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## Calcineurin signaling in the heart: The importance of time and place

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### Abstract

The calcium-activated protein phosphatase, calcineurin, lies at the intersection of protein phosphorylation and calcium signaling cascades, where it provides an essential nodal point for coordination between these two fundamental modes of intracellular communication. In excitatory cells, such as neurons and cardiomyocytes, that experience rapid and frequent changes in cytoplasmic calcium, calcineurin protein levels are exceptionally high, suggesting that these cells require high levels of calcineurin activity. Yet, it is widely recognized that excessive activation of calcineurin in the heart contributes to pathological hypertrophic remodeling and the progression to failure. How does a calcium activated enzyme function in the calcium-rich environment of the continuously contracting heart without pathological consequences? This review will discuss the wide range of calcineurin substrates relevant to cardiovascular health and the mechanisms calcineurin uses to find and act on appropriate substrates in the appropriate location while potentially avoiding others. Fundamental differences in calcineurin signaling in neonatal versus adult cardiomyocytes will be addressed as well as the importance of maintaining heterogeneity in calcineurin activity across the myocardium. Finally, we will discuss how circadian oscillations in calcineurin activity may facilitate integration with other essential but conflicting processes, allowing a healthy heart to reap the benefits of calcineurin signaling while avoiding the detrimental consequences of sustained calcineurin activity that can culminate in heart failure.

### Keywords

Calcineurin; Heart failure; Microdomains; Transmural gradient; Circadian rhythms

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None.

## 1. Introduction

It has been forty years since Claude Klee began her seminal work deciphering the structure and regulation of the calcium-activated protein phosphatase calcineurin [1–3] and almost twenty years since Jeff Molkentin and Eric Olson demonstrated that sustained activation of calcineurin in cardiomyocytes is sufficient to promote hypertrophic remodeling, decompensated failure, and arrhythmogenic death [4]. Numerous studies have subsequently verified that inhibition of calcineurin is an effective method for blunting hypertrophic growth and protecting the heart from both initial oxidative damage and subsequent pathological remodeling in response to a variety of insults [5–10]. Hundreds, if not thousands of papers have been published citing a role for calcineurin in cardiovascular disease, yet there is much we still do not understand regarding control and specificity of this enzyme that must function in the calcium-rich environment of a continually contracting heart without initiating a pathological signaling cascade. Here, we will review regulation of calcineurin and mechanisms it uses to target specific substrates within the myocardium. Key features unique to calcineurin signaling in the heart will be addressed including: fundamental differences in calcineurin signaling in neonatal versus adult cardiomyocytes and the importance of maintaining heterogeneity in calcineurin activity across the myocardium, particularly as it relates to ion channel activity and arrhythmogenesis. Finally, we will discuss how a circadian pattern of calcineurin activity may allow a healthy heart to reap the benefits of calcineurin signaling while avoiding the detrimental consequences of sustained calcineurin activity that can culminate in heart failure.

## 2. Calcineurin structure and regulation

Calcineurin is a heterodimer composed of a 60-kDa catalytic subunit (CnA) and a 19-kDa regulatory subunit (CnB). CnA contains an N-terminal catalytic domain, a CnB binding domain, a calmodulin binding domain, and a C-terminal autoinhibitory domain (AID). CnB has four EF-hand  $\text{Ca}^{2+}$ -binding sites: two structural sites that bind  $\text{Ca}^{2+}$  with high affinity in the nM range, that are always occupied [11, 12], and two regulatory sites that bind in the  $\mu\text{M}$  range (Fig. 1A). Binding of  $\text{Ca}^{2+}$  to the regulatory sites initiates a series of conformational changes that allow binding of a calmodulin/ $\text{Ca}^{2+}$  complex and a change in the orientation of the AID to expose the active site [13, 14] (Fig. 1B). Truncation of CnA to remove the AID yields a constitutively active phosphatase (CnA\*) that no longer responds to  $\text{Ca}^{2+}$ /calmodulin (Fig. 1C). It is this truncated form, under the control of the alpha myosin heavy chain ( $\alpha\text{MHC-CnA}^*$ ) that has now been used by many investigators to study the effects of calcineurin activity in the myocardium [4].

During the cardiac cycle, cytosolic  $\text{Ca}^{2+}$  raises from 0.1  $\mu\text{M}$  to 1  $\mu\text{M}$  [15], reaching well within the range to activate calcineurin, which requires only 0.6  $\mu\text{M}$  for half maximal activity in the presence of 20  $\mu\text{M}$  calmodulin [16]. It is not completely understood how calcineurin avoids beat-to-beat activation. However, many factors are likely involved, including the rate of association with calmodulin and regional differences in availability of this accessory protein [17]. An important concept that has emerged is the role of signaling microdomains that are defined by the local concentrations of  $\text{Ca}^{2+}$ , calmodulin, and target substrate [18–20]. Free calmodulin in an adult cardiomyocyte is estimated in the range of 50 to 100 nM [21].

This is low enough to impact calcineurin's threshold for activation by  $\text{Ca}^{2+}$ . For instance, at 30 nM calmodulin, half maximal activation of calcineurin requires  $1.3 \mu\text{M}\text{Ca}^{2+}$  [16]. Therefore, in a subcellular domain with limited free calmodulin, calcineurin might only be activated when there is an unusually high systolic  $\text{Ca}^{2+}$  transient, such as those following adrenergic input, whereas, calcineurin in a domain with free access to calmodulin would be able to respond to a lower  $\text{Ca}^{2+}$  input.

In mammals, three genes encode CnA ( $\alpha$ ,  $\beta$ , and  $\gamma$ ). Only CnA $\alpha$  (*PPP3CA*) and CnA $\beta$  (*PPP3CB*) are expressed in the heart. Of the two genes encoding the CnB regulatory subunit, only CnB $\alpha$ /CnB1 (*PPP3R1*) is expressed in the heart (Fig. 1D). Transcript and protein levels for CnA $\beta$ , but not CnA $\alpha$ , increase in response to mechanical stress or hypertrophic agonists in a calcineurin-dependent fashion [10, 22, 23]. Mice lacking CnA $\alpha$  have a severely shortened life span, therefore, a rigorous analysis of their cardiovascular phenotype has not been undertaken [24–26]. The hearts of mice lacking CnA $\beta$  are smaller than wild type littermates and show reduced hypertrophy in response to pressure overload [27]. Taken together, these findings suggest that CnA $\beta$  is the dominant isoform in the heart and that it may participate in a feed-forward amplification loop.

## 2.1. Cardiac expressed isoforms

Although CnA $\alpha$  and CnA $\beta$  isoforms are structurally similar, there are some notable differences. Purified enzyme composed of CnA $\beta$  is less sensitive to inhibition by FK506 [28]. CnA $\beta$  also contains a unique proline-rich N-terminal domain that increases its affinity for certain substrates [29]. Of particular note, the bHLH transcription factor ATOH8 was recently shown to interact specifically with CnA $\beta$  but not CnA $\alpha$  [30]. ATOH is involved in skeletal muscle and cardiac development [31, 32], however its role in heart disease has not yet been explored.

A unique splice variant of CnA $\beta$  was recently identified, CnA $\beta$ 1, that lacks the AID [33]. Similar to the C-terminal truncated CnA\* proteins, the CnA $\beta$ 1 variant is constitutively active. Remarkably, however, a cardiomyocyte-specific  $\alpha\text{MHC-Cn}\beta 1$  transgene does not provoke hypertrophy but exerts beneficial effects following myocardial infarction by promoting vascularization [34, 35]. How this is accomplished is not yet understood, however, interesting data is emerging that suggests CnA $\beta$ 1 may be important for proper localization and signaling of mTORC2 complexes [34, 36].

Cleavage of either CnA $\alpha$  or CnA $\beta$  by the  $\text{Ca}^{2+}$ -activated protease, calpain, generates constitutively active forms of calcineurin that lack the AID (Fig. 1C) [37–39]. Calpain activation following ischemia reperfusion (I/R) damages the myocardium by cleaving an array of proteins involved in contraction and its regulation [40–42]. Elevated levels of calpain-cleaved calcineurin are found in the hearts of patients with congestive heart failure [43, 44]. Once cleaved by calpain, calcineurin would remain active until removed by proteolysis or suppressed by interaction with an inhibitory protein.

Because CnB $\alpha$  is the only calcineurin regulatory subunit expressed in the heart, several groups have used tissue-specific deletion of CnB $\alpha$  to examine the consequences of eliminating calcineurin activity in the myocardium [45, 46]. Cardiac metabolism and

function decline in hearts lacking CnBa. The mice began to die of arrhythmias around 3 months of age, consistent with calcineurin playing a role in supporting cardiac electrophysiology.

Finally, the Fe—Zn active site of calcineurin is susceptible to reversible, Ca<sup>2+</sup>-dependent oxidative inactivation [47, 48]. This property may help limit unrestrained calcineurin activity in the setting of oxidative stress, which often occurs in conjunction with Ca<sup>2+</sup> overload.

## 2.2. Targeting of substrates to calcineurin

The catalytic cleft of calcineurin is relatively shallow and can accommodate a wide range of phospho-serine,-threonine and -tyrosine substrates [49, 50]. There is no conserved recognition motif surrounding the phosphorylated residue. Instead, targeting of most substrates relies on two docking motifs, PxIxIT and LxVP, found elsewhere in the target protein, that were initially identified in the well-characterized calcineurin substrate, Nuclear Factor of Activated T-cells (NFAT) [51, 52]. The PxIxIT domain binds to the catalytic domain of CnA, regardless of whether the enzyme is active or inactive, thereby increasing the effective local concentration of the substrate [53–55]. PxIxIT domains vary in their binding affinities, allowing for substrate selection based both on concentration and binding strength. The second docking motif, LxVP, binds to a hydrophobic pocket at the CnA/CnB interface, which is only accessible when calcineurin is active [14, 56]. An alternative model for LxVP binding has been proposed in which the LxVP docking site overlaps with the PxIxIT docking site [57]. Whether this mode of docking of substrates to CnA alone occurs when CnB is not present remains to be determined, but could impact interpretation of studies in which CnB has been deleted as a method of eliminating calcineurin activity.

## 2.3. Pharmacological inhibition of calcineurin

Calcineurin is the target of the major immunosuppressive drugs Cyclosporin A (CsA) and FK506 which form complexes with two different classes of immunophilins: cyclophilins and FK506 binding proteins respectively [58, 59]. The drug-immunophilin complexes bind in the same hydrophobic CnA/CnB groove used for docking of LxVP [56, 60–62], thereby blocking access of substrate proteins. The drug/immunophilin complexes do not actually block the catalytic domain, as the enzyme can still readily dephosphorylate p-nitrophenyl phosphate (pNPP), a non-proteinaceous, small molecule substrate often used in calcineurin assays [59]. Paradoxically, activity of calcineurin toward pNPP is stimulated as much as 4-fold by the presence of the drug complexes [59, 63, 64].

A number of small peptide inhibitors have been developed based on the PxIxIT, LxVP, and AID motifs (VIVIT) [65, 66]. A cell permeable derivative of PxIxIT (VIVIT) has been used successfully to inhibit pressure overload hypertrophy *in vivo* [67]. Because this targeted approach avoids the off-target side effects of CsA and FK506, it has been proposed as the basis for developing new therapeutic approaches aimed at cardiovascular disease [9]. For additional information Seiber and Baumgrass provide a comprehensive review of pharmacological calcineurin inhibitors [68].

## 2.4. Calcineurin inhibition by endogenous proteins

**2.4.1. CABIN-1/CAIN (calcineurin binding protein 1)**—CABIN-1 interacts with calcineurin *via* a PxIxIT-like domain [69, 70] as well as acting directly as a co-repressor of the transcription factor MEF2 [71, 72]. Although cardiomyocyte-specific over expression of CABIN-1 inhibits hypertrophy *in vivo* [8], it is uncertain whether the endogenous protein acts in this capacity, as calcineurin activity and NFAT translocation are unaltered in transgenic mice in which the calcineurin and MEF2-interacting domains of CABIN-1 have been deleted [73]. CABIN-1 is a very large, multidomain protein (N200-kDa), and therefore, may primarily function as a scaffold for calcineurin, facilitating its interaction with other proteins, rather than acting to inhibit calcineurin.

**2.4.2. Carabin (TBC1 domain family member 10C, TBC1D10C)**—Carabin is a bi-functional protein inhibiting both calcineurin and the small GTPase RAS [74, 75]. Carabin mRNA and protein levels decline in models of pressure overload and in human heart failure [76]. Loss of Carabin *in vivo* exacerbates pressure-overload hypertrophy and failure, whereas, cardiomyocyte-specific overexpression is protective [76, 77].

**2.4.3. Calcineurin homologous proteins (CHP1, 2 and 3)**—CHPs are structurally related to CnB and carry out a variety of cellular functions apparently unrelated to calcineurin [78], however, when over expressed, they are capable of both negatively [79] and positively [80] influencing calcineurin activity. The role of these proteins in cardiac function has not been explored.

**2.4.4. Regulators of calcineurin (RCAN1, 2 and 3)**—RCANs, also known as ADAPT78, DSCR1, MCIP1, and calcipressin [81] are relatively small proteins that inhibit calcineurin [82–84]. In addition to PxIxIT and LxVP motifs, RCANs contain a C-terminal domain unique to the RCAN family that is sufficient for calcineurin binding and inhibition [85–87]. Remarkably, in purified, *in vitro* assays, recombinant RCAN1 is capable of competitively inhibiting calcineurin-mediated dephosphorylation of both peptide substrates and pNPP [85, 87]. The ability to inhibit pNPP dephosphorylation makes them unique among calcineurin inhibitors, including CsA and FK506. Expression of the *Rcan1.4* isoform is under calcineurin/NFAT control and thus functions as a feedback inhibitor of calcineurin [88]. Expression of a cardiomyocyte-specific  $\alpha$ MHC-*Rcan1* transgene protects mice from a diversity of pathological stresses including pressure overload, damage from ischemia-reperfusion (I/R) and pathological remodeling following a myocardial infarction [5, 6, 89]. The hearts of animals deficient for RCAN1 (*Rcan1*<sup>-/-</sup>) are more susceptible to damage from I/R [89, 90]. Surprisingly, the hearts of *Rcan1*<sup>-/-</sup> mice are smaller and have a blunted hypertrophic response to pressure overload [90, 91]. This may be the result of physiological compensation or reflect RCAN1's impact on other pathways [92–94]. It has been suggested that RCAN1 may act as a chaperone or targeting protein that facilitates calcineurin signaling *in vivo*, although, rigorous biochemical evidence for this is currently limited [95, 96].

In addition, there are a wide variety of microRNAs relevant to cardiac remodeling and heart failure that have been proposed to act upon or be regulated by calcineurin that are beyond

the scope of this review. Their importance to control and integration of calcineurin signaling in cardiac health and disease should not be overlooked [97].

### 3. Methods for measuring calcineurin activity

Standard biochemical assays of calcineurin activity in tissue extracts are carried out under conditions of excess  $\text{Ca}^{2+}$  and calmodulin [98]. They therefore do not reflect actual *in vivo* activity but simply provide a measure of the total potential calcineurin activity available if all the calcineurin in the cell were activated. As an *in vivo* alternative, many groups have used quantification of *Rcan 1.4* transcript levels, as its expression is directly under the control of calcineurin/NFAT [88]. However, this approach also has limitations, as it only assesses calcineurin activity relative to NFAT-mediated transcription and therefore may not reflect the action of calcineurin on other substrates.

A widely used approach has been the use of luciferase or  $\beta$ -galactosidase reporters under the control of NFAT sites, delivered by transient transfection or adenoviral infection. Similarly, other groups have also used fluorescent proteins to monitor transcriptional activity of NFAT in the physiological context of living cells. The main advantages of the fluorescence-based assays over other traditional and classical luciferase assays are their application to individual living cells, as they do not require cell lysates for quantification. Based on the expression of GFP or RFP under the control of an NFAT-sensitive promoter, this assay displays a dose-sensitivity to different levels of NFAT and calcineurin activation [99]. To monitor activity *in vivo* a number of groups have developed transgenic mice carrying an NFAT-luciferase or  $\beta$ -galactosidase reporter [100, 101]. Even with these *in vivo* techniques it is important to remain aware of the potential impact of chromatin context as different reporters show different patterns of response dependent upon the location of the reporter within the genome.

A number of genetically-encoded, fluorescence resonance energy transfer (FRET) reporters have been developed recently that allow calcineurin activity to be assessed in live cells. The CaNAR reporter is based on the regulatory domain of NFAT tagged on opposing ends with a cyan and a yellow fluorescent protein. Dephosphorylation by calcineurin causes a conformational change that increases FRET transfer between the two ends [17]. This can be combined with a  $\text{Ca}^{2+}$  sensor to integrate  $\text{Ca}^{2+}$  dynamics with processing of a specific calcineurin substrate. Targeting this reporter to the cytosol, plasma membrane, mitochondria, or the ER, has been used to demonstrate that activation of the reporter in response to the same  $\text{Ca}^{2+}$  signal can be very different in different regions of a cell [17]. For instance, in a pancreatic  $\beta$  cell line, the cytoplasmic and plasma membrane reporter displayed a single, integrated response to an oscillatory  $\text{Ca}^{2+}$  input. In contrast, the ER and mitochondrial reporter oscillated directly in concert with the  $\text{Ca}^{2+}$  transient due to limited calmodulin availability proximal to the ER and rapid reversal of the reporter by cAMP activated protein kinase A (PKA) phosphorylation [17].

A new generation of FRET reporters, DuoCaN and UniCaN, has been tested in neonatal and adult cardiomyocytes and provides a direct readout of the calcineurin holoenzyme itself rather than its action on a particular substrate [102]. These reporters reveal important, fundamental differences in the pattern of calcineurin localization and activation at these two



stages of development. In neonatal cardiomyocytes the reporters were homogeneous throughout the cell and activated in response to a single  $\text{Ca}^{2+}$  pulse. Whereas, in isolated adult myocytes the reporters localized to T-tubules, primary sites  $\text{Ca}^{2+}$  release during contraction, consistent with the pattern described for endogenous calcineurin [103, 104]. Furthermore, in adult myocytes the reporters did not respond to either single  $\text{Ca}^{2+}$  transients or low frequency pacing, but required pacing frequencies sufficient to raise diastolic  $\text{Ca}^{2+}$  levels between beats. Thus, during early development of the heart,  $\text{Ca}^{2+}$  released during contraction may be sufficient to cause sustained activation of calcineurin, thereby promoting hypertrophic growth. Whereas, calcineurin in the adult heart remains unresponsive to normal, contraction coupled  $\text{Ca}^{2+}$  transients. This also means that mechanistic studies carried out in neonatal cardiomyocytes may not always be directly applicable to calcineurin signaling in the adult heart. It is important to note, however, that during failure, cardiomyocytes are thought to develop characteristics typical of immature cardiomyocytes. It is not known whether the pattern of calcineurin activation likewise reverts to a fetal pattern, or if this occurs in isolated adult cardiomyocytes that are maintained in culture over time.

#### 4. Calcineurin substrates relevant to cardiovascular health

Calcineurin has both immediate and long-term effects on cardiac function and adaptation. Rapid responses include direct changes in the function, activity, and/or localization of target proteins, changes that in general can be reversed rapidly by rephosphorylation. In contrast, calcineurin-dependent changes in transcription have longer-lasting consequences. First, we will discuss transcription factors controlled by calcineurin that have particular relevance to cardiovascular health. Then, cytoplasmic substrates not related to transcription will be discussed in the context of the specific subcellular domains in which they reside and the proteins that help organize these domains.

##### 4.1. Transcription factors controlled by calcineurin

**4.1.1. NFAT**—NFATs are the most fully characterized calcineurin substrates. They include: NFATc1 (*a.k.a.* NFAT2), NFATc2 (NFAT1), NFATc3 (NFAT4) and NFATc4 (NFAT3). The regulatory domains of NFATs are highly phosphorylated in resting cells and flanked by a PxIxIT and LxVP motif at either end [56, 105]. Dephosphorylation by calcineurin causes translocation to the nucleus to activate target genes. NFATs tend to function as heterodimers in cooperation with other transcription factors. Of particular note relative to cardiac growth and remodeling are MEF2 and GATA, which can also be directly activated by calcineurin-mediated dephosphorylation. Cardiac-specific over expression of an activated NFATc4 is sufficient to drive hypertrophy [4], whereas, forced expression of a dominant-negative NFATc4 will blunt calcineurin-dependent hypertrophy [106], demonstrating that calcineurin-mediated remodeling acts, at least in part, *via* NFAT-dependent transcriptional control. At the transcript level, NFATc2 is the most abundant NFAT isoform in the heart and indeed, pathological growth in response to pressure overload or angiotensin II (AngII) infusion is markedly blunted in *Nfatc2*<sup>-/-</sup> mice [107]. Physiological hypertrophy in response to voluntary exercise is unaltered in these mice, suggesting that either NFATc2 is not involved in this mode of hypertrophic growth or that the other NFATs are sufficient to compensate.

Pathological hypertrophy is also reduced in *Nfatc3*<sup>-/-</sup> mice but is unaltered in *Nfatc4*<sup>-/-</sup> mice [108]. Deficiency for *Nfatc1* is embryonic lethal due to defects in cardiogenesis [109] and as yet, a cardiomyocyte-specific deletion has not been studied in the context of hypertrophy. Although both NFATc2 and NFATc3 appear to mediate pathological hypertrophy, they differ in their affinity for calcineurin as well as in the dynamics of their response. For instance, in HEK293 cells, NFATc2 translocates to the nucleus in response to Ca<sup>2+</sup> released from plasma membrane channels and its subsequent exit from the nucleus is relatively slow. In contrast, translocation of NFATc3 to the nucleus requires an increase in both nuclear and cytoplasmic Ca<sup>2+</sup>, whereas, it exits from the nucleus rapidly [110]. Using NFAT-GFP fusion proteins in isolated adult atrial and ventricular cardiomyocytes, NFATc1 and NFATc3 showed different responses to AngII or endothelin-1 that depended upon both cell type and NFAT isoform [111]. Isoform-specific NFAT activation has also been reported in skeletal muscle [112, 113]. Although it is clear that NFAT-dependent changes in transcription are an important aspect of calcineurin-mediated cardiac remodeling, the identity of the target genes that are most relevant is less clear.

Calcineurin can regulate transcription through a number of other transcription factors in addition to NFAT. Of particular note, relative to cardiac remodeling, are the cAMP response element binding protein (CREB)-regulated transcriptional coactivators (CRTCs), the forkhead box protein 01 (FOXO1) and the transcription factor EB (TFEB).

**4.1.2. CRTC**—Similar to NFATs, dephosphorylation of CRTCs by calcineurin promotes translocation to the nucleus where they act in conjunction with CREB to promote gene expression. Although the role of CRTCs is yet to be studied in the heart, in other tissues they act to integrate hormonal and metabolic signals [114–116] and promote mitochondrial biogenesis [117–119]. The ability of CRTCs to integrate Ca<sup>2+</sup> and cAMP signals makes them ideal candidates for metabolic regulators, particularly in the setting of increased adrenergic stress.

**4.1.3. FOXO1**—FOXO1 is a key factor in metabolic remodeling of the heart in diabetic cardiomyopathy and post-ischemic heart failure [120–123]. In the ischemic brain, calcineurin has been shown to dephosphorylate FOXO1 forming a complex that translocates along with FOXO1 into the nucleus acting in conjunction with NFAT to drive expression of Fas-Ligand [124], which is also released by the ischemic heart [125]. In turn, there is evidence that FOXO1 activity can provide feedback inhibition of calcineurin [126–128], affording additional integration of these two pathways.

**4.1.4. TFEB**—TFEB is a basic-helix-loop-helix leucine-zipper transcription factor that is considered a master regulator of autophagic and lysosomal biogenesis [129, 130]. Dephosphorylation of TFEB by calcineurin causes activation and translocation to the nucleus to promote coordinate expression of target genes. This interaction may help integrate calcineurin-dependent hypertrophic growth with the degradative processes carried out by autophagy during structural and metabolic remodeling of the myocardium [131, 132]

It is important to note that calcineurin control of transcription factor activity is not necessarily always due to direct changes in phosphorylation of the transcription factor. For



instance, in response to hypertrophic agonists, there is a calcineurin-dependent increase in MEF2A and MEF2D protein levels due to an increase in the abundance of Polypyrimidine Tract Binding Protein (PTB) which then acts to increase translation of MEF2 proteins without altering transcript abundance [133]. It is not known whether PTB increases translation of other proteins with cardiovascular relevance.

#### 4.2. Targeting calcineurin to cytoplasmic domains

Both direct immunohistochemistry and the localization of FRET reporters indicate that in adult cardiomyocytes the majority of calcineurin protein is found in the vicinity of the T-tubules, which are deep invaginations of the sarcolemma allowing for the close association between voltage-activated L-type calcium channels (LTCC) in the sarcolemma and ryanodine receptors (RyR2) in the sarcoplasmic reticulum (SR). They form the fundamental dyad for  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$ -release involved in excitation-contraction coupling. Many other membrane channels and receptors are also found in T-tubules, which directly overlie the Z-line, or Z-disc of the sarcomere. Thus, calcineurin is in close proximity to many of the proteins involved in excitation-contraction coupling and mechanotransduction. This does not preclude the possibility of other pools of calcineurin, either free in the cytoplasm or tethered elsewhere.

Intracellular  $\text{Ca}^{2+}$  release from the SR is required for cardiac muscle contraction. Indeed, it is postulated that during heart failure impaired  $\text{Ca}^{2+}$  release results in decreased muscle contraction (systolic dysfunction) and defective  $\text{Ca}^{2+}$  removal hampers relaxation (diastolic dysfunction). There are numerous examples of animal models with diastolic dysfunction and hypertrophy associated with elevated calcineurin activity, [4, 134, 135]. Calcineurin specifically responds to sustained, low-amplitude  $\text{Ca}^{2+}$  but can ignore transient, high amplitude  $\text{Ca}^{2+}$  spikes [136], thus, the kind of sustained elevation in diastolic  $\text{Ca}^{2+}$  that occurs with diastolic dysfunction is ideally poised to support calcineurin activation and subsequent hypertrophic remodeling [137]. Diastolic  $\text{Ca}^{2+}$  levels depend upon the interplay between RyR release and reuptake by SERCA [138]. Normal  $\text{Ca}^{2+}$  handling is disrupted in patients with heart failure due to a chronic hyperadrenergic state that hyper activates then subsequently desensitizes beta-adrenergic receptors, which control RyR and SERCA function via PKA [139].

In the heart, calcineurin is often found in close association with PKA. A kinase anchor proteins (AKAPs) are scaffolding proteins that help to form multimolecular signaling complexes that contain PKA. Calcineurin binds to several AKAPs that play a prominent role in its subcellular targeting. In the heart these include: the beta isoform of AKAP6 (AKAP6 $\beta$ /mAKAP $\beta$ ), localized to the nuclear envelope; AKAP5 (AKAP79/AKAP150), AKAP12 (Gravin), and AKAP7 (AKAP15/AKAP18), localized to the plasma membrane, T-tubules, and ER/SR; and AKAP1 (AKAP121/AKAP84), localized to ER/SR and outer membrane of mitochondria (OMM) [140].

**4.2.1. AKAP6 (AKAP6 $\beta$ /mAKAP $\beta$ )**—Located at the nuclear envelope, AKAP6 does not use a PxIxIT motif to tether calcineurin [141]. As such, the interaction neither competes with substrates for binding nor inhibits catalytic activity. In neonatal cardiomyocytes and

C2C12 myoblasts, AKAP6 is essential for agonist induced NFAT translocation [142] and organization of a calcineurin/MEF2 regulatory complex [143]. AKAP6 also acts as a scaffold for HDAC4 and kinases that phosphorylate it, thereby releasing HDAC suppression of MEF2-dependent transcription [144]. AKAP6 has binding domains for both RYR2 [145] and the sodium calcium exchanger (NCX1) [146], a subset of which localize to the nuclear envelope. Mice with a cardiomyocyte-specific deletion of AKAP6 are resistant to pressure overload or agonist induced heart failure [144]. AKAP6 binds a wide array of other proteins and signaling molecules important in cardiac stress responses [147], therefore, AKAP6 provides a platform near the nucleus for integrating calcineurin signaling with a diversity of cytoplasmic signals known to mediate transcriptional responses to cardiac stress.

The AKAP6/PKA/calcineurin complex also mediates control over nuclear cytoplasmic shuttling of myopodin (synaptipodin 2), an actin-bundling protein that relocates from the nucleus to the Z-disc of myocytes upon differentiation but returns to the nucleus in response to stress [148]. Phosphorylation by PKA promotes release from the Z-disc and dephosphorylation by calcineurin prevents nuclear translocation [149]. Myopodin's role in heart failure is unknown.

**4.2.2. AKAP5 (AKAP79/AKAP150)**—AKAP5 mediates calcineurin association with several channels in the sarcolemma, including the LTCC. Quantitative analysis of a purified complex indicates that four calcineurin heterodimers bind per AKAP5 dimer [150]. Therefore, although the two PxIxIT motifs in the AKAP5 dimer would occupy two of the PxIxIT docking domains on calcineurin, two docking domains remain available for binding of calcineurin substrates, such as NFAT or nearby channel proteins. In adult cardiomyocytes there are two distinct pools of LTCCs, both of which are associated with AKAP5/calcineurin complexes [151]. The majority of LTCCs localize to T-tubules where the majority of calcineurin protein is also located. A small fraction of LTCCs are also found associated with AKAP5/calcineurin in caveolae, a specialized lipid raft domain defined by the presence of caveolin. Caveolae function both in endocytosis and as platforms for signalosomes [152, 153]. Inhibition of LTCC activity specifically within these Cav-3 containing domains has minimal impact on total LTCC current ( $I_{Ca,L}$ ) or myocyte contractility, but eliminates  $Ca^{2+}$  influx-induced nuclear translocation of NFATc3 [154]. Thus, input from LTCC localized to caveolae plays an important role in specifying LTCC/calcineurin/NFATc3 signaling. This is supported by studies demonstrating that cardiomyocytes isolated from mice lacking AKAP5 or expressing a mutant form of AKAP5 deleted for the calcineurin PxIxIT binding motif (AKAP5-PIX) are unable to sustain nuclear translocation of NFATc3 in response to adrenergic stimulation [155].

CIB1 ( $Ca^{2+}$ - and integrin-binding protein-1) is a small EF-hand protein similar in structure to CnB that is also involved in mediating an interaction between calcineurin and LTCC [156, 157]. CIB1 expression is elevated in the atria of patients with atrial fibrillation where it can be co-immunoprecipitated with CnB, LTCC, and NCX1 [158]. CIB1 levels increase in the setting of pathological hypertrophy, but not physiological hypertrophy [157]. Loss of CIB1 blunts, whereas over expression increases, the hypertrophic response to pressure overload [157].

In heart failure there is a loss of T-tubule structure [159, 160] and a decrease in the fraction of LTCC and NCX channels associated with T-tubule domains without a loss of total channel activity in individual myocytes [161–164]. This shift in LTCC pools during failure may increase the pool of LTCC available for flux through calcineurin/NFATc3 signaling in a feed-forward process. In turn, calcineurin increases LTCC activity in failing heart [165]. It is generally accepted that adrenergic stimuli increases  $I_{Ca,L}$  via PKA phosphorylation of the LTCC [166]. Yet, peak  $I_{Ca,L}$  is increased in  $\alpha$ MHC-CnA\* transgenic mice as well as in those subjected to pressure overload failure. Furthermore, this increase is can be reversed by calcineurin inhibition but not PKA inhibition [167, 168]. The finding that PKA activation of  $I_{Ca,L}$  is specific to T-tubules and is lost upon detubulation might explain this discrepancy [164, 169, 170]. One possibility is that modification of  $I_{Ca,L}$  by calcineurin occurs throughout the cell but is masked by the opposing action of PKA in T-tubules. Alternatively, calcineurin control of  $I_{Ca,L}$  may only occur outside of T-tubule domains.

AKAP5/calcineurin is also involved in the decline in fast transient outward  $K^+$  current ( $I_{to}$ ) that is associated with heart failure and pathological hypertrophy [171]. The  $\alpha$ MHC-CnA\* transgenic mice have a significant reduction in  $I_{to}$  density that can be reversed by treatment with CsA [172]. In isolated adult cardiomyocytes and intact hearts calcineurin decreases  $I_{to}$  in part through AKAP5-dependent activation of NFATc3 leading to transcriptional repression of the expression of several  $K^+$  channel subunits including Kv4.3, Kv4.2, and KChIP2 [155, 173, 174]. The mechanism of NFATc3-dependent repression is not fully understood and could be secondary to expression of an unidentified repressor. It is likely that calcineurin can also affect  $I_{to}$  independent of transcription. In neurons, AKAP5/calcineurin has been shown to form a complex with Kv channels where the opposing forces of phosphorylation by PKA and dephosphorylation by calcineurin regulate activity-dependent trafficking of Kv4.2 [175], with calcineurin promoting surface expression. This has not yet been demonstrated in cardiomyocytes, but changes in the ability to recycle channels appropriately could theoretically contribute to the calcineurin-dependent decline in  $I_{to}$  function. In cardiomyocytes the AKAP5/PKA/calcineurin complex has been shown to be required for internalization and recycling of  $\beta_1$ -adrenergic receptors ( $\beta_1$ -AR) [176]. Calcineurin's role in turnover of the many other ion channels found in lipid rafts and how this impacts cardiac function is an important avenue for future investigation.

**4.2.3. AKAP12 (Gravin/AKAP250)**—AKAP12 targets PKA, PKC and calcineurin to  $\beta_2$ ARs localized primarily to caveolae in the plasma membrane. The complex mediates desensitization and recycling of the receptor by controlling its phosphorylation [177]. Inhibition of calcineurin blocks resensitization following agonist removal [178]. In contrast, the interaction of AKAP5 with  $\beta_2$ ARs is thought to mediate the switch from stimulatory  $G_s$  to inhibitory  $G_i$  mediated signaling [179]. Whereas AKAP5 is absolutely required for the recycling of  $B_1$ ARs, which couple only via  $G_s$ , and is localized throughout the T-tubules and plasma membrane [176].

**4.2.4. AKAP7 (AKAP15/AKAP18)**—Differential splicing generates a variety of AKAP7 isoforms [180]. Although AKAP7 does not bind calcineurin directly, it can compete with AKAP5/calcineurin for binding LTCC [181]. However, mice lacking AKAP7 respond

normally to adrenergic stimulation [182], suggesting that the AKAP5 complex plays the primary role in adrenergic responses of the LTCC.

AKAP7 interacts indirectly with calcineurin by providing a scaffold for the protein phosphatase inhibitor 1 (I-1), a calcineurin substrate that controls the activity of the protein phosphatase 1 (PP1) [183]. Phosphorylation of I-1 at Thr35 by PKA induces selective inhibition of PP1 [184]. AKAP7/PKA/I-1 complexes are found associated with the SR and phospholamban (PLN) where PKA-mediated phosphorylation of I-1 helps prolong adrenergic responses at the SR by preventing their reversal by PP1. Perturbations in PP1 regulation by I-1 have been implicated in heart failure [185]. Calcineurin dephosphorylates I-1 at Thr35, releasing inhibition of PP1 [186], and providing a mechanism through which calcineurin impacts a wider range of cellular phosphatase activities in the heart. Because calcineurin has not been identified as a component of AKAP7 complexes, it may be that tethering I-1 to the SR *via* an AKAP7 scaffold helps to sequester this specific pool of I-1 so as to actually minimize reversal by calcineurin. Consistent with this model, PLN phosphorylation is significantly lower in cardiomyocytes deleted for CnB [46], suggesting that on the whole, calcineurin may act to promote PLN phosphorylation rather than decreasing it.

**4.2.5. AKAP1 (AKAP121/AKAP84) and calcineurin's impact on mitochondrial dynamics**—Mitochondrial dysfunction has emerged as a critical factor in the progression of heart failure [187–190]. Impaired mitochondrial biogenesis and loss of mitochondrial content is associated with the transition from compensated hypertrophy to failure in patients with heart disease [191]. Mitochondrial electron transport is impaired in  $\alpha$ MHC-CnA\* transgenic mice and superoxide production is increased both before and after I/R [192]. Mitochondrial changes reported in mice with a cardiomyocyte-specific deletion of CnB range from deficiencies in the ability to oxidize fatty acids [46] to aberrant mitochondrial architecture [45]. Taken together, these findings suggest that either sustained activation of calcineurin or insufficient calcineurin activity has a profound negative effect on cardiac mitochondrial function. Diverse mechanisms are likely involved, however, calcineurin's influence on mitochondrial dynamics is emerging as an important aspect.

Mitochondria are dynamic organelles that undergo a continuous process of fission and fusion that is essential for their repair and regeneration. Calcineurin promotes fission by dephosphorylating Dynamin Related Protein 1 (DRP1), which contains an LxVP calcineurin-docking motif [193], thereby activating it to initiate fission at the OMM [194, 195]. PKA rephosphorylates DRP1, thus, promoting fusion. Intermembranous mitochondria in the ventricular wall are often described as spanning from Z-band to Z-band, thereby placing sites of fission in alignment with the primary pool of calcineurin in adult cardiomyocytes. AKAP1 interacts with DRP1, PKA and calcineurin [196–198]. Although the nature of these interactions is not yet well defined. The extent to which intermembranous mitochondria undergo fission and fusion is currently debated [199] and may be limited due to the spatial constraints imposed by close proximity to the sarcomere. However, genetic data indicate that fission and fusion are essential to cardiovascular health as mice with cardiomyocyte-specific disruption of mitofusions 1 and 2 (*Mfn1/Mfn2*) (to prevent fusion) or *Drp1* (to prevent fission) progress to heart failure [200–203].

#### **4.2.5.1. Calcineurin-dependent mitochondrial fission is required for**

**hypertrophy:** Mitochondrial fission is required for hypertrophic growth of both neonatal [204, 205] and adult cardiomyocytes [206]. Expression of a dominant-negative DRP1 or treatment with mdivi-1, a DRP1 inhibitor, is sufficient to block induction of hypertrophic growth by norepinephrine. Conversely, depletion of the fusion protein MFN2 is sufficient to stimulate hypertrophic growth without agonist treatment [205]. On a certain level it is intuitive that fission must occur during maturation of neonatal cardiomyocytes to convert the continuous reticulum found in embryonic cells into an ordered intermyofibrillar array, however, treatment with mdivi-1 also blunts hypertrophic growth and preserves cardiac function in the adult heart in the setting of pressure overload induced heart failure [206]. Furthermore, mdivi-1 reduces infarct size and preserves cardiac function following ischemia reperfusion [207, 208]. The extent to which calcineurin-mediated mitochondrial fission contributes to other forms of ischemic and non-ischemic heart failure, and the potential for therapeutic targeting remains a promising avenue of research [209].

**4.2.5.2. Additional mitochondrial targets:** BAD, a pro-apoptotic Bcl2 protein, is dephosphorylated by calcineurin promoting its translocation to the OMM [210] where it initiates opening of the mitochondrial permeability transition pore (MPTP), release of cytochrome *c* and activation of a caspase-3 apoptotic cascade [211, 212]. This mechanism contributes to neuronal death in the hippocampus following ischemia [213], but its role in triggering death of cardiomyocytes is less well understood.

Cofilin is an actin-binding protein that translocates to mitochondria and induces cell death in response to oxidative stress [214, 215]. In cardiomyocytes, the cofilin phosphatase Slingshot 1L promotes cofilin translocation to mitochondria and increases damage from ischemia reperfusion [216]. Calcineurin activates Slingshot making cofilin-mediated damage an indirect target of calcineurin [217, 218]. Hyperphosphorylated cytoplasmic cofilin aggregates have been found in the hearts of patients with idiopathic cardiomyopathy [219]. Therefore, whether calcineurin's impact on cofilin is protective or pathological may depend on context.

**4.2.6. Calcineurin's impact on the AKAP/PKA interaction—**A varied range of signaling molecules are assembled by each AKAP. All AKAPs bind PKA, whereas only a subset of AKAP complexes also contain calcineurin. The catalytic domains of PKA (PKA<sub>cat</sub>) are bound to AKAPs in an inactive form by their regulatory subunits (either RI or RII). Binding of cAMP to the R subunits releases activated PKA<sub>cat</sub> from the complex. Phosphorylation of RII at Ser-96 can both increase affinity for AKAP [220] and decrease its affinity for PKA<sub>cat</sub> [221, 222]. The RI subunit is pseudo-phosphorylated at the corresponding residue and is therefore not regulated by changes in phosphorylation. RII Ser-96 is a calcineurin substrate and contains an LxVP targeting motif [56]. In fact, a phospho-peptide from the RII subunit is the standard peptide substrate used to assay calcineurin activity *in vitro*. Dissociation of PKA<sub>cat</sub> from RII upon activation exposes the phospho-Ser-96 site allowing access by calcineurin or other phosphatases. Thus, in AKAP complexes containing RII, calcineurin is posed to facilitate reassociation of PKA<sub>cat</sub> with RII

in the inactive form [223]. In heart extracts RII is primarily found in the phosphorylated form [224] and is decreased in human hearts with dilated cardiomyopathy [220].

### 4.3. Calcineurin and mechanotransduction

**4.3.1. Mechanotransduction at the plasma membrane**—Calcineurin activation in response mechanical stress lies downstream of  $\text{Ca}^{2+}$  entry through transient receptor potential canonical (TRPC) channels composed of homo or heterotetramers of either TRPC1/4/5 (stretch activated) or TRPC3/6/7 (activated by diacylglycerol downstream of G protein coupled receptors). Over expression of TRPC3/ 6/7 members promotes calcineurin/NFAT activation and hypertrophy [225, 226], whereas inhibition of either subtype blunts hypertrophy and failure in response to sustained agonist or pressure overload [227]. Expression of TRPC6 increases in response to pressure overload under the control of calcineurin/NFAT, thereby creating another feedforward amplification loop driving pathological hypertrophy [228]. The anti-hypertrophic effect of natriuretic peptides acts in part by inhibiting TRPC6 channel activity [229]. TRPC1/3/6 activation of calcineurin/NFAT signaling also occurs during stretch activation of myofibroblasts [230].

Polycystin-1 (PC-1), another member of the TRPC family of  $\text{Ca}^{2+}$  transporters, can act as a mechanosensor outside of its role as a component of primary cilia. In cardiomyocytes, PC-1 activates calcineurin/NFAT signaling in part by increasing LTCC stability [231]. Pressure overload hypertrophy and calcineurin activation are blunted in PC-1<sup>-/-</sup> mice.

Syndacan-4 is a transmembrane heparin sulfate proteoglycan that increases in the left ventricle following pressure overload [232, 233]. Syndacan-4 is required for activation of calcineurin/NFAT signaling in response to mechanical stress through TRPC6 in cardiac fibroblasts, promoting production of extracellular matrix and differentiation into activated myofibroblasts thereby increasing myocardial stiffness [233–235].

**4.3.2. Mechanotransduction at the Z-disc**—The Z-disc is emerging as a nodal point for cardiomyocyte signal transduction [236]. Calcineurin is tethered to the Z-disc *via* interactions with muscle LIM protein (MLP) [237] and calsarcin [104, 238]. Deficiency for MLP reduces activation of calcineurin following an adrenergic stimulus or biomechanical stress, whereas loss of calsarcin-1 increases mechanical stress activation of calcineurin, but has no effect on adrenergic activation of calcineurin.

PICOT (protein kinase C-interacting cousin of thioredoxin) interacts directly with MLP and can disrupt its interaction with calcineurin [239]. Over expression of PICOT inhibits calcineurin activation and blunts pressure overload-induced hypertrophy, whereas, mice heterozygous for a PICOT disruption have an exacerbated response to pressure overload [240].

LMCD1 (LIM and cysteine-rich domains 1) is another Z-disc protein that facilitates calcineurin activation. LMCD1 levels increase in pressure overload, following MI and in response to AngII infusion [241, 242]. Transgenic over-expression of LMCD1 increases calcineurin activation in response to pressure overload and accentuates pathological remodeling *in vivo*, whereas depletion of LMCD1 blunts NFAT activation *in vitro* [242].



Active mechanisms for controlling calcineurin protein levels are also present at the Z-disc. The muscle-specific RING finger 1 (MuRF1) is an E3 ubiquitin ligase that targets CnA for degradation *via* the proteasome [243]. *Murf1*<sup>-/-</sup> mice exhibit enhanced fibrosis and hypertrophy in response to pressure overload that can be normalized by calcineurin inhibition. For an expanded discussion of mechanotransduction at the Z-disc and the integration of calcineurin at this signaling juncture, several excellent reviews are available [236, 244–247].

#### 4.4. Targeting calcineurin to the Na<sup>+</sup>/H<sup>+</sup> exchanger 1 (NHE1)

Calcineurin binds directly to NHE1 in the plasma membrane through a PxIxIT motif [248]. Over expression of NHE1 in neonatal cardiomyocytes increased calcineurin activity by increasing intracellular pH. Moreover, this interaction required clustering of NHE1 into lipid rafts, creating a microdomain with higher pH. Transgenic over expression of NHE1 is sufficient to induce nuclear translocation of NFAT and cardiac hypertrophy [249]. NHE1 is found exclusively with caveolae in adult myocardium [152], whereas only a small portion of NCX1 is found in caveolae [250, 251]. Calcineurin can inhibit NCX1 activity [252, 253], thus the colocalization of NHE1 with NCX1 in lipid rafts provides a microdomain platform for calcineurin coupling to coordinate regulation of these exchangers.

The calcineurin targets highlighted here are by no means exhaustive of all known calcineurin substrates and targeting molecules that may be relevant to cardiovascular biology, however, we have tried to cover the majority of those for which molecular studies have been carried out in the context of cardiomyocytes. Given the broad-spectrum nature of phosphatase substrate selection in general, it is quite likely that some of targets most relevant to cardiovascular health are yet to be identified.

### 5. Not all calcineurin activity is pathological

Research on calcineurin's function in the diverse contexts described above has focused primarily on disease progression and pathological outcomes, however, the abundance of calcineurin in heart tissue suggests that it carries out fundamental processes needed to maintain normal function and health. This is supported by the observation that cardiac metabolism and function are severely compromised in mice with a cardiomyocyte-specific deletion of the regulatory subunit *PPP3r1* [45, 46]. Although a multitude of studies have demonstrated calcineurin's involvement in pathological hypertrophy, its role during physiological hypertrophy is not as clear. Cardiomyocyte-specific overexpression of an *Rcan1* transgene to inhibit calcineurin blunts exercise-induced physiological hypertrophy [5], yet exercise failed to activate a genetically encoded *NFAT-Luciferase* transgene reporter [100]. It may be that NFAT is not the pertinent calcineurin substrate during physiological hypertrophy. This would be consistent with the observation that pathological but not physiological hypertrophy is blunted in *Nfatc2*<sup>-/-</sup> mice [107]. Even in the context of pathological hypertrophy, some calcineurin-dependent processes may be beneficial. For instance, cardiomyocyte-specific overexpression of a *Rcan2* transgene inhibited hypertrophy in the setting of pressure overload but impaired relaxation and increased stiffness [254]. Pharmacological inhibition with CsA increased mortality in a model of severe pressure

overload [255]. Whether this was due to off target effects of the drug or inhibition of a beneficial calcineurin-dependent adaptation requires further study.

A recent study demonstrates that calcineurin is activated in models of both hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM) [256]. The HCM phenotype was caused by a mutation in cardiac troponin C (cTnC) that increased both  $\text{Ca}^{2+}$  affinity and tension, whereas the DCM cTnC mutation decreased  $\text{Ca}^{2+}$  affinity and myofilament tension. Although both models displayed calcineurin-dependent hypertrophic growth, the former manifest as concentric hypertrophy and the later as eccentric hypertrophy, suggesting that increased and decreased tension set in motion a different array of calcineurin-dependent responses. This study is an elegant illustration that pathological activation of calcineurin can derive from varied inputs and have diverse outcomes. Similarly, we propose that, although there are certain to be some aspects of similarity, the behavior and targets of calcineurin signaling in a healthy heart may be distinct from those set in motion by pathological stresses.

## 6. Maintaining the transmural gradient

Healthy hearts display variation in the morphology of the action potential across the ventricular wall such that the action potential duration (APD) is shorter in the sub-epicardial cardiomyocytes (EPI) than in the sub-endocardial cardiomyocytes (ENDO). This allows ventricular repolarization to proceed in a synchronized wave from EPI to ENDO, supporting efficient pump function and preventing arrhythmias. These transmural differences are due to gradients in the functional distribution of several membrane currents including the transient outward  $\text{K}^+$  current ( $I_{to}$ ), the LTCC ( $I_{Ca,L}$ ), and the Na/K ATPase pump current ( $I_p$ ) [257–259]. Models based on these known differences predict that both diastolic and systolic  $\text{Ca}^{2+}$  is higher in ENDO than in EPI [260] and indeed, actual measures of calcineurin and NFAT indicate higher activity in ENDO compared to in EPI [174]. Mechanical stress is likewise greater in ENDO than in EPI [261] and therefore represents a potential underlying mechanism for establishing a gradient in calcineurin activity across the ventricular wall.

In NFATc3-null mice the transmural gradient in  $I_{to}$  is lost due to an increase in Kv4.2 protein and  $I_{to}$  in ENDO, thereby shortening APD in ENDO to match that of EPI [174]. Chronic infusion of the  $\beta$  adrenergic agonist isoproterenol also leads to a loss of the in  $I_{to}$  gradient by reducing Kv4.2 and  $I_{to}$  in EPI, thus increasing APD in EPI to match ENDO [262]. This change requires both  $\beta$ 1-ARs and NFATc3. It therefore is likely mediated through AKAP5/calcineurin repression of  $I_{to}$  described above. Interestingly, loss of NFATc3 does not alter transmural expression of the transcription factor *Irx5* that has been linked to establishing the  $I_{to}$  gradient during development [263].

Calcineurin can also influence the gradient in  $I_{Ca,L}$ , which is likewise higher in ENDO than in EPI. In a model of compensated pressure overload hypertrophy, where the  $I_{to}$  gradient was not yet effected, there was a calcineurin-dependent increase in peak  $I_{Ca,L}$  across the ventricular wall that was greatest in ENDO [264]. Thus, in contrast to  $I_{to}$ , and its gradient, which are suppressed by calcineurin, the gradient in peak  $I_{Ca,L}$  is accentuated by calcineurin activity. One important difference in mechanism of control is that calcineurin interacts

directly with both the N and C termini of the LTCC [165, 167] whereas its control of  $I_{to}$  is indirect. Relative to the gradient in  $I_p$ , calcineurin activation increases expression of the Na/K ATPase in the heart [265, 266] and studies from brain demonstrate direct activation of the channel by calcineurin-mediated dephosphorylation [267]. Therefore, calcineurin activity may also control the transmural gradient in  $I_p$ , however, no studies have addressed this.

Also of note, miR-21 is among a limited number of microRNAs that display a transmural pattern of expression that peaks in the ENDO [268]. MiR-21 is also found upregulated in the hearts of young  $\alpha$ MHC-CnA\* mice before the onset of increased fibrosis, suggesting that MiR-21 expression is calcineurin-dependent [269]. Thus, calcineurin impacts transmural gradients through multiple mechanisms, some of which may remain to be identified.

Taken together, calcineurin activity is essential for establishing and maintaining an appropriate transmural gradient in ion channel activity and APD in a healthy heart. Whereas, sustained activation of calcineurin across the myocardium contributes to the collapse of the APD gradient increasing arrhythmogenic potential. It is relevant to note that  $\alpha$ MHC-CnA\* mice die of arrhythmias rather than overt pump failure.

## 7. Circadian activation of calcineurin in the heart

Timing of calcineurin activation may be as critical as location. The cardiovascular system displays significant circadian rhythms in many physiological processes and continual disruption of normal circadian rhythms predisposes humans to cardiovascular disease [270–274]. In a healthy rodent heart there is a circadian pattern of calcineurin activity that peaks at the end of the animal's active period and is at its lowest at the end of its rest period [275]. As much as a 20-fold change can be seen in nuclear translocation of NFAT, binding of NFAT to chromatin, and expression of the calcineurin/NFAT controlled gene, *Rcan1.4*, over the course of twenty-four hours. Therefore, although both sustained activation of calcineurin [4] and its complete absence [45] have pathological consequences, a diurnal pattern of calcineurin activation is compatible with heart health. The peak in calcineurin activity immediately precedes a time of day when the heart is less susceptible to damage from I/R [275, 276]. These diurnal changes in susceptibility appear to be mediated by changes in RCAN1 levels, as *Rcan1*<sup>-/-</sup> mice no longer display this temporal window of protection [89].

Circadian activation of calcineurin is not unique to the heart and has also been documented in skeletal muscle [277], as evidenced by changes in *Rcan1.4* transcript levels and activity of NFAT reporters. In a series of elegant experiments these investigators demonstrated that circadian activation of the calcineurin/NFAT pathway was independent of the transcriptional circadian clock mechanism, and was driven instead by muscle innervation and contractile activity. Related mechanisms are likely involved in circadian activation of calcineurin in the heart.

Sleep deprivation studies in rat indicate a prolongation in action potential duration (APD) attributable to a decrease in  $I_{to}$  [278]. These changes were significant after only one day demonstrating the immediate impact of proper circadian entrainment on fundamental

electrical properties of the heart. Whether the decline in  $I_{to}$  is directly attributable to disruption of a normal circadian pattern of signaling through AKAP5/ calcineurin/NFATc3 remains to be determined. It is interesting to note that several of the genes displaying transmural expression across the ventricular wall [279] also display a circadian pattern of expression the heart.

Circadian changes in signaling pathways controlled by protein phosphorylation are very often overlooked but are not unique to calcineurin. Some are directly under circadian entrainment, such as changes in adrenergic activity, while others may respond directly to feeding patterns, such as insulin release. Corresponding changes in the activity of PKA and protein kinase B (AKT) provide important signals regulating cardiac function, metabolism, and remodeling [280]. The circadian oscillations in calcineurin activity and other kinase/ phosphorylation cascades may allow temporal separation of processes that are incompatible but essential.

## 8. Calcineurin in neonatal versus adult cardiomyocytes

Isolated neonatal cardiomyocytes have proven an invaluable tool for studying the role of calcineurin in the heart. However, not all findings from neonatal cells may be directly translated to the adult heart as there are fundamental structural, metabolic, and functional differences [281, 282]. Of particular note are differences in  $Ca^{2+}$  handling, release, and storage. All of which can have a direct impact on the character and duration of the  $Ca^{2+}$  signal perceived by calcineurin. In early embryonic cardiomyocytes  $Ca^{2+}$  is released primarily from the perinuclear SR through the cooperative action of inositol-3-phosphate receptors ( $IP_3Rs$ ) and RyRs to drive contraction [283]. At the time of birth, some junctional  $Ca^{2+}$  release units or “dyads” have formed that position RyRs in close apposition to  $Ca^{2+}$  channels in the sarcolemma, however, these are primarily located in the periphery of the cell, as T-tubules do not form until 10 days after birth, simultaneous with the appearance of the protein junctophilin-2 [284]. At this point in development the majority of RyRs are internal and not associated with a dyad. These “orphaned” RyRs are triggered consecutively *via* a “fire-diffuse-fire” mechanism to propagate the  $Ca^{2+}$  wave [285]. This results in a slower time to peak and decay of both the action potential and the  $Ca^{2+}$  transient compared to in adult myocytes. In addition, after birth, there is a dramatic increase in the capacity for buffering cytosolic  $Ca^{2+}$ . This substantially slows the diffusion of  $Ca^{2+}$  relative to other ions, thus increasing the potential for establishing confined  $Ca^{2+}$  microdomains that may act to shield specific pools of calcineurin from activation. It is important to note that neonatal cardiomyocytes will spontaneously contract in culture whereas isolated adult cardiomyocytes do not, reflecting fundamental differences in  $Ca^{2+}$  handling. An excellent review provides details of the changes that occur during maturation from embryonic to adult cardiomyocytes [285] as well as discussing the consequences of T-tubule loss and increase in caveolae density in heart failure.

There are number of other notable differences between neonatal, adult, and failing cardiomyocytes that impact calcineurin activity. These include changes in the expression of calcineurin isoforms [10, 22, 23] and redistribution within the cell from cytosolic to colocalization with T-tubules [102–104]. In addition, compared to adult, the neonatal heart

has lower enzymatic activities for a number of antioxidant pathways [286] and is more susceptible to ischemic injury [287]. Taken together, changes that occur during maturation to an adult cardiomyocyte increase the total potential capacity for calcineurin activity while establishing environments that restrain activation and protect against uncontrolled signaling. During failure, loss of the T-tubule system and a shift toward caveolae, may release these domain constraints on calcineurin, allowing a pathological feed-forward signaling cascade.

Recent advances allowing differentiation of cardiomyocytes from embryonic stem cells or induced pluripotent stem cells (iPSCs) have provided powerful new tools for studying heart disease. While these models have the advantage of providing the genomic environment of a specific patient, it is important to note that, relative to  $\text{Ca}^{2+}$  release and handling, they may be more akin to embryonic cardiomyocytes than to adult. Thus, study of calcineurin dependent processes in these cells may be difficult to extrapolate to the adult heart [285].

## 9. Conclusion

The impact of calcineurin signaling on cardiac health and disease is far more complex than simple activation of NFAT-dependent hypertrophic gene expression. Here, we have focused primarily on activity localized to cardiomyocytes (Fig. 2). However, calcineurin mediates process in all cell types and tissues of the cardiovascular system. Many of these may be equally important to maintaining a healthy heart. Key points to keep in mind are: (1) The nature of calcineurin signaling in neonatal cardiomyocytes is profoundly different than that found in adult cardiomyocytes. (2) The responses of calcineurin and its substrates depend upon the nature and composition of specific subcellular domains. (3) Not all calcineurin activity is pathological. (4) Calcineurin activity in a healthy heart is heterogeneous relative to location and timing. (5) Sustained activation of calcineurin leads to fundamental changes in a number of substrates that often act in a feed-forward fashion that accelerates cardiac decline.

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## Abbreviations

<b>AID</b>	auto inhibitory domain of calcineurin
<b>AKAP</b>	A kinase anchor protein
<b>AngII</b>	angiotensin II
<b>APD</b>	action potential duration
<b><math>\beta_1</math>-AR</b>	$\beta_1$ -adrenergic receptors
<b>CABIN-1</b>	calcineurin binding protein 1

<b>Carabin</b>	TBC1 domain family member 10C
<b>CHP</b>	calcineurin homologous proteins
<b>CIB1</b>	Ca <sup>2+</sup> -and integrin-binding protein-1
<b>CN</b>	calcineurin holoenzyme
<b>CnA</b>	catalytic subunit of calcineurin
<b>CnB</b>	regulatory subunit of calcineurin
<b>CnA*</b>	constitutively active calcineurin
<b>CRTC</b>	CREB-regulated transcriptional coactivators
<b>CS1</b>	calsarcin 1
<b>CsA</b>	cyclosporin A
<b>DRP1</b>	dynamin related protein 1
<b>ENDO</b>	endocardium
<b>EPI</b>	epicardium
<b>FOXO1</b>	forkhead box protein 01
<b>FRET</b>	fluorescence resonance energy transfer
<b>I-1</b>	protein phosphatase inhibitor 1
<b>I<sub>Ca,L</sub></b>	LTCC current
<b>I<sub>p</sub></b>	Na/K ATPase pump current
<b>I<sub>to</sub></b>	fast transient outward K <sup>+</sup> current
<b>LMCD1</b>	LIM and cysteine-rich domains 1
<b>LTCC</b>	voltage-activated L-type calcium channel
<b>MLP</b>	muscle LIM protein
<b>MuRF1</b>	muscle-specific RING finger 1
<b>NCX1</b>	sodium calcium exchanger 1
<b>NFAT</b>	nuclear factor of activated T cells
<b>NHE1</b>	Na <sup>+</sup> /H <sup>+</sup> exchanger 1
<b>OMM</b>	outer membrane of mitochondria
<b>PC-1</b>	polycystin-1 PC-1
<b>PICOT</b>	protein kinase C-interacting cousin of thioredoxin



<b>PKA</b>	protein kinase A
<b>pNPP</b>	p-nitrophenyl phosphate
<b>PP1</b>	protein phosphatase 1
<b>PLN</b>	phospholamban
<b>RCAN</b>	regulator of calcineurin
<b>Rcan1.4</b>	exon four isoform of RCAN1
<b>RyR2</b>	ryanodine receptor 2
<b>SR</b>	sarcoplasmic reticulum
<b>TFEB</b>	transcription factor EB
<b>TRPC</b>	transient receptor potential canonical channel.

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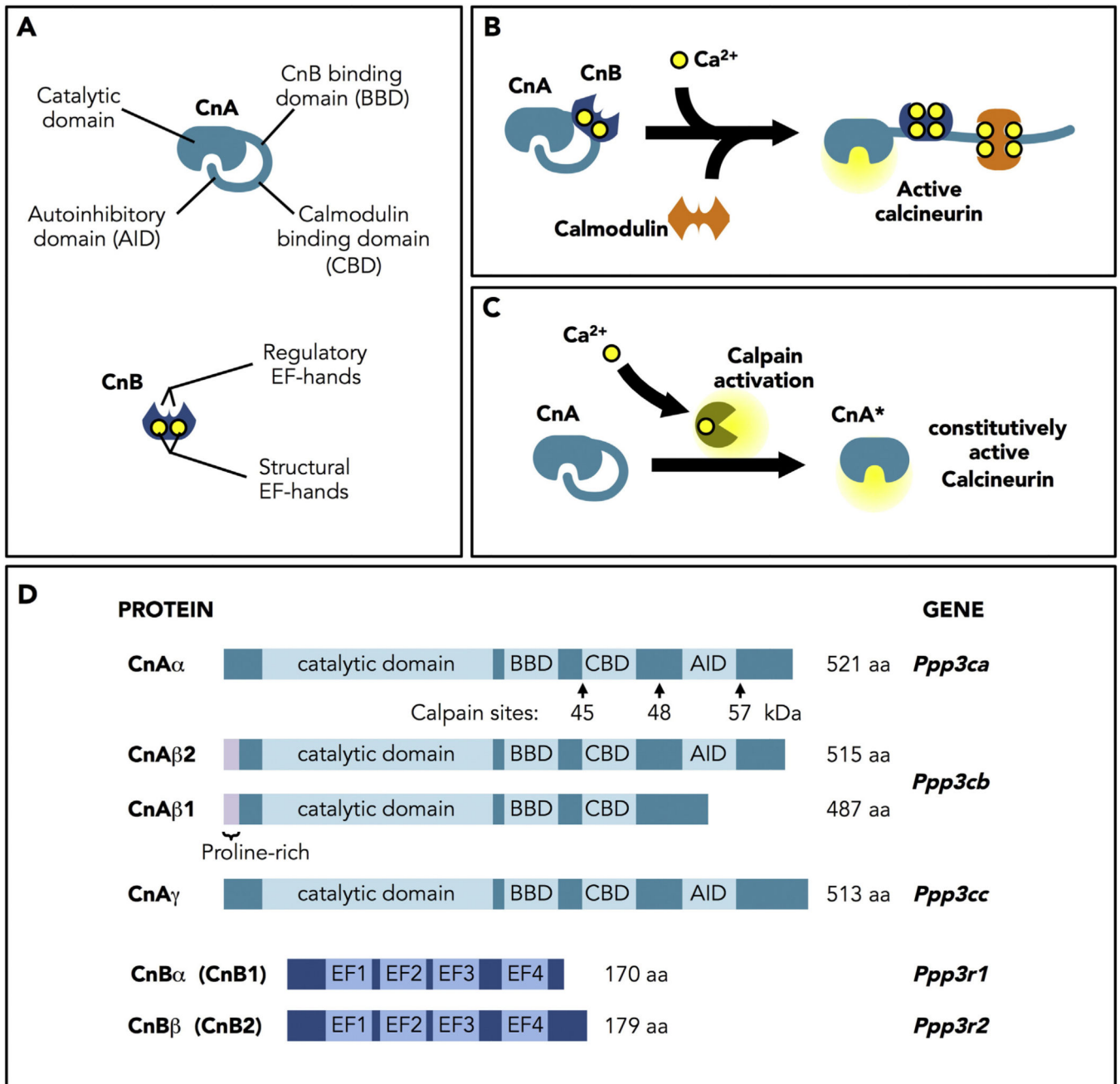
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**Fig. 1.**

Calcineurin structure, regulation and activation. A. Structure of the catalytic (CnA) and regulatory (CnB) subunits of calcineurin. CnA contains an N-terminal catalytic domain, a CnB binding domain, a calmodulin binding domain, and a C-terminal autoinhibitory domain (AID). CnB has four EF-hand  $\text{Ca}^{2+}$ -binding sites: two structural sites that bind  $\text{Ca}^{2+}$  with high affinity in the  $\eta\text{M}$  range, that are always occupied, and two regulatory sites that bind in the  $\mu\text{M}$  range. B. Model of calcineurin activation. Binding of  $\text{Ca}^{2+}$  to the regulatory sites initiates a series of conformational changes that allow binding of a calmodulin/ $\text{Ca}^{2+}$  complex and a change in the orientation of the AID to expose the active site. C.

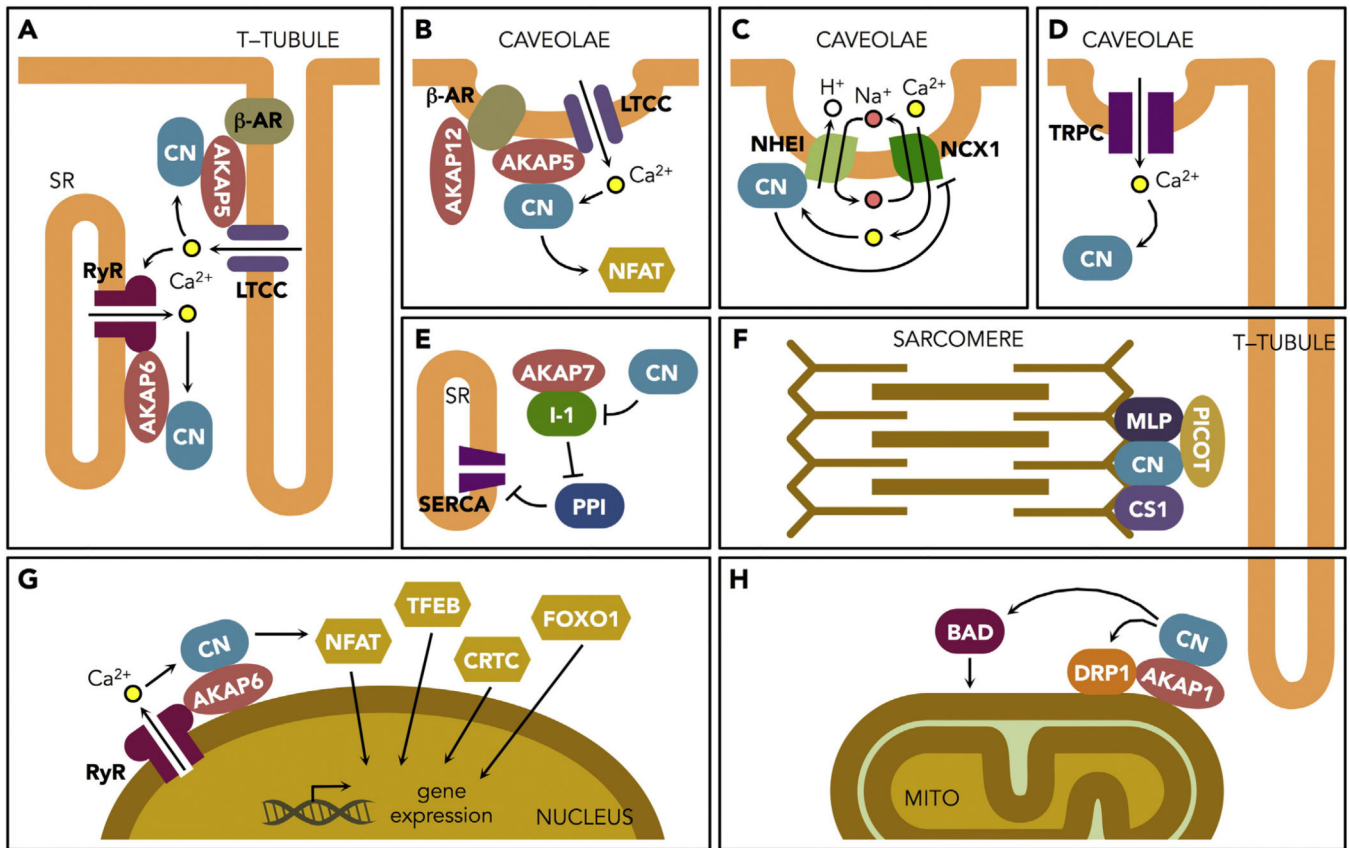
Constitutively active calcineurin. Truncation of CnA to remove the AID yields a constitutively active phosphatase (CnA\*) that no longer responds to  $\text{Ca}^{2+}$ /calmodulin. D. Calcineurin proteins and genes. In mammals, three genes encode CnA ( $\alpha$ ,  $\beta$ , and  $\gamma$ ). Only CnA $\alpha$  (PPP3CA) and CnA $\beta$  (PPP3CB) are expressed in the heart. Of the two genes encoding the CnB regulatory subunit, only CnB $\alpha$  (PPP3R1) is expressed in the heart.

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**Fig. 2.**

Targeting of calcineurin to specific domains and substrates in adult cardiomyocytes. Key points to note include the close association of calcineurin (CN) and many of its interacting partners with the T-tubule system. Dyads formed by RyR/LTCC at SR/T-tubule junctions (A), Z-band localized mechano-sensing complexes of the sarcomere (F), and prominent sites of mitochondrial fission (H) each contain specific tethering points for CN and sit in close proximity to the T-tubule system. These major pools of CN do not appear to couple to NFAT activation during  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release associated with contraction. In contrast, CN more readily couples to NFAT in response to  $\text{Ca}^{2+}$  release events in caveolae-associated microdomains (B, C, D). CN associated directly with the nucleus also directly couples to transcriptional regulation (G). Some mechanisms of CN targeting are yet to be defined, such as the pool that acts on I-1 (E). See text for further details and the abbreviations list for full definitions of symbols.