

 Open access • Journal Article • DOI:10.1038/NRC.2017.18

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**Published on:** 01 Jun 2017 - Nature Reviews Cancer (Nature Publishing Group)

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## Review

### The calcium-cancer signalling nexus

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Original available at Nature Reviews Cancer-

<https://doi.org/10.1038/nrc.2017.18>

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## Abstract

The calcium signal is a powerful and multifaceted tool by which cells can achieve specific outcomes. Cellular machinery important in tumour progression are often driven or influenced by changes in calcium ions, in some cases this regulation occurs within spatially defined regions. Over the past decade there has been a deeper understanding of how calcium signalling is remodeled in some cancers and the consequences of calcium signalling on key events such as proliferation, invasion and sensitivity to cell death. Specific calcium signalling pathways have also now been identified as playing important roles in the establishment and maintenance of multidrug resistance and the tumour microenvironment.

## Introduction

The study of calcium signalling had its historic roots in investigations focused on excitable cells such as those of the heart, where the calcium ion ( $\text{Ca}^{2+}$ ) was identified as having an important role in contraction over 130 years ago<sup>1</sup>. Subsequent to these early studies, the  $\text{Ca}^{2+}$  signal was identified as a key regulator of processes in other excitable cells such as neurons and in non-excitable cells<sup>2</sup>, including those of the epithelia, where it can control a diverse array of processes such as secretion, proliferation and gene expression<sup>3</sup>. It is therefore understandable that researchers can overlook a protein involved in  $\text{Ca}^{2+}$  homeostasis when identified as a protein of interest from cancer biopsies or a siRNA phenotypic screen, or discount  $\text{Ca}^{2+}$  signalling when it is identified as an altered pathway. The ubiquity of the  $\text{Ca}^{2+}$  signal can make it seem as if  $\text{Ca}^{2+}$  levels could have no possible role in the *selective* regulation of oncogenic machinery, and that modulation of a specific  **$\text{Ca}^{2+}$  channel** or  **$\text{Ca}^{2+}$  pump** would not be able to avoid drastic adverse effects on non-transformed cells.

However, the ubiquity of the  $\text{Ca}^{2+}$  signal and its ability to differentially regulate a variety of functions simultaneously in the same cell, has driven evolutionary complexity in  $\text{Ca}^{2+}$  signalling that is reliant on specific  $\text{Ca}^{2+}$  channels, pumps and **exchangers**<sup>4</sup>. The selective roles of specific proteins that transport  $\text{Ca}^{2+}$  are reflected in the clinical use of pharmacological inhibitors of L-type voltage gated  $\text{Ca}^{2+}$  channels in the treatment of hypertension<sup>5</sup>, the use of the N-type  $\text{Ca}^{2+}$  channel blocker ziconotide for the treatment of severe and chronic pain<sup>5</sup>, and drug development programs focused on producing specific pharmacological modulators of  $\text{Ca}^{2+}$  channels to treat a diverse array of conditions<sup>5</sup>. Indeed, the activity of many of the  $\text{Ca}^{2+}$  pumps and channels assessed in cancer can be modulated by pharmacological agents (Table 1).

In the context of cancer, there is often significant remodelling in the expression of proteins directly involved in  $\text{Ca}^{2+}$  signalling<sup>6,7</sup>. Moreover, important oncogenic machinery is sensitive to regulation by specific  $\text{Ca}^{2+}$  signals<sup>8,9</sup>. Some proteins that transport  $\text{Ca}^{2+}$  ions are not just regulators of oncogenic signalling but their altered expression can actually be an initiator of some cancers. Evidence for the direct role of proteins that regulate  $\text{Ca}^{2+}$  transport in the initiation of tumours is seen in mice heterozygous for the loss of the Golgi calcium pump secretory pathway  $\text{Ca}^{2+}$ -ATPase 1 (SPCA1; encoded by the gene *Atp2c1*) that show an increased occurrence of squamous cell tumours<sup>10</sup>.

In this Review, we provide a general overview of the more complex aspects of  $\text{Ca}^{2+}$  signalling for those outside the field and highlight the diversity of  $\text{Ca}^{2+}$  channels and  $\text{Ca}^{2+}$  pumps expressed in human cells. We will explore how these proteins regulate specific cellular processes, many of which intersect directly with processes important in cancer progression (e.g. proliferation, invasion and cell death). Some of the emerging fields, and areas requiring further study, such as the role of  $\text{Ca}^{2+}$  signalling in the context of the tumour microenvironment will also be discussed. Finally, new areas that have progressed significantly over the past decade will be described, such as the role of calcium signalling in controlling pathways important in therapeutic resistance.

## **[H1] Calcium channels, pumps and exchangers**

Unlike many other cellular signals,  $\text{Ca}^{2+}$  is not created from an enzymatic reaction or destroyed or converted to an inactive metabolite<sup>11</sup>. What controls the often complex changes in  $\text{Ca}^{2+}$  levels in the cytosol and sub-cellular organelles are the instruments of  $\text{Ca}^{2+}$  homeostasis –  $\text{Ca}^{2+}$  pumps, channels and exchangers<sup>11</sup>. These and other proteins involved in calcium homeostasis and signalling have been described as the calcium signalling “tool kit”<sup>12</sup> and are the means by which cells orchestrate specific biological events. Some of the key classes of proteins involved in the influx, efflux and sequestration of  $\text{Ca}^{2+}$  ions in mammalian cells are presented in Figure 1, and have been previously reviewed elsewhere<sup>1,4,12</sup>.

There is a low level of cytosolic free  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_{\text{Cyt}}$ ) ( $\sim 100$  nM) compared to the level of free  $\text{Ca}^{2+}$  in most extracellular fluids ( $> 1$  mM), creating a large

concentration gradient that promotes the influx of  $\text{Ca}^{2+}$  into the cell when  $\text{Ca}^{2+}$  permeable ion channels are open<sup>1</sup>.

$\text{Ca}^{2+}$  permeable ion channels are classified depending on the genes that encode their components and their functional properties. Voltage-gated  $\text{Ca}^{2+}$  channel component subunits are encoded by a variety of genes and can also be classified by their electrophysiological properties<sup>5</sup>. Transient receptor potential (TRP) ion channels are another diverse family, which include ion channels with high selectivity for  $\text{Ca}^{2+}$  and potential constitutive activity (e.g. TRPV6), as well as temperature sensitive channels such as the cold sensor TRPM8 and the heat and capsaicin (hot chilli component) sensitive TRPV1<sup>13</sup>. There are at least 20 ion channels encoded by TRP genes in mammals<sup>14</sup> and heteromeric associations of channel subunits may yield further functional diversity<sup>13</sup>. Another important  $\text{Ca}^{2+}$  channel is calcium release-activated calcium channel protein 1 (ORAI1), involved in a mechanism known as **store operated calcium entry (SOCE)**, which is detected by the endoplasmic reticulum (ER)  $\text{Ca}^{2+}$  sensor stromal interaction molecule 1 (STIM1). STIM1 proteins are redistributed upon calcium store depletion and subsequently, interact with ORAI1 proteins found at the plasma membrane leading to activation of  $\text{Ca}^{2+}$  influx<sup>15</sup> (Fig 1). ORAI1 related isoforms include ORAI2 and ORAI3, and heteromeric channels composed of ORAI isoforms may constitute channels with unique properties<sup>16</sup>.

Moving from influx to efflux, the efflux of  $\text{Ca}^{2+}$  ions occurs against a concentration gradient and therefore necessitates an active transport system using ATP cleavage or ion gradients. Plasma membrane  $\text{Ca}^{2+}$ -transporting ATPases (PMCAs) are an example of an efflux pump with four genes that generate a plethora of functionally and regulatory diverse transporters via alternative splicing<sup>17</sup>. Plasmalemmal sodium ( $\text{Na}^+$ )/ $\text{Ca}^{2+}$  exchangers are examples of  $\text{Ca}^{2+}$  efflux mechanisms using the  $\text{Na}^+$  gradient, and include SLC8A1 (also known as NCX1)<sup>18</sup>.

Subcellular organelles such as the ER, mitochondria and the Golgi also have mechanisms to increase and decrease  $\text{Ca}^{2+}$  levels including calcium permeable ion channels, pumps and exchangers (Fig 1). The ER plays a crucial role in the signalling of many G-protein coupled receptors (GPCRs) through the expression of  $\text{Ca}^{2+}$  permeable inositol 1,4,5-trisphosphate receptor (IP<sub>3</sub>R) channels that respond to IP<sub>3</sub> generated from phospholipase C (PLC) mediated cleavage of phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>)<sup>2</sup>. Following  $\text{Ca}^{2+}$  release into the cytoplasm through IP<sub>3</sub>Rs, an active transport system mediated by sarcoplasmic/ER  $\text{Ca}^{2+}$ -ATPases (SERCA1, SERCA2 and SERCA3) can sequester  $\text{Ca}^{2+}$  back into the  $\text{Ca}^{2+}$  rich ER lumen<sup>19</sup>. Similarly the Golgi has sequestration mechanisms via the pumps, SPCA1 and SPCA2 that can also transport manganese ( $\text{Mn}^{2+}$ ) ions<sup>19</sup>. Two pore channels (TPC1 and TRP2) are ion channels with a reported ability to regulate  $\text{Ca}^{2+}$  release from components of the endolysosomal system<sup>20</sup>.  $\text{Ca}^{2+}$  levels within mitochondria are controlled by key molecular components<sup>21</sup> that include multiple proteins contributing to the mitochondrial calcium uniporter (MCU) complex, and at least one molecularly defined  $\text{Ca}^{2+}$  efflux mechanism - a  $\text{Na}^+$ / $\text{Ca}^{2+}$  exchanger encoded by the gene *NCLX* (also known as *SLC8B1*)<sup>21</sup>.

## **[H1] Calcium: ubiquitous but selective**

When  $\text{Ca}^{2+}$  levels change in response to a stimulus, the nature of these changes are 'decoded' by cells to achieve specific cellular outcomes. The complexity of  $\text{Ca}^{2+}$  signals and how these can differentially regulate diverse cellular processes has been elegantly reviewed by a number of researchers<sup>12,22,23</sup>. Put simply,  $\text{Ca}^{2+}$  signals can vary in their magnitude as well as in their spatial and temporal characteristics. In a single cell, large sustained increases in  $\text{Ca}^{2+}$  may be associated with cell death, while highly localised changes in  $\text{Ca}^{2+}$  levels in the cytosol may regulate directional migration<sup>24,25</sup>.  $\text{Ca}^{2+}$  changes in mitochondria can control mitochondrial ATP levels, and the frequency and duration of  $[\text{Ca}^{2+}]_{\text{CYT}}$  oscillations can differentially activate transcription factors<sup>26,27</sup>. Examples of the diversity in  $\text{Ca}^{2+}$  signals and some of the consequences of such changes are depicted in Figure 2.

Recent studies using a variety of methods to assess  $\text{Ca}^{2+}$  signals (Box1) have provided insight into the molecular pathways by which nuanced changes in  $\text{Ca}^{2+}$  can achieve specific cellular outcomes. Two recent exemplars relate to gene transcription. The calcium dependent transcription factors nuclear factor of activated T cells 1 (NFAT1) and NFAT4 are differentially regulated by ORAI1-mediated  $\text{Ca}^{2+}$  influx through the spatial nature of changes in  $\text{Ca}^{2+}$ . Localised increases in  $\text{Ca}^{2+}$  around sites of ORAI1-mediated  $\text{Ca}^{2+}$  entry are sufficient to activate NFAT1, but NFAT4 activation requires this source of  $\text{Ca}^{2+}$  in combination with increases in the level of free  $\text{Ca}^{2+}$  within the nucleus<sup>28</sup>. The ability of the L-type voltage gated  $\text{Ca}^{2+}$  channel  $\text{Ca}_v1.2$  to effectively regulate gene transcription via cAMP responsive element-binding protein (CREB) requires local  $\text{Ca}^{2+}$  changes through  $\text{Ca}^{2+}$  influx then subsequent voltage-dependent conformational changes in the  $\text{Ca}_v1.2$  protein itself<sup>29</sup>. The temporal gap between these two events represents a further level of control (optimal at 10-20 s), and the misaligning of these two events is a feature of a mutation associated with autism spectrum disorder symptoms in patients with the multisystem disorder Timothy syndrome<sup>29</sup>. The aforementioned studies highlight a path for future work in cancer research, especially given the role of calcium dependent transcription factors such as NFAT in various aspects of cancer progression<sup>30</sup>.

As will be discussed below, we now have an understanding that the calcium toolkit is often remodelled with tumorigenesis and that the calcium signal can regulate key oncogenic and tumour suppressor machinery and pathways.

## **[H1] $\text{Ca}^{2+}$ signal remodelling in cancer**

### ***[H3] Expression remodelling***

Alterations in the expression of specific  $\text{Ca}^{2+}$  channels and pumps have been widely reported in several cancer types. Changes in expression can be an early event in cancer development, such as the down regulation of SERCA3 during colon carcinogenesis<sup>31</sup>. Expression changes in a single  $\text{Ca}^{2+}$  channel and/or pump can be a common feature across multiple cancer types, such as the reported

overexpression of TRPM8 in cancers of the prostate, breast, colon, pancreas and lung<sup>32,33</sup>, or expression changes may be specific to subtypes within cancers of the same origin, such as elevated *SPCA1* mRNA in basal molecular breast cancers compared with other molecular subtypes of breast cancer<sup>34</sup>. It is also clear that changes in expression of multiple Ca<sup>2+</sup> channels and/or pumps can occur in the same cancer type or even subtype, as reflected in elevated levels of TRPM8 and TRPV6 in prostate cancer and increased levels of ORAI1 and TRPV6 in basal molecular breast cancers (see Table 2).

Either directly or indirectly, the consequences of changes in Ca<sup>2+</sup> channel or pump expression can often be linked to Ca<sup>2+</sup>-sensitive oncogenic pathways. Examples include increased ORAI1 channel expression promoting Ca<sup>2+</sup> oscillations and proliferative pathways in oesophageal squamous cell carcinoma cells<sup>35</sup>, and *SPCA2* overexpression increasing the proliferation and growth in soft agar of a non-malignant breast epithelial cell line - MCF-10A<sup>36</sup>. Some cancers are associated with reduced expression of a calcium transporter and such changes may similarly promote specific hallmarks of cancer<sup>37</sup>. For example, the plasmalemmal calcium pump PMCA4, is down regulated in colon cancers, intensifying Ca<sup>2+</sup> sensitive proliferative stimuli through reduced Ca<sup>2+</sup> efflux<sup>38</sup>. Similarly, the down regulation of ORAI1 (and consequently SOCE), during the transition of prostate cancer to a hormone-refractory phenotype, bestows protection against diverse apoptosis-inducing pathways, such as those induced by the SERCA inhibitor thapsigargin (Table 1), tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) and cisplatin and/or oxaliplatin<sup>39</sup>.

In the past decade there have been major advances in the understanding of differences between subtypes of the same cancer and the molecular drivers behind alterations in the expression of proteins involved in Ca<sup>2+</sup> homeostasis. For example, TRPV6 is elevated in some but not all breast cancer subtypes<sup>33,40</sup>, and we now know that enhanced copy number of the *TRPV6* gene region is a feature of triple negative breast tumours and also breast cancers of the basal molecular subtype (which have a significant overlap with the triple negative subtype<sup>41,42</sup>). Triple negative breast cancer is a subtype for which new targeted therapies are required as this tumour type lacks the oestrogen receptor, a target for anti-oestrogen therapy and HER2 (also known as ERBB2), a target for the monoclonal antibody therapy trastuzumab<sup>41</sup>. Indeed women with tumours with higher levels of *TRPV6* mRNA have a poorer survival<sup>42</sup>. Similarly, in prostate cancer, *CACNA1D*, the gene encoding Cav1.3 was identified as hypo-methylated in TMPRSS2:ERG fusion positive prostate cancers compared with fusion negative cancers, and as a consequence leads to elevated *CACNA1D* mRNA levels in TMPRSS2:ERG fusion positive prostate cancers<sup>43</sup>. These results may be more than just correlative as there is evidence for a role for voltage gated Ca<sup>2+</sup> channels in processes which may be important in prostate cancer cells. For example, a previous study had shown not only an association between elevated *CACNA1D* and TMPRSS2:ERG fusion positive prostate cancers but also that pharmacological inhibition of L-type Ca<sup>2+</sup> channels (which includes Cav1.3) suppresses the proliferation of PC3 prostate cancer cells<sup>44</sup>. Moreover, other

voltage gated  $\text{Ca}^{2+}$  channels have been linked to processes in prostate cancer cells including neuroendocrine induced differentiation and proliferation<sup>45-48</sup>.

Recent studies have begun to identify a role for microRNA regulation in the remodelling of the expression of specific calcium transporters in some cancers. This is exemplified by the ability of the cancer related microRNA miR-25 when overexpressed in HeLa cells to dramatically reduce levels of MCU, as well as the association between high levels of miR-25 and low levels of MCU in colon cancer cells, the consequence of which may be reduced apoptosis sensitivity in some types of cancers<sup>49</sup>. Another example is the association between ORAI1 overexpression (implicated in increased proliferation) and reduced miR-519 levels in some colorectal cancer cell lines and colorectal cancer tissues<sup>50</sup>.

Despite the profound changes in the expression of  $\text{Ca}^{2+}$  channels and pumps that can occur in some cancers, it is important to note that changes in expression per se are not necessarily sufficient to make a contribution to oncogenic machinery. For example, the overexpression of a  $\text{Ca}^{2+}$  channel without constitutive activity may have no consequence on proliferative signalling or invasion pathways if it lacks the signals required for its activation. In terms of mechanism, the consequences to calcium homeostasis of a channel remaining open may be far more significant than changes in its expression.

### ***[H3] Activity remodelling***

$\text{Ca}^{2+}$  sensitive tumour promoting pathways could be effected even in the absence of altered expression of  $\text{Ca}^{2+}$  channels and pumps by the array of mechanisms regulating the activity of calcium channels and pumps, including activity regulating proteins (e.g. STIM1), post-translational modifications, splicing and trafficking<sup>36,51-55</sup>. For example the proteolytic cleavage of some  $\text{Ca}^{2+}$  channels and pumps can yield either inactive or more active forms<sup>56-59</sup>, and in the case of proteolytic cleavage of  $\text{Ca}_v1.2$  can produce a C-terminal fragment that acts as a transcription factor<sup>54</sup>. The role of proteolytic cleavage requires further investigation in the context of the potential roles of other  $\text{Ca}^{2+}$  channels and pumps in cancer models.

Further complexity is introduced when considering that activation mechanisms may be altered in a cancer or a cancer subtype. For example, ORAI3 is activated by  $\text{Ca}^{2+}$  store depletion and contributes to SOCE in oestrogen receptor positive but not in oestrogen negative breast cancer cell lines, however, the mechanism by which this occurs is still not fully understood<sup>60</sup>. In some prostate cancer cells, ORAI3 forms a heteromeric channel with ORAI1 which is activated by arachidonic acid<sup>61</sup>. The cellular signaling diversity offered by dynamic formation of various heteromeric channels compared with monomeric channels may provide some tumours with a growth advantage. Indeed the switch from ORAI1 monomeric channels to ORAI3-ORAI1 heteromeric channels in prostate cancer cells provides resistance to apoptosis through reduced SOCE (due to reduced Orai1 monomers) and increased proliferative signalling via arachidonic acid (as a result of increased ORAI3-ORAI1 heteromers)<sup>61</sup>. Clearly the calcium signal can be modulated via diverse mechanisms, not simply just by changes in expression.

In some cases the contribution of ion channels and pumps is attributed to indirect effects or even to functions independent of their own ion transport abilities<sup>36,62-65</sup>. ORAI1-mediated promotion of TRPV6 plasmalemmal localisation and subsequent enhancement of prostate cancer cell proliferation<sup>63</sup> is one such example, and cleavage of TRPM7 (an ion channel with a kinase domain) producing kinase domain containing fragments capable of chromatin remodelling and subsequent changes in gene expression<sup>66</sup> is another example.

### **[H1] Ca<sup>2+</sup> signal intersection with cancer**

Given the vital role of Ca<sup>2+</sup> signalling in so many cellular processes<sup>1,4</sup> it is unsurprising that the calcium signal can regulate cancer associated processes and pathways. However, studies have identified specific contributions of Ca<sup>2+</sup> homeostasis in selectively regulating oncogenic and tumour suppressor pathways in cancer cells. This is seen in the ability of BAPTA-AM-mediated chelation of [Ca<sup>2+</sup>]<sub>CYT</sub> to inhibit epidermal growth factor (EGF)-induced phosphorylation of signal transducer and activator of transcription 3 (STAT3) without inhibiting EGF-induced EGF receptor (EGFR), ERK1 and ERK2 or AKT1 phosphorylation<sup>67</sup>. Not surprisingly then, the examples described below often relate to nuanced regulation of tumourigenic pathways by Ca<sup>2+</sup>, frequently through spatially and/or temporally defined changes in Ca<sup>2+</sup>.

Specific examples of the intersection between oncogenes, tumour suppressors and the Ca<sup>2+</sup> signal include the association between H-RAS driven tumours and caveolin 1-dependent changes in Ca<sup>2+</sup> signalling<sup>68</sup>, and the role of p53 in increasing pro-apoptotic calcium signals within mitochondria through increases in Ca<sup>2+</sup> release from the ER via direct interactions with the Ca<sup>2+</sup> store pump SERCA2<sup>69</sup>. Figure 3 provides an overview of the variety of processes relevant to cancer which have been linked to changes in the Ca<sup>2+</sup> signal.

### **[H3] Ca<sup>2+</sup> and proliferative signalling in cancer cells**

Ca<sup>2+</sup> signalling is linked to specific cell cycle events and its importance in cellular proliferation including the mechanisms by which this regulation occurs have been previously described<sup>7,8,70</sup>. Changes in the level of [Ca<sup>2+</sup>]<sub>CYT</sub> occur during cell cycle progression and division<sup>71-73</sup> and key steps of the cell cycle are Ca<sup>2+</sup> signal dependent including early entry into G1 as well as progression through the G1/S and G2/M stages<sup>8</sup>. The cell cycle events that are Ca<sup>2+</sup>-sensitive during cell cycle progression include but are not limited to, the early induction of *FOS*, *JUN* and *MYC*, the phosphorylation of RB1, the activity of calmodulin and **Ca<sup>2+</sup>/calmodulin-dependent protein kinases** (CaMKs) and the activity of a variety of Ca<sup>2+</sup> dependent transcription factors including NFAT, CREB and nuclear factor- $\kappa$ B (NF- $\kappa$ B)<sup>7,8,74</sup>.

However, increased Ca<sup>2+</sup> is clearly not solely sufficient for proliferation, as it is the nature of the change in cytosolic Ca<sup>2+</sup> that is important in promoting proliferation. More specifically, if increasing Ca<sup>2+</sup> was all that was required for cell proliferation then interventions that increased global or localised Ca<sup>2+</sup> levels would always be pro-proliferative. This is plainly not the case, as pharmacological activation of TRPV4 channels reduces the proliferation of tumour endothelial cells<sup>75</sup> whilst silencing of the Ca<sup>2+</sup> efflux pump PMCA2



reduces the proliferation of SKBR3 breast cancer cells<sup>76</sup>. Hence, the role of the calcium signal and the intersection with proliferation is context dependent.

Changes in the expression or activity of specific calcium channels and pumps in some cancers could bestow increased sensitivity to genetic or pharmacological modulation of these proteins compared to the majority of normal cells in the body. This is particularly the case for calcium channels and pumps that are not ubiquitously expressed and therefore would not be regarded as universal regulators of cellular proliferation. This is highlighted by the anti-proliferative effects of experimental TRPM8 inhibition on prostate cancer cells<sup>77</sup>; this effect is achieved due to the higher levels of TRPM8 in many prostate cancers compared with the normal prostate and the relatively restricted distribution of this channel in other tissues<sup>32</sup>. Many studies have identified specific Ca<sup>2+</sup> channels or pumps whose reduced expression and/or pharmacological inhibition can suppress proliferation of cancer cell lines *in vitro* and *in vivo*<sup>6,8,78-82</sup>. One recent study showed that in a mouse model of HER2-overexpressing breast cancer, deletion of PMCA2 resulted in less tumour growth<sup>76</sup>. In addition to suggesting that PMCA2 may be a particularly appropriate therapeutic target for HER2 positive breast cancers, this study also highlights an effective and new approach for future studies wishing to define the role of other calcium channels and pumps in this and other transgenic animal cancer models.

### ***[H3] Ca<sup>2+</sup> signalling in the primary and metastatic tumour microenvironment***

The microenvironment of both the primary tumour and metastatic sites is a key player in tumor progression<sup>37,83</sup> and the calcium signal represents a potential means through which the tumour microenvironment (e.g. hypoxia, pH, immune cells, cancer associated fibroblast secreted factors) can signal to cancer cells. The important role of Ca<sup>2+</sup> signals in the diverse cell types in the stroma surrounding the primary tumour and at metastatic sites or even in the pre-metastatic niche and how these might contribute to disease progression is still relatively unexplored.

The tumour microenvironment is a driver of angiogenesis, which is required for the maintenance of tumour growth<sup>84</sup>. TRPV4 is crucial for processes important to tumour angiogenesis, as reflected in its upregulation in endothelial cells derived from breast cancers where it facilitates arachidonic acid-mediated increases in [Ca<sup>2+</sup>]<sub>CYT</sub>, mechanosensitivity and migration<sup>85,86</sup>. Indeed, pharmacological activation of TRPV4 enables sub-therapeutic doses of cisplatin to become effective in suppressing tumour growth in a syngeneic mouse model of Lewis lung carcinoma by partially reversing abnormal tumour vasculature<sup>86</sup>.

Alterations in Ca<sup>2+</sup> signalling in cancer cells invoked by immune cells and the role of Ca<sup>2+</sup> signalling in the regulation of key processes in immune cells is another example of the interplay between Ca<sup>2+</sup> signalling and cells of the tumor microenvironment. C-C motif chemokine 18 (CCL18), a chemokine released from tumor-associated macrophages and that promotes breast cancer metastasis, seems to act, at least in part, via Ca<sup>2+</sup> signals elicited downstream of the chemokine receptor membrane-associated phosphatidylinositol transfer protein

3 (PITPNM3) in breast cancer cells<sup>87</sup>. Interventions in ER Ca<sup>2+</sup> signalling in CD4<sup>+</sup>T-lymphocytes are a proposed mechanism to restore some immune function to the tumour microenvironment<sup>88</sup>. Furthermore, there is a direct role for calcium signals in regulating cancer cell death induced by cytotoxic T lymphocytes and natural killer cells (reviewed in detail in ref.<sup>89</sup>).

The calcium signal interacts with many microenvironmental factors such as oxygen tension, pH, growth factors and other signalling molecules. For example, STIM1 is an important driver of hypoxia-mediated changes that contribute to the progression of hepatocarcinoma; this occurs through a complex mutual interplay whereby STIM1-regulated Ca<sup>2+</sup> influx increases hypoxia-inducible factor-1 $\alpha$  (HIF1 $\alpha$ ) stability and HIF1 $\alpha$  in turn regulates STIM1 transcription<sup>90</sup>. The ability of Ca<sup>2+</sup> influx to regulate hypoxia-mediated increases in HIF1 $\alpha$  is also evident in glioma cells as TRPC6-mediated Ca<sup>2+</sup> influx regulates HIF1 $\alpha$  hydroxylation levels and thus HIF1 $\alpha$  stability<sup>91</sup>. The acidic nature of the tumour microenvironment also alters cytosolic and mitochondrial free Ca<sup>2+</sup> levels via acid-sensing ion channel 1 (ASIC1), the consequence of which is induction of reactive oxygen species (ROS) and activation of NF- $\kappa$ B in breast cancer cells. Indeed silencing of ASIC1 reduces tumour growth and metastasis in an *in vivo* mouse xenograft model<sup>92</sup>.

Cancer cells that metastasise will have different requirements for survival and growth at the metastatic site compared with that of the primary tumour. An integrated genomic and transcriptomic analysis reported that lower levels of the intracellular Ca<sup>2+</sup> store release channel IP<sub>3</sub>R1 (encoded by the gene *ITPR1*) is associated with metastasis to the brain but not to other organs<sup>93</sup>. A higher propensity for mutations which were predicted to be deleterious in *ITPR1* was also observed in brain metastases arising from breast cancers<sup>93</sup>. The very different growth environment in the brain may be favourable for the selection of breast cancer cells with reduced IP<sub>3</sub>R1 levels and/or activity, which may confer increased resistance to cell death from ER stress or apoptotic stimuli. A direct example of the interplay between cancer cells and the stromal cells of the metastatic niche is found in the establishment of abdominal metastasis in serous ovarian cancer<sup>94</sup>. This metastatic pathway involves Ca<sup>2+</sup> signalling in ovarian cancer cells induced by adipocytes at the metastatic niche. More specifically, Ca<sup>2+</sup> dependent phosphorylation of salt-inducible kinase 2 (SIK2) in ovarian cancer cells leads to the subsequent activation of fatty acid oxidation and AKT phosphorylation and as a consequence, augmentation of proliferative and pro-survival pathways<sup>94</sup>. Another example of the potential importance of the metastatic microenvironment and calcium is seen in the proposal that high levels of extracellular Ca<sup>2+</sup>, due to bone remodeling, is a driver for bone metastasis in cancers that express the **extracellular Ca<sup>2+</sup> sensing receptor (CaSR)**, which is also linked to the proliferation of some cancer cell lines such as BT-474 and MDA-MB-231 breast cancer cells and also PC-3 prostate cancer cells<sup>95,96</sup>. A better understanding of the remodelling of Ca<sup>2+</sup> signalling in cancer cells within different metastatic sites and calcium signalling induced by stromal cells and events in the metastatic niche, may help to define a novel set of molecular therapeutic targets and provide new insights into disease progression. Such studies should continue to be the focus of future research.

### ***[H3] Ca<sup>2+</sup> and pathways involved in cancer cell migration, invasion and metastasis***

The dissemination of cancer to other organs during metastasis requires the migration and invasion of cancer cells from the primary tumour<sup>37</sup>. Almost every step in the migration and invasion of cancer cells, from processes that influence the first responses to pro-migratory stimuli, to the rate of movement, directional control, and the release of enzymes that degrade the extracellular matrix have some degree of calcium signal dependence<sup>7,9,97</sup>. Here, we highlight some specific studies to provide examples of the mechanisms by which the calcium signal can contribute to these diverse metastatic processes.

Many pro-migratory signals act on GPCRs that couple to pathways that can mobilise Ca<sup>2+</sup> from internal stores. One example is the activation of the GPCR protease activated receptor-1 (PAR1) on cancer cells by matrix metalloprotease-1 (MMP-1) secreted by fibroblasts of the tumour stroma, which contributes to invasion of breast cancer cells<sup>98</sup>. Studies have consistently shown clear differences in the nature of [Ca<sup>2+</sup>]<sub>CYT</sub> signals between the leading and trailing edge of migrating cells, with often highly polarised and localised changes in free Ca<sup>2+</sup> and consistently higher Ca<sup>2+</sup> levels at the trailing edge<sup>99-102</sup>. In addition to contributing to forward motion through activating contractile proteins at the trailing edge, Ca<sup>2+</sup> signalling is also important in modifications in focal and peripheral adhesion molecules often through effecting turnover via Ca<sup>2+</sup> dependent phosphorylation or enzymatic cleavage. Furthermore, Ca<sup>2+</sup> changes can also affect key cytoskeletal proteins throughout the cell<sup>9</sup>. Silencing of components of SOCE (ORAI1 or STIM1) or pharmacological inhibition of this pathway modulates focal adhesion turnover in MDA-MB-231 cells and also reduces the invasiveness of this cell line<sup>103</sup>.

Localised calcium changes may also serve to maintain the polarised cell morphology required for cell migration<sup>104</sup>. Transient (~0.5 s) high levels of localised Ca<sup>2+</sup> at the leading edge of migrating cells, termed “Ca<sup>2+</sup> flickers”, control the direction in which cells move<sup>25</sup>. These studies in human WI-38 lung fibroblasts identified that Ca<sup>2+</sup> influx through TRPM7 in addition to IP<sub>3</sub> mediated Ca<sup>2+</sup> release from the ER was crucial in responding to platelet derived growth factor (PDGF) concentration gradients and subsequent directional migration<sup>25</sup>. The aforementioned studies place the calcium signal as both the navigator and a key component of the engine in some types of cell motility (Fig 2). Further work is now required to define the Ca<sup>2+</sup> channels involved in these specific migration modes in cancer cells; these studies will need to assess cancer cells originating from different organs as different cancers may exploit different types of Ca<sup>2+</sup> channels to achieve and control migration.

For invasion of cancer cells through the extracellular matrix, Ca<sup>2+</sup> influx pathways can induce the expression of pro-invasive enzymes, such as particular MMPs and cathepsin B as observed in prostate cancer cells via the Ca<sup>2+</sup> permeable ion channel TRPV2<sup>105</sup>. Ca<sup>2+</sup> influx is also important in the formation of **invadopodia** via ORAI1-dependent Ca<sup>2+</sup> oscillations in melanoma cells which occurs in part through promotion of SRC (pY416) phosphorylation<sup>106</sup>. Ca<sup>2+</sup> can

also contribute to steps in the metastatic cascade through augmentation of other pathways, such as ROS production, which subsequently increases cell migration in lung cancer cells<sup>107</sup>, and cAMP pathways, which increase the invasiveness of a highly metastatic variant of MDA-MB-231 cells *in vitro*<sup>108</sup>. Further evidence of the involvement of Ca<sup>2+</sup> in cancer cell migration, invasion and metastasis derives from silencing and/or pharmacological inhibition of proteins involved in Ca<sup>2+</sup> signalling, including some of the recently identified molecular components of mitochondrial Ca<sup>2+</sup> regulation, which have been shown to inhibit each of these processes (e.g. ref<sup>103,105,109,110</sup>). The demonstrated ability to inhibit the migration of MDA-MB-231 cells *in vitro* and the growth and metastasis of MDA-MB-231 cells to the lungs *in vivo* in a mouse xenograft model of breast cancer by silencing MCU (in this case likely through lower mitochondrial Ca<sup>2+</sup> levels reducing mitochondrial ROS production and subsequently HIF1 $\alpha$  levels) is just one example of studies that have defined roles for specific calcium transporting proteins during cancer progression<sup>110</sup>.

Specific tumour microenvironmental factors, such as EGF and hypoxia induce epithelial to mesenchymal transition (EMT) in cancer cells, which may promote increased invasiveness and therapeutic resistance<sup>111</sup>. Ca<sup>2+</sup> homeostasis is clearly different between the more epithelial and the more mesenchymal cell phenotypes<sup>112-114</sup>, with attenuation of both SOCE and ATP-mediated changes in [Ca<sup>2+</sup>]<sub>CYT</sub> occurring as a consequence of EMT in MDA-MB-468 breast cancer cells<sup>67,113-115</sup>. Such changes in Ca<sup>2+</sup> signalling are perhaps expected given the major changes in cellular morphology and protein expression that occur as a result of EMT<sup>111</sup>. However, the Ca<sup>2+</sup> signal itself is integral to the induction of EMT, and ion channels such as TRPM7 and TRPC6 can differentially contribute to the induction of specific EMT associated markers in different models, such as the regulation of EGF-induced vimentin expression by TRPM7 in MDA-MB-468 breast cancer cells<sup>67,116</sup>. It should be noted that the contribution of Ca<sup>2+</sup> channels and pumps to Ca<sup>2+</sup> changes during cancer cell migration and invasion will likely vary between cancer types and with the nature of the stimuli.

### **[H3] The Ca<sup>2+</sup> signal and cancer cell death**

Ca<sup>2+</sup> plays an important role in pathways relevant to cancer cell death<sup>2,7,8,24,82,117,118</sup> and the remodelling of calcium channel or pump expression may allow this relationship to be exploited to the benefit of the tumour. For example, in breast cancer cells, the overexpression of PMCA2 bestows increased resistance to cell death by increasing the ability of cells to extrude Ca<sup>2+</sup> after Ca<sup>2+</sup> overload induced by a **calcium ionophore**<sup>119</sup>. In some cases the contribution of a specific protein involved in calcium signalling may be through localised or more subtle changes in Ca<sup>2+</sup> signalling, such as the promotion of BCL-2 inhibitor (ABT-263)-induced cell death by silencing PMCA4 in MDA-MB-231 cells without obvious global changes in [Ca<sup>2+</sup>]<sub>CYT</sub> homeostasis<sup>120</sup>.

Although much remains to be understood about the precise relationship between Ca<sup>2+</sup> and the different modes of cell death, it is clear that sustained and large increases in [Ca<sup>2+</sup>]<sub>CYT</sub> can be associated with necrotic cell death. Ca<sup>2+</sup> overload by pharmacological activation of an overexpressed ion channel<sup>121-123</sup> or **calcium electroporation**<sup>124</sup> can induce necrosis in cancer cells. Accumulation of

intracellular  $\text{Ca}^{2+}$  can also trigger **necroptosis** in human neuroblastoma cells through a pathway involving phosphorylation of the death receptor kinase receptor interacting serine/threonine protein kinase 1 (RIPK1) by CaMKII<sup>125</sup>.

The accumulation of  $\text{Ca}^{2+}$  ions in mitochondria has long been associated with apoptotic cell death, with the increases in mitochondrial matrix  $\text{Ca}^{2+}$  induced by some stimuli or stresses contributing to the opening of the relatively non selective mitochondrial permeability transition pore (a collection of proteins) that contributes to the loss of mitochondrial membrane integrity and the subsequent release of pro-apoptotic factors from mitochondria<sup>117</sup>. Additionally there is a complex interplay between mitochondria, apoptotic regulating BCL-2 family members, and the  $\text{Ca}^{2+}$  release channels of the ER<sup>126,127</sup>. Some cancer cells (e.g. large B-cell lymphoma cells) are sensitive to experimental peptide-mediated disruption of the interaction between IP<sub>3</sub>Rs and BCL-2, the consequence of which is cell death<sup>128,129</sup>. The intersection between calcium stores, cell death and key players in tumor progression in cancer cells is not restricted to BCL-2. In addition to the association between p53 and ER  $\text{Ca}^{2+}$  levels via effects on SERCA activity discussed above<sup>69</sup>, oncogenic K-RAS (K-RASG13D) appears to attenuate  $\text{Ca}^{2+}$  release from the ER following apoptotic stimuli, which subsequently reduces  $\text{Ca}^{2+}$  accumulation in the mitochondria and promotes colon cancer cell survival<sup>130</sup>. Alternatively, the tumour suppressor BRCA1 can promote  $\text{Ca}^{2+}$  release from the ER during apoptosis through direct effects of IP<sub>3</sub>Rs<sup>131</sup>.

The relationship between the IP<sub>3</sub>R channels of internal  $\text{Ca}^{2+}$  stores and mitochondria is also evident in the regulation of **autophagy**. When the small constitutive  $\text{Ca}^{2+}$  release from IP<sub>3</sub>Rs of the ER to the mitochondria (which is required for resting mitochondrial bioenergetics) is interrupted (such as through pharmacological inhibition of IP<sub>3</sub>Rs by xestospongine B), pro-survival autophagy is induced<sup>132</sup>. However, in at least some cancer cell lines (e.g. MCF-7 and T47D breast cancer cells), the disruption of this ER to mitochondria  $\text{Ca}^{2+}$  transfer results in cell death<sup>133</sup>. Hence, the basal uptake of  $\text{Ca}^{2+}$  into mitochondria via this pathway is essential for the survival of some cancer cells and this observation adds a new dimension to the selective targeting of malignancies through the targeting of specific and localised  $\text{Ca}^{2+}$  signals. Clearly, there is complex interplay between mitochondria and the ER in the context of  $\text{Ca}^{2+}$  and cell death sensitivity. It appears, from the studies described above that many cancers differentially remodel this interaction to achieve a survival advantage. The significance of ER  $\text{Ca}^{2+}$  is not restricted to IP<sub>3</sub>Rs and the mitochondria. This is recently reflected by the role of ryanodine receptor calcium store release channels in TNF-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis in MDA-MB-231 cells, likely through calcineurin dependent activation of dynamin-1<sup>134</sup>.

### **[H1] The $\text{Ca}^{2+}$ signal and therapy resistance**

Some of the major recent advances in our understanding of the nexus between calcium signalling and cancer are provided by studies assessing how the calcium signal can regulate the response to therapeutic agents and the acquisition of therapeutic resistance. Work assessing the role of the calcium signal in augmenting or attenuating responses to clinically used agents has produced

some interesting observations. Recent examples include the promotion of the *in vitro* anti-proliferative effects of low concentrations of the chemotherapeutic agent doxorubicin by PMCA2 silencing in MDA-MB-231 cells<sup>135</sup>, and the sensitisation of human ovarian cells to carboplatin chemotherapy by the T-type  $\text{Ca}^{2+}$  channel blocker mibefradil in an *in vivo* xenograft mouse model<sup>136</sup>.

While this is an emerging area, more mechanistic detail is known for MCF-7 breast cancer cells that acquire resistance to doxorubicin. These cancer cells exhibit increased expression and function (as assessed by  $\text{Ca}^{2+}$  influx) of the ion channel TRPC5. As a consequence, TRPC5 upregulation results in increased expression of multidrug resistance protein 1 (MDR1), which is associated with resistance to several chemotherapeutic agents<sup>137</sup>. Disrupting TRPC5 expression or activity by gene silencing or antibody mediated functional inhibition suppresses the induction of MDR1, restoring doxorubicin sensitivity in resistant MCF-7 cells *in vitro* and in an *in vivo* xenograft mouse model of breast cancer<sup>137</sup>. TRPC5 may be gained by a cancer cell via intercellular transfer of extracellular vesicles containing TRPC5<sup>138</sup> leading to the acquisition of resistance. This represents a powerful way for cancer cells to propagate multidrug resistance within a tumour. The greater levels of TRPC5 containing extracellular vesicles in women with breast cancers receiving chemotherapy is further support for an important role for this calcium signalling mechanism in the development of multidrug resistance in some patients with breast cancer<sup>138</sup>. Importantly these results also identify an alternative therapeutic approach through targeting TRPC5, to overcome MDR1-mediated drug resistance without global pharmacological inhibition of MDR1 activity. The recent identification that TRPC5 silencing reverses the resistance to the chemotherapeutic agent 5-fluorouracil (5-FU) in HCT-8 and LoVo colorectal cancer cells<sup>139</sup>, suggests that the role of TRPC5 is not confined to breast cancer, and that further exploration of the role of TRPC5 and potentially other calcium channels in the acquisition and propagation of multidrug resistance would be an exciting avenue to explore.

### **[H1] Targeting the calcium signal**

Throughout this review we have highlighted examples of how the silencing and/or pharmacological inhibition of specific  $\text{Ca}^{2+}$  permeable ion channels or pumps attenuates the proliferation or invasiveness of cancers. We have also described examples where  $\text{Ca}^{2+}$  influx through channel activation or calcium electroporation can promote cancer cell death. Indeed, calcium electroporation is currently undergoing a clinical trial for the treatment of cutaneous metastases in comparison to standard electroporation with the chemotherapeutic agent bleomycin (NCT01941901)<sup>140</sup>. Studies have also shown that the chelation of  $[\text{Ca}^{2+}]_{\text{CYT}}$  greatly reduces the induction of apoptosis by photodynamic therapy *in vivo*, and this effect may be dependent in at least some cases on p53<sup>141</sup>. Besides these observations, other opportunities exist to target the calcium signal in cancer. For example, a recent study highlights an exciting avenue of exploration and is reflective of the expanse and potential of the calcium-cancer signalling field to provide novel therapeutic strategies to treat cancer. This study involved an epigenetic screen of over 1000 FDA-approved drugs, in addition to drugs already known to induce epigenetic changes. From this screen, 11 drugs with the

ability to alter Ca<sup>2+</sup> signalling were identified as being able to reverse epigenetic-mediated suppression of tumour suppressor genes in colon cancer cells likely through CaMKs<sup>142</sup>.

The demonstrated ability to pharmacologically modulate Ca<sup>2+</sup> channels and pumps (Table 1), provides a compelling argument that when Ca<sup>2+</sup> signaling is implicated in a tumour progression pathway, a specific Ca<sup>2+</sup> channel or pump should be seriously considered as a potential drug target. The viability and phenotypes of knockout animals for such identified targets, as well as the effects of pharmacological modulators (if available) in animal models, may act as early indicators of whether such proteins may be appropriate therapeutic targets. Indeed, the repurposing of existing drugs may represent rapid opportunities in this area.

One case in point is the interaction between K-RAS and calmodulin and the subsequent suppression of the non-canonical WNT (or WNT–Ca<sup>2+</sup> signalling) pathway, which is crucial for K-RAS oncogenesis<sup>143</sup>. Disrupting K-RAS–calmodulin binding was described as a unique and ‘drug-able’ opportunity to target K-RAS in pancreatic cancer<sup>143</sup>. However, the identification of Ca<sup>2+</sup> channels that when activated or inhibited can regulate the association between calmodulin and K-RAS would expand the therapeutic target repertoire of cancer treatments. Particularly given the often highly localised role of calcium channels in the regulation of CaMKII<sup>144</sup>, which is a key conduit in this K-RAS malignant pathway<sup>143</sup>.

There are clear examples (some of which have already been discussed in this Review), where specific isoforms of a channel or pump or a specific heteromeric channel is remodelled or contributes to a particular cancer relevant pathway. Hence, in many cases the optimal target may be a specific isoform of a Ca<sup>2+</sup> pump, channel or even a specific form of a heteromeric channel. For example, PMCA4 but not PMCA1 silencing promotes ABT-263 mediated cell death in MDA-MB-231 cancer cells, but this is not due to a more important role for PMCA4 in global Ca<sup>2+</sup> regulation<sup>120</sup>. Instead, this effect is likely due to either distinct regulators of PMCA1 and PMCA4, or the different contributions of the isoforms to localised Ca<sup>2+</sup> signalling due to their potentially different locations on the plasma membrane relative to other proteins and/or their different affinities for the Ca<sup>2+</sup> ion itself. All of these factors would have an impact on the activation of downstream Ca<sup>2+</sup>-sensitive proteins.

One of the newer areas addressed in this review has been the consideration of Ca<sup>2+</sup> signaling in the context of the microenvironment of the primary tumour and metastatic sites. These areas are a clear opportunity for therapeutic intervention worthy of further assessment. In addition to directly targeting those Ca<sup>2+</sup> channels or pumps within stromal cells, factors in the tumour microenvironment may also be directly targeted to induce Ca<sup>2+</sup>-mediated effects on cancer cells. Such is the case for a SERCA pump inhibitor in the form of a thapsigargin-based protease activated prodrug (mipsagargin, see Table 1), designed to be activated by prostate specific membrane antigen (PSMA). PSMA is rich in the microenvironment of many solid tumours and therefore, facilitating a localised

release of the SERCA pump inhibitor within a tumour would reduce cancer cell viability, whilst avoiding high systemic toxicity<sup>145</sup>. This agent is now in the first stages of clinical assessment for refractory prostate cancer therapy<sup>146</sup>.

## Conclusion

As the numerous examples outlined above attest to, the calcium signal is not a blunt instrument but is nuanced and contextual and is intricately involved in regulating key cellular mechanisms and pathways. How the signal is modulated and the downstream pathways affected may contribute to outcomes as varied as the evasion of cell death pathways, cancer cell growth, invasion and metastasis, cancer multi-drug resistance, and the regulation of tumour growth and survival by the primary and metastatic tumour microenvironments. The challenge is now to define how to best utilise the pharmacological opportunities offered by calcium channels and pumps to selectively target these processes and improve patient outcomes.

## References

**Acknowledgements:** This work is supported by the National Health and Medical Research Council of Australia (1079671 and 1079672). GM is supported by the Mater Foundation. The Translational Research Institute is supported by a grant from the Australian Government.

## Conflict of Interest

GM and SRT are associated with QUE-Oncology Inc.

## Box 1: Tools for the assessment of calcium signals in cancer cells.

Small molecule based probes undergo increases in fluorescence upon calcium ion ( $\text{Ca}^{2+}$ ) binding and can be delivered to cells as an acetomethyl (AM) ester<sup>147</sup>. Probes with affinities for  $\text{Ca}^{2+}$  suitable for the assessment of cytosolic free  $\text{Ca}^{2+}$  include ratio-metric probes (dual excitation, e.g. Fura-2, or dual emission, e.g. Indo-1), which are better suited to account for variances in dye loading<sup>148</sup> and single wavelength probes that typically have a superior dynamic range and a greater repertoire of emission and excitation wavelengths, which is often crucial with fluorescently tagged proteins. Single wavelength probes include Fluo-4 and Rhod-2, which have green and red fluorescence emissions, respectively<sup>149,150</sup>.

The development of genetically targeted fluorescent  $\text{Ca}^{2+}$  sensors has accelerated in recent years with a diversity of sensors with good dynamic ranges now available that can be targeted to specific cellular domains or organelles<sup>151,152</sup>.



Small molecule and genetically targeted  $\text{Ca}^{2+}$  sensors are available with different affinities for  $\text{Ca}^{2+}$ ; some are suited to the assessment of the high  $\text{Ca}^{2+}$  levels associated with certain forms of cancer cell death (e.g. Fura-2FF<sup>153</sup>), and those found within intracellular organelles, such as the endoplasmic reticulum (e.g. genetically targeted LAR-GECO and similar sensors) <sup>154</sup>.

$\text{Ca}^{2+}$  levels in cells can be assessed using a variety of methods from low throughput cuvette measurements to ultra-high throughput methods using microplates<sup>155</sup>. Imaging with confocal, multi-photon, total internal reflection fluorescence (TIRF) microscopy and fluorescence lifetime imaging microscopy (FLIM) are examples of other approaches<sup>156-159</sup>. Microscopic assessment can be carried out in cells lines, isolated tissue or even *in vivo*<sup>151</sup> including some studies in tumour masses in skin folds<sup>141</sup>. In addition to these light based approaches, electrophysiology methods often allow nuanced evaluation and characterisation of  $\text{Ca}^{2+}$  influx mechanisms<sup>60</sup>.

**Fig 1: Examples of  $\text{Ca}^{2+}$  permeable ion channels, pumps and exchangers of the plasma membrane and intracellular organelles.** The level of free  $\text{Ca}^{2+}$  [ $\text{Ca}^{2+}$ ] is much higher in the extracellular fluid (ECF) (> 1 mM) compared with that of the resting free calcium in the cytosol ([ $\text{Ca}^{2+}$ ]<sub>CYT</sub>; ~ 100 nM). This gradient is maintained by the active transport of  $\text{Ca}^{2+}$  across the plasma membrane via the plasma membrane  $\text{Ca}^{2+}$ -transporting ATPase (PMCA) pumps. This pump can also contribute to the decline in [ $\text{Ca}^{2+}$ ]<sub>CYT</sub> levels after cell stimulation. One mechanism to increase [ $\text{Ca}^{2+}$ ]<sub>CYT</sub> is the opening of plasma membrane  $\text{Ca}^{2+}$  permeable ion channels such as those that are voltage gated (subdivided into L-type, T-type, P/Q-type, R-type and N-type; an example of an L-type channel is shown), or members of the transient receptor potential (TRP) family, such as the cool and menthol (found in mint) activated TRPM8, the heat and capsaicin (found in hot chilli peppers) activated TRPV1, and the highly  $\text{Ca}^{2+}$  selective channel, TRPV6 that has constitutive activity in some cells. [ $\text{Ca}^{2+}$ ]<sub>CYT</sub> levels can also increase by  $\text{Ca}^{2+}$  store release, such as via inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ), which activates the  $\text{IP}_3$  receptors ( $\text{IP}_3\text{Rs}$ ) of the endoplasmic reticulum (ER). This  $\text{IP}_3$  can be generated by stimulation of some G-protein-coupled receptors (GPCRs) or receptor tyrosine kinases (such as the epidermal growth factor receptor (EGFR), through activation of phospholipase C $\beta$  (PLC $\beta$ ) and PLC $\gamma$  isoforms, respectively. The depletion of these intracellular  $\text{Ca}^{2+}$  stores is detected by the  $\text{Ca}^{2+}$  sensor stromal interaction molecule 1 (STIM1), which can activate an calcium release-activated calcium channel protein 1 (ORAI1) dependent  $\text{Ca}^{2+}$  influx pathway to promote  $\text{Ca}^{2+}$  store refilling through the  $\text{Ca}^{2+}$  sequestration pump - the sarcoplasmic/ER  $\text{Ca}^{2+}$ -ATPase (SERCA).  $\text{Ca}^{2+}$  sequestration into the Golgi is mediated by a secretory pathway  $\text{Ca}^{2+}$ -ATPase (SPCA), which can also actively transport  $\text{Mn}^{2+}$  ions. Other intracellular organelles have  $\text{Ca}^{2+}$  transporting proteins, such as the mitochondrial calcium uniporter (MCU) and the mitochondrial  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (NCLX) and the two pore channels (TPC) of lysosomes. Note that some of the transporters shown have multiple isoforms, and not all regulators nor all families of  $\text{Ca}^{2+}$  permeable ion channels, pumps or exchangers are depicted<sup>79,82,160-164</sup>. NAADP, nicotinic acid adenine dinucleotide

phosphate.

**Fig 2: Ca<sup>2+</sup> signal diversity.** This figure illustrates the diversity in Ca<sup>2+</sup> signalling and how this may contribute to processes that are relevant in the context of cancer progression. This diversity in Ca<sup>2+</sup> signalling and its consequences include, but are not limited to: A) The degree of increase in cytosolic free calcium ([Ca<sup>2+</sup>]<sub>CYT</sub>) (amplitude modulation - AM), which may control cell fate (e.g. proliferation or cell death); the different coloured lines on the graph represent cytosolic Ca<sup>2+</sup> increases of different amplitude. B) The frequency of [Ca<sup>2+</sup>]<sub>CYT</sub> oscillations (frequency modulation - FM), which may control the nature of gene transcription and hence different cellular outcomes and expression remodelling; the different coloured lines on the graph represent cytosolic Ca<sup>2+</sup> increases of different oscillation frequencies. C) Highly localised and transient increases in Ca<sup>2+</sup> at the edge of some cells (Ca<sup>2+</sup> flickers), which control directional migration; D) Free Ca<sup>2+</sup> levels within the endoplasmic reticulum (ER) that may influence cell stress and the sensitivity to apoptotic stimuli, which act in part through the release of Ca<sup>2+</sup> from internal stores; E) the confined transfer of Ca<sup>2+</sup> ions from the ER to the mitochondria that when reduced may induce autophagy or even death in some cancer cells; F) Levels of Ca<sup>2+</sup> ions within the mitochondrial matrix where modest changes may promote ATP synthesis but sustained high levels may promote cell death; G) Localized very high levels of Ca<sup>2+</sup> (known as Ca<sup>2+</sup> microdomains) achieved upon the opening of plasmalemmal Ca<sup>2+</sup> channels can specifically regulate gene transcription pathways. In some cells, Ca<sup>2+</sup> bound calmodulin (CaM) produced near the opening of Cav1 channels can be delivered by Ca<sup>2+</sup>/calmodulin-dependent protein kinase II subunit gamma (CaMKII $\gamma$ ) to the nucleus to promote the phosphorylation of cAMP responsive element-binding protein (CREB) and gene transcription; in other cellular systems, localized Ca<sup>2+</sup> increases produced upon the opening of ORAI1 channels is sufficient via a series of signalling pathways (indicated by the small dashed arrow and the '+' symbol) to produce the translocation of nuclear factor of activated T cells 1 (NFAT1) to the nucleus. In contrast ORAI1-activated gene transcription via NFAT4 also requires increases in nuclear Ca<sup>2+</sup> (not shown on this figure). Notes:  $\Delta$  denotes changes in a pathway or levels,  $\uparrow$  denotes an increase in levels or activity. Refs<sup>22,25,28,126,133,165-168</sup>.

**Fig 3. The calcium signal and tumor progression.** The calcium signal often intersects through specific calcium permeable ion channels and calcium pumps, with a variety of processes relevant to the growth, metastasis and death of cancer cells. Refs<sup>8,9,67,68,82,85,86,89,93,94,117,124,127,133</sup>.

**Table 1: Available pharmacological modulators of some of the calcium (Ca<sup>2+</sup>) permeable ion channels, pumps and exchangers.** Where possible the IUPHAR/BPS Guide to pharmacology<sup>169</sup> and associated material has been used to construct this table. Agents that have been widely reported to have effects on multiple targets (other than closely related isoforms) have not been listed here (refs<sup>79,82</sup>).

| Target                 | Example Inhibitor(s) | Example Activator(s) |
|------------------------|----------------------|----------------------|
| Ca <sup>2+</sup> pumps |                      |                      |

|   |   |   |
|---|---|---|
| PMCA  | Caloxin 2A1 <sup>170</sup> , caloxin 1b3 (reported to be more selective for PMCA1) <sup>171</sup> , caloxin 1b1 (more selective for PMCA4) <sup>172</sup> | -   |
| SERCA   | Thapsigargin, cyclopiazonic acid, and BHQ <sup>173</sup> , mipsagargin (prodrug) <sup>145</sup> .   | Ochratoxin A <sup>173</sup> .   |
| <b>Ca<sup>2+</sup> permeable plasma membrane ion channels</b>       |   |   |
| L-type voltage gated Ca <sup>2+</sup> channel                       | Diltiazem, nifedipine verapamil <sup>174</sup>  | (-)-(S)-BayK8644, FPL64176, SZ(+)-(S)-202-791 <sup>174</sup>                    |
| T-type voltage gated Ca <sup>2+</sup> channel                       | Mibefradil <sup>174</sup>   | -   |
| TRPC5   | ML204 <sup>175</sup>  | (-)-englerin A <sup>122</sup> .   |
| TRPC6   | Larixyl Acetate <sup>176</sup>  | Hyperforin <sup>177,178</sup>   |
| TRPV1   | AMG517, SB366791, JNJ17203212 <sup>177</sup>  | Capsaicin, Resiniferatoxin <sup>177</sup> .                                     |
| TRPV4   | HC067047 <sup>177</sup>   | GSK1016790A <sup>177</sup> .  |
| TRPV6   | cis-22 a <sup>179</sup>   | -   |
| TRPM7   | Waixenicin A <sup>180</sup> , NS8593 <sup>181</sup>   | Mibefradil <sup>181</sup> (note also a T-type Ca <sup>2+</sup> channel blocker) |
| TRPM8   | PF-05105679 <sup>182</sup> , M8-B <sup>183</sup>  | WS-12 <sup>177,184</sup>  |
| Orai1-dependent SOCE  | YM-58483 <sup>185</sup> , Synta66 <sup>186</sup> , GSK-7975A <sup>187</sup>   | -   |
| <b>Endoplasmic Reticulum Ca<sup>2+</sup> permeable ion channels</b> |   |   |
| IP <sub>3</sub> Rs  | Xestospongins B <sup>188</sup>  | Adenophostin A <sup>188</sup>   |

IP<sub>3</sub>Rs, inositol 1,4,5-trisphosphate receptors; Orai1, calcium release-activated calcium channel protein 1; PMCA, plasma membrane Ca<sup>2+</sup>-transporting ATPases; SERCA, sarcoplasmic/endoplasmic reticulum Ca<sup>2+</sup>-ATPases; SOCE, store-operated calcium entry; TRP, transient receptor potential.

**Table 2: Examples of similar and diverse expression remodeling of Ca<sup>2+</sup> permeable ion channels in breast, prostate and colon cancer:** Although expression changes have been previously tabulated<sup>6,82</sup>, ion channel expression studies have still not yet been assessed sufficiently across different cancer types to make a comprehensive identification of which channels if any may be universally altered. However, it is already clear that some ion channels such as TRPV6 have increased expression across a variety of cancer types. In contrast, Orai1 is upregulated in basal molecular breast cancers where it may contribute to the invasiveness of this subtype, yet it is down regulated in prostate cancer which may contribute to apoptotic resistance. Microenvironmental factors (hypoxia, growth factors, pH) may also contribute to expression diversity within tumours and/or during disease progression.

| Ca <sup>2+</sup> permeable ion channel | Cancer Type   |   |                                     |
|--|---|---|-------------------------------------|
|  | Breast  | Prostate  | Gastric, Colorectal                 |
| <b>ORAI1</b>                           | <b>Increased (basal subtype)<sup>189</sup></b>                                | <b>Decreased (castration-resistant)<sup>190</sup></b> | <b>Increased<sup>191, 192</sup></b> |
| <b>ORAI3</b>                           | <b>Increased (oestrogen receptor positive)<sup>60</sup></b>                   | <b>Increased<sup>61</sup></b>                         | <b>not assessed</b>                 |
| <b>TRPV6</b>                           | <b>Increased (basal subtype and oestrogen receptor negative)<sup>42</sup></b> | <b>Increased<sup>193</sup></b>                        | <b>Increased<sup>194, 195</sup></b> |
| <b>TRPC6</b>                           | <b>Increased<sup>196</sup></b>  | <b>Increased<sup>197</sup></b>                        | <b>Reduced<sup>198</sup></b>        |
| <b>TRPM8</b>                           | <b>Increased<sup>32,33,199,200</sup></b>                                      | <b>Increased<sup>32,201,202</sup></b>                 | <b>Increased<sup>32</sup></b>       |

ORAI, calcium release-activated calcium channel protein; TRP, transient receptor potential

## Glossary

**Ca<sup>2+</sup> channel:** A protein or group of proteins which form a Ca<sup>2+</sup> ion permeable pore across a membrane which can be opened or closed by different stimuli.

**Ca<sup>2+</sup>/calmodulin-dependent protein kinases (CaMKs):** Serine/Threonine protein kinases whose activation is usually dependent on binding to calmodulin (CaM) in the Ca<sup>2+</sup> bound state.

**Ca<sup>2+</sup> pump:** A protein which through the hydrolysis of ATP can transport Ca<sup>2+</sup> ions against a concentration gradient also referred to as Ca<sup>2+</sup>-ATPases.

**Exchanger:** A transporter of ions which involves the exchange of one type of ion or ions for another type across a membrane which does not involve the direct cleavage of ATP.

**Store operated calcium entry (SOCE):** A Ca<sup>2+</sup> influx pathway activated upon the depletion of intracellular endoplasmic reticulum Ca<sup>2+</sup> stores. The canonical pathway involves Ca<sup>2+</sup> influx through ORAI1 after action by the endoplasmic reticulum Ca<sup>2+</sup> sensor STIM1.

**Invadopodia:** Plasma membrane protrusions associated with degradation of the extracellular matrix which is important in cancer cell invasion.

**Calcium ionophore:** A chemical moiety that can facilitate increases in [Ca<sup>2+</sup>]<sub>CYT</sub> independent of Ca<sup>2+</sup> channel activation.

**Autophagy:** A physiological process involving degradation of the cell's own components, which can be initiated by nutrient deficiency and can aid cell survival.

**Extracellular calcium-sensing receptor:** A plasmalemmal G-protein coupled receptor activated by changes in levels of extracellular free Ca<sup>2+</sup>.

**Calcium electroporation:** A process whereby an electrical field which increases the permeability of the plasma membrane of cells is applied in the presence of raised extracellular Ca<sup>2+</sup>.

**Necroptosis:** A regulated form of necrotic cell death.

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### Author Biographies

Greg Monteith is a professor in the School of Pharmacy and Mater Research Institute at The University of Queensland, Australia. He heads the Calcium Signaling in Cancer Research Laboratory. His current research is focused on the identification and characterization of calcium channels and pumps as novel therapeutic targets for the treatment of triple negative breast cancers.

Sarah Roberts-Thomson is a professor of pharmacy in the School of Pharmacy, The University of Queensland, Australia. She is also Associate Dean (Academic), Faculty of Health and Behavioural Sciences. She is a registered pharmacist. Her laboratory seeks to identify new drug targets that exploit altered calcium signalling in cancer cells.

Natalia Prevarskaya is a professor of physiology at the University of Lille, France. She is the director of the Cell Physiology Unit, INSERM U1003, certified by the INSERM (National Institute for Health and Medical Research). Her group is part of the Laboratory of Excellence in Ion Channels Science and Therapeutics. She leads the calcium signatures of prostate cancer team certified by the National League Against Cancer. Her scientific interests include the function and regulation of ion channels and the role of ion channels and calcium signaling in carcinogenesis.

### Key Points

- Calcium is a ubiquitous but nuanced cellular signal; it regulates functions as diverse as cell motility, cell division and cell death. Precise control of the temporal and spatial aspects of calcium changes allow the signal to achieve specific cellular outcomes.
- The nature of the calcium signal is controlled by a diverse array of calcium pumps, channels and exchangers present on the plasma membrane and membranes of intracellular organelles. Certain cancers are associated with the remodeling of the expression of some of these proteins.
- Calcium channels and pumps are amenable to targeting by pharmacological agents.
- Calcium and calcium regulating proteins contribute to many of the processes key to cancer cells, including proliferation, invasion and cell death. A number of oncogenes and tumour suppressors have effects on calcium homeostasis.
- Calcium signalling in the tumour microenvironment is likely to be a complex interplay between a number of different stromal cell types and cancer cells and represents new opportunities for therapeutic intervention.
- The calcium signal is a critical regulator of processes associated with tumour progression including epithelial to mesenchymal transition and the acquisition of specific pathways important in therapeutic resistance.
- The application of new methods to assess calcium signalling *in vivo* and over long periods of time will provide new insights into the remodelling

of calcium signalling in cancer.

### Subject Categories

[Biological sciences / Cell biology / Cell signalling / Calcium signalling](#)

[URI /631/80/86/1999]

[Biological sciences / Cancer / Metastasis](#)

[URI /631/67/322]

[Biological sciences / Cancer / Cancer microenvironment](#)

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[Biological sciences / Cancer / Oncogenes](#)

[URI /631/67/395]

[Biological sciences / Cancer / Cancer therapy](#)

[URI /631/67/1059]

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Changes in the calcium signal allow cells to achieve specific outcomes. In this Review, Monteith *et al.* describe how the remodelling of the expression and activity of calcium channels and pumps in cancer cells can influence tumour progression.

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