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Connexin43 Cardiac Gap Junction Remodeling: Lessons from Genetically Engineered Murine Models

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Abstract Sudden cardiac death is responsible for several hundred thousand deaths each year in the United States. Multiple lines of evidence suggest that perturbation of gap junction expression and function in the heart, or what has come to be known as cardiac gap junction remodeling, plays a key mechanistic role in the pathophysiology of clinically significant cardiac arrhythmias. Here we review recent studies from our laboratory using genetically engineered murine models to explore mechanisms implicated in pathologic gap junction remodeling and their proarrhythmic consequences, with a particular focus on aberrant posttranslational phosphorylation of connexin43.

Keywords Arrhythmia · Connexins · Gap junctions · Mouse model · Phosphorylation

Connexins and Gap Junctions

Connexins comprise a family of proteins encoded by as many as 20 genes in most mammalian species (Willecke et al. 2002). Substantial experimental evidence has demonstrated that connexins oligomerize into channels, which are organized into arrays at the gap junction. Channels formed from single connexin isoforms (homomeric/homotypic channels) have distinct biophysical properties, which can most easily be studied in heterologous expression systems (Harris 2001). Characteristic biophysical parameters include such properties as unitary conductance, voltage dependence, as well as size and charge selectivity (Spray and Burt 1990; Spray et al. 1992). There is also evidence that individual connexin isoforms may mix and match to form complex heteromeric and/or heterotypic channels; these more complex assemblies may have biophysical properties that differ from those formed from only a single connexin isoform (Harris 2001). Indeed, this molecular diversity is postulated to provide a mechanism for physiological diversity and regulation (Giovannone et al. 2011; Kanno and Saffitz 2001). Importantly, this combinatorial complexity may be directly relevant to understanding the pathophysiology of gap junctions, as aberrant regulation and/or mutations of a single connexin isoform may exert dominant effects on alternative connexin isoforms.

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Gap Junction Remodeling and Arrhythmogenesis

There is compelling experimental evidence linking abnormalities in gap junctions with a highly proarrhythmic substrate (reviewed in Severs et al. 2008). These data include pathologic studies of hearts from patients with a broad assortment of acquired arrhythmic syndromes including ischemic and hypertrophic cardiomyopathies, inherited diseases such as arrhythmogenic right ventricular cardiomyopathy (ARVC) (Saffitz 2009; Severs 2002; Severs et al. 2004, 2006, 2008), human genetic studies of patients with somatic (Gollob et al. 2006; Thibodeau et al. 2010) or germ line (Paznekas et al. 2003) mutations in connexin genes, as well as genetically engineered murine models created by our own group (Gutstein et al. 2001) and others (van Rijen et al. 2004). Indeed, in recent years a growing body of literature suggests that gap junction remodeling represents a "final common pathway"

predisposing to arrhythmias in response to diverse pathologic insults to the heart. The mechanistic relationship between gap junction remodeling and the increased propensity for arrhythmic activity is multifactorial (Kanno and Saffitz 2001). Abnormal localization and/or gating of intercellular channels disturbs the highly orchestrated temporal and spatial pattern of cardiac excitation, with slow and oftentimes heterogeneous conduction conducive to reentrant activity. Heterogeneous gap junction remodeling may also enhance the dispersion of repolarization (Poelzing et al. 2004), another highly proarrhythmic factor.

Genetically Engineered Murine Models

The carboxyl-terminus of Cx43 contains numerous sites that are subject to posttranslational phosphorylation and these modifications are thought to regulate virtually all aspects of the Cx43 life cycle, including translation, trafficking, degradation, and gating, as reviewed in (Lampe and Lau 2000, 2004; Solan and Lampe 2009). Altered phosphorylation of Cx43 has been observed in response to a variety of pathologic stimuli, including acute ischemia (Beardslee et al. 2000), hypoxic stress (Matsushita et al. 2006), rapid pacing (Akar et al. 2007)

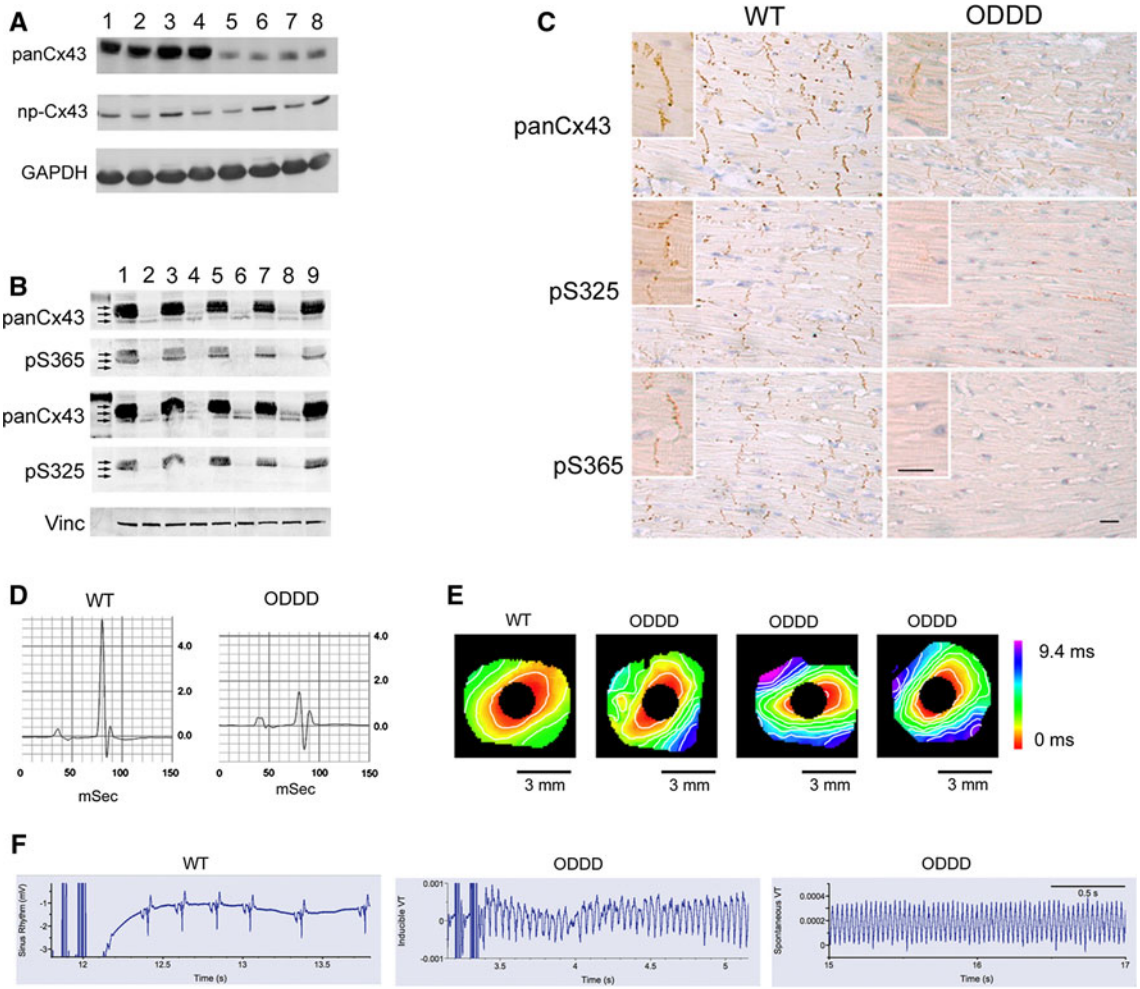

Fig. 1 Aberrant posttranslational phosphorylation of Cx43 in ODDD mutant hearts. **a** Western blot analysis using antibodies recognizing all forms of Cx43 (panCx43) and nonphosphorylated Cx43 (np-Cx43) showing specific loss of phosphorylated Cx43. *Lanes 1–4* are from wild-type hearts and *lanes 5–8* are from ODDD hearts. **b** Western blot analysis using antibodies recognizing all forms of Cx43 (panCx43), phosphoS365-Cx43 (pS365) and phosphoS325/328/330-Cx43 (pS325), showing specific loss of PS365 and pS325. *Lanes 1*, *3*, *5*, *7* and *9* are from wild-type hearts and *lanes 2*, *4*, *6* and *8* are from ODDD hearts. **c** Immunohistochemical staining of wild-type and ODDD mutant hearts with panCx43, pS325 and pS365 antibodies. Phosphorylated forms of Cx43 are virtually absent in ODDD mutant hearts. *Bar* 20 μm. **d** Representative signal-averaged surface electrocardiograms (lead II) from a wild type (WT) and an ODDD mutant mouse. Note the diminished QRS amplitude in the mutant. **e** Optical mapping of the *left* ventricular surface of a representative WT heart and 3 individual ODDD mutant hearts showing significant slowing of conduction in the mutant hearts. **f** Programmed electrical stimulation showing return of sinus rhythm after premature beats in a wild type heart, but induction of sustained VT in an ODDD heart (*middle*), and spontaneous VT in an ODDD heart (*right*). Adapted from Kalcheva et al. (2007)

and other stressors. Several years ago we developed a murine model of the human syndrome oculodentodigital dysplasia (ODDD), an autosomal-dominant systemic disorder caused by mutations in the Cx43 gene (Kalcheva et al. 2007; Paznekas et al. 2003). Unexpectedly, because the missense mutation (I130T) was at a distance from the serine-rich carboxy-terminus, we observed a profound defect in the posttranslational phosphorylation of Cx43 at both serine 365 (a PKA-dependent site) and the triplet of serines at 325, 328, and 330 (CK1δ-dependent sites), as defined by using phospho-specific antibodies generated by the Lampe laboratory. These mice also demonstrated significant abnormalities in cardiac impulse propagation and increased susceptibility to induced cardiac arrhythmias (Fig. 1).

We next sought to extend these results to a more common and arguably more relevant model of cardiac disease, pressure-overload hypertrophy induced by transverse aortic constriction (TAC). Imposition of hemodynamic overload causes a similar time-dependent reduction in posttranslational phosphorylation of Cx43 at these same sites, especially the CK1δ-dependent sites (Qu et al. 2009), and as with the ODDD mutant mice, we observed significant slowing of cardiac impulse propagation and increased arrhythmogenicity (Fig. 2). Importantly, treatment with the aldosterone receptor antagonist spironolactone, a drug which has been shown to diminish sudden arrhythmic death in human clinical trials (Pitt et al. 1999), blunted the development of gap junction remodeling and reversed the functional abnormalities as well. Taken together with our findings in the ODDD mutant mice, these data suggested that aberrant posttranslational phosphorylation of Cx43 might be a common mechanism through which both intrinsic (i.e., genetic) and extrinsic (acquired) stressors result in pathologic gap junction remodeling.

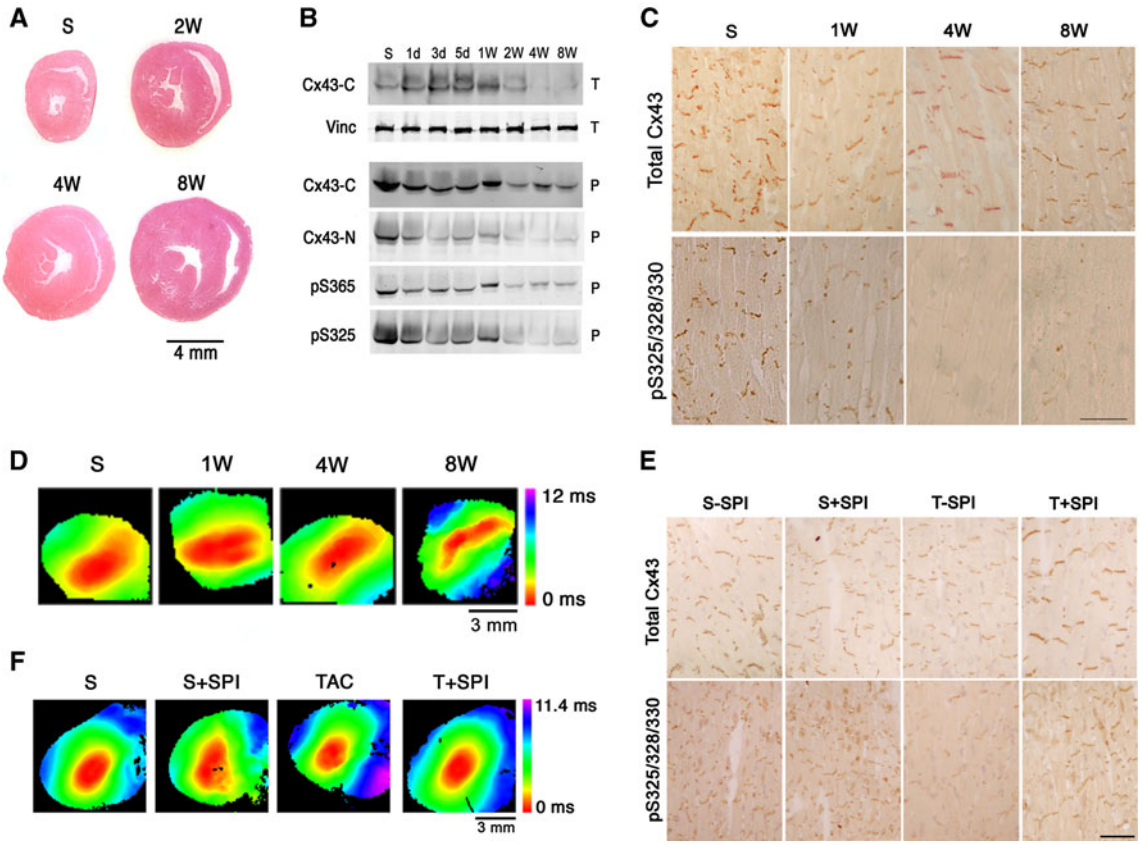

Fig. 2 Structural, molecular and functional gap junction remodeling with pressure overload hypertrophy. **a** Cross-sections of hearts showing progressive hypertrophy after transverse aortic constriction (TAC). **b** Western blot analysis demonstrating progressive reduction in total and phosphoCx43 expression in total cellular lysates (T) or Triton X-100 insoluble pellet fractions (P). Antibodies recognized the Cx43 C-terminus (Cx43-C); Cx43 amino-terminus (Cx43-N); vinculin (Vinc); S365-phosphoCx43 (pS365) or S325/328/330-phospho-Cx43 (pS325). **c** Immunostaining showing progressive loss of junctional Cx43 with TAC, especially S325/328/330-pCx43. **d** Representative optical maps demonstrating progressive slowing of conduction velocity after TAC. **e** Immunostaining showing loss of Cx43 gap junction plaques in TAC mice treated with vehicle alone (T-SPI), but substantial improvement in mice treated with spironolactone (T + SPI), comparable to sham-operated mice receiving vehicle alone (S-SPI) or spironolactone (S + SPI). *Scale bar* 50 μm. **f** Representative optical maps from each of the 4 groups. Adapted from Qu et al. (2009)

Nonetheless, given the multiplicity of kinase target sites that might be affected during acute and chronic stress, these studies alone did not establish a *direct* link between aberrant phosphorylation of Cx43, gap junction remodeling and arrhythmic susceptibility. Therefore, to unequivocally determine the importance of CK1δ-dependent phosphorylation, we created two new strains of mutant mice, in which the serine 325, 328 and 330 (the CK1δ target sites) were mutated to either nonphosphorylatable alanines (S3A mice) or phosphatase-resistant, phosphomimetic glutamic acid residues (S3E mice) (Remo et al. 2011). Both strains of mutant mice were grossly indistinguishable from wild-type (WT) controls at birth and throughout development, and there were no significant differences with regards to baseline physiological and echocardiographic measurements. For many years it has been known that posttranslational phosphorylation of Cx43 influences its electrophoretic mobility by SDS-PAGE (Crow et al. 1990). Interestingly, immunoblotting of total heart homogenates and junctional membrane enriched samples from the mutant mice demonstrated that mutations in the triplet of serines significantly influenced Cx43 mobility. Cx43 immunoreactive bands from Cx43-S3E mutant mice migrated more slowly and conversely those from Cx43-S3A mutant mice migrated more rapidly than those observed in WT hearts. Moreover, immunofluorescent staining demonstrated that Cx43-S3A mice had significantly less junctional Cx43 compared to WT or S3E mice (Fig. 3). These results suggest that the inhibition of CK1δ-dependent phosphorylation of Cx43, as demonstrated in vivo by the Cx43-S3A mutant mice, interferes with either trafficking of Cx43 to the junctional membrane or its stability after assembly into gap junction plaques. These molecular changes were associated with significant functional sequelae. The Cx43-S3A mutant mice displayed significantly increased susceptibility to inducible ventricular tachycardia whereas the Cx43-S3E mice were relatively resistant. Moreover, the Cx43-S3E mutant mice produced gap junctions that were resistant to pathologic gap junction remodeling associated with TAC (Fig. 3). Taken together, these data clearly confirmed a mechanistic link between posttranslational phosphorylation of Cx43 and gap junction formation, pathologic gap junction remodeling and arrhythmic susceptibility within the context of the intact organism.

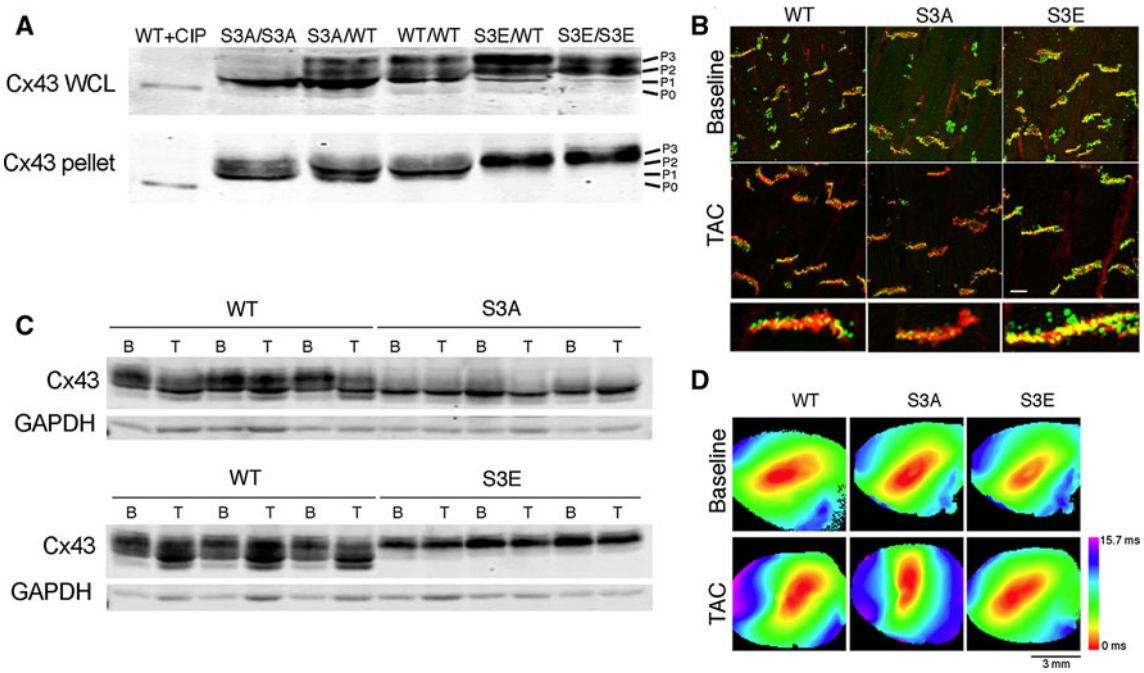

Fig. 3 Molecular and functional analysis of CK1δ mutant mice. **a** High-resolution Western blot analysis of whole cell lysates (WCL) or Triton X-100 insoluble pellets (pellet) prepared from ventricles of mice with the indicated genotypes, probed with polyclonal panCx43 antisera. Wild type Cx43 lysate treated with calf intestine phosphatase (CIP) migrates at P0 and is shown for comparison to various major phosphorylated forms of Cx43 (P1, P2, P3). **b** Representative immunofluorescent staining with panCx43 (Cx43, *green*) and N-cadherin (N-cad, *red*) antibodies at baseline and 4 weeks after TAC. *Scale bar* 10 μm. Magnified views of individual gap junction plaques for each genotype after TAC are shown below. **c** Representative immunoblots of whole cell lysates at baseline (B) and after TAC (T) from each of the indicated genotypes, probed with polyclonal panCx43 and GAPDH antibodies. **d** Representative activation maps from each of the indicated genotypes at baseline and after TAC, showing blunting of conduction slowing in S3E mutant mice. Adapted from Remo et al. (2011)

Regulation by the Nonreceptor Tyrosine Kinase Src

More recently, we have turned our attention to the role of Src kinase-dependent phosphorylation of Cx43 in the heart. Atkinson et al. (1981) originally reported that infection of cells with an avian sarcoma virus resulted in junctional uncoupling; this effect was subsequently shown to be due to the activity of the viral tyrosine kinase *v-src* (Chang et al. 1985) and more specifically, to phosphorylation of Cx43 on tyrosine residues in the carboxy terminus (Swenson et al. 1990; Filson et al. 1990). Subsequently, Toyofuku et al. (1999) reported that endogenous, or cellular c-Src was increased in myopathic BIO 14.6 hamsters and that activated phospho-Src reduced gap junctional coupling between cardiac myocytes, suggesting a role for dysregulated Src signaling and pathologic gap junction remodeling within the context of the intact organism. Moreover, they also showed that Src could directly phosphorylate Cx43 and this posttranslational modification diminished the interactions of Cx43 with ZO-1 (Toyofuku et al. 2001). Conversely, Giepmans et al. (2003) showed that the receptor protein tyrosine phosphatase mu (RPTPmu) also interacted with the carboxy-terminus of Cx43 and could prevent Src-mediated closure of gap junction channels, suggesting dynamic regulation of Cx43 by the opposing actions of tyrosine kinases and phosphatases. Subsequent studies by Pointis and colleagues, although performed in nonexcitable Sertoli cells, have not only confirmed that activated pSrc can bind to Cx43 and displace ZO-1, but also that this molecular reorganization promotes rapid, dynamin2-dependent endocytic internalization of Cx43 GJs (Gilleron et al. 2008, 2011). Sorgen and colleagues have begun to examine the relevance of these molecular events within the context of the intact heart, using the canine infarct model. They found that the pathologic gap junction remodeling in the epicardial border zone was characterized by activation of Src and a molecular reorganization of Src and ZO-1 (Kieken et al. 2009), although the posttranslational status of Cx43 was not examined in detail. Moreover, in apparent contrast to the model of Pointis et al (Gilleron et al. 2008), their studies suggested that activated Src preferentially bound to ZO-1, resulting in an "untethering" of Cx43 from its ZO-1 scaffold, allowing it to migrate from the intercalated disc to the lateral myocyte membrane.

Interestingly, recent evidence suggests that aldosterone signaling, acting through either a genomic or nongenomic mechanism, may increase Src activity (Kobayashi et al. 2006; Shi et al. 2011). These data are intriguing given our recent observation that treatment of aortic banded mice with the aldosterone receptor antagonist spironolactone resulted in significant amelioration of pathologic gap junction remodeling (Qu et al. 2009). Further supporting a fundamental role for Src in pathologic gap junction remodeling is the recent report by Dudley's group examining angiotensin converting enzyme overexpressing transgenic mice, in which Cx43 remodeling and arrhythmias are prevalent. Treatment of ACE-overexpressors with the Src inhibitor PP1 resulted in partial normalization of Cx43 levels and diminished arrhythmic burden (Sovari et al. 2011). Not only is the renin–angiotensin–aldosterone system pathway implicated in pathologic gap junction remodeling, but additional circulating factors including endothelin-1, lipopolysaccharide and TNFα have all been reported to induce Cx43 tyrosine phosphorylation and uncoupling (Huang et al. 2003; Lidington et al. 2002; Postma et al. 1998). To date, gene-targeted murine models that might elucidate the role of Src in cardiac physiology and pathology have not been revealing. Unfortunately, germ line knockout of Src produces osteopetrotic, runted mice that die in the perinatal period, complicating an analysis of potential arrhythmic behavior (Soriano et al. 1991). Recently, however, mice harboring a floxed Src allele have been created by the Muller laboratory (Marcotte et al. 2012), and analysis of their cardiac phenotype should be instructive.

New Directions

Paralleling our strategy to elucidate the role of CK1δ-dependent phosphorylation, we have recently begun replacing tyrosines 247 and 265 in the carboxy-terminus of Cx43 with either nonphosphorylatable phenylalanine residues or phosphomimetic glutamic acids. Mice harboring these mutations should provide a definitive approach to determine the importance of tyrosine-dependent phosphorylation of Cx43 and the role of this modification in the cardiac gap junction lifecycle, both in health and in response to pathologic stressors. Moreover, we have generated mice expressing a Cx43-eGFP fusion protein in the heart. Our preliminary analysis reveals appropriate colocalization of the fusion protein with endogenous Cx43 at the cardiac intercalated disc. Real time fluorescent imaging of cardiomyocytes and myocardial tissue slices from these mice can be used to characterize the numerous signaling pathways (i.e., kinases, phosphatases, acetylases, ubiquitin ligases, etc.) that are thought to regulate gap junction formation and internalization. In the future, we anticipate that these and other genetically engineered murine models will provide important insights into the mechanisms and consequences of pathologic cardiac gap junction remodeling.

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