tails amputated again (in just water, with no further perturbation), the same two-headed phenotype results, and this is repeated upon subsequent amputations. Thus a transient perturbation of physiological cell:cell communication stably changes the pattern to which the animal regenerates upon damage, despite normal genomic sequence. This again illustrates the potential divergence of genetic versus physiological information, especially since the phenotype is stable across fission (this animal's most frequent reproductive mode), and thus could have significant implications for evolution. Although epigenetic processes may be involved, chromatin modification mechanisms alone are not a sufficient explanation, since the ectopic heads (tissue that might be suggested to have been epigenetically reprogrammed into a head state from its original tail identity) are *thrown away* at each generation of cutting. What remains is a gut fragment, which somehow knows that it is to form two heads, not one, upon further

cutting; the information about basic anatomical polarity and body organization must be stored in a distributed form throughout the animal. Quantitative, field-like models of this circuit remain to be developed to understand precisely how information guiding specific shape outcomes is encoded in (represented by) bioelectric states among cells.

CONCLUSION: NEXT STEPS AND BEYOND

Major open questions for future progress include the mechanisms by which cells compare bioelectric state across distances, additional molecular details of the interactions of bioelectrical signals with chemical gradients and physical forces, and the development of quantitative models of bioelectric circuits that store stable patterning information during morphogenesis. Expansions of the toolkit of synthetic biology will soon allow the rational top-down programming of bioelectric circuits, which will have important implications for regenerative medicine, cancer biology, and bioengineering (Reid *et al.*, 2011a; Levin, 2013). Optogenetics, once expanded to facilitate the control of stable V_{mem} in large, nonexcitable cell groups, will play a large part, and there is significant room for advances in better voltage reporters and techniques for in vivo modulation of bioelectric state. One hypothesis for the development of deep, quantitative theory in this field is that pattering information may be stored within nonneural bioelectric cell networks using the same molecular mechanisms and information-processing algorithms that underlie behavioral memory in the nervous system. This is being tested in our lab. It is thus possible that the techniques such as those now used to extract mental imagery from electrical measurements of living human brains (Nishimoto *et al.*, 2011) may shed crucial light on the encoding of anatomical pattern in the electrical circuits of somatic cells; conversely, the cracking of the bioelectric code in development and regeneration may have important benefits for the understanding of the semantics of electric states in the brain.

In practical terms, the molecular biologist needs to consider not only transcriptional and protein profiles when working to understand regulation of single-cell behavior and pattern formation. Significant instructive information is generated at the level of bioelectricity; ion channels and gap junctions are the molecular elements of such circuits, but bioelectrical signaling has its own unique dynamics that will become increasingly tractable with development of new technology specifically targeting stable V_{mem} states. The existence of bioelectric signaling among most cell types, not only neurons, suggests that the field of applicability of electroceuticals (Famm *et al.*, 2013; Sinha, 2013; Birmingham *et al.*, 2014) is much wider than anticipated by current plans to target neural function. More broadly, to the extent that the data of developmental bioelectricity are erasing artificial distinctions between neural and nonneural cell types, the insights of computational neuroscience and cognitive science will become relevant to cell and developmental biology. It is possible that the most effective ways to understand high-order (anatomical-level) outcomes will involve not only bottom-up models of molecular pathways but also top-down models in which information and control theory concepts play central roles. In this way, molecular bioelectricity may be revealing a mechanistic path toward understanding the intelligence exhibited by cell behavior and harnessing it toward transformative advances in biomedicine and the information sciences (Albrecht-Buehler, 1985; Rubenstein *et al.*, 2009; Marshall, 2011; Aur, 2012).

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