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Transcriptional Mechanisms Regulating Ca^{2+} Homeostasis

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Abstract

Ca^{2+} is a dynamic cellular secondary messenger which mediates a vast array of cellular responses. Control over these processes is achieved via an extensive combination of pumps and channels which regulate the concentration of Ca^{2+} within not only the cytosol but also all intracellular compartments. Precisely how these pumps and channels are regulated is only partially understood, however, recent investigations have identified members of the Early Growth Response (EGR) family of zinc finger transcription factors as critical players in this process. The roles of several other transcription factors in control of Ca^{2+} homeostasis have also been demonstrated, including Wilms Tumor Suppressor 1 (WT1), Nuclear Factor of Activated T cells (NFAT) and c-myc. In this review, we will discuss not only how these transcription factors regulate the expression of the major proteins involved in control of Ca^{2+} homeostasis, but also how this transcriptional remodeling of Ca^{2+} homeostasis affects Ca^{2+} dynamics and cellular responses.

Control of Ca^{2+} homeostasis

Cytosolic Ca^{2+} concentration is maintained at a level ~4 orders of magnitude lower than either the extracellular space or the lumen of the endoplasmic reticulum (ER). This remarkably high Ca^{2+} gradient is maintained via the near constant activity of several highly conserved families of pumps and exchangers located both on the plasma membrane (plasma membrane Ca^{2+} /ATPase; PMCA and the sodium- Ca^{2+} exchanger; NCX) and the ER (Sarco/endoplasmic reticulum Ca^{2+} /ATPase; SERCA) (Fig 1). The activity of these pumps and exchangers serves to both clear cytosolic Ca^{2+} and, in the case of SERCA, also to maintain ER Ca^{2+} concentration at or near 500 μM . This function is, at least in part, assisted by the store-operated Ca^{2+} entry (SOCE) [1-3]. The ER Ca^{2+} sensors and activators of SOCE are STIM1 and STIM2, two type 1A transmembrane proteins containing low Ca^{2+} affinity luminal EF hands [4]; when ER Ca^{2+} content is high, their EF hands are bound to Ca^{2+} and the proteins are inactive [5-7]. Decreases in ER Ca^{2+} concentration cause dissociation of Ca^{2+} from the STIM EF hands, resulting in a conformational change [7-9] that leads to STIM aggregation in regions of the ER that are adjacent to the PM [10-12], where they interact with and activate the highly Ca^{2+} -selective channel Orai1 [10, 11, 13-20] and, perhaps, members of the TRPC family of cation channels [21-26], although the latter relationship remains a topic of much debate [27-31].

Recent investigations have revealed that, although constitutively expressed, the level of expression of proteins involved in Ca^{2+} homeostasis is modulated at the transcriptional level, with significant implications to the nature of Ca^{2+} signaling. The current review focuses precisely on this type of transcriptional control, with an emphasis on the roles of members of the Early Growth Response (EGR) family of zinc finger transcription factors and the closely related protein Wilms Tumor Suppressor 1 (WT1).

The zinc finger transcription factors EGR1 and WT1

The Early Growth Response (EGR) family of transcription factors consists of 4 closely related members (EGR1-4) that, through conserved zinc finger domains, bind to GC-rich DNA motifs and enhance or repress the expression of wide variety genes which regulate growth, differentiation or cell remodeling [32-36]. While the EGR transcription factors are only transiently induced, the impact of this expression has a relatively long-term impact. Hence, EGR1-induced gene products initiate subsequent waves of gene expression which can ultimately result in cell differentiation, changes in the rate of proliferation or apoptosis [37].

Activation of the EGR transcription factors occurs in nearly all cell types through a wide array of physiological and pathophysiological stimuli which facilitate the activation of the transcription factors Ets-Like gene-1 (ELK-1) and cAMP response element-binding protein (CREB), the primary transcriptional activators of the EGR genes [38-42]. Hence, multiple pathological conditions including hypoxia, dysregulation of redox balance, UV genotoxic stress, viral infection and mechanical strain activate ELK-1/CREB-mediated EGR transcription via ERK1/2, p38 and JNK1 paradigms [40, 43-49]. Similarly, activation of tyrosine-kinase receptors, such as Insulin-like Growth Factor-1 Receptor (IGF-1R) or Epidermal Growth Factor Receptor (EGFR) leads to the induction of EGR transcription via analogous MAPK paradigms and/or the PI3K/Akt cascade [42, 50, 51]. Interestingly, increases in intracellular Ca^{2+} concentration can also lead to CREB induction, thereby potentially inducing EGR transcription [52-54]. Considered collectively then, crosstalk amongst multiple receptor-mediated signal transduction pathways leads to convergence at the promoters of EGR genes, making their induction common components of multiple cellular stimuli.

WT1 is structurally very similar to EGR1 and the array of WT1 and EGR1-regulated genes extensively overlaps, although usually with mutually opposing consequences [55, 56]. Despite these similarities, because WT1 is non-responsive to growth factor stimulation and predominantly a developmentally regulated gene [57, 58], we do not consider it to be a member of the EGR family. A key feature of WT1 is the existence at least 2 sites for alternative splicing, resulting in 4 major splice variants (see Fig 2) [59]. The first and most significant alternative splice donor site results in the addition of the amino acids KTS (Lys-Thr-Ser) between zinc fingers three and four [59]. WT1 variants A and B, which lack this KTS site are the forms best described as transcription factors; the functions served by WT1 variants C and D (KTS+) is a subject of ongoing controversy. Whereas these proteins have been thought to function post-transcriptionally as RNA splicing proteins [59], KTS+ forms of WT1 have been reported to regulate gene transcription, albeit with distinct binding characteristics [60]. The other major alternative splice donor site results in the inclusion of 17 amino acids in the middle of the protein in WT1 B and D, which is thought to regulate interactions between WT1 and cofactors [61].

WT1 and EGR1 regulate STIM1 expression

Our interest in WT1 and EGR1 as transcription factors regulating the expression of Ca^{2+} signaling proteins was initiated with the observation that they regulate the expression of STIM1 [56]. Thus, based on a combination of knockdown and overexpression approaches, we determined that EGR1 drives STIM1 expression, while WT1 inhibits its expression. In an effort to distinguish between direct and indirect roles for WT1 and EGR1, we analyzed the region of the genome at or around the STIM1 transcriptional start site using the Transcriptional Element Search System (TESS; University of Pennsylvania), uncovering 4 potential response elements. We then tested the validity of these sites using chromatin

immunoprecipitation (ChIP), comparing the binding efficacy of EGR1 in WT1-null G401 tumor cells with that in WT1-expressing HEK293 cells. Intriguingly, both EGR1 and WT1 bound to response element 1 (RE1; see Fig 3), although binding of EGR1 was considerably weaker in the WT1-expressing cells. Further, EGR1, but not WT1 also bound to RE3, which was only detectable in the WT1-null cells. Based on these observations, we propose that binding of WT1 to RE1 inhibits both the binding of EGR1 to the STIM1 promoter and loss of STIM1 expression (Fig 3).

An important question that remains only partially answered is how WT1/EGR1-dependent control of STIM1 expression modulates Ca^{2+} signals. In our recent study [56], we showed that loss of STIM1 expression due to either overexpression of WT1 or knockdown of EGR1 resulted in decreased thapsigargin-induced SOCE. However, the physiological implications of the finding were not fully elucidated. As discussed above, transient upregulation of EGR1 is a fairly common signaling event [62, 63], while WT1 is heavily expressed in the developing kidney, mammary glands and hematopoietic system [57, 58]. Considered in combination with our findings then, we would predict STIM1 expression to be upregulated in response to multiple receptor-mediated signaling pathways, although predominantly in mature rather than undifferentiated WT1-expressing cell types. Precisely how STIM1 upregulation would impact Ca^{2+} signals would likely also be highly dependent on stoichiometry, as STIM1 upregulation does not increase Ca^{2+} signals unless Orai1 is in excess [14, 64]. Current investigations in our laboratory are focused on defining and characterizing this concept of dynamic receptor-mediated transcriptional control of store-operated Ca^{2+} entry via upregulation of EGR1.

Transcriptional control of SERCA2 expression

SERCA proteins are Ca^{2+} pumps found on the ER in every type of eukaryotic cell. These proteins are critical for the maintenance of both cytosolic and ER Ca^{2+} levels. The three SERCA paralogs (SERCA 1, 2, and 3) are encoded by separate genes and produce more than 10 isoforms through alternative splicing mechanisms [65]. Whereas SERCA1 isoforms are exclusively expressed in fast-twitch skeletal muscle, SERCA3 can be found in multiple cell types, particularly those of the hematopoietic system, exocrine and endocrine glands [65]. The SERCA2 gene produces two distinct isoforms; SERCA2a which is found in cardiac and slow-twitch skeletal muscle and SERCA2b, a ubiquitously expressed isoform found at varying levels in all cell types [65].

Interestingly, while a relationship between EGR1 and SERCA2 expression has been demonstrated [66, 67], the precise nature of this relationship remains somewhat unclear [68]. The first indication that EGR1 might be involved in control of SERCA2 expression was based on examination of doxorubicin-induced cardiomyopathy [67]. This type of cardiomyopathy is a well documented side effect of this otherwise useful chemotherapeutic agent. In this study, the investigators linked doxorubicin (DOX) treatment of cardiomyocytes to EGR1-mediated repression of the SERCA2 gene. Specifically, incubation of cardiomyocytes with DOX led to a p44/42 MAPK-dependent increase in EGR1 expression (as expected since EGR1 is a redox-activated transcription factor [69, 70]) with correlative decreases in SERCA2 mRNA and protein. This phenomenon was attributed to direct EGR1-mediated transcriptional repression based on the identification of EGR1 binding sites in the 5' flanking region of the SERCA2 gene. In a more recent study by the same group, they showed that EGR1 also mediates prostaglandin-induced inhibition of SERCA2 expression in cultured neonatal rat cardiomyocytes [66]. However, this issue has also been addressed by the Fuller group (Imperial College London, UK), who came to a contradictory conclusion [68]. Hence, their analysis of a SERCA2 promoter-driven

luciferase construct failed to support any functional role for these EGR1-binding sites, leading the authors to conclude that they were inactive [68].

While it is obvious that both conclusions cannot be correct, our own investigations in this area support the conclusion that EGR1 is a negative regulator of SERCA2 expression, although we see little evidence for a direct relationship. Hence, overexpression of EGR1 has a relatively minor effect on SERCA2 mRNA levels in multiple cell types, yet leads to an approximately 90% reduction in SERCA2 protein content (with WT1 having the opposite effect) [71]. Further, we obtained the SERCA2 luciferase vector from Dr. Stephen Fuller (Imperial College London, UK; [68]) and saw no evidence of EGR1-dependent luciferase expression (unpublished observations). Finally, our attempts to perform ChIP using primers targeting EGR1 consensus sequences in the SERCA2 promoter found no evidence of binding (unpublished observations). Based on this combination of findings, we are confident that EGR1 does indeed regulate SERCA2 expression, although it seems likely that this relationship is indirect and quite possibly post-transcriptional.

While EGR1 functions as a negative regulator of SERCA2 expression, SERCA2 transcription is induced via the combination of several other transcription factors. Basal SERCA2 expression is primarily driven by the ubiquitously-expressed transcription factors Sp1 and Sp3, which bind directly to proximal regions of the SERCA2 promoter to transactivate the SERCA2 gene [68]. In addition, there is considerable circumstantial evidence that Nuclear Factor of Activated T cells (NFAT) is a positive regulator of SERCA2 transcription [72-75], despite the lack of evidence of direct transcriptional control. Hence, NFAT activation leads to induction of SERCA2 transcription [72, 74, 76], while induction of glycogen synthase kinase 3 β (GSK3 β ; a negative regulator of NFAT nuclear translocation) leads to downregulation of SERCA2 [77, 78]. Furthermore, GSK3 β transgenic mice exhibit impaired post-natal cardiomyocyte growth, abnormal cardiac contractile function and impaired diastolic relaxation [78], all consistent with defective SERCA2 expression.

Interestingly, there are also several examples of SERCA2 regulation by early or upstream signaling molecules. Hence, treating primary β cells with IL-1 or IFN- γ for 24 hours substantially reduces SERCA2 expression and reduces the ER Ca²⁺ pool enough to induce the ER stress response [79]. Furthermore, direct activation of protein kinase C (PKC) by the addition of phorbol-12-myristate-13-acetate (PMA) to neonatal cardiomyocytes significantly reduces SERCA2 expression [80]. Although these modes of SERCA2 down regulation were not attributed to an EGR-1 dependent mechanism, IL-1, IFN- γ and PKC are all potent activators of EGR1 [81-83]. Thus, given previous investigations on EGR1-dependent inhibition of SERCA expression, these effects may well be dependent on EGR1 activity [67, 84].

Transcriptional Control of PMCA

Although often described simply as PMCA, there are, in fact, 4 different PMCA genes, each of which exists in multiple isoforms [85]. While virtually all cell types express PMCA, these isoforms have distinct expression patterns and functional characteristics. Hence, PMCA1 and PMCA4 are present in virtually every tissue type, while PMCA2 and PMCA3 are expressed predominantly in excitable cells [85-87]. The functional differences among PMCA isoforms correspond to the differences in Ca²⁺ dynamics between excitable and non-excitable cells. Hence, PMCA2 and PMCA3 are the most quickly activated PMCA isoforms, enabling them to clear Ca²⁺ within the rapid time course of changes in Ca²⁺ concentration observed in excitable cells [88]. PMCA4, in contrast, is both activated and inactivated

relatively slowly, permitting relatively long changes in cytosolic Ca^{2+} concentration, as observed in non-excitable cells [89].

Unlike for SERCA, a published relationship between WT1, EGR1 and the expression of PMCA has not yet been elucidated. However, the involvement of several different transcription factors in PMCA expression have been investigated, particularly c-myc. Hence, in B lymphocytes, activation of the B cell receptor (BCR) by agonists such as IL-7 and IL-4 leads to both elevated cytosolic Ca^{2+} concentration and induction of c-myc [90, 91]. Interestingly, c-myc has been shown to repress PMCA4 expression, resulting in decreased Ca^{2+} clearance from the cytosol, augmented NFAT and, ultimately, increased B cell activation [92]. Similar observations have been made in pancreatic β cells, in which glucose both induces c-myc expression and downregulates both PMCA1 and PMCA2 [93, 94]. Hence, downregulation of PMCA may be a common component of “activation” signals in a wide variety of different cell types.

While the ability of c-myc to suppress PMCA expression seems clear, precisely what drives their expression has yet to be fully elucidated, although there have been several interesting clues. Thus, vitamin D3 induces expression of PMCA1 in kidney epithelial cells, osteoblasts and duodenal cells [95-97]. Induction of PMCA4b has also been demonstrated in duodenal cells in response to the synthetic anti-inflammatory steroid, dexamethasone [98]. However, in neither case, has direct transcriptional control been demonstrated. Given the dynamic roles that PMCA proteins play in shaping not only Ca^{2+} dynamics, but also both normal and pathophysiological function of numerous critical cell types, future investigations directed at characterizing the transcriptional mechanisms in control of PMCA expression are clearly a necessity.

EGR1-mediated remodeling of Ca^{2+} homeostasis in cardiac hypertrophy

In addition to control of SERCA2 expression, EGR1 negatively regulates both NCX [99] and calsequestrin expression [100]. This has led to several studies addressing the role of EGR1 in cardiac remodeling. Hence, not only is EGR1 upregulated during cardiac hypertrophy [99-102], but EGR1-KO mice exhibit increased cardiac damage in response to catecholamine infusion [99, 100, 103]. EGR1-mediated loss of SERCA2 and NCX (the major proteins mediating cytosolic calcium clearance in cardiomyocytes) would lead to attenuated cytosolic Ca^{2+} clearance and amplification of Nuclear Factor of T cells (NFAT) activation, ultimately the critical mediator of myocardial thickening [104-106]. However, as the major Ca^{2+} binding protein in the sarcoplasmic reticulum (SR), loss of calsequestrin expression would tend to have the opposite effect, making the role of EGR1 in cardiac remodeling somewhat more difficult to fully quantify.

Interestingly, multiple cell systems, including cardiac models, increase PMCA expression in response to loss of SERCA2 expression [107, 108]. Additionally, there is growing evidence to support the notion that while PMCA plays a limited role in the excitation-contraction coupling process of cardiac systems, it modulates Ca^{2+} dependent signal transduction pathways [109, 110]. Hence, upregulation of PMCA attenuates the calcineurin-dependent hypertrophic response [111]. Thus, increases in PMCA expression might mitigate hypertrophic Ca^{2+} signaling cascades in response to SERCA2 deficiency and protect a cell from hypertrophy, although this concept yet to be validated.

In addition to regulation of Ca^{2+} clearance mechanisms, EGR1 also activates STIM1 expression [56], which could potentially increase Ca^{2+} influx, thereby further augmenting the aforementioned Ca^{2+} dynamics. However, while STIM1-mediated Ca^{2+} entry is observed in neonatal cardiomyocytes, it is downregulated in adult cardiomyocytes [112, 113]. This is somewhat intriguing, in that the reactivation of fetal gene programs is thought

to be a component of induction of cardiac hypertrophy [114, 115]. Further, inhibition of STIM1-mediated Ca^{2+} entry abrogates hypertrophic characteristics in studies performed in vitro in neonatal cardiomyocytes [116, 117].

In summary, induction of EGR1 results in inhibition of Ca^{2+} clearance, increased Ca^{2+} entry and decreased ER Ca^{2+} load. Precisely what role these changes have on cardiac remodeling is not currently clear, however, considered collectively, it is difficult to discount EGR1 as a critical player in this process.

Redox-state regulation of Calcium dynamics: a potential role for EGR1

Reactive oxygen species (ROS) can directly modulate both the activity and expression of several key proteins involved in Ca^{2+} homeostasis. The primary means whereby ROS affects protein function are via modifications of the sulfhydryl (SH) moieties within cysteine residues leading to formation of disulfide bonds and/or oxidation of reduced glutathione leading to S-glutathionylation. We have recently shown that S-glutathionylation of cysteine 56 on STIM1 leads to constitutive, store-independent Orai1 activation [118]; hence, the initial response to elevated ROS is elevated Ca^{2+} entry. However, this effect can be compensated in part by direct ROS-induced stimulation of both SERCA [119] and NCX [120] activity. In addition, H_2O_2 -induced decreases L-type Ca^{2+} channel activity may also partially compensate for ROS-induced Ca^{2+} elevation in excitable cells [121, 122]. This is particularly relevant in muscle, where elevated ROS is a relatively common event during exercise [123, 124]. Although the mechanism whereby H_2O_2 changes L-type Ca^{2+} channel function was not described, recent demonstrations by us and others that STIM1 inhibits L-type channel activity [30, 125] may provide an explanation for this phenomenon.

Interestingly, ROS is well known to induce EGR1 expression [49, 126, 127]. Given the host of different Ca^{2+} homeostasis proteins regulated by EGR1 described in this review, it seems reasonable to speculate that EGR1-dependent changes in their expression may greatly impact ROS-dependent changes in Ca^{2+} homeostasis, ultimately determining physiological outcomes. Hence, EGR1-dependent downregulation of SERCA2 and NCX as well as upregulation of STIM1 would exacerbate ROS-dependent Ca^{2+} elevation and induce cell death via collapse of mitochondrial membrane potential [128, 129] and/or induction of NFAT [130, 131]. However, similar to direct ROS-dependent changes in the activity of Ca^{2+} regulatory proteins, there are compensatory mechanisms in place to limit damage caused by changes in their expression. For example, acute elevations in cytosolic Ca^{2+} concentration stimulate cAMP response element-binding protein (CREB) activity [132] which induces the expression of both antioxidants and ROS detoxifying enzymes [133-137]. Similarly, ROS stimulates the expression of Down syndrome critical region gene 1 (DSCR1) [138] which inhibits calcineurin-dependent NFAT activity [139-141]. Finally, as mentioned in preceding sections, decreased SERCA2 expression leads to increased PMCA expression. Interestingly, PMCA recruits calcineurin from the cytoplasm to the plasma membrane and inhibits its ability to effectively activate NFAT [143].

In summary, ROS-mediated changes in Ca^{2+} homeostasis via either direct or transcriptional mechanisms lead to elevated Ca^{2+} concentration while, paradoxically stimulating multiple compensatory pathways. Presumably, these observations reflect not only cell type-dependent differences in tolerance to oxidative stress, but also the difference between normal physiological ROS signaling and pathophysiological ROS levels.

Summary and perspective

We have delineated several mechanisms by which transcription factors regulate Ca^{2+} signaling through modifications in the gene expression of the key families of Ca^{2+}

homeostasis proteins SERCA, STIM1, PMCA and NCX. While most of the investigations demonstrating these relationships were performed in isolation, it is important to recognize that these events do not occur that way. This is particularly true of EGR1, which has been separately demonstrated to modulate the expression of SERCA2 [66, 67, 71], NCX [99] and STIM1 [56] (see Fig 4). These reports point to dramatic EGR1-dependent changes in how Ca^{2+} dynamics are regulated. Given that EGR1 is a major receptor-dependent transcription factor, it seems reasonable to speculate that receptor-dependent remodeling of Ca^{2+} signals via transcriptional control of Ca^{2+} homeostatic proteins is a common event in cellular physiology. However, since EGR1-dependent gene transcription is negatively regulated by WT1, it is likely that these responses are both cell-type dependent and highly contextual. Nevertheless, this remodeling is by no means limited to EGR1; both NFAT and c-Myc regulate SERCA2 and PMCA, respectively, and are also exceedingly common receptor-dependent transcription factors. Considered collectively then, it seems reasonable to propose that transcriptional control of Ca^{2+} homeostasis proteins represents a new paradigm in receptor-dependent control of Ca^{2+} signaling. Future investigations directed at characterizing precisely how this receptor-dependent remodeling of Ca^{2+} homeostasis impacts Ca^{2+} signals will provide important new insight into Ca^{2+} -dependent changes in cellular physiology.

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Figure 1. Model depicting control of Ca^{2+} homeostasis

The major proteins involved in control of Ca^{2+} homeostasis are depicted. Resting cytosolic Ca^{2+} concentration is maintained primarily by the combined action of the sodium/calcium exchanger (NCX) the plasma membrane Ca^{2+} /ATPase (PMCA) and the sarco/endoplasmic calcium ATPase (SERCA). The SERCA pump also serves as the primary regulator of the Ca^{2+} concentration in the endoplasmic reticulum (ER). Activation of the inositol 1,4,5-triphosphate receptor (IP_3R) results in release of Ca^{2+} from the ER into the cytosol. This depletion of ER Ca^{2+} content is sensed by STIM proteins, which aggregate near the plasma membrane (PM) to interact with Orai1, thereby initiating store-operated Ca^{2+} entry.

Figure 2. The domain structure of the 4 WT1 splice variants

WT1 is a zinc finger transcription factor with 2 distinct sites for RNA splicing. WT1A is the shortest form of WT1, in that it lacks both inserts. WT1B and WT1D contain exon 5 (Ex5), a 17 aa insert located immediately 3' of the activation domain. In addition, the tripeptide lysine-threonine-serine (KTS) is inserted immediately after zinc finger 3 (ZF3) in both WT1C and WT1D. Amino acids 1-180 are critical for dimerization of WT1, which is a prelude to transcriptional activation; amino acids 84-124 repress WT1 activity by blocking dimerization. Finally, there is a putative RNA Recognition Motif (RRM) near the N-terminus of WT1 thought to contribute to the RNA splicing functions of the KTS+ forms of WT1 (WT1C and WT1D).

Figure 3. Model for control of *STIM1* expression by WT1 and EGR1

Functional WT1/EGR1 response elements are depicted relative to the start site for transcription of *STIM1* mRNA (marked by the blue arrow). WT1-mediated inhibition of EGR1 binding at RE1 is marked with an arrow.

Figure 4. Regulation of Ca^{2+} homeostasis by WT1 and EGR1

Early Growth Response 1 (EGR1) activation is achieved through multiple receptor-dependent signal transduction pathways. Activated EGR1 rapidly enters the nucleus where it induces STIM1 expression, yet negatively regulates the expression of the sodium/calcium exchanger (NCX) and the sarco/endoplasmic calcium ATPase (SERCA); the potential relationship between EGR1 and PMCA expression remains currently undefined. However, in cells expressing Wilms Tumor Suppressor 1 (WT1), EGR1-mediated changes in the expression of these Ca^{2+} signaling proteins are greatly attenuated.