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Resting potential, oncogene-induced tumorigenesis, and metastasis: the bioelectric basis of cancer *in vivo*

Maria Lobikin, Brook Chernet, Daniel Lobo and Michael Levin[1]

Biology Department and Tufts Center for Regenerative and Developmental Biology, and Department of Biology, Tufts University, 200 Boston Avenue, Medford, MA 02155, USA

E-mail: michael.levin@tufts.edu

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Abstract

Cancer may result from localized failure of instructive cues that normally orchestrate cell behaviors toward the patterning needs of the organism. Steady-state gradients of transmembrane voltage (V_{mem}) in non-neural cells are instructive, epigenetic signals that regulate pattern formation during embryogenesis and morphostatic repair. Here, we review molecular data on the role of bioelectric cues in cancer and present new findings in the *Xenopus laevis* model on how the microenvironment's biophysical properties contribute to cancer *in vivo*. First, we investigated the melanoma-like phenotype arising from serotonergic signaling by 'instructor' cells—a cell population that is able to induce a metastatic phenotype in normal melanocytes. We show that when these instructor cells are depolarized, blood vessel patterning is disrupted in addition to the metastatic phenotype induced in melanocytes. Surprisingly, very few instructor cells need to be depolarized for the hyperpigmentation phenotype to occur; we present a model of antagonistic signaling by serotonin receptors that explains the unusual all-or-none nature of this effect. In addition to the body-wide depolarization-induced metastatic phenotype, we investigated the bioelectrical properties of tumor-like structures induced by canonical oncogenes and cancer-causing compounds. Exposure to carcinogen 4-nitroquinoline 1-oxide (4NQO) induces localized tumors, but has a broad (and variable) effect on the bioelectric properties of the whole body. Tumors induced by oncogenes show aberrantly high sodium content, representing a non-invasive diagnostic modality. Importantly, depolarized transmembrane potential is not only a marker of cancer but is functionally instructive: susceptibility to oncogene-induced tumorigenesis is significantly reduced by forced prior expression of hyperpolarizing ion channels. Importantly, the same effect can be achieved by pharmacological manipulation of endogenous chloride channels, suggesting a strategy for cancer suppression that does not require gene therapy. Together, these data extend our understanding of the recently demonstrated role of transmembrane potential in tumor formation and metastatic cell behavior. V_{mem} is an important non-genetic biophysical aspect of the microenvironment that regulates the balance between normally patterned growth and carcinogenesis.

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[1] Author to whom any correspondence should be addressed.

1. Introduction

1.1. Cancer as a developmental disorder

Cancer may be fundamentally a developmental disorder (Baker *et al* 2009, Potter 2001, 2007, Rowlatt 1994, Rubin 1985, Tsonis 1987), and may occur when cells stop obeying the normal patterning cues of the body (Needham 1936, 1963, Waddington 1935). Cancer is thus 'part of an inexorable process in which the organism falls behind in its ceaseless effort to maintain order' (Rubin 1985). The signals that establish and maintain anatomy during embryogenesis and adult life comprise both genetic and epigenetic pathways, and there has been much debate about the relative contributions of genetic versus epigenetic disruptions to cancer (Bissell and Hines 2011, Bissell and Labarge 2005, Hanahan and Weinberg 2011, Hendrix *et al* 2007, Kasemeier-Kulesa *et al* 2008, Roskelley and Bissell 2002, Sonnenschein and Soto 1999, 2004, Vaux 2011). The view of cancer as a reversible physiological state has significant medical implications because learning to modulate the impact of the cellular environment on neoplastic progression could impact prevention and detection strategies. Moreover, a mechanistic dissection of pathways by which the host reboots cancer cells may give rise to strategies that normalize cancer (Del Rio-Tsonis and Tsonis 1992, Ingber 2008, Kulesa *et al* 2006), in contrast to current approaches that seek to kill tumors and thus risk a compensatory proliferation response by any remaining cancer cells (Fan and Bergmann 2008).

The phenomenon of tumor reversion (e.g., observed when cancer cells are placed in normal embryonic or regenerative environments) contradicts irreversible, cell-autonomous genetically-deterministic models of the origin of cancer, and emphasizes the role of the tissue structure (Bissell and Radisky 2001, Bizzarri *et al* 2011, Bizzarri *et al* 2008, Ingber 2008, Weaver and Gilbert 2004). Biologists are beginning to explore (Dinicola *et al* 2011) the idea that cancer is a kind of attractor in a multi-dimensional transcriptional state space. 'The topology of the attractor is the "invisible hand" driving the system functions into coherent behavioral states: they are self-organizing structures and can capture the gene expression profiles associated with cell fates' (Huang *et al* 2009). However, such models are not only compatible with transcriptional states but also with state spaces in which the dimensions correspond to physical properties. If cancer is indeed best understood as part of the interplay between the host organism and individual cell regulation, then it thus becomes crucial to dissect the endogenous physiological signals used to coordinate cell growth with the large-scale patterning needs of the body.

1.2. Gradients of transmembrane potential mediate patterning cues

In addition to the biochemical gradients and gene-regulatory networks that underlie cell–cell communication, the complex field of patterning information that impinges upon all cells within a host organism (Levin 2011b) also contains an important biophysical component. 'Bioelectricity' refers to the slowly-changing gradients of transmembrane (resting) potential, ion fluxes and electric fields produced and sensed by non-excitable cells (Levin 2011a, McCaig *et al* 2005, McCaig *et al* 2009). While classical work has long suggested the importance of bioelectric gradients for regeneration, development and cancer (Borgens 1986, Burr 1940, Burr *et al* 1938, Jaffe 1979, Lund 1947), molecular-resolution tools have recently been developed for real-time detection and manipulation of bioelectrical properties *in vivo* (Adams and Levin 2012a, 2012b). This work has identified novel roles for bioelectricity in cellular regulation and dissected the pathways linking ion flows to transcriptional responses and changes in cell behavior (Levin 2009, Pu *et al* 2007, Pullar *et al* 2007, Zhao *et al* 2006). Indeed, transmembrane voltage gradients are now known to control cell proliferation, migration, differentiation and orientation (Blackiston *et al* 2009, Sundelacruz *et al* 2009). Moreover, the information stored in physiological networks (dynamic spatio-temporal patterns of ion flows through cell membranes and among connected cell groups) is instructive for anatomical identity of newly produced tissue, and mediates size control and positional information during organ formation, large-scale organ/appendage regeneration and axial patterning (Adams *et al* 2007, 2006, Beane *et al* 2011, Levin *et al* 2002, Pai *et al* 2012, Tseng *et al* 2010).

1.3. Bioelectricity as a non-genetic aspect of cancer microenvironment

The view that cancer is a developmental disorder predicts that molecular mechanisms known to be important mediators of the morphogenetic field would be involved in tumorigenesis. Indeed, there is mounting evidence that the bioelectric cues that establish normal pattern can go awry and result in cancerous growth. The unique bioelectrical properties of tumor tissue have been long-recognized (Aberg *et al* 2004, Burr 1941, Burr *et al* 1938, Cameron *et al* 1979a, 1979b, 1980, Cameron and Smith 1980, 1989, Gupta *et al* 2004, Jeter *et al* 1982, Koch and Leffert 1979, Leffert and Koch 1980, Rozengurt and Mendoza 1980, Smith *et al* 1978, Sparks *et al* 1983, Stojadinovic *et al* 2005); specifically, cancer cells are generally depolarized with respect to normal healthy tissue (Arcangeli *et al* 1995, Binggeli and Weinstein 1986, O'Grady and Lee 2005, Pardo 2004). Modern molecular data have confirmed the physiological observations, and several pathways of high relevance to cancer have now been shown to be under bioelectrical control, including apoptosis (Wang 2004), epigenetic chromatin modification (Carneiro *et al* 2011, Tseng and Levin 2012), stem cell regulation (Lange *et al* 2011, Sundelacruz *et al* 2008, Yasuda and Adams 2010) and the transfer of signals through gap junctions (Levin and Mercola 1998). A number of ion channel, pump and gap junction genes are now recognized as bona-fide oncogenes (table 1). Importantly, ion translocators are not only markers associated with neoplastic processes, but are also functional determinants of cancerous progression.

Table 1. Known ion translocators as oncogenes. Several ion transporters are now recognized as causal agents in carcinogenesis, consistent with the role of V_{mem} in regulating cell proliferation, migration, and differentiation. Future work remains to test the hypothesis that the patterning roles of voltage gradients are an important component of pattern dysregulation as a fundamental cause of neoplasia (Dinicola *et al* 2011, Potter, 2007).

Protein	Species	Reference
NaV1.5 sodium channel	Human	Onkal and Djamgoz, 2009
EAG-1 potassium channel	Human	Pardo *et al* 1999
KCNK9 potassium channel	Mouse	Pei *et al* 2003
Ductin (proton V-ATPase component)	Mouse	Saito *et al* 1998
SLC5A8 sodium/butyrate transporter	Human	Gupta *et al* 2006
KCNE2 potassium channel	Mouse	Roepke *et al* 2010
Connexin26 (gap junctions)	Mouse	Temme *et al* 1997

1.4. Recent molecular data implicates ion translocator proteins in cancer

The proliferation of some tumor cells is dependent on voltage-gated potassium channels (Conti 2004, Fraser *et al* 2000). hERG channels are particularly implicated (Arcangeli 2005, Bianchi *et al* 1998, Lastraioli *et al* 2004, Lin *et al* 2007, Wang *et al* 2002, Wang 2004), as are 2-pore channels such as KCNK9 (Kim *et al* 2004, Mu *et al* 2003). While roles other than ion transport have been proposed for some channels, in the case of KCNK9, it is known that its oncogenic potential depends on K^+ transport function, and not some other role of the protein (Pei *et al* 2003). A screen of several cervical cancers found the K^+ channel EAG expressed in 100% of the biopsies analyzed, and overexpression of EAG in human cells resulted in more quickly dividing progeny in culture; this result was replicated *in vivo* using mice implanted with human EAG-expressing CHO cells (Farias *et al* 2004, Pardo *et al* 1999). hEAG-1 is a true oncogene since its overexpression drives mammalian cells into uncontrolled proliferation and favors tumor progression in cells injected into immune-suppressed mice (Pardo *et al* 1999). Likewise, hERG is not normally present in most differentiated cells other than in the heart, but has been observed in a number of human cancers and during neoplastic transformation in prostate epithelium (Klezovitch *et al* 2008, Wang *et al* 2002). In these cells, hERG appears to recruit tumor necrosis factor receptor (TNFR) to the plasma membrane and cause a subsequent increase in NFκB, a known regulator of proliferation. In addition to modulations of single channels, some cancers are characterized by the activation of multiple potassium currents, such as human melanoma lines that express both hEAG1 and Ca^{2+}-activated K^+ channels (Meyer *et al* 1999). Indeed, complex interactions by multiple channels likely exist, and trans-membrane currents driven by diverse families of potassium channels (including calcium activated, inward rectifying K_{ir}, EAG and ERG) have all been correlated with cancerous tissue.

In addition to potassium, a number of other ions can play similar roles (Becchetti 2011, Prevarskaya *et al* 2010). Manipulation of membrane H^+ flux confers a neoplastic phenotype upon cells (Perona and Serrano 1988), while studies in glioma cell lines have revealed a role of chloride channels (Habela *et al* 2008, Volkov *et al* 2012). Inhibition of Clc-3 though hairpin RNA constructs resulted in the loss of premitotic condensation and arrest of the cell cycle in glioma cells (Habela *et al* 2008). In studies of human prostate cancer lines, these results support the role of chloride channels as key regulators of proliferation through cell size regulation (Jirsch *et al* 1993, Shuba *et al* 2000).

Sodium channels are a particularly important set of targets for cancer. The human knockout $APC^{min/+}$ cell line shares a mutation found in many human colorectal cancers, and when introduced into mice subsequently results in the development of multiple intestinal neoplasias (Ousingsawat *et al* 2008). *In vivo* transepithelial voltage recordings in this line revealed an increase in Na^+ levels compared to wild-type mice that was the result of an increase in the expression of the ENaC Na^+ channel. Metastatic potential correlates with voltage-gated inward sodium current, and it has been convincingly argued that some sodium channels may be oncofetal genes (Brackenbury and Djamgoz 2006, Diss *et al* 2005, Fraser *et al* 2005, Onganer and Djamgoz 2005, Onganer *et al* 2005). Indeed, a sodium-channel gene was identified as the top node of a genetic network that regulates colon cancer invasion (House *et al* 2010).

1.5. Investigating the biophysics of cancer in vivo: the frog model

Ion translocators are both predictive markers (Prevarskaya *et al* 2010), and an important set of targets for new cancer drugs (Arcangeli *et al* 2009, Kometiani *et al* 2005). Dissecting the molecular mechanisms by which biophysical properties regulate oncogenesis and metastatic processes during morphogenesis requires a model system that is tractable to both biophysical/physiological techniques and state-of-the-art molecular genetics. Recent work in the *Xenopus* tadpole has both confirmed the role of ion flow in oncogenesis *in vivo*, and identified bioelectricity as an important aspect of non-local signaling of the cellular microenvironment that can induce and suppress cancer-like cell behavior.

An expression analysis revealed a widely distributed, sparse population of cells expressing glycine-gated chloride channels (GlyCl). By exposing embryos to the specific GlyCl channel activator ivermectin, and controlling the extracellular levels of chloride, the membrane potential of these specific cells could be set to any desired level (and confirmed with voltage-reporting fluorescent dyes; strategy is shown in figure 1(*A*)). We took advantage of this finding to probe the consequences of bioelectrical dysregulation *in vivo* and to investigate the consequences of depolarizing select cell groups in the tadpole (Blackiston *et al* 2011).

When depolarized, these cells signal, at significant distance, to melanocytes—pigment cell derivatives of the neural crest (Morokuma *et al* 2008). The melanocytes then acquire three properties commonly associated with metastasis: they hyper-proliferate, change to a highly dendritic morphology and invade tissues throughout the animal (such as blood vessels, gut and neural tube) in a matrix metalloprotease-dependent manner. The ability of these cells to direct the

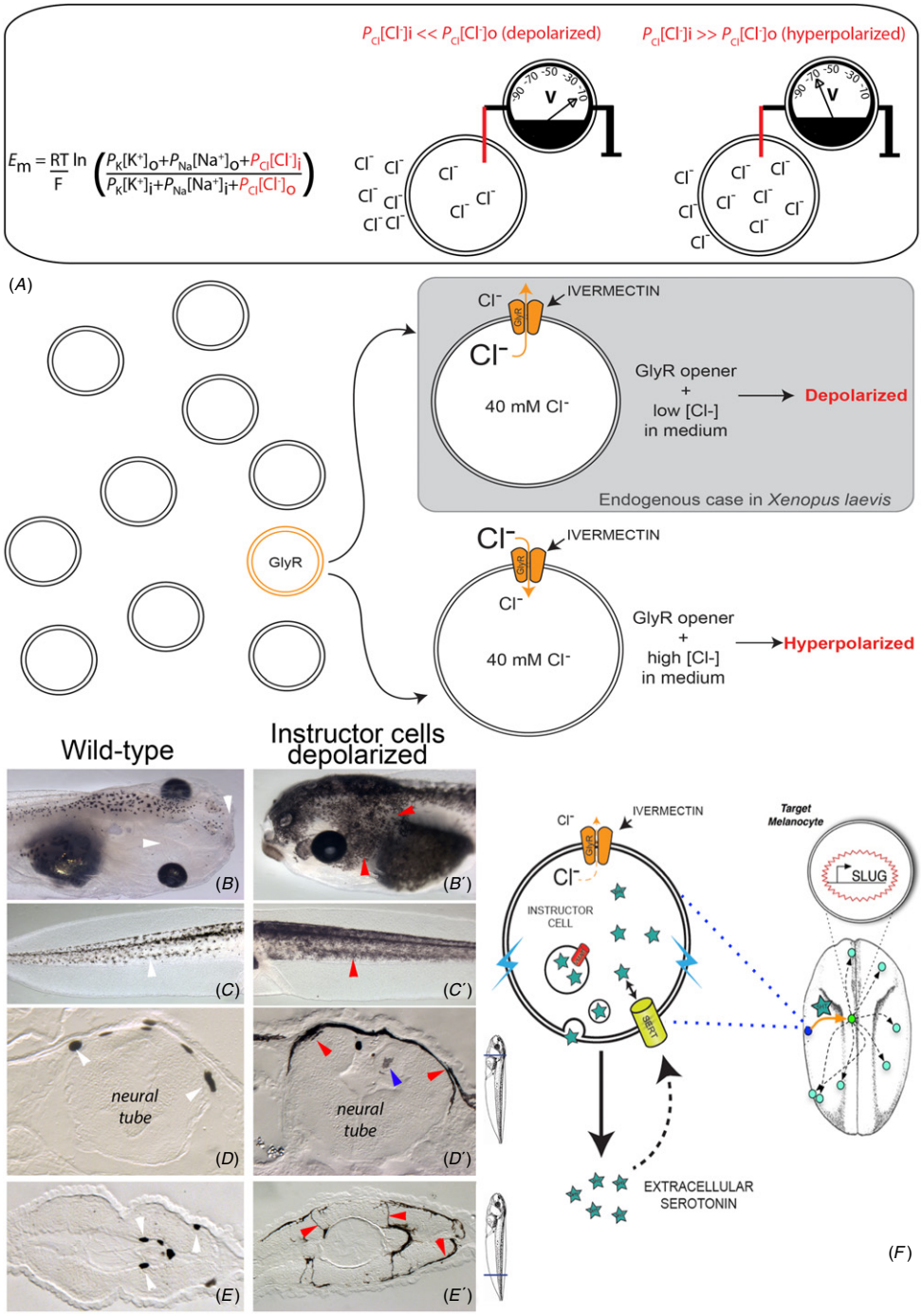

Figure 1. Depolarization of instructor cells induces metastatic phenotype in melanocytes. The Goldman equation reveals how resting potential V_{mem} depends on the internal and external K^+, Na^+ and Cl^- concentrations, ambient temperature (T), and permeability of each ion (P). (*A*) Manipulation of endogenous chloride channels as a means for selective probing of V_{mem}-based signaling. Ivermectin treatment targets GlyCl-expressing ('instructor') cells, opening these chloride channels. Then, by manipulating external chloride levels, chloride ions can be made to enter or exit the GlyCl-expressing cells, thus controlling their transmembrane potential. Normal tadpoles exhibit small numbers of black melanocytes over their neural tubes but not around their eyes or mouth ((*B*), white arrowheads). In contrast, tadpoles whose instructor cells were depolarized for as little as 12 h exhibit excess melanocytes, which possess a much more arborized appearance, and colonize areas normally devoid of melanocytes ((*B′*), red arrowheads). The same change in shape is seen in the tail (compare normal tail in panel C with the tail of a depolarized tadpole in (*C′*)); this change was quantified, and mitotic indices were calculated in (Blackiston *et al* 2011). Sectioning of these tadpoles reveals that in contrast to the small number of round melanocytes normally present in the neural tube ((*D*), white arrowheads), depolarization of instructor cells causes melanocytes to acquire a highly aberrant spread-out morphology ((*D*), red arrowheads) as well as to invade the nervous tissue (blue arrowhead). This is also seen in more posterior sections through the tail/trunk, where the normally round melanocytes ((*E*), white arrowheads) are far more numerous and extend long abnormal projections ((*E′*), red arrowheads). (F) Because the phenotype can be recapitulated by gain-of-function of serotonin signaling, and prevented by blockade of the serotonin transporter SERT, the data suggest a model of how instructor-cell signaling affects melanocyte morphology: depolarized instructor cells' SERT runs backwards, secreting serotonin into the extracellular space where it can diffuse and activate melanocytes to acquire three basic properties of metastasis: overgrowth, abnormal cell shape, and invasion of other tissues.

activity of an entirely different set of cells led us to call the depolarizing GlyCl-expressing population 'instructor cells'. Lineage and marker analysis was used to show that the hyperpigmentation phenotype did not result from other cells being abnormally shifted into becoming melanocytes, but rather that this change affected mature, existing and differentiated melanocytes (Blackiston *et al* 2011). Crucially, the phenotype could also be induced with the native ligand of GlyCl (glycine), ruling out off-target effects of ivermectin. Indeed, the effect could be induced or suppressed by modulating extracellular chloride levels, precisely as predicted by the Goldman equation (which links overall transmembrane potential to the concentrations and permeabilities of various ion species), ruling out ion-independent roles of GlyCl protein. Finally, it was found that the misexpression of mRNAs encoding depolarizing sodium, potassium or proton translocators could also phenocopy the highly specific change in melanocyte behavior induced by the efflux of chloride through GlyCl. Moreover, the effect of GlyCl opening in the presence of low extracellular chloride could be rescued by expressing a hyperpolarizing potassium channel in the exposed embryos. Together these results demonstrated that the melanocyte-transforming effect is truly initiated by a change in voltage and independent of any specific ion or channel protein. This finding is consistent with the published data in mammalian systems, which implicate many different ion channels in cancer (as all of them can contribute to the regulation and dysregulation of resting potential). One interesting aspect of the phenotype is its all-or-none character: in the induction and rescue/suppression experiments, some percentage of the treated population become hyperpigmented while the rest do not, but any one tadpole is either hyperpigmented or not—there appear to be no in-between (partial) states.

It was shown that depolarization induces the up-regulation of cancer-relevant genes such as Sox10 and SLUG (Morokuma *et al* 2008), but how do depolarized instructor cells signal to the distant melanocytes to induce the metastasis-like phenotype? A suppression screen tested known mechanisms by which transmembrane voltage changes were transduced into transcriptional responses, and implicated serotonergic signaling (a mechanism which also mediates long-range bioelectric signaling in left–right patterning (Adams *et al* 2006, Carneiro *et al* 2011, Fukumoto *et al* 2005)). Inhibition of the voltage-regulated serotonin transporter SERT abolished the hyperpigmenting defect of depolarization, and SERT was seen to co-localize with the GlyCl channel (be expressed in instructor cells). Moreover, direct application of serotonin recapitulated the metastatic phenotype, suggesting a model in which membrane voltage regulated the dynamics of serotonin secretion by instructor cells, allowing non-cell-autonomous regulation of the melanocyte function (figure 1(*F*)).

The above data illustrated the power of depolarized V_{mem} as an epigenetic initiator of widespread metastatic behavior in the absence of a centralized tumor. More recently (Chernet and Levin 2012a), we investigated the role of bioelectricity in tumor-like foci induced by canonical oncogenes. Tumors induced in *Xenopus* by oncogenes such as Gli1 and Rel3, or mutant tumor suppressors such as DNp53 and $KRAS^{G12D}$ (Dahmane *et al* 1997, Le *et al* 2007, Wallingford *et al* 1997, Yang *et al* 1998) exhibit the predicted depolarized potential, but additional metrics are needed to refine a more narrow physiological signature that can distinguish prospective tumor sites from normal depolarized cells (e.g., stem cells). Further, while depolarization has been associated with cancer in the literature, it is now necessary to explore the extent to which forced hyperpolarization could prevent or revert tumor development *in vivo*.

1.6. Open questions and new data

Thus, transmembrane potential can both induce a metastatic phenotype in widespread normal somatic cells, and participate in localized carcinogenesis induced by canonical pathways. Here, we investigated several key questions brought up by the remarkable effects of localized depolarization in *Xenopus*. What other cell types, besides the melanocytes, may be affected by depolarization of instructor cells? How many depolarized instructor cells are sufficient to induce hyperpigmentation (metastatic conversion of normal melanocytes) throughout the animal? What serotonin receptors mediate the non-cell-autonomous signaling between the instructor cells and melanocytes, and what model can explain the puzzling all-or-none character of melanoma-like transformation in depolarized tadpoles? What bioelectric changes are induced by carcinogens and oncogene expression? Are such changes localized to the tumor site, and how consistent are these changes among individual animals? Can such tumors be identified by a specific physiological signature, and could artificial control of the resting potential (either by transgenes or by pharmacological modulation of native ion channels) change the incidence of induced tumors? Here we present new data that fill in important details of this fascinating epigenetic pathway and highlight novel aspects of the bioelectric control of cancer.

2. Materials and methods

2.1. Animal husbandry

Xenopus embryos were maintained according to standard protocols (Sive *et al* 2000) in 0.1 × Modified Marc's Ringers (MMR), pH 7.8, plus 0.1% gentamycin. *Xenopus* embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber 1967).

2.2. Microinjection

Capped, synthetic mRNAs were dissolved in water and injected into embryos at cleavage stages in 3% Ficoll using standard methods (Sive *et al* 2000). mRNA injections were made into the locations indicated using borosilicate glass needles calibrated to bubble pressures of 50–70 kPa in water, delivering 70 ms pulses. After 30 min, embryos were washed in 0.75 × MMR for 30 min and cultured in 0.1 × MMR until desired stages. Constructs used included GlyCl-A288G-tom

(Lynagh and Lynch, 2010), Gli1 (Dahmane *et al* 1997), Xrel3 (Yang *et al* 1998), KRASG12D (Le *et al* 2007) and Kir4.1 (Fakler *et al* 1996).

2.3. Transgenics

PT2xflk-1:GFP transgenic embryos were prepared using the *Sleeping Beauty* transposon system (Doherty *et al* 2007). Transgenic embryos were treated with 1 μM ivermectin and imaged on an Olympus BX-61 using a FITC filter. Adobe Illustrator was used to define a 200 μm region on the tail tip region, and vascular cells in this region were counted.

2.4. Drug exposure

Stocks of ivermectin (Sigma) were stored at 10 mM concentration in dimethyl sulfoxide (DMSO). Embryos were exposed in 0.1 X MMR for the stages indicated to ivermectin, 0.05–1 μM; reserpine 100 μM; methiothepin 10 nM; cyanopridolol 50 μM, altanserin, 10 μM; tropisetron, 10 μM; GR113808, 1 μM; SB 699551, 2.5 μM; Ro 04–6790, 50 μM; SB 258719, 50 μM. All compounds were obtained from Tocris unless otherwise noted. A stock of 4-nitroquinolin-1-oxide (4NQO) (Sigma) was stored at 175 mM concentration in acetone. Embryos were exposed to 42 μM 4NQO in 0.1 $\times$ MMR for the stages indicated.

2.5. Voltage and sodium dye imaging

Transmembrane potential (figure 5(*E*)) was imaged as previously described (Adams and Levin 2012b, 2012c, Blackiston *et al* 2011). For sodium imaging, a stock of CoroNa green (Invitrogen) was stored at 5 mM concentration in DMSO. Embryos were incubated for 1 h in 100 μM of CoroNa Green in 0.1 $\times$ MMR. Embryos were washed with fresh 0.1 $\times$ MMR and directly visualized using an FITC filter set of a fluorescent microscope.

2.6. Predictive screening

Gli1 injected *Xenopus* embryos were collected at the neurula stage (st. 15), before ITLSs were morphologically and histologically apparent. Collected embryos were imaged using CoroNa green (as described above), and divided into two categories based on the presence of group of cells with high Na^+ content. The effectiveness of unique Na+ content in predicting ITLS formation was quantified by calculating false positives (how many of the embryos exhibited increased Na^+ foci but never developed ITLSs), false negatives (how many did our technique miss), sensitivity (how many of the ITLS were preceded by unique Na^+ content) and specificity values (how many of the non-ITLS forming cells were not preceded by unique Na^+ content).

2.7. Electroporation

The method used is described in detail in Chernet and Levin (2012b). Here, poring and driving pulse parameter values of volt (V) = 35/7; length (ms) = 50/50; interval (ms) = 50/50 and repeat count = 3/10 were used.

3. Results

3.1. Depolarization-induced metastasis-like conversion of melanocytes

In order to better understand the neoplastic-like phenotype induced by changes in the transmembrane potential (V_{mem}) *in vivo*, we took advantage of the glycine gated chloride (GlyCl) channel, specifically expressed in a sparse yet widely distributed cell population, whose V_{mem} can be specifically modulated by pharmacologically opening the channel and then altering the extracellular chloride levels (figure 1(*A*)). To open these GlyCl channels, we utilized ivermectin, a well-characterized antiparisitic that is known to specifically affect GlyCl channels (Blackiston *et al* 2011, Ottesen and Campbell 1994). In unperturbed *Xenopus laevis* embryos, the extracellular medium contains a lower chloride concentration than the cytoplasm inside the cell. Thus, treatment of *Xenopus* embryos with 1 μM ivermectin depolarizes cells expressing the GlyCl channel (because negative chloride ions leave those cells down their concentration gradient). We first confirmed the remarkable hyperpigmentation phenotype in almost all treated individuals, resulting from a change of normal melanocyte behavior in three ways characteristic of cancerous transformation: an increase in proliferation, a change in their morphology (extensive arborization) and an extensive colonization of ectopic regions including blood vessels and neural tissues (figures 1(*B*)–(*E*)). This effect is quite specific and the embryos appear to have no abnormalities of morphogenesis in any of the major organ systems or overall axial development. Furthermore, this effect is non-cell autonomous, the GlyCl-expressing instructor cells are being depolarized, whereas melanocyte behavior is being affected. However, the strong melanocyte phenotype suggests the possibility that other cell types are affected but not easily detected if they lack pigment. We specifically asked whether another important aspect of cancer—vascularization—might also be affected.

3.2. Depolarization of instructor cells causes dysregulation of vascular patterning

Transgenic frog embryos (figures 2(*A*) and (*B*)) whose endothelial cells are labeled with fluorescent protein using the flk-1 promoter (Doherty *et al* 2007) were exposed to 1 μM ivermectin from gastrulation to organogenesis (stages 10–46), and the blood vessel-specific fluorescence was imaged and compared to control embryos of the same stage. In untreated embryos, the intersomitic vessels extending between the major tail veins resemble evenly spaced rungs on a ladder (figures 2(*C*) and (*C*′)). However, embryos whose GlyCl-expressing cells had been depolarized by ivermectin treatment (electrophysiology shown in (Blackiston *et al* 2011)) displayed disorganized intersomitic vessels (figure 2(*D*)). This phenotype was marked by a decrease in the overall number of vessels (figure 2(*G*)) as well as an increase in vessels that displayed aberrant morphologies (branching, tilting and truncations; figure 2(D)). An increase in amount of small, blind-end vessels extending from the posterior cardinal vein

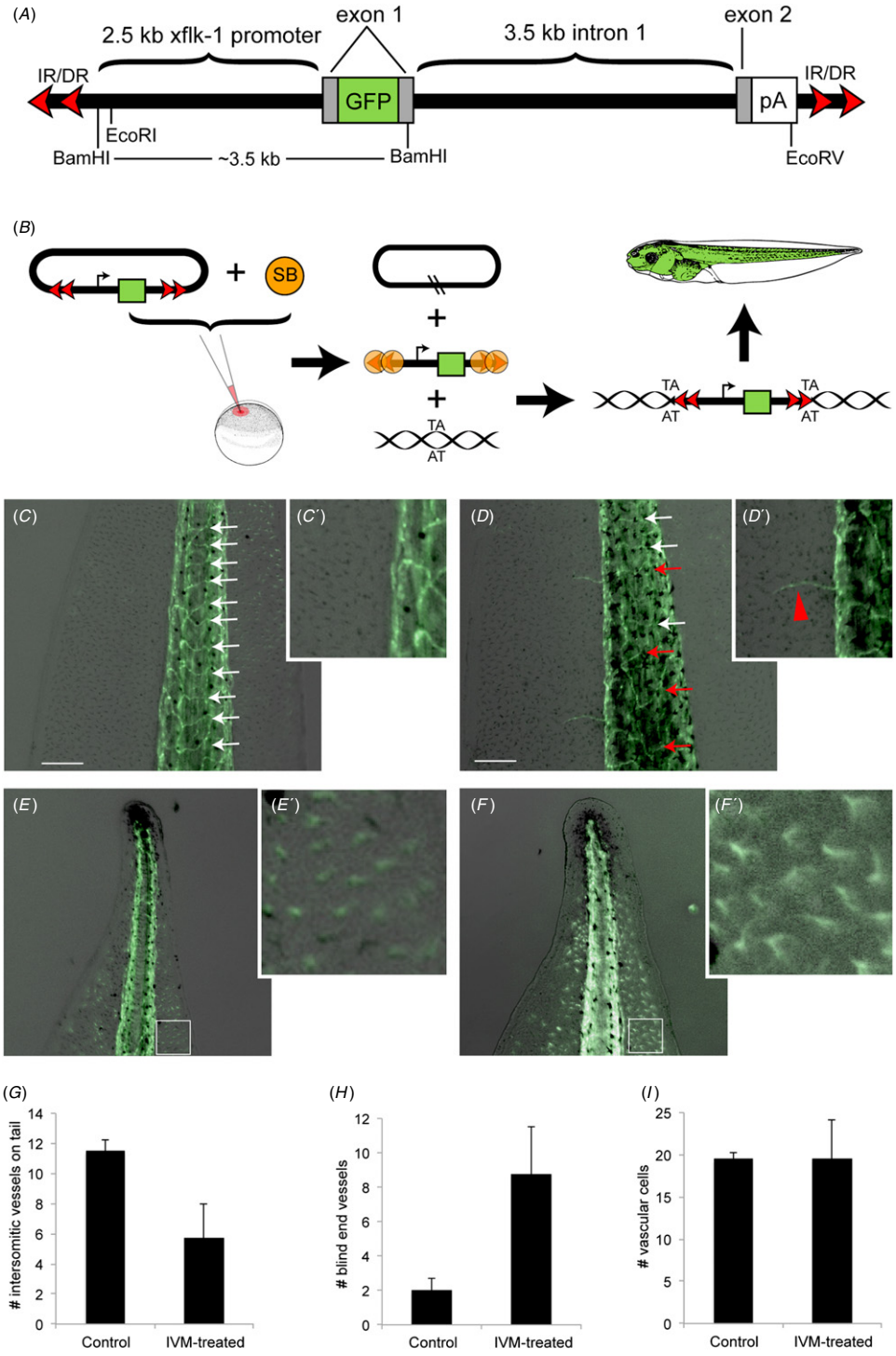

Figure 2. GlyCl-mediated depolarization induces abnormal vascular structure *in vivo*. (*A*) Schematic of the pT2xflk-1:GFP transposon gene, adapted from (Doherty *et al* 2007) and not drawn to scale. The green fluorescent protein (GFP) on exon 1 is driven by an flk-1 promoter 2.5 kb upstream. A polyadenylation signal (pA) was cloned to maintain the integrity of the splice acceptor. The xflk-1:GFP construct was cloned into the pT2 non-autonomous transposon between the invert-direct repeats (IR/DR). (*B*) Mechanism of SB-mediated transposition. The pT2flk-1:GFP was injected together with *Sleeping Beauty* (SB) transposase mRNA into fertilized eggs at the one-cell stage. SB transposase binds to the IR/DRs as shown and cuts the transposon (containing the flk-1 promoter driving GFP expression) out of the plasmid (the cut sites are indicated by the two black slashed lines in the remaining plasmid). A DNA molecule with a 'TA' sequence becomes the recipient of a transposed transposon; transgenic animals bearing this fluorescent marker of vasculature were used in experiments where instructor cells were depolarized with ivermectin. ((*C*), (*C*′)) In untreated pT2flk-1:GFP *Xenopus laevis* transgenic tadpoles, the intersomitic vessels on the tail are evenly spaced (white arrows) and tail regions largely lack blind end vessels. ((*D*), (*D*′)) Ivermectin-treated embryos display an overall decrease in the number of intersomitic vessels (*G*), as well as an increase in disrupted intersomitic vessels (red arrows), and an increase in the amount of small blind-end vessels extending into the tail fin region (*H*). To determine whether there was an increase in vascular cells in the tail tip between control and ivermectin-treated embryos ((*E*), (*F*)), the number of GFP-positive cells in a 200 μm square region (white boxes) was counted. While there is no difference in the number of vascular cells in the tail tip between treatments (I), the shape of the vascular cells in ivermectin-treated embryos (*F*′) compared to controls (*E*′) reveals an increase in arborization. Scale bars, 250 μm.

on the tail was also observed in embryos whose instructor cells had been depolarized (figures 2(*D*′) and (*H*)). To determine whether there was an overall increase in endothelial precursors of blood vessels in the tail tip of the tadpoles, the numbers of GFP-positive cells in 200 μm square regions were counted. While ivermectin treatment did not affect the number of vascular cells present (figure 2(*I*)), the vascular cells did appear more arborized and larger following depolarization (compare figures 2(*E*) and (*F*)). We conclude that the instructor cells regulate not only the behavior of melanocytes, but also the morphogenesis of the vasculature.

3.3. Depolarization of very few instructor cells is sufficient to transform melanocytes

Hyperpigmentation is an all-or-none effect: if affected, the whole tadpole becomes colonized and all of the melanocytes abandon their normal round appearance in favor of a highly arborized morphology. Interestingly, prior work showed that it is not necessary to depolarize all of the instructor cells to induce the hyperpigmentation phenotype: it is sufficient to misexpress depolarizing channels in parts of the embryo (Morokuma *et al* 2008). Hyperpigmentation is initiated by global depolarization of a very sparse but widespread cell population, but what is not known is the size of the minimal signaling unit: How small an area can be depolarized, and how far from the dorsal neural tube (the site of the melanocyte precursors) can it be, in order to activate the transformation of all (or most) of the melanocytes in the embryo?

We selectively depolarized cell groups of differing size and location to determine which can produce the hyperpigmented phenotype. In order to do so, we took advantage of a mutant GlyCl-channel (GlyCl-A288G) that has increased sensitivity to ivermectin. GlyCl channels normally have a threshold of activation of roughly 100–300 nM for ivermectin (Shan *et al* 2001). However, the introduction of the A288G mutation increases ivermectin sensitivity almost 100-fold (Lynagh and Lynch 2010). Thus, through microinjection of the GlyCl-A288G mutant mRNA into *Xenopus* embryos followed by exposure to doses of ivermectin too low to activate native GlyCl receptors, we were able to systematically depolarize only those cells expressing the injected super-sensitive mutant, and not endogenous GlyCl-expressing cells (figure 3(*A*)).

Synthetic mRNA encoding the GlyCl-A288G mutant fused to a tdTomato fluorescent tag (tom) was injected into *Xenopus* embryo blastomeres at the 32-cell stage (allowing for the targeting of progressively smaller regions of the developing embryo) as schematized in figure 3(*B*). These embryos were then treated 0.05 μM ivermectin. At this concentration, ivermectin does not affect endogenous GlyCl channels (confirmed by lack of hyperpigmented effect in uninjected tadpoles treated with this low ivermectin dose); however, we observed hyperpigmentation when embryos were pre-injected with the GlyCl-A288G-tom mutant and then exposed to the lower ivermectin level. Embryos were reared to stage 22, at which point they were sorted for tdTomato expression (figure 3(*C*)) to determine which regions had been affected by the depolarizing treatment, and allowed to develop to tadpole stage (stage 45), at which point they were scored for hyperpigmentation.

By correlating the degree of hyperpigmentation with the location and size of the region rendered sensitive to low ivermectin-induced depolarization, we performed a survey of the spatial limits of the depolarization → hyperpigmentation effect. tdTomato expression in hyperpigmented tadpoles was highly variable. However, even very small depolarized regions at the very posterior of the tadpole were able to produce embryo-wide hyperpigmentation (figures 3(*D*)–(*F*)). Depolarization of small regions of GlyR-A288G-tom injected cells produced hyperpigmentation in 48.3% (N = 267) of embryos treated with low dose of ivermectin. Thus, depolarization-induced hyperpigmentation of large and distant body regions only requires a small number of instructor cells to be depolarized.

3.4. Monoamine vesicle transporter regulates transformation-relevant serotonin levels

Depolarization functions non-cell-autonomously to affect melanocyte behavior. The long-range signal is mediated by serotonin (Blackiston *et al* 2011). Serotonin is an important signaling molecule that participates in a wide range of physiological systems. Increasing evidence also indicates that serotonin plays a developmental role before it becomes a neurotransmitter, affecting craniofacial, gastrointestinal and cardiovascular morphogenesis (Buznikov *et al* 2001, Levin *et al* 2006, Moiseiwitsch, 2000, Nguyen *et al* 2001). Our prior work showed that the regulation of serotonin levels by the voltage-regulated serotonin transporter SERT is essential for the transduction of V_{mem} changes into changes in cell behavior observed during hyperpigmentation (Blackiston *et al* 2011a). One model (figure 1(*F*)) is that when the membrane of instructor cells is depolarized, SERT not only fails to clear the extracellular space of excess serotonin, but exports additional serotonin (Adams and DeFelice, 2002, Hilber *et al* 2005). The excess serotonin level surrounding the developing melanocytes induces their neoplastic-like behavior (Blackiston *et al* 2011).

We next sought to determine whether intracellular stores of serotonin were also involved in the depolarization-induced hyperpigmentation. The vesicular monoamine transporter (VMAT) is responsible for the translocation of monoamines (including serotonin) from the cytoplasm into storage vesicles (Wimalasena 2011). As part of a suppression screen (Adams and Levin 2006, 2012a), we applied a VMAT antagonist together with ivermectin treatment to determine whether the VMAT function was required for depolarization to be effectively transduced into changes in melanocyte behavior. Treatment with the VMAT blocker, reserpine, reduced ivermectin-induced hyperpigmentation significantly (table 2), without other apparent effects on overall patterning or embryo health. It is likely that the intracellular packaging of serotonin in GlyCl-expressing cells is important, as it is in synapses, for cooperating with SERT to regulate tissue serotonin levels needed to activate melanocytes.

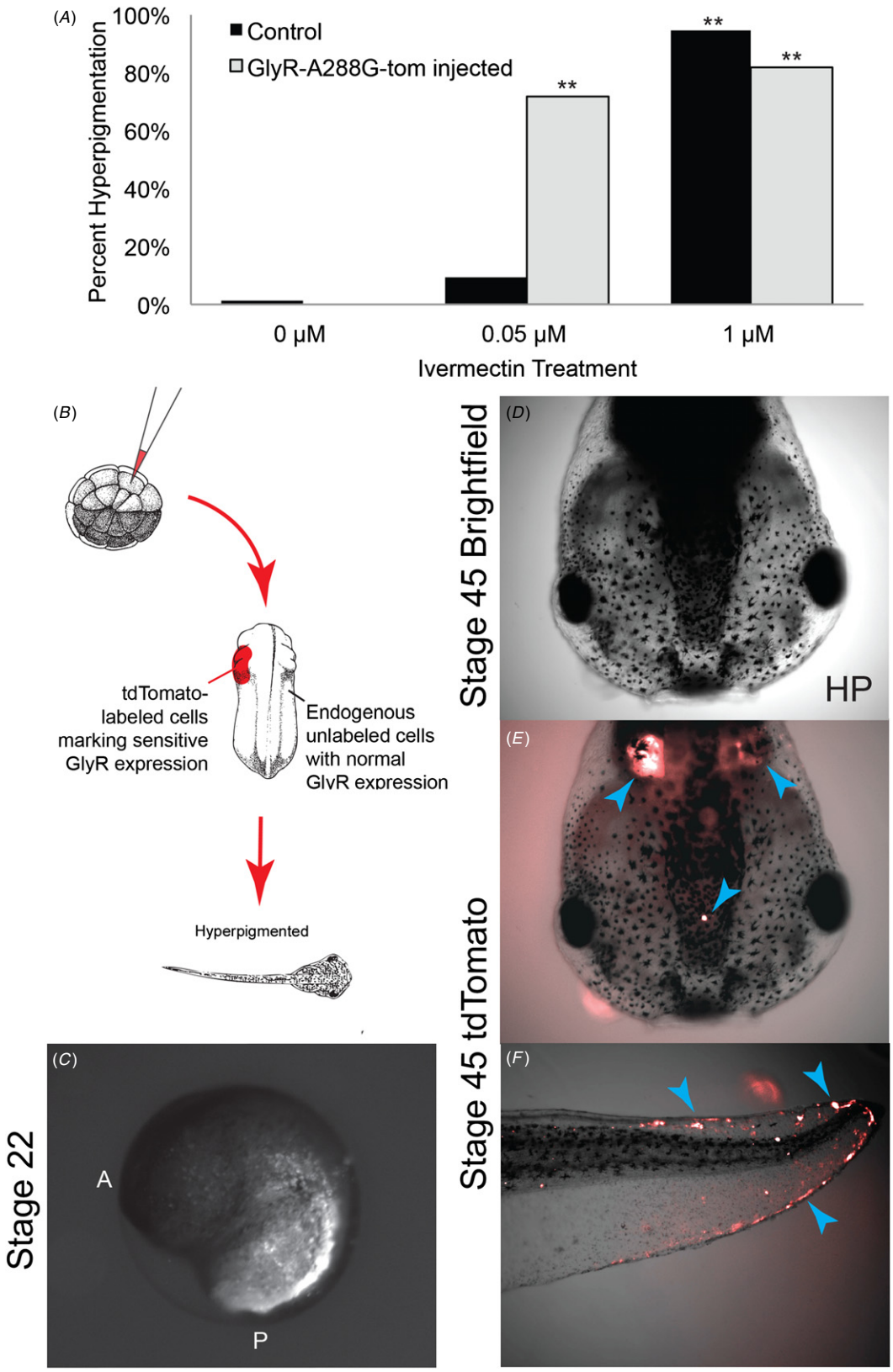

Figure 3. Hyperpigmentation requires the depolarization of only a small number of instructor cells. (*A*) Embryos that had been injected at the 1-cell stage with GlyCl-A288G-tom, a GlyCl channel with increased sensitivity to ivermectin fused to a tdTomato fluorescent label, were treated with 0.05 μM ivermectin. This resulted in significant rates of hyperpigmentation, while uninjected control embryos had no phenotype, demonstrating that this level of ivermectin does not trigger native GlyCl in instructor cells. (*B*) Injections of GlyCl-A288G-tom were done into a randomly chosen 1 cell of stage 6 *Xenopus* embryos. Embryos were then treated with 0.05 μM ivermectin, sorted at stage 22 for discrete tdTomato signal localization (*C*), then reared to stage 45 and scored for hyperpigmentation. (*D–F*) Analysis of stage 45 hyperpigmented tadpoles reveals that only a small number of GlyCl-A288G-tom-expressing cells (blue arrows) are necessary to induce hyperpigmentation.

Table 2. Hyperpigmentation involves VMAT function. Embryos were exposed to either 1 μM ivermectin, 100 μM reserpine (a VMAT antagonist), or a combination of both agents from stage 10 to 46 (from gastrulation to organogenesis). Ivermectin exposure alone resulted in a high incidence of hyperpigmentation, not seen in control embryos. Concurrent exposure to ivermectin and reserpine significantly reduced the rate of ivermectin-induced hyperpigmentation.

	Control	Ivermectin	Reserpine	Ivermectin + Reserpine
Normally-pigmented tadpoles	124	5	46	16
Hyperpigmented tadpoles	1	97	0	54
% hyperpigmented	0.8%	95.1%	0%	77.1%*
n	125	102	46	70

*$p < 0.01$ compared to ivermectin treatment alone, students *t*-test.

Table 3. Blocking multiple 5-HT receptors induces hyperpigmentation, but inhibits depolarization-induced hyperpigmentation. Embryos were exposed to either 1 μM ivermectin, 10 nM methiothepin (antagonist to serotonin receptors 1, 2, 5, 6 and 7), or a combination of both agents from stage 10 to 46 (from gastrulation to organogenesis). Ivermectin exposure alone resulted in a high incidence of hyperpigmentation, not seen in control embryos. Exposure to methiothepin significantly reduced ivermectin-induced hyperpigmentation, but was also able to induce hyperpigmentation on its own.

	Control	Ivermectin	Methiothepin	Ivermectin + Methiothepin
Normally pigmented tadpoles	331	7	67	27
Hyperpigmented tadpoles	8	151	129	78
% hyperpigmented	2.4%	95.6%	65.8%#	74.3%*
N	339	158	196	105

* $p < 0.01$ compared to ivermectin treatment alone, # $p < 0.01$ compared to control, students *t*-test.

3.5. A model of serotonin receptors for signal transduction during hyperpigmentation

The levels of serotonin are regulated by SERT and VMAT, but the activation of melanocytes must be transduced by a receptor. Thus, we next sought to deduce which serotonin receptor is required for the depolarization of instructor cells to be effectively transduced into changes in melanocyte behavior. Extracellular serotonin receptors can be divided into seven classes based on their pharmacological profiles, primary sequences and signal transduction mechanisms; with the exception of 5-HT_3, a ligand-gated ion channel, all other 5HT receptors are G-coupled protein receptors that activate intracellular second messenger cascades (Barnes and Sharp 1999). Using the same suppression screen strategy that implicated both SERT and VMAT, individual inhibitors of serotonin receptors were used together with ivermectin treatment to investigate whether receptor inhibition could suppress the depolarization-induced hyperpigmentation.

We began the screen with the broad-spectrum receptor antagonist, methiothepin, which blocks 5-HT receptor types 1, 2, 5, 6 and 7 (Monachon *et al* 1972). Treatment with methiothepin was successful in significantly reducing hyperpigmentation rates when embryos were exposed to both this serotonin antagonist and depolarizing ivermectin (table 3). However, surprisingly, treating embryos with this receptor antagonist alone (i.e. without ivermectin) resulted in hyperpigmentation in 65.8% of embryos! The ability of a receptor blocker to suppress the transformation phenotype when it is being induced by depolarization, but also to induce it on a wild-type background animal suggested the involvement of multiple serotonin receptors that antagonized each other's activity. We thus went on to test the involvement of each serotonin receptor subtype by targeting them individually using receptor specific antagonists. Treatment with blockers of receptors 3, 4, 5, 6 or 7 (10 μM tropisetron, 1 μM GR 113808, 2.5 μM SB 699551, 50 μM Ro 04–6790 and 50 μM SB 258719, respectively) did not result in any reduction in ivermectin-induced hyperpigmentation (table 4). However, exposure to both 5-HT receptor 1 and receptor 2 antagonists (50 μM Cyanopridolol and 10 μM Altanserin, respectively), significantly reduced hyperpigmentation in embryos also treated with ivermectin. Treatments of receptor antagonists without ivermectin revealed that blocking 5-HTR-5 and blocking 5-HTR-1 led to significant levels of hyperpigmentation (30.4% with 2.5 μM SB 699551, and 22% with 10 μM cyanopridolol). Taken together, these data presented a complex serotonergic signal transduction network operating between changes in V_{mem} and hyperpigmentation.

The results of the suppression screen revealed that blocking serotonin receptors could both suppress depolarization-induced hyperpigmentation, and hyperpigment on its own. Even more puzzling was the stochastic nature of the all-or-none phenotype; some individuals in the treated population would become hyperpigmented, and some would not, but none exhibited a partial phenotype: the whole body behaves as a 'unit' with respect to melanocyte behavior, but the decision whether to express the transformed melanocyte phenotype is variable among the identically treated population. In order to formulate a model that quantitatively predicts and explains this complex counter-intuitive dataset, we undertook a computational approach. The specific details of the method will be published in a subsequent manuscript highlighting the application of network searches to difficult problems in developmental signaling. We began with a network of possible functional relationships between serotonin and its receptors (see supplementary data—supplement 1 (available from stacks.iop.org/PhysBio/9/065002/mmedia)), and constrained it using known data on the interplay between antagonistic serotonin receptors in mammary cell development (Pai and Horseman, 2008, Stull *et al* 2007). Using simulated annealing to find connectivity values that correctly predict the results of all of our experiments, we arrived at a model consistent with our data (figure 4). It explains the observation that using an antagonist to multiple serotonin receptors can suppress hyperpigmentation when used with ivermectin treatment, yet, induce hyperpigmentation even when instructor cells are not depolarized. In the endogenous case, when instructor cells

Table 4. Rescue of hyperpigmentation reveals involvement of 5-HT receptors 1, 2 and 5. Embryos were exposed to either 1 μM ivermectin concurrent with a selective serotonin (5-HT) receptor antagonist, or a serotonin receptor antagonist alone from stage 10 to 46 (from gastrulation to organogenesis). Ivermectin exposure alone resulted in a high incidence of hyperpigmentation (HP), not seen in control embryos. Exposure to tropisetron, GR113808, Ro 04–6790, or SB 258719 did not inhibit hyperpigmentation or induce hyperpigmentation. However, exposure to either cyanopridolol or altanserin effectively reduced ivermectin-induced hyperpigmentation, while treatment of SB699551 was able to hyperpigment on its own.

5-HT Receptor Blocked	Control	R1	R2	R3	R4	R5	R6	R7
Antagonist	None	Cyano-pridolol	Altan-serin	Trop-isetron	GR 113808	SB 699551	Ro 04–6790	SB 258719
% HP w/ivermectin	95.6%	83.6%*	70.9%*	96.2%	96.6%	92.0%	92.6%	93.8%
% HP w/o ivermectin treatment	2.4%	5.9%	3.5%	1.4%	0%	30.4%#	0%	0%

* $p < 0.01$ compared to ivermectin treatment alone, # $p < 0.01$ compared to control, students *t*-test.

are not depolarized, extracellular serotonin levels remain low. Under these conditions, blocking multiple receptors also blocks the serotonin receptor that normally maintains correct melanocyte morphology (receptor 5—a suppressor of transformation), inducing hyperpigmentation in a significant proportion of treated embryos. Following instructor cell depolarization, excess serotonin is expelled into the milieu of the developing melanocytes, resulting in binding to receptors that are not normally activated (receptors 1 and 2). Binding to receptors 1 and 2 overcomes baseline inhibitory activity of receptor 5. Thus, blocking receptors 1 and 2 with a broad serotonin receptor antagonist can decrease hyperpigmentation rates, but not completely, since it is also blocking the serotonin receptor involved in maintaining melanocyte behavior. The multiple competing levels of inhibition and activation among elements in this model and the sigmoidal response curve of the transforming transcription factors Slug and Sox10 (Morokuma *et al* 2008) to the cAMP output of the serotonin receptor account for the pharmacology data and the bi-modal behavior of the output (all-or-none character of the hyperpigmentation phenotype); this scheme makes a number of specific, quantitative predictions that can be tested to refine the model in subsequent investigations.

3.6. Global voltage change during chemically-induced tumorigenesis

Our previous data focused on metastatic-like phenotypes induced by voltage change in the absence of chemical or genetic insult. We next sought to characterize the voltage changes that may function during the processes initiated by traditional carcinogens. Exposure to 42 μM 4NQO, a known carcinogen (Boroughs *et al* 2011, Hirose *et al* 1976, Li *et al* 2011, Pai *et al* 2009), induced global hyperpigmentation (figures 5(*A*) and (*B*)), consistent with the above-described effects of depolarization (figure 1(B′)). Unlike the instructor-cell depolarization, this also caused discrete tumor-like structures (ITLSs) to be formed (figure 5(*C*), yellow arrowhead), which also attracted vasculature (figure 5(*D*), red arrowheads, supplementary movie 1 (available from stacks.iop.org/PhysBio/9/065002/mmedia)). We specifically examined transmembrane potential in the exposed tadpoles using voltage-sensitive fluorescent reporter dye, $DiBAC_4$ (3) (Adams and Levin 2012c, Pai *et al* 2012). We observed an embryo-wide depolarization, but only in approximately half of the treated tadpoles (figures 5(*E*) and (*E*′)). We conclude that exposure to carcinogens affects the bioelectrical state of many more cells than those in the resulting tumor; however, this effect is highly variable among a population of individuals.

3.7. Sodium content reveals tumors

It has long been noted that tumor cells can be distinguished from normal cells by their aberrant transmembrane potential (Binggeli and Weinstein 1986, Burr *et al* 1938). However, a number of normal patterning events are also driven by localized depolarizations (Lange *et al* 2011, Pai *et al* 2012, Sundelacruz *et al* 2008); thus, non-invasive detection of incipient tumors will depend on the discovery of additional biophysical criteria that would allow the defining of a narrower physiological signature by which to distinguish prospective cancer sites from normal.

Gli1 mRNA, encoding a known human oncogene (Dahmane *et al* 1997, Kasper *et al* 2006), was injected into one blastomere of *Xenopus* embryos at the 2-cell stage. Embryos were then raised to early tadpole stages (stage 35), at which point ITLSs were clearly visible (figure 6(*A*)). We then imaged these *Gli1*-induced tumors with the sodium reporter dye CoroNa Green, which revealed that such tumors are indeed enriched for sodium (figures 6(*A*)′ and (*A*″)). To determine whether elevated sodium levels could be a predictor of future tumor development, we imaged neurula-stage (stage 15) embryos with CoroNa Green. At this stage, there were no histological or morphological signs of incipient tumors; however, *Gli1*-injected embryos exhibited foci with unique sodium content (compare figures 6(*B*) and (*C*)). Out of the ∼30% of *Gli1*-injected embryos displaying CoroNa foci ($N = 81$), 46% (sensitivity value) formed morphologically apparent ITLSs by stage 35 (figure 6(*D*)). Other predictive parameters tested include specificity (82%), false positive (44%) and false negative (18%). Together, data collected suggest that unique bioelectric sodium signal is indicative of the prospective tumor formation.

3.8. Forced hyperpolarization reduces tumor incidence

If transmembrane potential is a causal factor in tumorigenesis, as it is in metastatic transformation of melanocytes, then

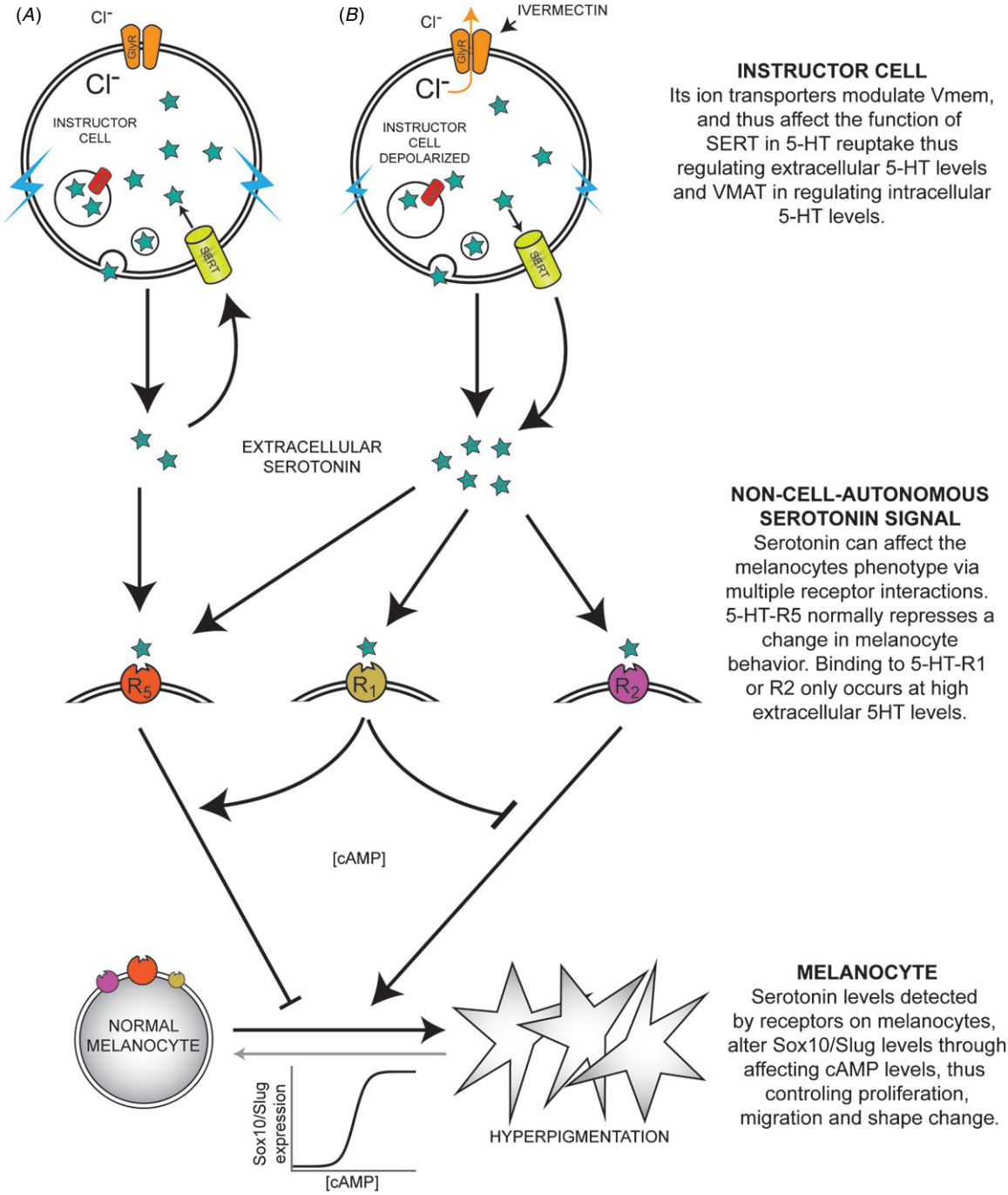

Figure 4. A model of melanocyte control by serotonergic signaling downstream of voltage change. (*A*) In unperturbed embryos, ion transporters keep the plasma membrane in a state where it is able to power the reuptake of extracellular 5-HT through its transporter, SERT, and regulate intracellular 5-HT levels with VMAT. With normal extracellular 5-HT levels, binding to 5-HT-R5 maintains normal melanocyte behavior by regulating cAMP levels, which in turn maintain low Sox10 and Slug levels and suppress melanocyte transformation. (*B*) When the instructor cell population is depolarized, VMAT no longer functions properly to sequester serotonin inside vesicles, and SERT runs backwards, exporting additional 5-HT into the microenvironment of the melanocytes. At high extracellular 5-HT levels, 5-HT activates 5HT-R1 and 5HT-R2. Binding to 5-HT-R2 induces the transformation of melanocytes. R1-binding (which induces hyperpigmentation) functions to partially suppress the effects of R2-binding, and increase the effects of R5-binding through the regulation of intracellular cAMP levels.

artificial hyperpolarization of cells ought to reduce the incidence of tumorigenesis. We used two canonical oncogenes that induce tumors (rhabdomyosarcoma, lymphoma and various solid cancers) in *Xenopus*, Xrel3 (Yang *et al* 1998) and KRASG12D (Le *et al* 2007). Electroporation of either mRNA consistently ITLSs in ∼45% of electroporated embryos (figures 7(*A*)–(*C*)). Remarkably, pre-injection of the embryos with mRNA encoding a hyperpolarizing Kir4.1 channel (previously characterized and shown to persist in the animal for days and hyperpolarize cells (Aw *et al* 2008, Pai *et al* 2012)) significantly reduced the percentage of embryos that develop tumors (by roughly 25% and 75% in Xrel3 and

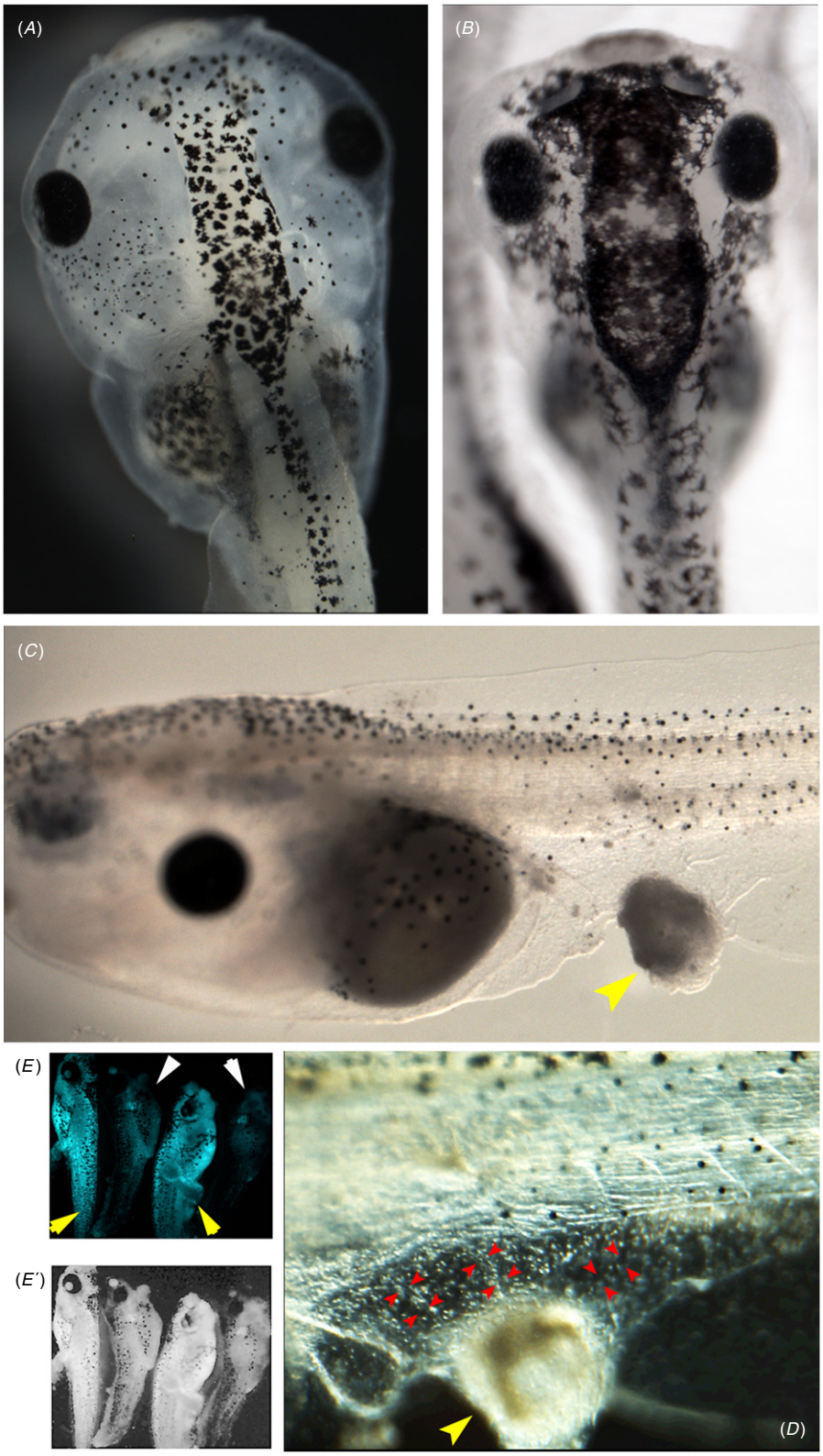

Figure 5. Exposure to carcinogen 4NQO induces hyperpigmentation, embryo-wide depolarization, and tumor-like structures. (*A*) Unperturbed stage 45 embryo showing normal pigmentation in the dorsoanterior region. (*B*) Stage 45 embryos treated with 42 μM 4NQO for 24 h exhibited the hyperpigmentation. Following exposure, hyperproliferative melanocytes migrated and colonized regions that are normally devoid of melanocytes. (*C*) A stage 47 embryo with a typical induced tumor-like structure (ITLS, yellow arrowhead). To generate this phenotype, embryos were treated with 42 μM 4NQO for 24 h; ITLSs were observed 48 h post termination of 4NQO treatment. (*D*) A stage 48 embryo showing vascularization (red arrowheads) of an ITLS (yellow arrowhead). See supplementary movie 1 (available from stacks.iop.org/PhysBio/9/065002/mmedia) for a movie of the blood flow that more clearly reveals the attraction of vasculature by this tumor-like structure. (*E*) Imaging with $DiBAC_4(3)$ (a fluorescent V_{mem} reporter) using fluorescent confocal microscopy as previously described (Adams and Levin 2012c) reveals that 53.3% of hyperpigmented embryos ($n = 21$) are relatively depolarized (the panel shows four embryos, side by side, as shown by white ring-light view of the same 4 embryos in panel *E*′).

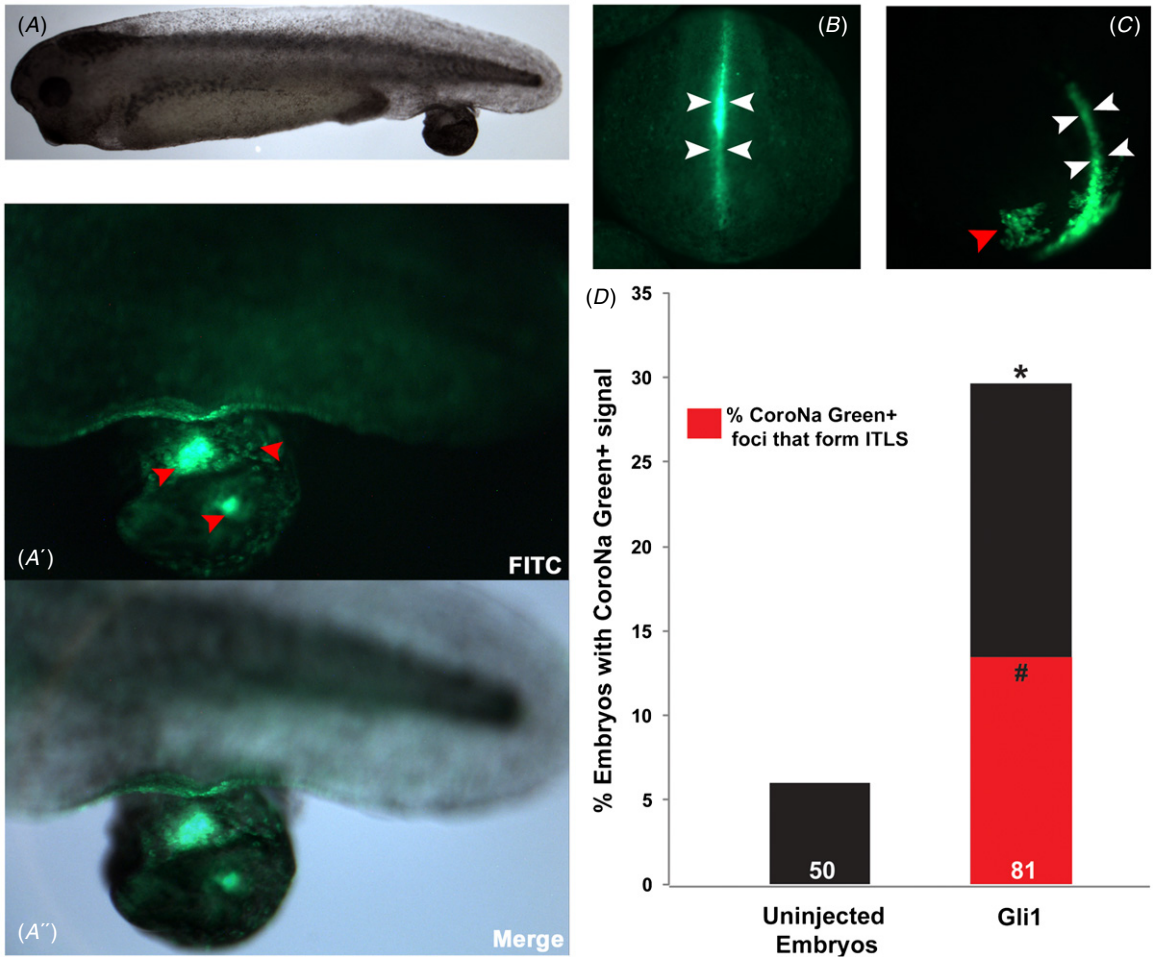

Figure 6. Overexpression of *Gli1* results in ITLSs with unique Na^+ signature. (*A*) A stage 35 embryo showing *Gli1* ITLS on its tail. To generate this phenotype, embryos were injected with 2ng of *Gli1* mRNA into 1 of 2 cell stage embryos. Injected embryos were raised to stage 35 and were imaged using bright-field microscopy. (*A′*) Imaging using CoroNa green (fluorescent Na+ content reporter) reveals that tumor cells have relatively higher (but heterogeneous) Na^+ content as compared with healthy surrounding tissue. (*A″*) Overlay of bright field and FITC (CoroNa green) images showing co-localization of ITLS and unique Na^+ fluorescent signal, confirming that foci of ITLS and unique Na^+ signature are identical. (*B*) An unperturbed stage 15 embryo showing the normal Na^+ content along the neural tube (white arrowheads). (*C*) At stage 15, when there are no histological and morphological signs of ITLS, *Gli1*-injected embryos exhibit foci with unique Na^+ content (red arrowheads) as well as the normal Na^+ signal within the neural tube (white arrowheads). (*D*) At stage 15, unique Na^+ content foci were present in 29.6% (black bar) of *Gli1* injected embryos ($n = 81$), 45.8% of which (red bar) formed morphologically apparent ITLSs by stage 35. In unperturbed embryos, unique Na^+ content foci were observed in only 6% (black bar) of all treatments ($n = 50$), and no ITLS formation was observed. #$p < 0.001$; *$p < 0.001$; $\chi 2$ test compared to uninjected embryos.

KRAS12D, respectively; figure 7(*D*)). We conclude that depolarized transmembrane potential is a causative factor in tumor induction by two canonical oncogenes, and that this signal can be counteracted by hyperpolarizing ion flows.

3.9. Hyperpolarization without gene therapy

If hyperpolarization is to become a viable treatment modality to reduce tumor incidence, then it will be beneficial to avoid the need for the introduction of transgenes, as biomedical gene therapy involves a number of safety and efficacy hurdles. Following the same strategy we used to regulate voltage levels to induce organ regeneration without introduction of transgenes (Tseng *et al* 2010), we sought to use pharmacological reagents to capitalize on ion channels already natively expressed in the relevant tissues. Overexpression of *Xrel1* using mRNA injections directed into one blastomere of 2-cell embryos results in ITLSs in 65% of embryos. Raising the extracellular chloride level of the tadpoles' medium, thus hyperpolarizing via chloride channels, significantly reduced the incidence of tumors in embryos that had been pre-injected with *Xrel3* (figure 8), suggesting the presence of an endogenous depolarizing chloride channel that contributes to tumorigenesis. Consistently with this, application of indanyloxy acetic acid (IAA), a potent pharmacological blocker of chloride channels, abolished the high-chloride inhibition of tumorigenesis. We conclude that endogenous chloride channels contribute to the voltage regulation that mediates oncogene-induced tumor formation, and these channels represent a potentially convenient target for modulation of this process.

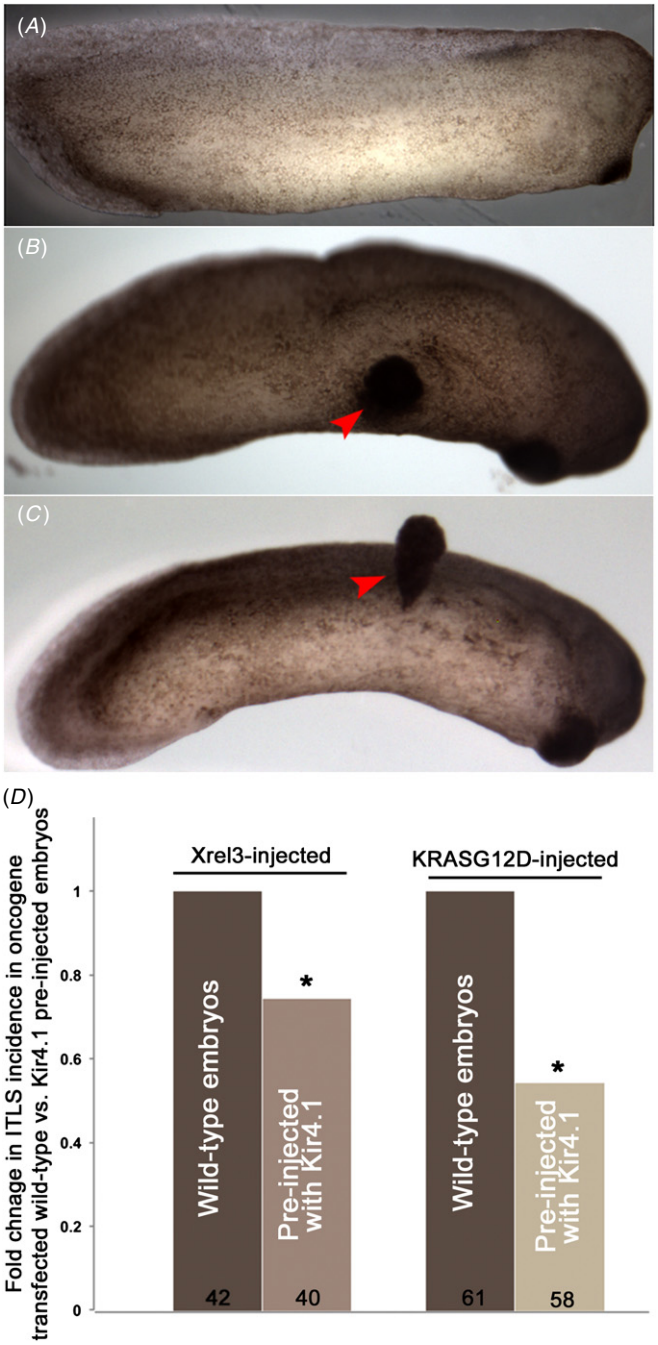

Figure 7. Oncogene-induced ITLSs can be suppressed by prior injection of hyperpolarizing channel mRNA. (*A*) An unperturbed (control) stage 29 embryo showing normal development. (*B*) Embryos that are electroporated with Xrel3 DNA at stage 10 exhibit ITLSs by stage 29 (red arrowhead). (*C*) Embryos that are electroporated with KRASG12D DNA at stage 10 exhibit ITLSs by stage 30 (red arrowhead). (*D*) Xrel3 and KRASG12D ITLS formation can be partially blocked by forced pre-hyperpolarization via molecular expression of *Kir*4.1 (a hyperpolarizing channel). Injection of *Kir*4.1 mRNA at the one-cell stage followed by electroporating with oncogene DNA at stage 10 results in 25.8% fewer embryos with ITLSs compared with Xrel3 electroporation on a wild-type background and 46% fewer embryos with ITLSs compared with KRASG12D electroporation on a wild-type background. $^{*}p < 0.05$ One-sample *t*-test to a normalized ITLS incidence (set to 1) in Xrel3- or KRASG12D-electroporated embryos.

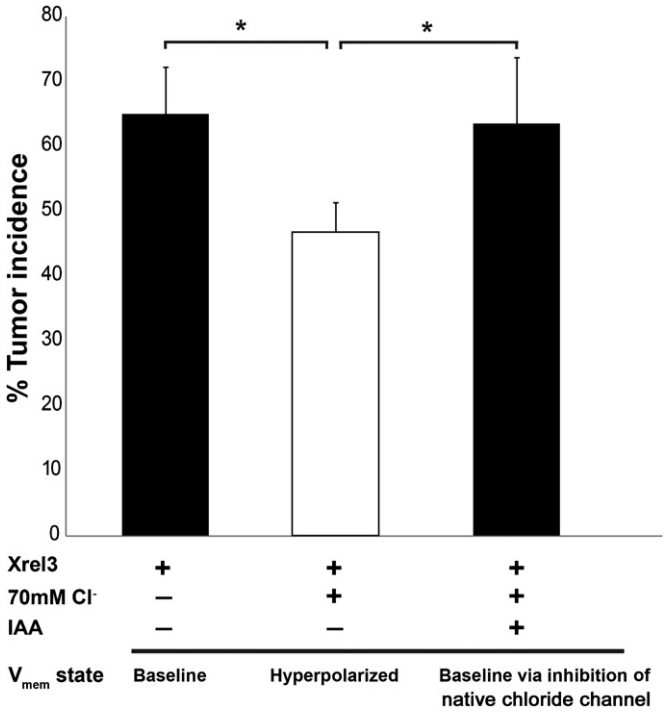

Figure 8. Pharmacological targeting of endogenous Cl^- channels suppresses *Xrel3* ITLSs. Overexpression of *Xrel3* in 1 of 2 cell stage embryos results in ITLSs in 65% of the embryos. When *Xrel3*-injected embryos are exposed to a medium containing high chloride levels (70 mM), ITLS incidence is reduced to 46.8%, suggesting the presence of a native chloride channel that would mediate the hyperpolarization by influx of Cl^- ions. The ITLS suppression effect can be blocked by pharmacological blockade of Cl^- channels – exposure to indanyloxy acetic acid (IAA) along with the high chloride results in the baseline ITLS incidence of 63.5%, similar to that of *Xrel3* only treatment. $^{*}p < 0.01$; One-way ANOVA; Bonferroni post hoc.

4. Discussion

4.1 Cancer in an in vivo model: developmental dysregulation

The *Xenopus* tadpole is an ideal model for understanding the interplay between normal developmental cues and the defects of morphostasis that manifest as cancer. Its amenability to physiology, molecular genetics, cell biology and pharmacology techniques allowed us to investigate two very different aspects of malignancy. The first was a widespread metastatic conversion affecting many mature cells (melanocytes) throughout the body with no definable 'tumor' at its source. Our data suggest that even in the absence of a primary tumor, the main properties of metastasis (overproliferation, cell shape change that facilitates invasion, and colonization of other organs and tissues) can be conferred upon a mature cell type. While originally the phenotype was discovered by its obvious effect on melanocytes, we found that vascular patterning was also affected (figure 2), consistent with the well-known role of aberrant vasculogenesis as a hallmark of cancer. While tadpoles with depolarized instructor cells have no obvious anatomical defects, it is possible that other, even more subtle changes still remain undetected. Future studies in transgenic animals with other fluorescently-marked cell types,

or detailed marker analysis in sections, may identify additional cell populations that are regulated by the instructor cells.

4.2. Bioelectric aspects of the microenvironment—melanoma at a distance

Resting potential across the plasma membrane is a crucial parameter regulating many aspects of cell behavior (Blackiston *et al* 2009, Cone 1974, Cone and Cone 1976, Sundelacruz *et al* 2009). Spatio-temporal changes in V_{mem} occur due to the opening and closing of existing ion translocators (post-translational regulation by physiological cues). These alterations of the biophysical cell state (bioelectric signals) are thus a source of non-genetic information during development and regeneration, and are now increasingly understood as an important factor in cancer. The signal from depolarized neighbors that confers metastatic-like behavior upon melanocytes is mediated by serotonin. This neurotransmitter has been shown to mediate bioelectric signals into transcriptional cascades in other aspects of developmental regulation (Carneiro *et al* 2011, Levin *et al* 2006), and has been linked to carcinogenesis through studies of serotonergic drugs and activity of serotonin pathway enzymes in tumors (Brandes *et al* 1992, Dizeyi *et al* 2004, Kannen *et al* 2011, Manda *et al* 1988, Meredith *et al* 2005, Pai *et al* 2009, Vicaut *et al* 2000). Interestingly, only a very small region of the embryo has to be depolarized for all of the melanocytes to become activated (figure 3). Indeed, the minimal signaling unit is likely to be even somewhat smaller than revealed by the fluorescence in figures 3(*E*) and (*F*) because not every targeted cell is going to be maximally depolarized.

While the few millimeter length of a tadpole is not considered a very long distance on the size scale of human tumors, the ability of a few cells on the tail to influence all of the melanocytes in the animal's head region is a remarkably long-range developmental signal. Serotonin is difficult to image *in vivo* because any tag placed on could significantly change its size and thus diffusion/transport dynamics; the development of novel techniques that would allow molecular tracking of serotonin movement in living embryos represents an important area for future work, since dissecting the dynamics of cross-body transfer of this important signaling molecule is likely to shed light on both developmental regulation and cancer. Interestingly, it is now known that transglutaminase is an important component of transformation-conferring microvesicles from cancer cells (Antonyak *et al* 2011, Li *et al* 2011); transglutaminase is important for serotonin signaling (Dale *et al* 2002, Paulmann *et al* 2009, Walther *et al* 2003), and future work will test the hypothesis that serotonin is involved in microvesicle-mediated passage of transformation among cells in biomedical settings.

The endpoint of the serotonergic signaling triggered by depolarization is a change in behavior of melanocytes toward metastatic melanoma. Because melanocytes are derivatives of the migratory neural crest, they have conserved significant neuronal characteristics, including production of neurotransmitters and expression of their functional receptors (Slominski 2009, Slominski *et al* 2003). Indeed, the skin of amphibians, rodents and humans has been shown to contain endogenous serotonin receptors (English *et al* 1992, Potenza and Lerner 1994, Slominski *et al* 2004). Human cutaneous melanocytes are also immunoreactive to serotonin (Johansson *et al* 1998) and express multiple serotonin receptor subtypes (Slominski *et al* 2003). Taken together, melanocytes can be thought to act as the 'neurons of the skin'—a hypothesis that is quite similar to the 'acquisition of excitability' that has been suggested as underlying the role of onco-fetal sodium channels in cancerous transformation (Brackenbury *et al* 2008, Onganer *et al* 2005). We hypothesize that the extension of abnormal long projections (figures 1(*E*) versus (*E*′)) may be another indicator that melanocytes have assumed some nerve-like properties as part of this transformation, much as melanoma cells have been described to mimic vascular cells (Hendrix *et al* 2003, Lee *et al* 2005, Maniotis *et al* 1999).

4.3. Serotonin: transducing voltage change into cellular effectors

In addition to its roles in development, serotonin also has well-established functions as a regulator of mitosis and tumor growth in adult human tissue. The ability of serotonin signaling to cause melanocytes to proliferate is consistent with its proposed role as an unconventional mitogen (Fanburg and Lee 1997, Gustafsson *et al* 2006, Lee *et al* 1999, Nebigil *et al* 2000), while the induced change of shape to a highly arborized appearance has been also observed in human melanocytes (Blackiston *et al* 2011) and breast cells (Pai *et al* 2009). Our pharmacological data relied on well-characterized inhibitors to suggest VMAT as an important regulator of serotonin availability for export by instructor cells, as well as several receptor proteins; future studies using gene-specific knockdown will be necessary to conclusively confirm the identity of the gene products involved.

The inhibitor data painted a complex picture: How can inhibition of a receptor suppress hyperpigmentation from depolarizing treatments but induce hyperpigmentation when used on its own? A model (figure 4) based on the competing interactions between serotonin receptors and the downstream cAMP signaling in the breast cell field (Pai *et al* 2009) was our basis for a model that explains these data. Our model also has the advantage that it explains the bistable (all-or-none) hyperpigmentation phenotype: the dynamics of such networks, together with the known variable initial serotonin concentration among animals (Fukumoto *et al* 2005), results in two stable 'attractor' states with respect to final output (Ferrell and Xiong 2001, Gallaher *et al* 2010, Sachdeva *et al* 2010, Xiong and Ferrell 2003), corresponding to the activation of transformation or maintenance of normal melanocyte state. Our future work will be focused on refining a quantitative model of this network, developing our method into a general-purpose bioinformatics tool to help scientists formulate predictive models from puzzling functional data and building a detailed picture of cAMP signaling among cells *in vivo*, using optogenetic control of cAMP levels (Weissenberger *et al* 2011). Regardless of the specifics of the model, the opposing activity of several serotonin receptors suggests that therapeutic

approaches must be based on the quantitative simulation of the relevant physiology—the desired outcome will unfortunately not be as simple as applying serotonergic blocker drugs to melanoma.

4.4. Localized tumorigenesis and bioelectric cues

Transmembrane voltage level is not only an initiator of dispersed metastasis but is involved in formation of discrete tumor-like structures. Exposure to well-known carcinogen 4NQO results in tumors with functional blood vessels (figures 5(*C*) and (*D*), supplementary movie 1 (available from stacks.iop.org/PhysBio/9/065002/mmedia)); interestingly, the directly-detected (figure 5(*E*)) change of transmembrane potential throughout the organism, and its attendant hyperpigmentation (figure 5(*A*)) reveal a kind of field carcinogenesis (Brink *et al* 2012, Volkov *et al* 2011a, Volkov *et al* 2011b)—a change in the physiology of cells well outside of the region containing an anatomically-apparent tumor. While the channels responsible for cancer-relevant sodium (figure 6) and chloride (figure 7) fluxes remain to be genetically identified in this system, all of the data indicate that it is the voltage and ion content, not the molecular identity of the translocator protein, that is crucial for determining cell behavior. Thus, systems of fluorescent voltage- and sodium-reporter dyes may be a useful modality for the identification of the transformed field prior to, and around the site of, tumor formation in clinical settings.

Interestingly, we observed considerable heterogeneity in the voltage responses of animals treated with 4NQO. We found about half the number of animals to be largely depolarized throughout their bodies, while the other half appeared unaffected. This bi-stability, similar to that discussed for the hyperpigmentation serotonin signaling pathway above, may be a common feature of physiological networks (Williams *et al* 2002) and could underlie the well-known heterogeneity among human patients with respect to cancer susceptibility and response to treatments. We suggest that in addition to the epigenetic profiling currently under way to help understand differences among the population, the states of physiological (not genetic) networks should play an important part in understanding inherent variability in cancer-relevant processes (Rubin 1985, 1990).

4.5. Conclusions and future prospects

These studies have provided important details on the fascinating novel area of voltage regulation of cancer; however, many new questions now arise. The spatial properties of voltage-regulated serotonergic long-range signaling, and the complex dynamics of physiological networks upstream of key cell decisions are both likely to be of relevance not only to developmental patterning, but also to a new understanding of the biophysics of the tumor microenvironment. In addition to the basic biology revealed by this pathway, these data suggest a number of approaches with biomedical applications. The search for new types of instructor cells adds an interesting dimension to the current focus on stem cells as a uniquely important cancer cell population. Moreover, the ability to visualize tumor and carcinogen-modified cells via physiological dyes likely represent interesting non-invasive diagnostic modalities to identify tissues at risk or tumor margins during surgery. Most excitingly, forced hyperpolarization (figures 7 and 8) via either molecular-genetic or pharmacological means can functionally reduce tumor incidence. It is likely that capitalizing on the functional significance of transmembrane potential as a mediator of pattern maintenance and tumor suppression *in vivo* represents an exciting and important future approach for cancer management. It is hoped that by unraveling fundamental roles of bioelectricity in pattern formation, biomedicine will someday be able to activate at will the remarkable pathways that highly regenerative model species use to normalize, not kill, tumor tissue (Donaldson and Mason 1975, Rose and Wallingford 1948, Seilern-Aspang and Kratochwill 1965, Wolsky 1978).

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References

Aberg P, Nicander I, Hansson J, Geladi P, Holmgren U and Ollmar S 2004 Skin cancer identification using multifrequency electrical impedance—a potential screening tool *IEEE Trans. Biomed. Eng.* **51** 2097–102

Adams S V and DeFelice L J 2002 Flux coupling in the human serotonin transporter *Biophys. J.* **83** 3268–82

Adams D S and Levin M 2006 Inverse drug screens: a rapid and inexpensive method for implicating molecular targets *Genesis* **44** 530–40

Adams D S and Levin M 2012a Endogenous voltage gradients as mediators of cell-cell communication: strategies for investigating bioelectrical signals during pattern formation *Cell Tissue Res.* at press (doi:10.1007/s00441-012-1329-4)

Adams D S and Levin M 2012b General principles for measuring resting membrane potential and ion concentration using fluorescent bioelectricity reporters *Cold Spring Harb. Protoc.* at press

Adams D S and Levin M 2012c Measuring resting membrane potential using the fluorescent voltage reporters DiBAC4(3) and CC2-DMPE *Cold Spring Harb. Protoc.* at press

Adams D S, Masi A and Levin M 2007 H+ pump-dependent changes in membrane voltage are an early mechanism necessary and sufficient to induce Xenopus tail regeneration *Development* **134** 1323–35

Adams D S, Robinson K R, Fukumoto T, Yuan S, Albertson RC, Yelick P, Kuo L, McSweeney M and Levin M 2006 Early, H+-V-ATPase-dependent proton flux is necessary for

consistent left-right patterning of non-mammalian vertebrates *Development* **133** 1657–71
Antonyak M A, Li B, Boroughs L K, Johnson J L, Druso J E, Bryant K L, Holowka D A and Cerione R A 2011 Cancer cell-derived microvesicles induce transformation by transferring tissue transglutaminase and fibronectin to recipient cells *Proc. Natl Acad. Sci. USA* **108** 4852–7
Arcangeli A 2005 Expression and role of hERG channels in cancer cells *Novartis Found. Symp.* **266** 225–32 (discussion 232–224)
Arcangeli A, Bianchi L, Becchetti A, Faravelli L, Coronnello M, Mini E, Olivotto M and Wanke E 1995 A novel inward-rectifying K+ current with a cell-cycle dependence governs the resting potential of mammalian neuroblastoma cells *J. Physiol.* **489** 455–71
Arcangeli A, Crociani O, Lastraioli E, Masi A, Pillozzi S and Becchetti A 2009 Targeting ion channels in cancer: a novel frontier in antineoplastic therapy *Curr. Med. Chem.* **16** 66–93
Aw S, Adams D S, Qiu D and Levin M 2008 H,K-ATPase protein localization and Kir4.1 function reveal concordance of three axes during early determination of left-right asymmetry *Mech. Dev.* **125** 353–72
Baker S G, Soto A M, Sonnenschein C, Cappuccio A, Potter J D and Kramer B S 2009 Plausibility of stromal initiation of epithelial cancers without a mutation in the epithelium: a computer simulation of morphostats *BMC Cancer* **9** 89
Barnes N M and Sharp T 1999 A review of central 5-HT receptors and their function *Neuropharmacology* **38** 1083–152
Beane W S, Morokuma J, Adams D S and Levin M 2011 A chemical genetics approach reveals H,K-ATPase-mediated membrane voltage is required for planarian head regeneration *Chem. Biol.* **18** 77–89
Becchetti A 2011 Ion channels and transporters in cancer. 1. Ion channels and cell proliferation in cancer *Am. J. Physiol. Cell Physiol.* **301** C255–65
Bianchi L *et al* 1998 herg encodes a K+ current highly conserved in tumors of different histogenesis: a selective advantage for cancer cells? *Cancer Res.* **58** 815–22
Binggeli R and Weinstein R 1986 Membrane potentials and sodium channels: hypotheses for growth regulation and cancer formation based on changes in sodium channels and gap junctions *J. Theor. Biol.* **123** 377–401
Bissell M J and Hines W C 2011 Why don't we get more cancer? A proposed role of the microenvironment in restraining cancer progression *Nature Med.* **17** 320–9
Bissell M J and Labarge M A 2005 Context, tissue plasticity, and cancer: are tumor stem cells also regulated by the microenvironment? *Cancer Cells* **7** 17–23
Bissell M J and Radisky D 2001 Putting tumours in context *Nature Rev. Cancer* **1** 46–54
Bizzarri M, Cucina A, Biava P M, Proietti S, D'Anselmi F, Dinicola S, Pasqualato A and Lisi E 2011 Embryonic morphogenetic field induces phenotypic reversion in cancer cells. Review article *Curr. Pharm. Biotechnol.* **12** 243–53
Bizzarri M, Cucina A, Conti F and D'Anselmi F 2008 Beyond the oncogene paradigm: understanding complexity in cancerogenesis *Acta Biotheor.* **56** 173–96
Blackiston D, Adams D S, Lemire J M, Lobikin M and Levin M 2011 Transmembrane potential of GlyCl-expressing instructor cells induces a neoplastic-like conversion of melanocytes via a serotonergic pathway *Dis. Models Mech.* **4** 67–85
Blackiston D J, McLaughlin K A and Levin M 2009 Bioelectric controls of cell proliferation: ion channels, membrane voltage and the cell cycle *Cell Cycle* **8** 3519–28
Borgens R B 1986 The role of natural and applied electric fields in neuronal regeneration and development *Prog. Clin. Biol. Res.* **210** 239–50
Boroughs L K, Antonyak M A, Johnson J L and Cerione R A 2011 A unique role for heat shock protein 70 and its binding partner tissue transglutaminase in cancer cell migration *J. Biol. Chem.* **286** 37094–107
Brackenbury W J and Djamgoz M B 2006 Activity-dependent regulation of voltage-gated Na +channel expression in Mat-LyLu rat prostate cancer cell line *J. Physiol.* **573** 343–56
Brackenbury W J, Djamgoz M B and Isom L L 2008 An emerging role for voltage-gated Na +channels in cellular migration: regulation of central nervous system development and potentiation of invasive cancers *Neuroscientist* **14** 571–83
Brandes L J, Arron R J, Bogdanovic R P, Tong J, Zaborniak C L, Hogg G R, Warrington R C, Fang W and LaBella F S 1992 Stimulation of malignant growth in rodents by antidepressant drugs at clinically relevant doses *Cancer Res.* **52** 3796–800
Brink P R, Valiunas V, Gordon C, Rosen M R and Cohen I S 2012 Can gap junctions deliver? *Biochim. Biophys. Acta* **1818** 2076–81
Burr H S 1940 Biologic organization and the cancer problem *Yale J. Biol. Med.* **12** 277–82
Burr H S 1941 Changes in the field properties of mice with transplanted tumors *Yale J. Biol. Med.* **13** 783–8
Burr H S, Strong L C and Smith G M 1938 Bioelectric correlates of methylcolantherene-induced tumors in mice *Yale J. Biol. Med.* **10** 539–44
Buznikov G A, Lambert H W and Lauder J J 2001 Serotonin and serotonin-like substances as regulators of early embryogenesis and morphogenesis *Cell Tissue Res.* **305** 177–86
Cameron I L, Pool T B and Smith N K 1979a An x-ray microanalysis survey of the concentration of elements in the cytoplasm of different mammalian cell types *J. Cell Physiol.* **101** 493–501
Cameron I L and Smith N K 1980 Energy dispersive x-ray microanalysis of the concentration of elements in relation to cell reproduction in normal and in cancer cells *Scan. Electron Microsc.* **Pt2** 463–74 PMID: 6999606
Cameron I L and Smith N K 1989 Cellular concentration of magnesium and other ions in relation to protein synthesis, cell proliferation and cancer *Magnesium* **8** 31–44
Cameron I L, Smith N K and Pool T B 1979b Element concentration changes in mitotically active and postmitotic enterocytes. An x-ray microanalysis study *J. Cell Biol.* **80** 444–50
Cameron I L, Smith N K, Pool T B and Sparks RL 1980 Intracellular concentration of sodium and other elements as related to mitogenesis and oncogenesis *in vivo Cancer Res.* **40** 1493–500
Carneiro K, Donnet C, Rejtar T, Karger B L, Barisone G A, Diaz E, Kortagere S, Lemire J M and Levin M 2011 Histone Deacetylase activity is necessary for left-right patterning during vertebrate development *BMC Dev. Biol.* **11** 29
Chernet B and Levin M 2012a Transmembrane potential is an instructive factor in cancerous transformation and normalization submitted
Chernet B T and Levin M 2012b A versatile protocol for mRNA electroporation of Xenopus laevis embryos *Cold Spring Harb. Protoc.* **4** 447–52
Cone C D 1974 The role of the surface electrical transmembrane potential in normal and malignant mitogenesis *Ann. New York Acad. Sci.* **238** 420–35
Cone C D and Cone C M 1976 Induction of mitosis in mature neurons in central nervous system by sustained depolarization *Science* **192** 155–8
Conti M 2004 Targeting K+ channels for cancer therapy *J. Exp. Ther. Oncol.* **4** 161–6
Dahmane N, Lee J, Robins P, Heller P and Ruiz i Altaba A 1997 Activation of the transcription factor Gli1 and the Sonic hedgehog signalling pathway in skin tumours *Nature* **389** 876–81
Dale G L, Friese P, Batar P, Hamilton S F, Reed G L, Jackson K W, Clemetson K J and Alberio L 2002 Stimulated platelets use serotonin to enhance their retention of procoagulant proteins on the cell surface *Nature* **415** 175–9

Del Rio-Tsonis K and Tsonis P A 1992 Amphibian tissue regeneration—a model for cancer regulation *Int. J. Oncol.* **1** 161–4

Dinicola S, D'Anselmi F, Pasqualato A, Proietti S, Lisi E, Cucina A and Bizzarri M 2011 A systems biology approach to cancer: fractals, attractors, and nonlinear dynamics *OMICS* **15** 93–104

Diss J K, Stewart D, Pani F, Foster C S, Walker M M, Patel A and Djamgoz MB 2005 A potential novel marker for human prostate cancer: voltage-gated sodium channel expression *in vivo Prostate Cancer Prostatic Dis.* **8** 266–73

Dizeyi N, Bjartell A, Nilsson E, Hansson J, Gadaleanu V, Cross N and Abrahamsson P A 2004 Expression of serotonin receptors and role of serotonin in human prostate cancer tissue and cell lines *Prostate* **59** 328–36

Doherty J R, Johnson Hamlet M R, Kuliyev E and Mead PE 2007 A flk-1 promoter/enhancer reporter transgenic Xenopus laevis generated using the Sleeping Beauty transposon system: an *in vivo* model for vascular studies *Dev. Dyn.* **236** 2808–17

Donaldson D J and Mason J M 1975 Cancer-related aspects of regeneration research: a review *Growth* **39** 475–96

English K B, Wang Z Z, Stayner N, Stensaas L J, Martin H and Tuckett R P 1992 Serotonin-like immunoreactivity in Merkel cells and their afferent neurons in touch domes from the hairy skin of rats *Anat. Rec.* **232** 112–20

Fakler B, Bond C T, Adelman J P and Ruppersberg J P 1996 Heterooligomeric assembly of inward-rectifier K+ channels from subunits of different subfamilies: Kir2.1 (IRK1) and Kir4.1 (BIR10) *Pflugers Arch.* **433** 77–83

Fan Y and Bergmann A 2008 Apoptosis-induced compensatory proliferation. The cell is dead. Long live the cell! *Trends Cell Biol.* **18** 467–73

Fanburg B and Lee S 1997 A new role for an old molecule: serotonin as a mitogen *Am. J. Physiol.* **272** L795–806

Farias L M *et al* 2004 Ether a go-go potassium channels as human cervical cancer markers *Cancer Res.* **64** 6996–7001

Ferrell J E and Xiong W 2001 Bistability in cell signaling: How to make continuous processes discontinuous, and reversible processes irreversible *Chaos* **11** 227–36

Fraser S P *et al* 2005 Voltage-gated sodium channel expression and potentiation of human breast cancer metastasis *Clin. Cancer Res.* **11** 5381–9

Fraser S P, Grimes J A and Djamgoz M B 2000 Effects of voltage-gated ion channel modulators on rat prostatic cancer cell proliferation: comparison of strongly and weakly metastatic cell lines *Prostate* **44** 61–76

Fukumoto T, Kema I P and Levin M 2005 Serotonin signaling is a very early step in patterning of the left-right axis in chick and frog embryos *Curr. Biol.* **15** 794–803

Gallaher J, Bier M and van Heukelom J S 2010 First order phase transition and hysteresis in a cell's maintenance of the membrane potential-An essential role for the inward potassium rectifiers *Biosystems* **101** 149–55

Gupta D, Lammersfeld C A, Burrows J L, Dahlk S L, Vashi P G, Grutsch J F, Hoffman S and Lis C G 2004 Bioelectrical impedance phase angle in clinical practice: implications for prognosis in advanced colorectal cancer *Am. J. Clin. Nutr.* **80** 1634–8

Gupta N, Martin P M, Prasad P D and Ganapathy V 2006 SLC5A8 (SMCT1)-mediated transport of butyrate forms the basis for the tumor suppressive function of the transporter *Life Sci.* **78** 2419–25

Gustafsson B I, Thommesen L, Stunes A K, Tommeras K, Westbroek I, Waldum H L, Slordahl K, Tamburstuen M V, Reseland J E and Syversen U 2006 Serotonin and fluoxetine modulate bone cell function *in vitro J. Cell. Biochem.* **98** 139–151

Habela C W, Olsen M L and Sontheimer H 2008 ClC3 is a critical regulator of the cell cycle in normal and malignant glial cells *J. Neurosci.* **28** 9205–17

Hanahan D and Weinberg R A 2011 Hallmarks of cancer: the next generation *Cell* **144** 646–74

Hendrix M J, Seftor E A, Hess A R and Seftor R E 2003 Vasculogenic mimicry and tumour-cell plasticity: lessons from melanoma *Nature Rev. Cancer* **3** 411–21

Hendrix M J, Seftor E A, Seftor R E, Kasemeier-Kulesa J, Kulesa P M and Postovit L M 2007 Reprogramming metastatic tumour cells with embryonic microenvironments *Nature Rev. Cancer* **7** 246–55

Hilber B, Scholze P, Dorostkar M M, Sandtner W, Holy M, Boehm S, Singer E A and Sitte H H 2005 Serotonin-transporter mediated efflux: a pharmacological analysis of amphetamines and non-amphetamines *Neuropharmacology* **49** 811–9

Hirose M, Takahashi M, Hananouchi M, Tatematsu M and Kinoshita H 1976 Transplantation of chemically induced gastric cancer in Wistar rats *Gann* **67** 365–9

House C D *et al* 2010 Voltage-gated Na +channel SCN5A is a key regulator of a gene transcriptional network that controls colon cancer invasion *Cancer Res.* **70** 6957–67

Huang S, Ernberg I and Kauffman S 2009 Cancer attractors: a systems view of tumors from a gene network dynamics and developmental perspective *Semin. Cell Dev. Biol.* **20** 869–76

Ingber D E 2008 Can cancer be reversed by engineering the tumor microenvironment? *Semin. Cancer Biol.* **18** 356–64

Jaffe L F 1979 Control of development by ionic currents *Membrane Transduction Mechanisms* ed R A Cone and J E Dowling (New York: Raven)

Jeter J R Jr, Cameron I L, Smith N K, Steffens W L and Wille J J 1982 Cell cycle-fluctuations in concentration of various elements in cytoplasm and in nucleus/chromatin of *Physarum polycephal*um *Cytobios* **35** 47–62

Jirsch J, Deeley R G, Cole S P, Stewart A J and Fedida D 1993 Inwardly rectifying K+ channels and volume-regulated anion channels in multidrug-resistant small cell lung cancer cells *Cancer Res.* **53** 4156–60

Johansson O, Liu P Y, Bondesson L, Nordlind K, Olsson M J, Lontz W, Verhofstad A, Liang Y and Gangi S 1998 A serotonin-like immunoreactivity is present in human cutaneous melanocytes *J. Invest. Dermatol.* **111** 1010–4

Kannen V *et al* 2011 Fluoxetine induces preventive and complex effects against colon cancer development in epithelial and stromal areas in rats *Toxicol. Lett.* **204** 134–40

Kasemeier-Kulesa J C, Teddy J M, Postovit L M, Seftor E A, Seftor R E, Hendrix MJ and Kulesa P M 2008 Reprogramming multipotent tumor cells with the embryonic neural crest microenvironment *Dev. Dyn.* **237** 2657–66

Kasper M, Regl G, Frischauf A M and Aberger F 2006 GLI transcription factors: mediators of oncogenic Hedgehog signalling *Eur. J. Cancer* **42** 437–445

Kim C J, Cho Y G, Jeong S W, Kim Y S, Kim S Y, Nam S W, Lee S H, Yoo N J, Lee J Y and Park W S 2004 Altered expression of KCNK9 in colorectal cancers *APMIS* **112** 588–94

Klezovitch O, Risk M, Coleman I, Lucas J M, Null M, True L D, Nelson P S and Vasioukhin V 2008 A causal role for ERG in neoplastic transformation of prostate epithelium *Proc. Natl Acad. Sci. USA* **105** 2105–10

Koch K S and Leffert H L 1979 Increased sodium ion influx is necessary to initiate rat hepatocyte proliferation *Cell* **18** 153–63

Kometiani P, Liu L and Askari A 2005 Digitalis-induced signaling by Na+/K+-ATPase in human breast cancer cells *Mol. Pharmacol.* **67** 929–36

Kulesa P M, Kasemeier-Kulesa J C, Teddy J M, Margaryan N V, Seftor E A, Seftor R E and Hendrix M J 2006 Reprogramming metastatic melanoma cells to assume a neural crest cell-like phenotype in an embryonic microenvironment *Proc. Natl Acad. Sci. USA* **103** 3752–7

Lange C, Prenninger S, Knuckles P, Taylor V, Levin M and Calegari F 2011 The H(+) vacuolar ATPase maintains

neural stem cells in the developing mouse Cortex *Stem Cells Dev.* **20** 843–50
Lastraioli E *et al* 2004 herg1 gene and HERG1 protein are overexpressed in colorectal cancers and regulate cell invasion of tumor cells *Cancer Res.* **64** 606–11
Le X, Langenau D M, Keefe M D, Kutok J L, Neuberg D S and Zon L I 2007 Heat shock-inducible Cre/Lox approaches to induce diverse types of tumors and hyperplasia in transgenic zebrafish *Proc. Natl Acad. Sci. USA* **104** 9410–5
Lee L M, Seftor E A, Bonde G, Cornell R A and Hendrix M J 2005 The fate of human malignant melanoma cells transplanted into zebrafish embryos: assessment of migration and cell division in the absence of tumor formation *Dev. Dyn.* **233** 1560–70
Lee S L, Wang W W, Finlay G A and Fanburg B L 1999 Serotonin stimulates mitogen-activated protein kinase activity through the formation of superoxide anion *Am. J. Physiol.* **277** L282–91
Leffert H L and Koch K S 1980 Ionic events at the membrane initiate rat liver regeneration *Ann. New York Acad. Sci.* **339** 201–15
Levin M 2009 Bioelectric mechanisms in regeneration: Unique aspects and future perspectives *Semin. Cell Dev. Biol.* **20** 543–56
Levin M 2011a Molecular bioelectricity in developmental biology: new tools and recent discoveries *Bioessays* at press
Levin M 2011b The wisdom of the body: future techniques and approaches to morphogenetic fields in regenerative medicine, developmental biology and cancer *Regenerative Med.* **6** 667–73
Levin M, Buznikov G A and Lauder J M 2006 Of minds and embryos: left-right asymmetry and the serotonergic controls of pre-neural morphogenesis *Dev. Neurosci.* **28** 171–85
Levin M and Mercola M 1998 Gap junctions are involved in the early generation of left-right asymmetry *Dev. Biol.* **203** 90–105
Levin M, Thorlin T, Robinson K R, Nogi T and Mercola M 2002 Asymmetries in H+/K+-ATPase and cell membrane potentials comprise a very early step in left-right patterning *Cell* **111** 77–89
Li B, Cerione R A and Antonyak M 2011 Tissue transglutaminase and its role in human cancer progression *Adv. Enzymol. Relat. Areas Mol. Biol.* **78** 247–93
Lin H, Xiao J, Luo X, Wang H, Gao H, Yang B and Wang Z 2007 Overexpression HERG K(+) channel gene mediates cell-growth signals on activation of oncoproteins SP1 and NF-kappaB and inactivation of tumor suppressor Nkx3.1 *J. Cell Physiol.* **21** 137–47
Lund E 1947 *Bioelectric Fields and Growth* (Austin, TX: University of Texas)
Lynagh T and Lynch J W 2010 An improved ivermectin-activated chloride channel receptor for inhibiting electrical activity in defined neuronal populations *J. Biol. Chem.* **285** 14890–7
Manda T, Nishigaki F, Mori J and Shimomura K 1988 Important role of serotonin in the antitumor effects of recombinant human tumor necrosis factor-alpha in mice *Cancer Res.* **48** 4250–5
Maniotis A J, Folberg R, Hess A, Seftor E A, Gardner L M, Pe'er J, Trent J M, Meltzer P S and Hendrix M J 1999 Vascular channel formation by human melanoma cells *in vivo* and *in vitro*: vasculogenic mimicry *Am. J. Pathol.* **155** 739–52
McCaig C D, Rajnicek A M, Song B and Zhao M 2005 Controlling cell behavior electrically: current views and future potential *Physiol. Rev.* **85** 943–78
McCaig C D, Song B and Rajnicek A M 2009 Electrical dimensions in cell science *J. Cell Sci.* **122** 4267–76
Meredith E J *et al* 2005 The serotonin transporter (SLC6A4) is present in B-cell clones of diverse malignant origin: probing a potential antitumor target for psychotropics *FASEB J.* **19** 1187–9
Meyer R, Schonherr R, Gavrilova-Ruch O, Wohlrab W and Heinemann S H 1999 Identification of ether a go-go and calcium-activated potassium channels in human melanoma cells *J. Membr. Biol.* **171** 107–15
Moiseiwitsch J R 2000 The role of serotonin and neurotransmitters during craniofacial development *Crit. Rev. Oral Biol. Med.* **11** 230–9
Monachon M A, Burkard W P, Jalfre M and Haefely W 1972 Blockade of central 5-hydroxytryptamine receptors by methiothepin *Naunyn Schmiedebergs Arch. Pharmacol.* **274** 192–7
Morokuma J, Blackiston D, Adams D S, Seebohm G, Trimmer B and Levin M 2008 Modulation of potassium channel function confers a hyperproliferative invasive phenotype on embryonic stem cells *Proc. Natl Acad. Sci. USA* **105** 16608–13
Mu D *et al* 2003 Genomic amplification and oncogenic properties of the KCNK9 potassium channel gene *Cancer Cells* **3** 297–302
Nebigil C G, Launay J M, Hickel P, Tournois C and Maroteaux L 2000 5-hydroxytryptamine 2B receptor regulates cell-cycle progression: cross-talk with tyrosine kinase pathways *Proc. Natl Acad. Sci. USA* **97** 2591–6
Needham J 1936 New advances in the chemistry and biology of organized growth: (section of pathology) *Proc. R. Soc. Med.* **29** 1577–1626
Needham J 1963 *Chemical Embryology* (New York: Hafner)
Nguyen L, Rigo J M, Rocher V, Belachew S, Malgrange B, Rogister B, Leprince P and Moonen G 2001 Neurotransmitters as early signals for central nervous system development *Cell Tissue Res.* **305** 187–202
Nieuwkoop P D and Faber J 1967 *Normal Table of Xenopus Laevis (Daudin)* (Amsterdam: North-Holland)
O'Grady S M and Lee S Y 2005 Molecular diversity and function of voltage-gated (Kv) potassium channels in epithelial cells *Int. J. Biochem. Cell Biol.* **37** 1578–94
Onganer P U and Djamgoz M B 2005 Small-cell lung cancer (human): potentiation of endocytic membrane activity by voltage-gated Na(+) channel expression *in vitro J. Membr. Biol.* **204** 67–75
Onganer P U, Seckl M J and Djamgoz M B 2005 Neuronal characteristics of small-cell lung cancer *Br. J. Cancer.* **93** 1197–201
Onkal R and Djamgoz M B 2009 Molecular pharmacology of voltage-gated sodium channel expression in metastatic disease: clinical potential of neonatal Nav1.5 in breast cancer *Eur. J. Pharmacol.* **625** 206–19
Ottesen E A and Campbell W C 1994 Ivermectin in human medicine *J. Antimicrob. Chemother.* **34** 195–203
Ousingsawat J, Spitzner M, Schreiber R and Kunzelmann K 2008 Upregulation of colonic ion channels in APC (Min/+) mice *Pflugers Arch.* **456** 847–55
Pai V P, Aw S, Shomrat T, Lemire J M and Levin M 2012 Transmembrane voltage potential controls embryonic eye patterning in Xenopus laevis *Development* **139** 313–23
Pai V P and Horseman N D 2008 Biphasic regulation of mammary epithelial resistance by serotonin through activation of multiple pathways *J. Biol. Chem.* **283** 30901–10
Pai V P, Marshall A M, Hernandez L L, Buckley A R and Horseman N D 2009 Altered serotonin physiology in human breast cancers favors paradoxical growth and cell survival *Breast Cancer Res.* **11** R81
Pardo L A 2004 Voltage-gated potassium channels in cell proliferation *Physiology* **19** 285–92
Pardo L A, del Camino D, Sanchez A, Alves F, Bruggemann A, Beckh S and Stuhmer W 1999 Oncogenic potential of EAG K(+) channels *EMBO J.* **18** 5540–7
Paulmann N *et al* 2009 Intracellular serotonin modulates insulin secretion from pancreatic beta-cells by protein serotonylation *PLoS Biol.* **7** e1000229
Pei L, Wiser O, Slavin A, Mu D, Powers S, Jan L Y and Hoey T 2003 Oncogenic potential of TASK3 (Kcnk9) depends on K+ channel function *Proc. Natl Acad. Sci. USA* **100** 7803–7
Perona R and Serrano R 1988 Increased pH and tumorigenicity of fibroblasts expressing a yeast proton pump *Nature* **334** 438–40

Potenza M N and Lerner M R 1994 Characterization of a serotonin receptor endogenous to frog melanophores *Naunyn Schmiedebergs Arch. Pharmacol.* **349** 11–9
Potter J D 2001 Morphostats: a missing concept in cancer biology *Cancer Epidemiol. Biomarkers Prev.* **10** 161–70
Potter J D 2007 Morphogens, morphostats, microarchitecture and malignancy *Nature Rev. Cancer* **7** 464–74
Prevarskaya N, Skryma R and Shuba Y 2010 Ion channels and the hallmarks of cancer *Trends Mol. Med.* **16** 107–21
Pu J, McCaig C D, Cao L, Zhao Z, Segall J E and Zhao M 2007 EGF receptor signalling is essential for electric-field-directed migration of breast cancer cells *J. Cell Sci.* **120** 3395–403
Pullar C E, Zhao M, Song B, Pu J, Reid B, Ghoghawala S, McCaig C and Isseroff R R 2007 Beta-adrenergic receptor agonists delay while antagonists accelerate epithelial wound healing: evidence of an endogenous adrenergic network within the corneal epithelium *J. Cell Physiol.* **211** 261–72
Roepke T K, Purtell K, King E C, La Perle K M, Lerner D J and Abbott G W 2010 Targeted deletion of Kcne2 causes gastritis cystica profunda and gastric neoplasia *PLoS One* **5** e11451
Rose S M and Wallingford H M 1948 Transformation of renal tumors of frogs to normal tissues in regenerating limbs of salamanders *Science* **107** 457
Roskelley C D and Bissell M J 2002 The dominance of the microenvironment in breast and ovarian cancer *Semin. Cancer Biol.* **12** 97–104
Rowlatt C 1994 Some consequences of defining the neoplasm as focal self-perpetuating tissue disorganization *New Frontiers in Cancer Causation.* ed O H Iversen (Washington, DC: Taylor and Francis) pp 45–58
Rozengurt E and Mendoza S 1980 Monovalent ion fluxes and the control of cell proliferation in cultured fibroblasts *Ann. New York Acad. Sci.* **339** 175–90
Rubin H 1985 Cancer as a dynamic developmental disorder *Cancer Res.* **45** 2935–42
Rubin H 1990 The significance of biological heterogeneity *Cancer Metastasis Rev.* **9** 1–20
Sachdeva G, Kalyanasundaram K, Krishnan J and Chakravarthy V S 2010 Bistable dynamics of cardiac cell models coupled by dynamic gap junctions linked to Cardiac Memory *Biol. Cybern.* **102** 109–21
Saito T, Schlegel R, Andresson T, Yuge L, Yamamoto M and Yamasaki H 1998 Induction of cell transformation by mutated 16K vacuolar H+-atpase (ductin) is accompanied by down-regulation of gap junctional intercellular communication and translocation of connexin 43 in NIH3T3 cells *Oncogene* **17** 1673–80
Seilern-Aspang F and Kratochwill L 1965 *Relation Between Regeneration and Tumor Growth, Regeneration in Animals and Related Problems* (Amsterdam: North-Holland) pp 452–473
Shan Q, Haddrill J L and Lynch J W 2001 Ivermectin, an unconventional agonist of the glycine receptor chloride channel *J. Biol. Chem.* **276** 12556–64
Shuba Y M, Prevarskaya N, Lemonnier L, Van Coppenolle F, Kostyuk P G, Mauroy B and Skryma R 2000 Volume-regulated chloride conductance in the LNCaP human prostate cancer cell line *Am. J. Physiol. Cell Physiol.* **279** C1144–54
Sive H, Grainger R M and Harland R M 2000 *Early Development of Xenopus Laevis: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory Press)
Slominski A 2009 Neuroendocrine activity of the melanocyte *Exp. Dermatol.* **18** 760–3
Slominski A, Pisarchik A and Wortsman J 2004 Expression of genes coding melatonin and serotonin receptors in rodent skin *Biochim. Biophys. Acta* **1680** 67–70
Slominski A, Pisarchik A, Zbytek B, Tobin D J, Kauser S and Wortsman J 2003 Functional activity of serotoninergic and melatoninergic systems expressed in the skin *J. Cell Physiol.* **196** 144–53
Smith N R, Sparks R L, Pool T B and Cameron I L 1978 Differences in the intracellular concentration of elements in normal and cancerous liver cells as determined by x-ray microanalysis *Cancer Res.* **38** 1952–9
Sonnenschein C and Soto A M 1999 *The Society of Cells : Cancer Control of Cell Proliferation* (New York: Springer)
Soto A M and Sonnenschein C 2004 The somatic mutation theory of cancer: growing problems with the paradigm? *Bioessays* **26** 1097–107
Sparks R L, Pool T B, Smith N K and Cameron I L 1983 Effects of amiloride on tumor growth and intracellular element content of tumor cells *in vivo Cancer Res.* **43** 73–7
Stojadinovic A *et al* 2005 Electrical impedance scanning for the early detection of breast cancer in young women: preliminary results of a multicenter prospective clinical trial *J. Clin. Oncol.* **23** 2703–15
Stull M A, Pai V, Vomachka A J, Marshall A M, Jacob G A and Horseman N D 2007 Mammary gland homeostasis employs serotonergic regulation of epithelial tight junctions *Proc. Natl Acad. Sci. USA* **104** 16708–13
Sundelacruz S, Levin M and Kaplan DL 2008 Membrane potential controls adipogenic and osteogenic differentiation of mesenchymal stem cells *PLoS One* **3** e3737
Sundelacruz S, Levin M and Kaplan D L 2009 Role of membrane potential in the regulation of cell proliferation and differentiation *Stem Cell Rev. Rep.* **5** 231–46
Temme A, Buchmann A, Gabriel H D, Nelles E, Schwarz M and Willecke K 1997 High incidence of spontaneous and chemically induced liver tumors in mice deficient for connexin32 *Curr. Biol.* **7** 713–6
Tseng A S, Beane W S, Lemire J M, Masi A and Levin M 2010 Induction of vertebrate regeneration by a transient sodium current *J. Neurosci.* **30** 13192–200
Tseng A-S and Levin M 2012 Transducing bioelectric signals into epigenetic pathways during tadpole tail regeneration *Anat. Rec.* at press
Tsonis P A 1987 Embryogenesis and carcinogenesis: order and disorder *Anticancer Res.* **7** 617–23
Vaux D L 2011 Response to 'The tissue organization field theory of cancer: a testable replacement for the somatic mutation theory' *BioEssays* **33** 660–1
Vicaut E, Laemmel E and Stucker O 2000 Impact of serotonin on tumour growth *Ann. Med.* **32** 187–94
Volkov A G, Baker K, Foster J C, Clemmons J, Jovanov E and Markin V S 2011a Circadian variations in biologically closed electrochemical circuits in Aloe vera and Mimosa pudica *Bioelectrochemistry* **81** 39–45
Volkov A, Waite A J, Wooten J D and Markin V S 2012 Circadian rhythms in biologically closed electrical circuits of plants *Plant Signaling Behav.* **7** 282–4
Volkov A G, Wooten J D, Waite A J, Brown C R and Markin V S 2011b Circadian rhythms in electrical circuits of *Clivia miniata J. Plant Physiol.* **168** 1753–60
Waddington C H 1935 Cancer and the theory of organisers *Nature* **135** 606–8
Wallingford J B, Seufert D W, Virta V C and Vize P D 1997 p53 activity is essential for normal development in Xenopus *Curr. Biol.* **7** 747–57
Walther D J *et al* 2003 Serotonylation of small GTPases is a signal transduction pathway that triggers platelet alpha-granule release *Cell* **115** 851–62
Wang Z 2004 Roles of K+ channels in regulating tumour cell proliferation and apoptosis *Pflugers Arch.* **448** 274–86
Wang H, Zhang Y, Cao L, Han H, Wang J, Yang B, Nattel S and Wang Z 2002 HERG K+ channel, a regulator of tumor cell apoptosis and proliferation *Cancer Res.* **62** 4843–8

Weaver V M and Gilbert P 2004 Watch thy neighbor: cancer is a communal affair *J. Cell Sci.* **117** 1287–90

Weissenberger S, Schultheis C, Liewald J F, Erbguth K, Nagel G and Gottschalk A 2011 PACalpha–an optogenetic tool for *in vivo* manipulation of cellular cAMP levels, neurotransmitter release, and behavior in Caenorhabditis elegans *J. Neurochem.* **116** 616–25

Williams S R, Christensen S R, Stuart G J and Hausser M 2002 Membrane potential bistability is controlled by the hyperpolarization-activated current I(H) in rat cerebellar Purkinje neurons *in vitro J. Physiol.* **539** 469–83

Wimalasena K 2011 Vesicular monoamine transporters: structure-function, pharmacology, and medicinal chemistry *Med. Res. Rev.* **31** 483–519

Wolsky A 1978 Regeneration and cancer *Growth.* **42** 425–426

Xiong W and Ferrell J E Jr 2003 A positive-feedback-based bistable 'memory module' that governs a cell fate decision *Nature* **426** 460–5

Yang S, Lockwood A, Hollett P, Ford R and Kao K 1998 Overexpression of a novel Xenopus rel mRNA gene induces tumors in early embryos *J. Biol. Chem.* **273** 13746–52

Yasuda T and Adams D J 2010 Physiological roles of ion channels in adult neural stem cells and their progeny *J. Neurochem.* **114** 946–59

Zhao M *et al* 2006 Electrical signals control wound healing through phosphatidylinositol-3-OH kinase-gamma and PTEN *Nature* **442** 457–60