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EGR-mediated control of STIM expression and function

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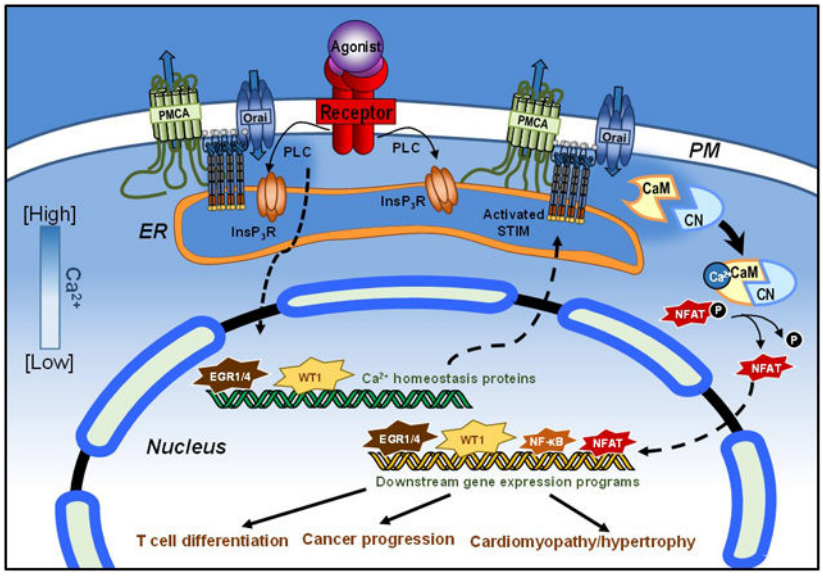
Summary

Ca^{2+} is a ubiquitous, dynamic and pluripotent second messenger with highly context-dependent roles in complex cellular processes such as differentiation, proliferation, and cell death [1]. These Ca^{2+} signals are generated by Ca^{2+}-permeable channels located on the plasma membrane (PM) and endoplasmic reticulum (ER) and shaped by PM- and ER-localized pumps and transporters. Differences in the expression of these Ca^{2+} homeostasis proteins contribute to cell and context-dependent differences in the spatiotemporal organization of Ca^{2+} signals and, ultimately, cell fate. This review focuses on the Early Growth Response (EGR) family of zinc finger transcription factors and their role in the transcriptional regulation of Stromal Interaction Molecule (STIM1), a critical regulator of Ca^{2+} entry in both excitable and non-excitable cells.

Graphical Abstract



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Section 1: Introduction

Store-operated Ca^{2+} entry (SOCE) is a ubiquitous Ca^{2+} entry pathway, with important roles in both excitable and non-excitable cells. The principal means of SOCE to the cytosol is via Ca^{2+}-Release Activated Ca^{2+} (CRAC) channels formed by Orai proteins and activated by Stromal Interaction Molecule (STIM). STIM is a single-pass membrane protein located primarily in the ER membrane that senses ER Ca^{2+} content via its EF hand domains. Following a drop in ER Ca^{2+}, STIM translocates within the ER membrane toward the PM where it associates directly with Orai1 (reviewed in [2-4]). Although STIM1 and Orai1 are the primary mediators of SOCE, each has isoforms with high sequence homology and similar overall structural organization. Hence, similar to STIM1, STIM2 has an ER Ca^{2+} sensor and a capacity to activate CRAC channels, although it is prone to activation with minimal ER Ca^{2+} depletion [5-8] and activates CRAC with less efficiency [7, 9]. Further, both STIM proteins can activate Orai2 and Orai3 [10-12], which share biophysical characteristics with Orai1, but poorly described physiological roles. The regulation of STIM1 and Orai1 expression contributes directly to Ca^{2+} signaling in non-excitable cell types. Thus, transcriptional control of Ca^{2+} homeostasis proteins is critical to understanding how Ca^{2+} signals contribute to development and disease.

Section 2: Zinc finger transcription factors EGR and WT1

Early Growth Response (EGR) proteins are a family of transcription factors that bind to the GC-rich regions of the promoters of hundreds of genes in multiple cell types, including leukocytes from both the lymphoid and myeloid lineages, fibroblasts, neurons, and endothelial cells. The EGR genes are located on separate chromosomes (5q31 for EGR1, 10q21 for EGR2, 8p21 for EGR3 and 2p13 for EGR4), but are dynamically upregulated together in response to a variety of cell stimuli, including growth and pro-differentiation factors [13]. The four EGR proteins have highly conserved zinc finger domains, and generally share the recognition sequence GCG (G/T)GG GCG [14-16], although some differences in binding affinities for specific nucleotides at each position exist (Fig 1). Further, isoform-specific flanking regions and cell type-specific expression support distinct functions for each protein, which can both positively and negatively regulate target gene expression [17]. For example, EGR1 and EGR4 exhibit the capacity to drive STIM1 expression while EGR2 and EGR3 do not [18]. This is similar to the link between EGR1 and EGR4 for control of Luteinizing Hormone expression [19], indicative of greater targeting similarity between these two family members.

Although Wilms Tumor Suppressor 1 (WT1) exhibits substantial overlap in its consensus targets with EGR1, it is not considered a member of the EGR family. Unlike EGRs which are immediate early genes, WT1 expression is primarily developmentally regulated [20, 21]. Over 20 isoforms of WT1 exist due to multiple transcriptional start sites, alternative splicing, and RNA editing. The most striking difference between these isoforms results from an alternative splice site in exon 9 that leads to the insertion of a lysine-threonine-serine (KTS) sequence between the 3rd and 4th zinc fingers. This has ramifications for their DNA binding affinities. Whereas KTS- splice variants readily bind DNA and act as transcription factors, KTS+ WT1 isoforms have a low affinity for DNA and regulate RNA processing [22, 23].

The extent to which the RNA splicing and gene transcription functions of WT1 overlap is not well established, although we have observed opposing regulation of STIM1 expression between the KTS+ and KTS- forms of WT1 (data not shown).

While WT1 and EGR1 share many transcriptional targets, WT1 frequently binds to and represses gene targets that EGR1 upregulates [24]. The frequently opposing effects of EGR1 and WT1 on their shared targets reflects important differences in how the proteins identify targets and recruit transcriptional machinery. They also exhibit differences in their sensitivity to epigenetic modifications. Briefly, within the mammalian genome, 70 to 80% of cytosines found within CpG sites (stands for 5'-C-phosphate-G-3') (CpG) sites are methylated. Given the EGR1 consensus sequence, methylation is common and has been correlated with reduced EGR1 binding and downregulation of EGR1 target genes in rodent models of cardiomyopathy and bladder cancer [25, 26]. However, X-ray crystallography and affinity studies showed that the EGR1 and WT1 zinc finger domains were able to accommodate fully methylated DNA, and their binding kinetics were insensitive to methylation [27, 28]. Rather, EGR1 and WT1 differ in their ability to bind oxidized forms of cytosine, generally considered intermediate states in epigenetic reprogramming. Hence, a glutamate residue within EGR1 precludes binding to 5-carboxylcytosine, whereas the homologous residue in WT1 is a glutamine which favors binding to carboxylated cytosine (Fig. 2) [28]. This preferential binding to 5-carboxylcytosine is potentially informative for understanding how EGR1/WT1 targets could diverge in neoplasias, as elevated 5-carboxylcytosine has been observed in breast cancer and gliomas and may serve as a distinct epigenetic mark in its own right [29, 30]. The effect of methylation on other EGR isoforms will be equally important to understand how EGR transcriptional activity interacts with epigenetic marks.

Although EGR1 and WT1 are largely insensitive to methylation, their binding may be subject to regulation by, or competition with, other zinc finger transcription factors. These include Sp1, which is a ubiquitously expressed zinc finger DNA-binding transcription factor whose recognition sequence overlaps with EGR1 [31, 32]. The sequence of Sp1/EGR1 binding sites contributes to the binding affinity of the transcription factors, and therefore the extent to which a downstream gene may be regulated by EGR1 and/or Sp1 [33]. Conversely, EGR proteins, with the exception of EGR4, share a repressor binding domain, and are inhibited by NGFI-A binding (NAB) proteins NAB1 and NAB2 [34]. NAB2, which binds to EGR1 and regulates EGR1 target gene expression, is itself regulated by EGR1, forming a negative feedback loop by which EGR1 activity is controlled [35-38]. Whereas NAB1 inhibits EGR1, the role of NAB2 is more nuanced. In the context of T cell activation, NAB2 is upregulated downstream of costimulation and acts as a coactivator of EGR1-mediated IL-2 expression [39]. A better understanding of how these transcription factors simultaneously target genes with overlapping binding sites is an intriguing and important question for future studies [33, 40-42].

EGR1 and WT1 are implicated across cell type and developmental stage. This reflects the several layers of regulation required for the function in the isoforms and splice variants that are expressed in a cell type-specific manner, as well as in differences in DNA binding, and interactions with epigenetic marks, and the expression levels of repressor proteins and other transcription factors.

Section 3: Regulation of EGR expression

EGRs mediate many of the expression changes during diverse types of cell stimulation, including cytokines, mitogens, or oxidative stress [43-45]. EGR1 activity is largely effected by inducing EGR1 upregulation and by post-translational modifications [17, 46-48]. The human EGR1 promoter contains 5 serum response elements (SREs) and a cAMP response element (CRE) [49, 50]. As further reviewed by Veyrac *et al.*, a number of transcription factors can be recruited to the *EGR1* promoter in different cell types [51]. EGR1 is able to regulate its own transcriptional activity in a negative feedback loop, both at the expression level by binding to its promoter, and by inducing the expression of its repressor NAB2 [17]. Elk-1, which is activated by phosphorylation downstream of ERK, JNK, and p38 MAPK signaling pathways, associates with serum factors at SREs [52, 53]. EGR1 is also regulated by PI3K/Akt, which phosphorylates and disrupts the DNA-binding ability of FoxO1, a negative regulator of EGR1 expression [54].

In pre-synaptic neurons, EGR1 expression is repressed by transcriptional repressor CtBP1 until neuronal activity relieves repression by CtBP1 [55]. In cells exposed to UVB-induced genotoxic stress, NF-κB binds to the EGR1 promoter to initiate an apoptosis response [56]. Additionally, during T cell activation, EGR1-induced upregulation of STIM1 promotes increases in cytosolic Ca^{2+} content [18] which also may contribute to EGR1 upregulation (reviewed in [57]). This is supported by the observation that chelation of cytoplasmic Ca^{2+} by BAPTA-AM downregulates EGR1 expression, and that it may be required for recruiting transcriptional machinery to SREs and CRE within the EGR1 promoter [58-60]. Additional support for the role of Ca^{2+} signals in EGR1 upregulation came from investigations in vascular smooth muscle revealing mediatory roles of CaMKII and CREB in Angiotensin II-mediated EGR1 expression [61].

EGR1 may be modified post-translationally by phosphorylation, glycosylation and sumoylation with distinct functional outcomes. Low levels of EGR1 phosphorylation in quiescent cells have been shown to contribute to decreased EGR1 stability [17]. In contrast, phosphorylation by casein kinase II negatively modulated EGR1's DNA binding activity [62]. This likely reflects multiple phosphorylation sites on EGR1, each with a distinct impact on EGR1 function. Phosphorylation of EGR1 by Akt has been revealed as a requirement for sumoylation, a critical precursor to EGR1 ubiquitination and degradation [63]. O-glycosylation of the EGR1 transactivation domain has also been shown [64], although the functional implications were not determined. Future investigations dissecting the coordination of these disparate post-translational mechanisms are needed to understand how the stability and targeting of EGR1 may be regulated through crosstalk mechanisms with other signaling pathways.

EGR4 is also upregulated in response to trophic factors in an ERK1/2-dependent manner that has been characterized in the context of neuronal development and spermatogenesis [65-67]. While EGR4 appears to compensate for EGR1 in some cases, SRF binding to the *EGR4* promoter was shown to occur in neuronal cells [68] supported by indirect evidence that serum may regulate the EGR4 promoter generated in Jurkat T cells [69]. Interestingly, the EGR4 promoter is regulated by both EGR1 and 4, with EGR4 opposing its own

transcription and EGR1 modestly enhancing EGR4 expression [70]. Hence, despite the many similarities between EGR consensus sequences, they are not interchangeable, and their expression can lead to mutually opposing outcomes.

Section 4: WT1 and EGR1/4 regulate STIM1 expression and function

Our investigations over the last 10 years have revealed modulation of Ca^{2+} homeostasis due to EGR1-, EGR4- and WT1-mediated control of STIM1 expression. Our first observation ultimately leading us in this direction was that WT1-expressing Wilms Tumor cells exhibit decreased STIM1 expression and SOCE [71]. Using a combination of knockdown and overexpression strategies in HEK293 and G401 cells we subsequently established that EGR1 positively regulates STIM1 expression and WT1 suppresses it [71]. Recognizing that EGRs are primarily immediate early genes, subsequent work focused on dynamic control of STIM1 expression using T cells as a model system (Figure 4) [18, 72]. In Jurkat T cells, we observed that EGR1 and STIM1 are both upregulated within 2 hours of TCR engagement by either the lectin PHA [72] or anti-CD3/CD28 antibodies [18]. However, EGR1 knockdown failed to eliminate STIM1 upregulation, leading to the discovery that EGR1 and EGR4 cooperatively regulate STIM1 expression in T cells [18]. Hence, loss of STIM1 expression was only observed when both EGR1 and EGR4 were knocked down. Further, while chromatin immunoprecipitation (ChIP) analysis showed that TCR engagement leads to EGR1 binding to DNA, EGR4 knockdown decreased EGR1 binding to the STIM1 promoter [18]. These observations reveal a cooperative model by which EGR1 and EGR4 control STIM1 expression during T cell activation.

Because WT1-mediated STIM1 downregulation led to SOCE suppression, our preliminary hypothesis was that EGR-mediated STIM1 upregulation would lead to increased SOCE. However, comparisons of SOCE magnitude in resting and activated T cells revealed only marginal differences in SOCE [18, 72]. In retrospect, this is perhaps not surprising since, although required for SOCE, STIM1 upregulation generally does not increase SOCE unless accompanied by coincident changes in Orai1 expression [10, 73-75]. We also observed PMCA4 upregulation upon T cell activation and significant inhibition of Ca^{2+} clearance [72]; we have since attributed PMCA4 upregulation to EGR1/4-dependent transcription based upon the identification of two EGR binding sites by ChIP within the PMCA4 promoter [18]. EGR1/4-mediated upregulation of both STIM1 and PMCA4) [18] is accompanied by the translocation of both proteins towards the immunological synapse (IS) where STIM1 and PMCA4 colocalize [72, 76-78] (Fig. 3). Although this only minimally affects the initial rate of Ca^{2+} clearance, attenuation of the “slow” phase of Ca^{2+} clearance was observed. Notably, this could be mimicked by STIM1 overexpression without T cell activation and blocked by partial inhibition of SOCE through STIM1 knockdown or pharmacological inhibition [72]. Although which PMCA4 domains are required for STIM1 binding were not determined, the proline/serine-rich domain of STIM1 was required for PMCA inhibition and co-immunoprecipitation. Hence, STIM1-mediated control of Ca^{2+} entry and clearance are independent events that can be effectively separated under different conditions. This inhibition of PMCA4 by STIM1 facilitates increases in intracellular Ca^{2+} levels sufficient to engage NFAT transcription, a necessary requirement of T cell activation

[18, 79]. Thus, STIM1 sustains elevated Ca^{2+} levels in activated T cells using multiple mechanisms, namely, via Orai1 activation and PMCA4 regulation (Fig. 4).

EGR1 has previously been shown to be upregulated following strong TCR activation, leading to disruption of development from the αβ to the γδ lineage [80]. Further, Lohoff *et al.* reported that EGR1 is preferentially expressed in Th2 cells and upregulates IL-4 expression downstream of NFAT activation [44]. The extent to which EGR1/4 regulate STIM1 expression to shape T cell activation and differentiation is under active investigation by our group.

Another component of the intracellular signaling pathway under EGR transcriptional control is the sarco/endoplasmic reticulum ATPase 2 isoform (SERCA2). SERCA family proteins are ATP-driven pumps that drive Ca^{2+} from the cytosol to the ER. SERCA pumps concentrate at ER-PM junctions upon store-depletion to facilitate store-refilling upon engagement of Orai channels by STIM proteins. Of the three SERCA family members, SERCA1 is expressed exclusively in fast-twitch muscle while SERCA2 and 3 are ubiquitously expressed [81]. The relationship between EGR1 and SERCA2 is still somewhat controversial. A link between EGR1 and SERCA2 was first demonstrated in the context of doxorubicin-induced cardiomyopathy, whereby EGR1 was upregulated downstream of the p44/42 MAPK pathway and a concurrent decrease in SERCA2 expression was observed [82]. While this was initially attributed to direct EGR1 transcriptional regulation, another group found no evidence of functional EGR1 binding sites in the SERCA2 promotor [83]. More recently a study in mitogen-activated protein kinase (MAPK)-activated protein kinase 2/3 knock-out mice found a decrease in EGR1 expression driven by MK2, correlated with an increase in SERCA2a in cardiomyocytes [84]. The group found that SERCA2 expression is promoted by Sp1 binding to its promoter, with EGR1 occluding these sites to act as a negative regulator, under the control of MK2. Therefore, EGR1 may negatively regulate SERCA2 expression through an indirect mechanism. Conversely, NFAT positively regulates SERCA2 expression through a similar indirect mechanism [85, 86]. How this may affect T cell activation is not well established, although increased ER Ca^{2+} uptake via SERCA2 would support the increased Ca^{2+} cycling necessary for the Ca^{2+} oscillations required for T cell activation [87].

EGR1 has also been shown to downregulate sodium-calcium exchanger (NCX), a plasma membrane protein that uses the electrochemical gradient of sodium to extrude one Ca^{2+} for every three sodium ions imported. ChIP analysis determined that EGR1 could directly bind the promoter of NCX1 and EGR1 over-expression in cardiomyocytes lead to decreased NCX1 expression [88]. EGR1 has been shown to be upregulated during cardiac hypertrophy [99-102] and the corresponding loss of NCX- and SERCA2-mediated Ca^{2+} clearance in cardiomyocytes would lead to increased cytosolic Ca^{2+} levels and enhanced NFAT activity, thereby contributing to the myocardial thickening that defines this disease.

Section 5: Cooperativity of transcription factors in T cell activation

EGR family members frequently interact with other transcription factors, leading to fundamental differences in the genes that are regulated. For example, the C-terminal region

of zinc finger III in EGR4 directly interacts with the NF-κB family member p65, leading to robust activation of the IL-2 promoter [89]. There is also growing evidence of a Ca^{2+}-dependent feedback loop between NF-κB and STIM1/Orai1 expression in which 1) SOCE drives IκB degradation [90], 2) Ca^{2+} regulates NF-κB nuclear localization via p65 S536 phosphorylation [90] and 3) NF-κB is recruited to binding sites in the promoters of STIM1 and Orai1 [91-93]. Since both EGR and NF-κB both regulate STIM1 expression and yet are themselves regulated by Ca^{2+}, their co-activation would be expected to be relatively common. If so, one might expect EGR-NF-κB complexes to be similarly common, fundamentally altering gene expression patterns in cell types of interest.

Whereas EGR1 and EGR4 contribute primarily to early steps in T cell activation, EGR2 and EGR3 serve as negative regulators of effector differentiation [39]. Hence, *Egr2−/−Egr3−/−* lymphocytes exhibit impaired AP-1 activation during antigen-induced activation, resulting in lethal autoimmune syndrome [94]. Further, EGR2/3 inhibit T-bet-mediated IFN-γ production, facilitating clonal expansion and suppressing effector differentiation [95-97]. Consistent with this immunosuppressive role, EGR2 drives TGF-β expression during induction of $LAG3^+$ regulatory T cells (Tregs); in the absence of EGR2, loss of $LAG3^+$ Tregs leads to loss of protection in a mouse model of systemic lupus erythematosus (SLE) [98]. Human $LAG3^+$ Tregs also suppress antibody production and SLE is correlated with both decreased EGR2 expression and decreased LAG3+ Treg numbers [99, 100]. While the molecular mechanisms controlling EGR2/3 expression are not fully elucidated, NF-κB binding to its recognition sequence in the proximal promoter of EGR2 has been observed [89, 101]. Further, Ca^{2+} dependent expression of EGR2 and EGR3 has been observed [39]. Hence, despite their fundamentally different roles, the EGR family members share some of the mechanisms by which they are regulated. It seems likely that signaling by different EGRs is primarily due to temporal control of EGR isoforms, with EGR1 and EGR4 being expressed during early events followed by negative regulation by EGR2 and EGR3 at later time points.

Section 6: Dysregulation of SOCE in cancer

As mitogen-activated transcription factors, EGRs have a multifaceted role in tumorigenesis and progression, including as regulators of SOCE components. Our group has shown that melanoma cells expressing known oncogene Wnt5a exhibit decreased SOCE and increased invasive ability compared to non-Wnt5a-expressing cells [102]. Further, SOCE promotes apoptotic cell death in prostate cancer [103, 104], providing a potential explanation for why loss of SOCE might be favorable for tumor progression. However, SOCE has been shown to have oncogenic properties as well, enhancing tumor angiogenesis through the production of vascular endothelial growth factor (VEGF) in cervical cancer [105], and cyclooxygenase-2 (COX-2) [106] and nuclear factor of activated T cells (NFAT) [107] in a variety of pathophysiological responses. Furthermore, upregulation of STIM1 and Orai1 has been reported in patients exhibiting a variety of cancers including lung, liver, and colorectal cancers among others [103]. Here, we will address potential some of these seeming contradictions to better define the pleiotropic roles of Ca^{2+} signals in cancer initiation and progression.

Interestingly, EGR/WT1 transcription factors also play dual roles in cancer progression. EGR1 upregulation is downstream of some established oncogenic pathways such as TGF-β, NF-κB, and MAPKs, however its contributions to cancer remain poorly defined [108, 109]. Although WT1 was originally described as a pro-apoptotic tumor suppressor, WT1 contributes to cancer progression in some contexts [110-114]. We previously showed that WT1 interferes with Ca^{2+} homeostasis by blocking expression of STIM1 in Wilms tumor cells [71]. Interestingly, aberrant expression of the STIM1-independent Ca^{2+} channel (CaV2.3) is associated with Wilms tumor relapse through MAPK-related pathways [115]. Further, decreased EGR levels have been correlated with limited response to chemotherapy in patients with Wilms tumor [116]. Therefore, in Wilms tumor, it appears both WT1 expression and loss of Ca^{2+} signals are associated with tumor suppression, whereas both EGR1 and increased Ca^{2+} signals are associated with tumor resilience.

EGR/WT1 transcription factors are implicated in other cancers in both tissue-specific and tissue-agnostic roles. In cervical cancer, EGR1 has been shown to promote tumor progression through downregulating telomerase [117]. In hepatocellular carcinoma (HCC), EGR1 downregulation reduces pro-apoptotic and growth inhibiting pathways [108, 118]. However, EGR1 also contribute to chemotherapy resistance by regulating microtubule function and autophagy [119-121]. Interestingly, although it frequently acts in opposition to EGR1, WT1 expression in HCC cell lines was also associated with chemotherapy resistance [122]. In several cancers, including ovarian and colon cancer, EGR1 has been shown to play a role in EMT and metastasis [123, 124]. WT1 plays an intriguing role in renal cell carcinoma (RCC). In a study on RCC, WT1 induced a hybridized state of EMT and MET (EMHT) in which the mesenchymal marker, Snail, was simultaneously expressed with epithelial marker E-cadherin [125]. This exciting finding suggests that EMT and MET are not mutually exclusive, and that WT1 could be an important modulator of the equilibrium between the two states. Interestingly Orai1 expression has been shown to be associated with favorable prognosis in clear cell RCC where genetic and epigenetic alterations in EGR1 expression were associated with survival [126, 127]. However, the extent to which EGR1/WT1-mediated regulation of Ca^{2+} signaling components contributes to EMT/MET transitions remains unknown.

During both development and tumorigenesis, the expression of EGR/WT1 transcription factors, and the control of Ca^{2+} signaling machinery are critical. ER- breast cancer exhibits deletion of *EGR1* particularly in higher-grade carcinomas [128]. *EGR1* deletions also occur in gastric carcinoma and are associated with conferring invasive properties, since they are found primarily in distant metastases [129]. Since EGR deletion attenuates STIM1 expression and interferes with Ca^{2+} signals [113], these data may be consistent with a link between SOCE suppression and metastatic properties. In prostate cancer, this may be the case since Orai1 and androgen receptor regulate each other's expression in a negative feedback loop and STIM1 and Orai1 expression were inversely correlated with Gleason score [130]. On the other hand, STIM1 and Orai1 were linked to focal adhesion formation in breast cancer where SOCE suppression decreased focal adhesions leading to increased metastasis *in vivo* [131]. For the purposes of illustration, the context-specific roles of EGR1, WT1 and SOCE discussed in this section are shown in figure 5. Further investigations delineating the features of these distinct diseases that contribute to differential roles of these

factors are needed to better understand the relationship between Ca^{2+} signals and cell growth, migration, invasion and survival.

Section 7: WT1 and EGR1 in cardiovascular and pulmonary pathophysiology

An important regulator of vascular disease is the inflammatory immune response induced upon vascular injury. EGR1 is strongly up-regulated in macrophages and other immune cells during wound healing [132] and vascular injury [133], the latter of which is strongly associated with cardiovascular disease [134]. Outlined below are some of the strides made in recent years in understanding the role of EGR1 in vascular disease.

EGR1 is strongly associated with promoting atherosclerosis progression [135]. In smooth muscle cells from both LDL-receptor deficient [136] and apolipoprotein E (ApoE)-null mice [137]. EGR1 is upregulated in atherosclerotic lesions. Further, statins inhibit EGR1 expression in atherosclerotic lesions [138], further supporting the existence of a link between EGR1 expression and atherosclerosis. EGR1 expression in vascular smooth muscle cells (VSMCs) is primarily driven by NADPH-dependent heme oxidation, amongst other things [139, 140]. These observations support the notion EGR1 target genes are involved in cellular responses to oxidative conditions. This is pertinent because atherosclerotic plaques frequently produce hypoxic conditions, exacerbating damage and inflammation in the vasculature [141]. Following hypoxic conditions, multiple tissue types upregulate EGR1, including neural tissue, cells in peri-infarct regions, and renal cells [135, 142-145]. There is evidence to suggest that this is mediated at least in part through regulation of TGFβ signaling by EGR1, in the context of pulmonary fibrosis, and through cyclin D and EGF signaling in pulmonary adventitial fibroblasts during vascular remodeling [146-148]. These studies establish the beginning of an understanding into how EGR1 regulates vascular remodeling following hypoxic injury.

Similar to what we have described in neoplastic disease, EGR1 is upregulated under ischemic conditions, such as during lung or myocardial reperfusion injury, and has been implicated in numerous aspects of these conditions. In some contexts, EGR1 could be seen as beneficial following some types of cardiovascular injury. For example, EGR1-mediated promotion of angiogenesis improves cardiac remodeling in a rat model of heart failure concurrent with dietary obesity [149]. However, despite its role in vascular remodeling, EGR1 is involved in many pro-inflammatory processes and can drive disease progression. In rodent models of cardiac reperfusion injury following myocardial infarct, treatments that reduce infarct size act by suppressing EGR1 expression [150, 151]. Thus, the ability to repress EGR1 inhibits multiple means of damage from cardiac infarct, including subsequent inflammation, apoptosis, and autophagy. Furthermore, in a mouse model of lung transplant, EGR1 deletion improved graft function by attenuating neutrophil recruitment [152]. Of note, downregulating EGR1 in cardiomyocytes also appears to be beneficial. Rat cardiomyocytes treated *in vivo* and *in vitro* with Ca^{2+} channel blockers had significantly reduced damage. Interestingly, WT1 is also expressed in response to hypoxia and ischemia, however this aspect of vascular injury is only beginning to be elucidated *in vivo* [153, 154]. This suggests

WT1 and EGR1 work in separate pathways to induce hypoxia and ischemia; future studies focused on delineating their roles are needed to better understand how their actions are coordinated, particularly considering the mutually exclusive nature of EGR/WT1-mediated gene transcription.

Section 8: EGR1 and WT1 in cardiomyopathy

In cardiomyocytes, Ca^{2+} is utilized in both excitation-contraction coupling and non-excitable signaling. However, it is aberrant non-excitable Ca^{2+} signaling that has been identified in the etiology of cardiomyopathies in response to pathological stimuli, such as aberrant adrenergic signaling or volume overload [155-157].

As discussed in section 4, SERCA2 expression is negatively regulated by EGR1. Reduced replenishment of the sarcoplasmic reticulum (SR) Ca^{2+} store by SERCA had long been recognized as contributing to abnormal Ca^{2+} cycling in cardiomyocytes [158-160]. The predominant splice variant in cardiomyocytes, SERCA2a, is downregulated during heart failure, and loss of SERCA2a activity in animal models leads to heart failure [161, 162]. Because of this, the regulation of SERCA2a activity has been developed as a target in the treatment of heart failure. Recent clinical trials, notably the Ca^{2+} Upregulation by Percutaneous Administration of Gene Therapy in Cardiac Disease (CUPID) phase I and II trials, demonstrated mixed success with a high dose of a SERCA2a-expressing adeno-associated viral vector. While the smaller scale CUPID phase I trial saw fewer cardiovascular events, the CUPID phase II trial did not see significant differences in time-to-recurrent heart failure-related hospitalization, or time to terminal event [163]. Future approaches targeting SERCA2a activity have also looked at regulating SERCA2a inhibitor phospholamban, or increasing SERCA2a stability through increasing sumoylation [164, 165].

In recent years, our understanding of EGR1 as a prominent marker of cardiac hypertrophy has expanded considerably [166]. In addition to regulating SERCA2a expression, EGR1 upregulation induces transcription of the miR-99 family and downregulates Akt signaling in an *in* vivo model of pathologic hypertrophy [167]. The consequences of both EGR1 upregulation and Akt downregulation are consistent with the reemergence of fetal heart gene expression programs, and the loss of normal adult heart signaling during pathologic cardiac hypertrophy [168]. Further in a mouse model of heart failure, NAB1, which negatively regulates EGR1 function, was upregulated, facilitating compensatory protection against cardiac hypertrophy [169]. Intriguingly, WT1, which has principally been described in the context of cardiac development, is also upregulated following myocardial infarction [129, 154]. Cardiac-resident colony-forming cells thought to aid in tissue repair following infarction originate from WT1-positive cells [170, 171]. Thus, the role of WT1 in differentiation appears to persist to a limited degree in the adult heart.

Cumulatively, these studies establish EGR1 as a driver of apoptosis and an inflammatory response after injury to endothelial cells, or during the chronic development of a cardiomyopathy. As a corollary, the repression of EGR1 signaling, by NAB1, WT1, or

others, have shown protective effects, and are appealing targets to prevent damage and perhaps encourage healthy remodeling and restore cardiac function.

Conclusions

In non-excitable cells, the generation of Ca^{2+} signals begins with the production of $InsP_3$ and depletion of the ER store and is maintained by mechanisms controlling the movements of Ca^{2+} across the plasma membrane. The ability of Ca^{2+} signals to modulate the expression of transcription factors such as EGRs, NFAT and NF-κB is now well understood. However, it is increasingly clear that the expression of these Ca^{2+} homeostasis proteins themselves is regulated in response to receptor activation by a host of transcription factors including EGRs, NFAT and NF-κB. How these differences shape long-term Ca^{2+} responses over time are less clear. While complex, future investigations addressing these questions may lead to new insights into fundamental mechanisms driving T cell activation, cancer progression and cardiovascular disease.

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Highlights

- EGR family members and WT1 translocate to shared DNA consensus sites, where differences in binding to epigenetic marks and binding partners regulate transcriptional activity.
- During T cell activation, gene expression changes are enabled by EGR1/4- and WT1-mediated regulation of the expression and function of proteins involved in Ca^{2+} homeostasis such as STIM1, STIM2, PMCA4 and SERCA.
- The roles of EGR1/4 and WT1 in inflammation contributes to cardiovascular pathologies
- EGR1/4-mediated control of Ca^{2+} homeostasis can contribute to tumor formation and progression through crosstalk with multiple pathways.

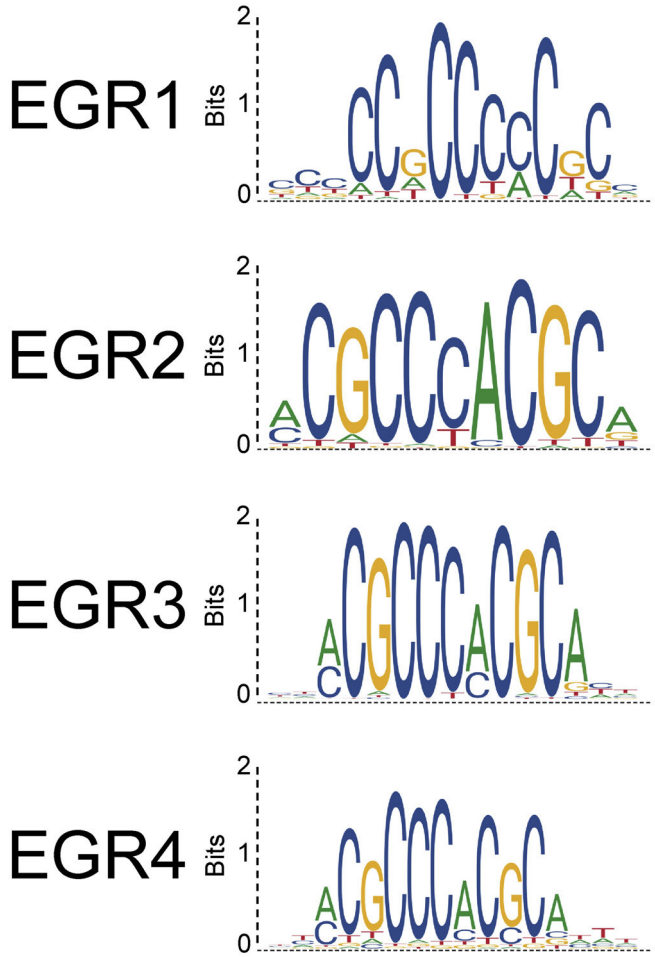

Figure 1: Homology of EGR consensus sequences.

Binding profile of EGR family members based on reported experimental data generated by chromatin immunoprecipitation with next generation DNA sequencing. Source: JASPAR database.

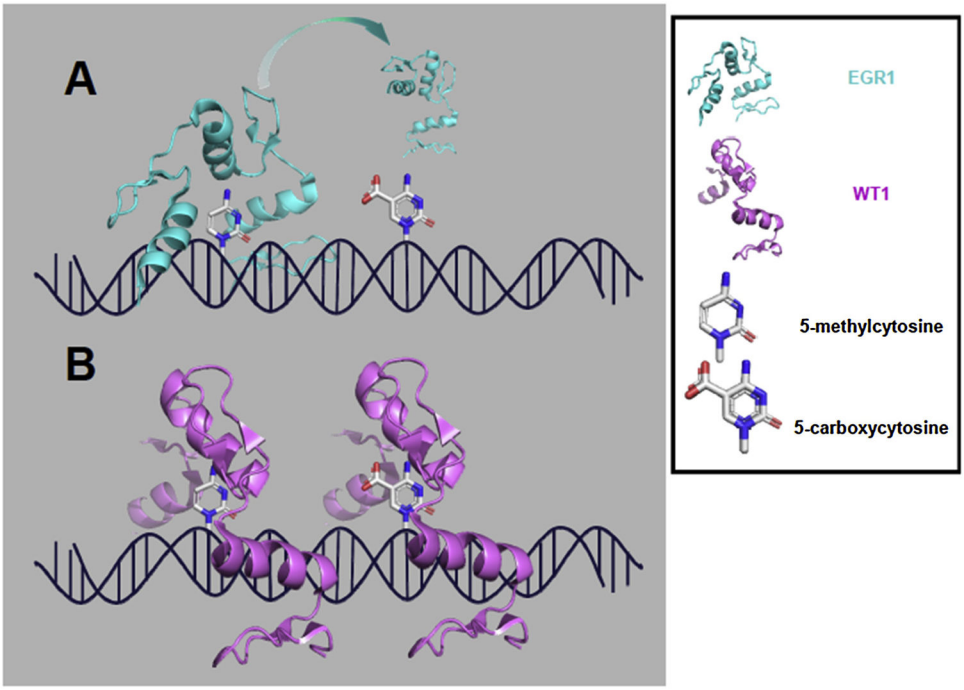

Figure 2: EGR1/WT1 binding to methylation marks.

(A) EGR1 can accommodate and bind DNA with 5-methylcytosine but not 5-carboxylcytosine (PDB: 4R2A); **(B)** WT1 can accommodate both stages of cytosine oxidation (PDB: 4R2E).

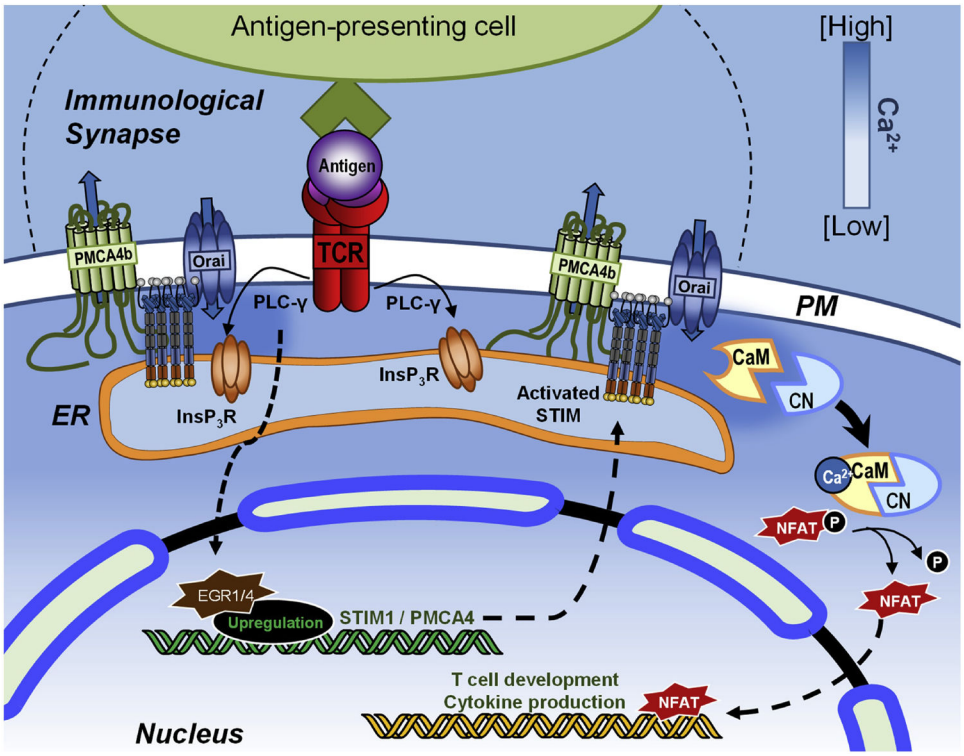

Figure 3: EGR1-mediated regulation of Ca^{2+} signaling in T cell activation.

The TCR is activated upon engagement with its antigenic peptide presented by an antigen-presenting cell. With TCR engagement, phospholipase Cγ (PLCγ) is activated, leading to the rapid release of ER Ca^{2+} through the $InsP_3R$ and the entry of Ca^{2+} through Orai1 Over time, the zinc finger transcription factors EGR1 and EGR4 are activated, driving the expression of Ca^{2+} homeostasis proteins including STIM1 and PMCA4 to facilitate long-term Ca^{2+} signals. These sustained Ca^{2+} signals contribute to CaM/CN activation, critical for driving NFAT translocation to the nucleus where it facilitates the expression of T cell differentiation and proliferation gene programs.

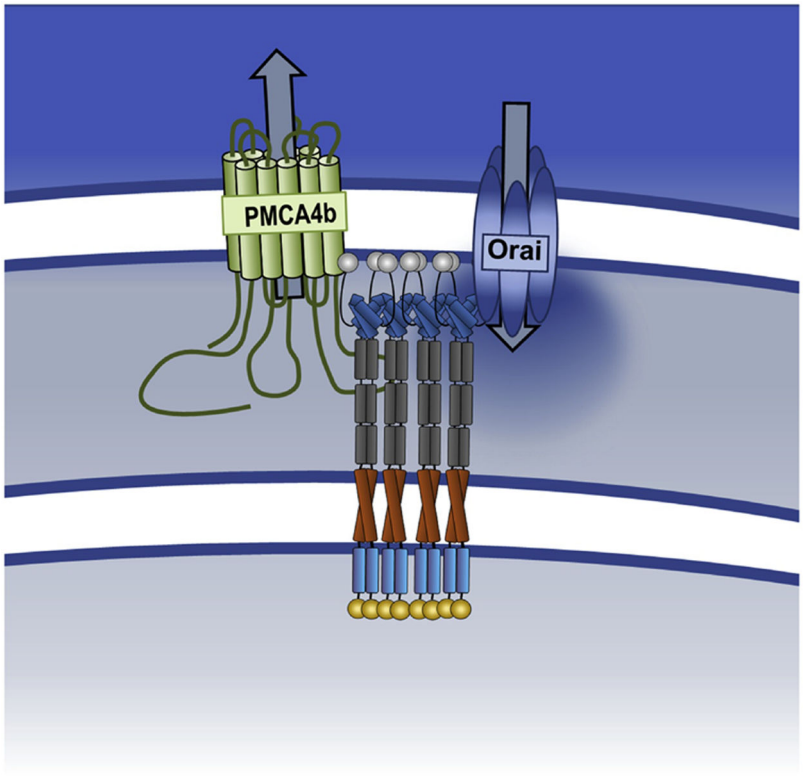

Figure 4: STIM1-mediated control of Ca^{2+} entry and clearance.

ER Ca^{2+} depletion causes STIM1 to oligomerize and extend towards the PM to activate Ca^{2+} entry via Orai channels. STIM1 also interacts with PMCA through its serine/threonine-rich domain causing decreased function.

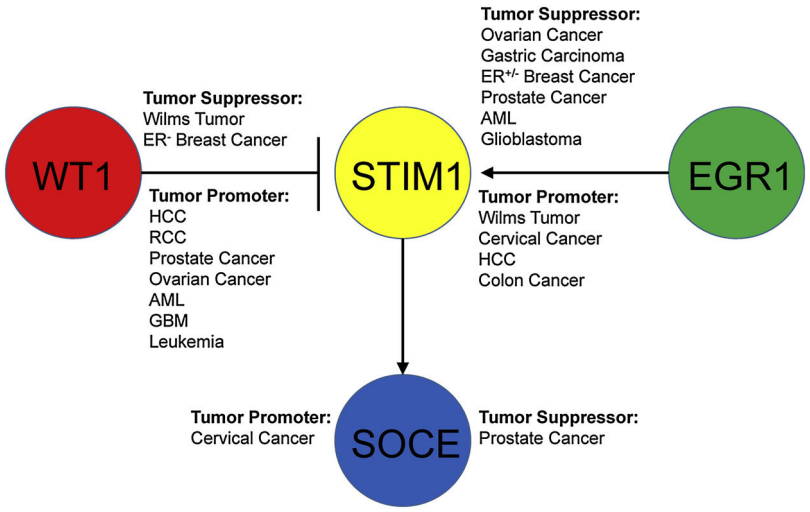

Figure 5: Dual and opposing roles of WT1 and EGR1 in cancer progression.

WT1 and EGR1 play opposing roles in regulating STIM1 during cancer progression. WT1 acts in a tumor suppressing nature in Wilms tumor and ER+ breast cancer, but in a tumor-promoting in ER- breast cancer, and other cancers. EGR1 acts as a tumor promoting nature in both ER+/− breast cancer and other types of cancer.