Review Article

Cardiac Signaling and the 'Convergent Hypothesis': Probing the Structural-Functional Nexus of the Heart

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Cardiovascular diseases (CVD), including heart failure and primary cardiomyopathies, are worldwide medical and public health problems that together constitute the leading cause of death and disability in the western world.[1-7] While much study has been dedicated to dissecting the complex web of signaling pathways that interact to regulate cardiac function, studies of the intersection of cardiac signaling with the function of structural proteins of the heart are decidedly deficient. The intersection of the cardiac signaling milieu and the functional output of sarcomeric proteins is well-deserving of special attention in dissecting the molecular underpinnings of CVD.

Cellular signaling provides the cardiovascular system with critical regulatory mechanisms that control both physiological and pathophysiological processes. The various intricate intracellular signaling apparatuses in the heart facilitate the transduction of extracellular stimuli into internal signals that drive the inner workings of the normal and diseased heart. These crucial regulators of cardiac function have been the subject of much study, owing to their aptness as molecular targets for therapeutic intervention in the management of cardiovascular diseases.[1-3] The juncture of cardiac signaling and functional output of sarcomeric proteins will be presented using the well-characterized studies of troponin T (TnT) shifts that are noted to occur in TnT isoforms, their effects on cardiovascular disease, and it will be shown how cardiac signaling pathways stand interposed as the molecular mediators of cardiac function.

The fundamental function of the heart as a systemic 'pump' is attributable to the fact that it is a muscular organ whose contractile efficiency is a direct measure of its health: i.e. ejection fraction. Cardiac muscle, along with skeletal muscle, is 'striated muscle', whose basic unit is the sarcomere. Indeed, sarcomeric proteins subserve both structural and functional roles,[4] and in fact, differential sarcomeric protein isoform expression has been established as a key component of cardiovascular disease. In support of this, several independent epidemiologic studies have evaluated and confirmed that sarcomeric protein isoforms are clinically useful in predicting the risk of cardiovascular disease.[5] Aside from the well-characterized role of sarcomeric proteins in genetic diseases of the myocardium (the inherited cardiomyopathies), recent research suggests that these physiological 'struts' serve as an important nexus that defines the functional significance of normal and diseased cardiac signaling. Such investigations have suggested that aberrant expression of specific sarcomeric proteins is associated with hemodynamic stress sec-

ondary to cardiac disease, including primary cardiomyopathies as well as heart failure.[6] The changes in cellular signaling that occur in cardiovascular disease parallel to functional deviations in sarcomeric proteins have been traditionally viewed as independent spheres; however, recent studies support an aptly termed "*convergent hypothesis*", wherein extrinsic stimuli trigger signaling cascades that target structural proteins of the sarcomere, resulting in measurable, cardiac functional changes (Figure 1).

Cardiac muscle contraction and sarcomeric proteins – the case of the TnT isoform switch

Striated muscular contraction requires a concerted regulation of sarcomeric Ca^{2+} levels. Specifically, force development is triggered by Ca^{2+} binding to the Ca^{2+}-specific, low affinity regulatory sites of troponin C (TnC).[4,8] The regulatory unit of the contractile apparatus is formed by the orchestrated interactions of the ternary troponin complex – composed of the Ca^{2+}-binding subunit, TnC, troponin I (TnI), and troponin T (TnT) – along with the thin-filament protein tropomyosin (Tm).

In the human, three separate genes encode TnT, the slow- and fast-twitch skeletal and the human cardiac genes, which confer tissue-specific and developmental regulatory control of TnT function. The four identified human cardiac TnT (HCTnT) isoforms, generated by alternative mRNA splicing of two exons, differ predominantly in their N-terminal regions.[9] The hypothesis that the HCTnT

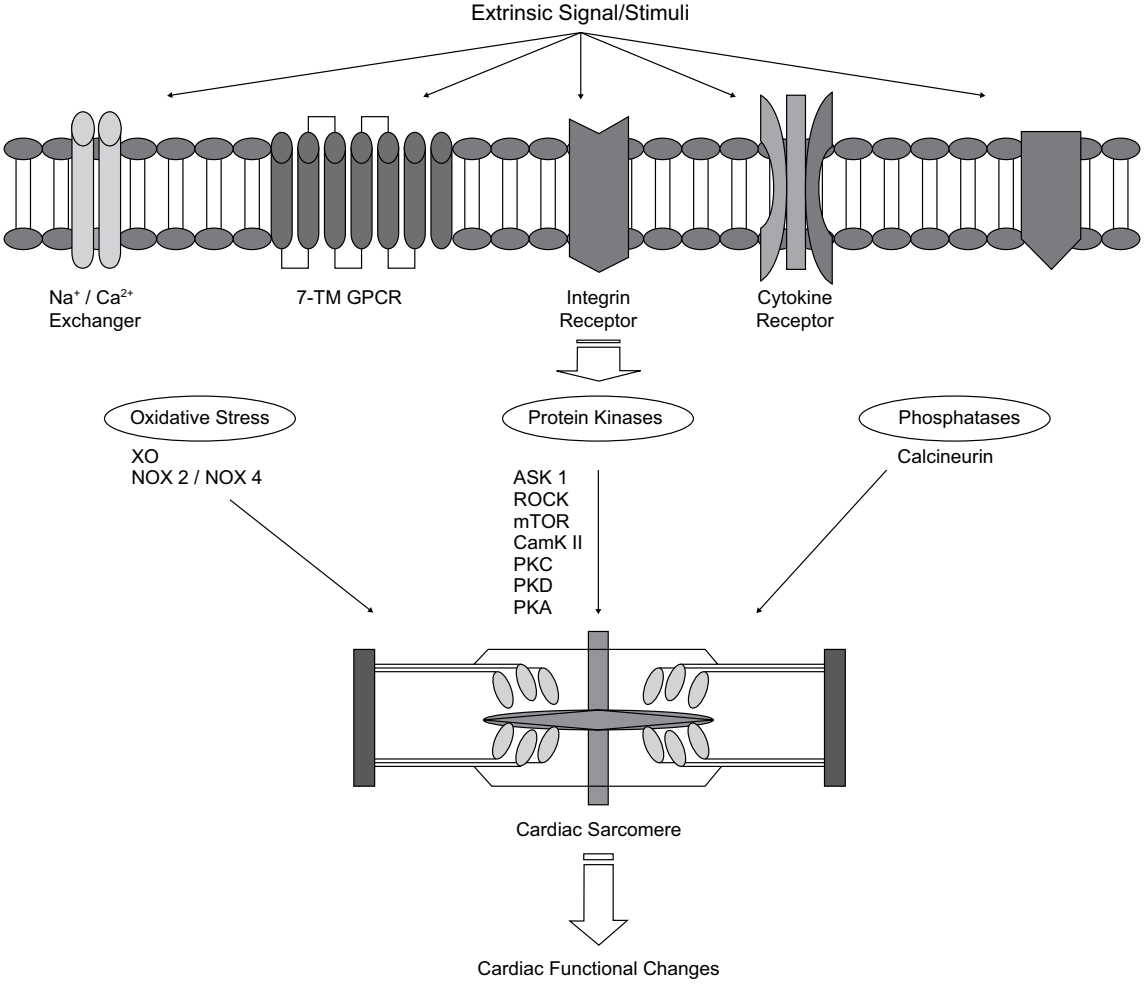

Figure 1. The convergent hypothesis of cardiac signaling. Depicted here are the various intracellular signaling mediators that regulate cardiac (patho)physiology. Sarcomeric proteins have been demonstrated to be the end targets of various cardiac signaling pathways, conferring functional significance on alterations in cardiac signaling.

N-terminal region (specifically the hypervariable region, HVR) may confer functionally significant differences in spatial or ontogenically-regulated force development is predicated on studies that have demonstrated that thin filaments constituted with different isoforms result in unique contractile properties.[10,11]

The probable functional importance of HCTnT isoform-specific expression is supported by developmental studies that have demonstrated the strict fetal/adult dichotomous nature of the four HCTnT isoforms. For instance, HCTnT 1, 2, and 4 are all fetal isoforms, with HCTnT1 being the predominant TnT isoform expressed in the fetal myocardium.[11] In the fetal/adult transition of myocardial development, however, there is a notable inverse shift: the splice variant encoding HCTnT1 is down-regulated and that for HCTnT3 is up-regulated, eventually yielding HCTnT3 as the predominant HCTnT isoform expressed in the normal adult heart.

In pathological states of the heart, such as left ventricular hypertrophy, which is characteristic of hypertrophic cardiomyopathy (HCM) as well as myocardial failure in both animal models and humans, several studies, albeit conflicting in some cases, have documented a *reverse ontogenic* shift in HCTnT isoform expression (the pathological "*HCTnT isoform switch*").[11-13] Remarkably, such studies have found an increased cardiac expression of the fetal isoform HCTnT4 (4% to 12% global HCTnT levels) in the adult diseased myocardium and a similar decrease in the expression of the predominantly adult isoform, HCTnT3.[10,11,14-18] Consideration that regional variation in cardiac sarcomeric protein isoform expression occurs as a consequence of local cardiac dysfunction[19] can result in a potentially near-geometric increase in the relative HCTnT4 expression profile and, therefore, sarcomeric incorporation, in diseased regions of the myocardium. These findings lend further, although inconclusive, credence to the hypothesis that differential HCTnT isoform expression results in functionally significant changes in cardiac contractile function.

Pathophysiological regulation of TnT expression

The cardiac troponin T gene (TNNT2, 1q32) generates different transcripts and, consequently, unique isoforms via a combinatorial alternative splicing mechanism whereby exons 4, 5, 10, and 13[10,11] are either included or excluded. There are four most prevalent isoforms that are formed by the inclusion or exclusion of exons 4 and 5, termed HCTnT1, HCTnT2, HCTnT3, and HCTnT4 (Figure 2). As described earlier, HCTnT isoform expression is developmentally regulated, with HCTnT1, HCTnT3, and HCTnT4 being predominantly fetal isoforms, whereas during development HCTnT1 expression declines and that of HCTnT3 increases until in the adult HCTnT3 is the major isoform.[14]

TnT isoform expression varies greatly across species, confounding the interpretation of experimental characterization of animal models.[15] Some clinical reports document the pathological HCTnT isoform switch in animal and human heart failure and HCM. Originally, animal models of adult heart failure (rabbit and guinea pig)[16,17] showed a marked expression of fetal HCTnT isoforms, which was later confirmed to occur also in human heart tissue, with up to 20% increased expression of an HCTnT isoform that had a faster mobility than the single band observed in non-failing heart tissue,[10] corresponding to the fetal HCTnT4 isoform. Later studies by Mesnard-Rouiller et al,[18] however, scrutinized mRNA and protein levels of different isoforms in normal and failing human heart tissue and found that there was a similar HCTnT isoform expression profile in diseased and normal hearts, with insignificant differences in isoforms. Thus, the HCTnT isoform switch has been reported in heart failure by some,[18] but was not observed by others, suggesting that it may perhaps be a phenomenon particular to subsets of heart failure patients.

Based on these findings, the physiological and functional impact of the troponin T isoform switch seems negligible as a direct and primary cause of contractile dysfunction. However, an emerging hypothesis is that the switch may not be a primary cause of myocardial pathology, but may be a downstream effect of pathological processes and remodeling due to an initial insult triggering a signaling cascade. Interestingly, pathological signaling pathways, i.e. redox sensitive signaling pathways, may in fact target specific isoforms of HCTnT and may amplify or dampen (possibly compensate for) aberrant contractility. Specifically, O_2^- sources, such as NADPH-oxidase, iNOS, xanthine oxidase and mitochondrial oxidases, may effect sarcomeric protein phosphorylation states. Current evidence favors alterations in troponin phosphorylation as the predominant cause of contractile dysfunction in heart failure.[20]

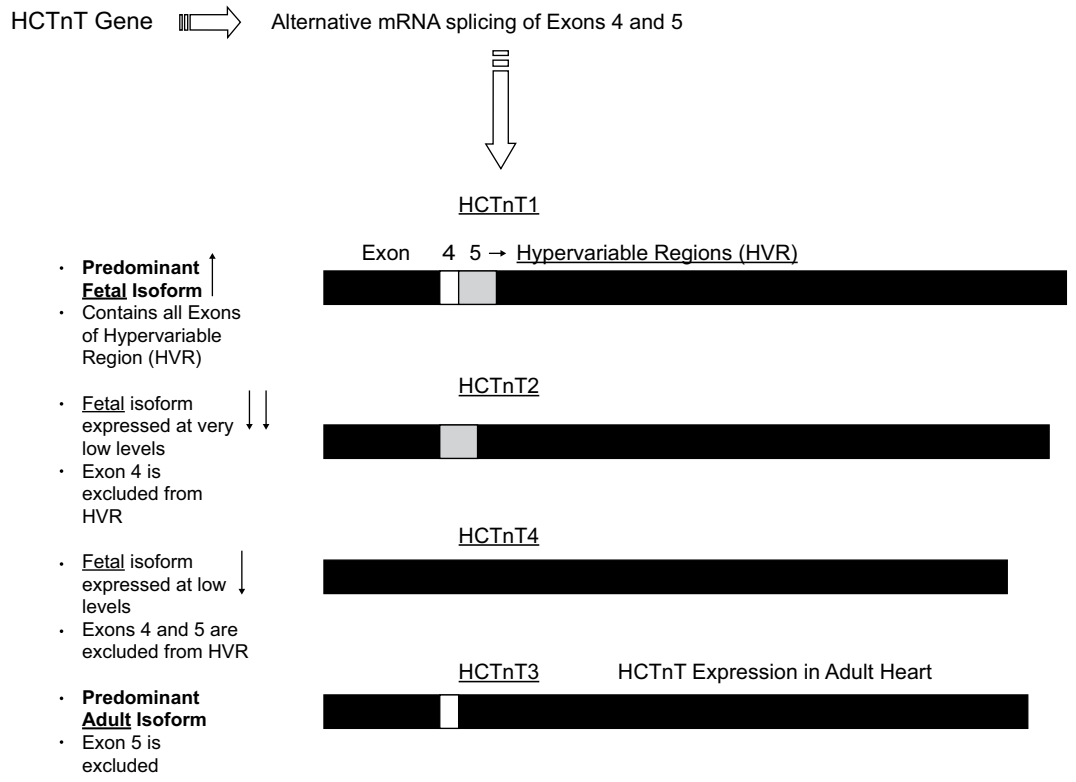

Figure 2. Schematic of HCTnT isoform expression. This figure shows the differential HCTnT isoform expression via alternative mRNA splicing of the hypervariable region (HVR) of the HCTnT gene. Some reports have demonstrated a marked re-expression of HCTnT4 in diseased adult human and animal myocardium (reverse ontogenic shift).

Sarcomeric mutations and cardiomyopathies – the case of HCM

Differential sarcomeric protein expression plays a key role in the attenuated cardiac pump function in heart failure. Alterations in sarcomeric proteins in cardiac disease range from altered isoform expression to post-translational protein changes, such as proteolysis and phosphorylation. *In vitro* and *in vivo* studies confirm that cardiac pathologies, including heart failure, have sarcomeric manifestations, i.e. including altered maximum force development, Ca^{2+} sensitivity, and increased passive stiffness. The etiology of such sarcomeric dysfunction is thought to be altered protein phosphorylation, caused by neurohumoral-induced alterations in the kinase-phosphatase balance inside the cardiomyocytes.

HCM is the leading cause of sudden cardiac death in athletes and young people, characterized by excessive thickening, i.e. hypertrophy, of the left ventricular wall or interventricular septum. HCM exists as both a primary and familial cardiomyopathy, with heterogeneous expression, unique pathophysiology, and a varied clinical course. As a consequence of inordinate hypertrophic remodeling of the left ventricular and septal walls, the early-stage HCM heart retains a supramaximal contractile capacity and therefore maintains a high ejection fraction. Chronic hypertrophic remodeling in HCM, however, leads to rigid, inflexible, almost fibrotic myocardial tissue, resulting in uncoordinated and impaired systolic function as well as an inability to fully relax (diastolic dysfunction), exhibiting 'restrictive' characteristics, which serve to increase afterload.

There are several pathologically relevant consequences of increased afterload due to remodeled cardiac tissue. Specifically, the hypertrophic heart requires inordinately greater pressures to expand with the inflow of incoming blood, has increased end-systolic volume, translating to a decrease in stroke volume, cardiac output, and ejection fraction,[12,21,22] all of which act to decrease perfusion and may precipitate cardiac failure. Hence, positive inotropic compensation can theoretically counter the physiological parameters characteristic of heart failure.[12]

Etiologically, HCM is an autosomal dominant genetic disorder that can be attributed to dysfunction of the cardiac sarcomere. The HCTnT isoform shift has been reported to occur in both familial and non-familial forms of HCM. To date, a number of loci, including thick- and thin-filament associated proteins, have been identified as being causative agents of HCM.[20-22] HCM-linked mutations in HCTnT, although representing approximately 15% of all cases of HCM, are characterized clinically by a greatly increased likelihood of sudden cardiac death. Notably, patients with HCM-linked HCTnT mutations have hearts with extensive myocardial disarray, but significantly less hypertrophy.[12]

Physiologically, most HCM-causing sarcomeric mutations result in a potentiated Ca^{2+} sensitivity of force development.[20-21] This finding translates functionally to enhanced contractile force generation at a given Ca^{2+} concentration. Beta-adrenergic stimulation and subsequent PKA phosphorylation of HCTnI, however, works in opposition to decrease Ca^{2+} sensitivity.[20] Another common feature among many of the HCM mutations is a defect in the ability to fully relax muscle fibers in *in vitro* systems.[20,21] This failure, as well as the slow relaxation of HCM fibers due to increased Ca^{2+} sensitivity, would both contribute to severe diastolic dysfunction. Additionally, an increase in the Ca^{2+} sensitivity of force seems to correlate with the severity of disease seen in HCM.[21] Interestingly, PKA phosphorylation of HCTnI—resulting in an attenuated force-Ca^{2+} relationship—would appear to assist diastolic function in HCM.

Pathological cardiac signaling pathway activation and the HCTnT isoform switch

Aberrant cardiac signaling has been demonstrated to be associated with the initiation and progression of cardiac disease (Figure 3). Specifically, cardiac hypertrophy, especially in HCM, is coincident with activation of $G_{\alpha}q$ signaling and protein kinase C (PKC), resulting in phosphorylation of hypertrophy-associated transcription factors but also in pathological phosphorylation of myofilament proteins that affect contractility. A recent study demonstrated that the signaling effector, ROCK-II, of the small GTPase Rho-A, phosphorylates TnT and consequently yields a reduction in ATPase activity and force production with an associated decrease in the calcium-dependence of these parameters.[23] Specifically, Vahebi et al showed that ROCK-II phosphorylation of murine CTnT at S278 and T287 induced a depression in maximal ATPase rate and force, which was independent of ROCK-II phosphorylation of MLC. Additionally, exchange of ROCK-II–phosphorylated Tn complex with the native Tn complex in the fiber bundles resulted in inhibition of maximal Ca^{2+} activation of tension and ATPase activity. It is hypothesized that altered sarcomeric function in HCM may give rise

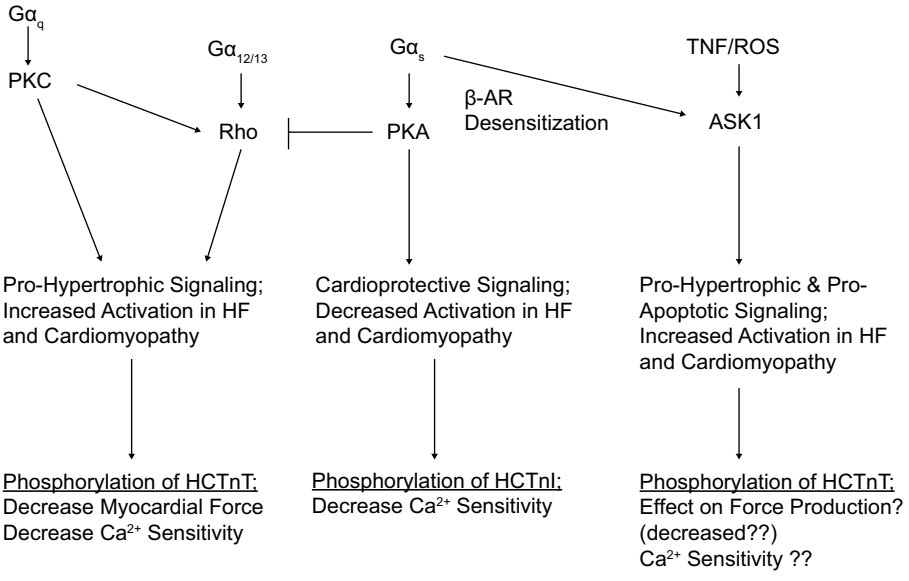

Figure 3. Cardiac signaling pathways targeting troponin. Shown here are some of the several signaling cascades that are activated in the diseased heart and that target the troponin complex. Some pathways are cardioprotective, i.e. compensatory under physiological stress conditions. HF – heart failure.

to a vicious cycle in which hypertrophy is associated with depressed contractility, eventually precipitating heart failure. Alternatively, as Rho activation and signaling is also involved in the *progression* to heart failure and HCM, it is also hypothesized that Rho-kinase mediated phosphorylation of TnT may initially oppose the functional deficits of early-stage HCM, i.e. supramaximal force production and contractility; hence, Rho activation may *initially* serve as a compensatory mechanism.

Additionally, recent studies have shown that another signaling molecule, apoptosis signal-regulating kinase 1 (ASK1), may be implicated in the pathogenesis of HCM.[24] Specifically, several lines of evidence suggest that increased production of proinflammatory mediators, such as tumor necrosis factor (TNF) and reactive oxygen species (ROS), contribute to the pathogenesis of cardiac dysfunction via ASK1 interaction with target proteins.[25,26] ASK1 is highly expressed in cardiac muscle[25] and is an important mediator in the signaling pathways induced by TNF, interleukin-1, and ROS. ASK 1 has been demonstrated to specifically phosphorylate cardiac TnT *in vitro* and *in vivo*, resulting in depressed contractility. The specific functional significance of this pathological modification of HCTnT in actomyosin ATPase activity and calcium sensitivity of force development has not been explored. It is hypothesized that ASK-1 phosphorylation of HCTnT may affect the calcium dependence of force development and ATPase activity and hence participate in cytokine/ROS-induced pathogenesis of cardiomyopathy and heart failure. Additionally, this modification of HCTnT may be impacted by the HCTnT isoform present and/or the degree of the HCTnT isoform shift.

Apoptotic cardiac signaling and TnT – does heart disease trigger contractile dysfunction and not *vice versa?*

The traditional view held by some muscle physiologists has been that changes in myofilament calcium responsiveness are a direct result of changes in the expression or stability of calcium cycling proteins *and* that these effects constitute primary causes of cardiovascular disease. While this hypothesis is supported by studies identifying sarcomeric protein mutations as causative for various inherited cardiomyopathies, current evidence suggests that the majority of non-familial disease exhibits alterations in calcium sensitivity as an effect, not the cause of the primary disorder.

Programmed cell death, or apoptosis, is a critical event in the onset and progression of several diseases of the heart, including HCM and heart failure. Recent studies indicate that caspases, enzymatic catalysts that can degrade myofibrillar proteins, can target TnT, yielding an impaired force-calcium relationship and ATPase (measure of ATP consumption) activity.[27] Therefore, such findings have bolstered the hypothesis that pathological remodeling in the heart due to an initial injury may in fact induce contractile dysfunction, challenging the traditional, myopic view that minor differences in calcium sensitivity due to sarcomeric protein variation are the primary causes of cardiomyopathy (Figure 4).

Apoptosis is a common molecular event in several forms of cardiovascular pathologies and strong evidence suggests that cardiac myocyte apoptosis plays a crucial role in disease progression, loss of ventricular functional indicators, and may be a critical event precipitating congestive heart failure.[28] Additionally, the apoptotic process may target cytoplasmic and nuclear substrates, permitting myocellular damage and remodeling via a continual degradation of cytoplasmic proteins, most of which are contractile proteins, in the presence of an intact nuclear membrane. The abundance of caspases in the failing heart,[28] coupled with findings that sarcomeric proteins, including TnT, are subject to progressive cleavage coincident with impaired calcium-sensitized contractility, lends strong credence to the hypothesis that contractile dysfunction is the result, not the cause of cardiovascular disease.

Troponin T and other proteins of the cardiac sarcomere have mostly been studied and examined in their structural context, as 'biological struts' that connect tropomyosin to the TnI-TnC complex, but troponin also plays a functional role, conferring calcium regulatory control and cooperativity on the activity of the troponin complex on actomyosin ATPase.[4] Specifically, the caboxyl terminus of TnT is necessary for submaximal activation of ATPase activity, whereas the amino terminus is essential for maximal activation. Communal et al found that caspase-mediated TnT cleavage occurred in fully complexed Tn complexes and that such cleavage disrupts both structural and functional roles of TnT, resulting in substantial contractile impairment.[27]

The clinical relevance of these findings is evident in other studies that showed that after ischemia-reperfusion injury, caspase inhibition blocked the breakdown of TnI and the subsequent precipitant contractile dysfunction.[29] Additionally, in stunned myocar-

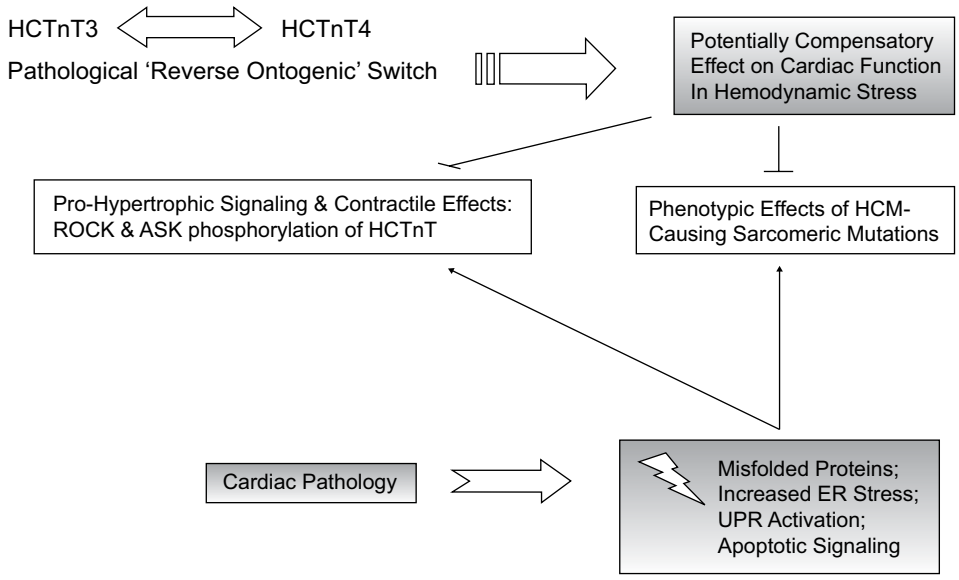

Figure 4. The HCTnT isoform switch and apoptotic signaling – possible 'negative feedback' regulation. Physiological and cellular stresses (misfolded proteins) can lead to pathological apoptotic signaling and subsequent contractile dysfunction. Some of these effects are attributable to targeting, and thereby modifying, troponin T. The reverse ontogenic HCTnT shift may, in fact, be triggered by such pathological signaling and serve to counter the deleterious effects.

dium, TnI is specifically induced and its cleavage is directly dependent on calpain activation.[29] Caspases also target proteins of the Z-band, such as α-actinin, which structurally unites the thin filament[30] and ensures cooperativity in both systole and diastole. In myocardial disease processes that target α-actinin for degradation, i.e. ischemia, this results in contractile dysfunction by disrupting inter-filament spacing and thereby negatively affecting the transmission of force from the actin-myosin reaction. Hence, several studies support the view that pathological signaling processes in the heart induce contractile dysfunction via exuding effects on sarcomeric proteins.

Summary and perspectives

Recent evidence suggests that differential sarcomeric protein expression occurs concomitant with signaling pathway derangements in CVD. Based on exciting findings that demonstrate a linkage between troponin mutations, differences in isoform expression profile, cardiac signaling, and the ensuing cardiovascular pathology, it is anticipated that such knowledge will be exploited for usage clinically in both the diagnosis and treatment of CVD. These studies have shown that cardiac signaling pathways serve as the molecular mediators of cardiac pathology, with sarcomeric protein targets being the crucial nexus that bridges the structural-functional molecular divide. In the near future, it is likely that the intersection of specific signaling cascades and sarcomeric isoform-specific post-translational modifications will play an increasingly important role in our understanding of the complexities of cardiovascular function and will yield extensive insight into disease processes and the development of future targeted therapeutics.

It is expected that future experiments should elucidate novel mechanistic insight into the role of troponins as the link between deranged cardiac signaling and the macroscopic defects observed in the diseased myocardium. The results of these studies may yield insight into the potential role of apoptotic signaling, i.e. ASK1, activation and TnT phosphorylation as a potential pathological signaling event occurring in response to increased endoplasmic reticulum (ER) stress. This interpretation is plausible, as studies have shown that there is an increased unfolded protein response activation in subjects with cardiac hypertrophy/cardiomyopathies. Hence, in late stages of cardiomyopathy, the increased ER stress due to the accumulation of misfolded proteins may activate ASK1, which phosphorylates HCTnT, resulting in contractile

dysfunction and eventually precipitating heart failure (Figure 4). Similarly, it is expected that future studies will clearly establish the role of signaling proteins as the regulators of the reverse ontogenic TnT isoform switch employed by the heart to fine tune cardiac sarcomeric function in response to cardiac stress.

It is anticipated that such experiments and their results will fill a significant portion of the gap in our current understanding of the complexities of the molecular pathogenesis of heart disease and the intrinsic mechanisms employed by the heart to restore cardiac function in the context of cardiac pathology. It is expected that future advances will support the hypothesis that cardiac signaling not only occurs concomitantly with sarcomeric protein functional deviation, but directs that process, eventually resulting in cardiac dysfunction. These studies will be critical in the identification of novel mechanistic pathways in cardiovascular disease management and treatment. The most promising avenues of research suggest that neurohumoral-induced alterations in the kinase-phosphatase balance within cardiomyocytes control sarcomeric protein phosphorylation and thereby regulate contractility. Hence, novel therapies, which specifically target phosphorylation sites within sarcomeric proteins or the kinases and phosphatases involved, are expected to improve cardiac function in heart failure.

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