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Designer Gap Junctions that Prevent Cardiac Arrhythmias

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Abstract

Cardiac gap junctions are specialized membrane structures comprised of arrays of intercellular channels responsible for propagation of the cardiac impulse. These channels are formed by oligomerization of individual protein subunits known as connexins. In response to a broad array of pathologic stressors, gap junction expression is disturbed, resulting in aberrant cardiac conduction and increased propensity for rhythm disturbances. In this article we review some of the recently identified molecular regulators of connexin assembly, membrane targeting and degradation, focusing on the role of post-translational phosphorylation of connexin43, the major gap junctional protein expressed in ventricular myocardium. We also describe efforts to engineer “designer” gap junctions that are resistant to pathologic remodeling.

Introduction

Cardiac gap junctions are areas of membrane specialization that harbor arrays of intercellular channels formed from connexin proteins. Within adult mammalian ventricular myocardium, gap junctions are enriched at the intercalated discs, whereas in atrial tissue gap junctions may also be found along the lateral surfaces of cardiomyocytes (Dewey and Barr 1964; Gourdie et al. 1991). Each channel is composed of two hemichannels, or connexons - one contributed by each cell. Each hemichannel, in turn, is formed by oligomerization of six connexin monomers into a hexameric structure with a central permeation pathway (Figure 1). Multiple connexin isoforms have been identified, each forming channels with distinct biophysical properties such as unitary conductance and voltage dependence (Spray et al. 1992). Moreover, there is electrophysiological and some biochemical evidence indicating that different connexin isoforms may oligomerize into heteromeric and/or heterotypic channels (Harris 2001), although the rules governing these complex assemblies remain incompletely characterized (Moreno 2004).

Connexin 43 (Cx43) is the major ventricular gap junction in the mammalian heart (Beyer et al. 1987) and numerous lines of evidence have demonstrated that gap junction mediated intercellular coupling is essential for normal cardiac impulse propagation (Gutstein et al. 2001; Peters et al. 1997; Saffitz 2009; Severs et al. 2008). Over the past several decades, studies from numerous laboratories have described substantial dysregulation of gap junction expression in association with many forms of heart disease. This pathologic process, first described by Severs and colleagues (Smith et al. 1991) and now referred to as gap junction

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remodeling (GJR), is typically characterized by a significant reduction in the abundance of gap junctional protein, and oftentimes a redistribution from junctional to non-junctional membrane (i.e., lateralization). GJR has been observed in a variety of acquired and inherited arrhythmic syndromes, including ischemic heart disease, hypertrophic cardiomyopathy, and arrhythmogenic right ventricular cardiomyopathy (Saffitz 2009; Severs et al. 2008). Furthermore, data from human genetic studies in patients with somatic and germline mutations in connexin proteins, as well as data from engineered mouse models support the close relationship between GJR and a highly proarrhythmic substrate (Gollob et al. 2006; Gutstein et al. 2001; Thibodeau et al. 2010; van Rijen et al. 2004). The mechanistic basis by which gap junction remodeling increases arrhythmic susceptibility is multifactorial. In normal myocardium, the resistance of gap junctions relative to cytoplasmic resistance is such that there is discontinuous conduction at the microscopic level; although at the macroscopic scale conduction appears uniform (Spach et al. 1988). However, when gap junctional coupling is reduced, conduction becomes highly discontinuous and this may set the stage for reentrant arrhythmias (Kanno and Saffitz 2001; Kleber and Rudy 2004). Additionally, gap junction uncoupling can increase the incidence of arrhythmic triggers and their propagation into adjacent myocardium (Gutstein et al. 2005; Kanno and Saffitz 2001).

Cardiac Cx43 has a relatively short half-life, on the order 1–2 hours (Berthoud et al. 2004; Darrow et al. 1995; Laird et al. 1991), suggesting that synthesis and degradation of gap junctions is a very dynamic process and regulation of protein stability may be a major mechanism involved in GJR. Cx43 is translated in the rough endoplasmic reticulum (ER), undergoes oligomerization in the post-ER/ Golgi compartment, and then Cx43 containing vesicles are thought to be transported to the plasma membrane at the periphery of existing gap (Gaietta et al. 2002), in a domain recently termed the perinexus. Here, undocked connexons may aggregate into the gap junction proper in a ZO-1 dependent manner (Hunter et al. 2005; Rhett et al. 2011). While a complete understanding is lacking, recent data suggest that the forward trafficking mechanism may utilize a microtubule and actin-based transport system that delivers connexons to the adherens junction complex (Shaw et al. 2007; Smyth et al. 2012). Channel assembly and forward trafficking are tightly controlled by a variety of regulatory mechanisms; pathological triggers, such as oxidative stress (Shaw et al. 2007; Smyth et al. 2010) or hemodynamic overload (Chkourko et al. 2012) can disrupt components of the forward trafficking machinery and perturb gap junction formation and promote pathologic remodeling.

Additionally, Cx43, like other cardiac connexins (Cx40 and Cx45), is a phosphoprotein, with a carboxy-terminal domain rich in serines, threonines and tyrosines. Virtually all aspects of the gap junction life-cycle, including assembly of connexons, trafficking to the sarcolemmal membrane, and degradation appear to be regulated by post-translational phosphorylation. In addition, channel gating is also subject to regulation by phosphorylation (Moreno and Lau 2007). Sequence analysis has identified consensus phosphorylation sites for numerous kinases including protein kinase A, Akt (protein kinase B), mitogen activated protein kinase, cdc2 kinase, Src kinase and casein kinase 1 δ and substantial effort has been directed toward biochemical validation of these predicted sites and correlation with functional sequelae. For instance, protein kinase A, Akt (protein kinase B), and casein kinase 1 δ (CK1 δ)-dependent phosphorylation of Cx43 result in increased gap junction communication, thought to be mediated through enhanced channel assembly and trafficking, as well as channel stabilization at the membrane (Moreno and Lau 2007) (Dunn et al. 2012; Solan and Lampe 2009). On the other hand, protein kinase C, Src kinase, mitogen activated protein kinase, and cdc2-dependent phosphorylation lead to decreased gap junction communication, thought to be mediated through increased internalization and degradation through lysosomal and/or proteasomal pathways that involve CIP75, Nedd4, and Eps15, as well as reduced channel open probability (Berthoud et al. 2004; Gilleron et al. 2009; Girao

et al. 2009; Kjenseth et al. 2010; Laing et al. 1997; Lampe and Lau 2000; Lampe and Lau 2004; Solan and Lampe 2009; Su et al. 2010; Toyofuku et al. 2001). To date, the majority of studies of phosphorylation-dependent regulation of Cx43 have utilized *in vitro* model systems and the extension of these studies to *in vivo* physiology and disease has lagged. Here, we describe our recent analysis of genetically engineered mice harboring mutations in the putative CK1 δ target sites of Cx43.

CK1 δ dependent phosphorylation of Cx43 and GJR

CK1 δ is a constitutively active kinase that phosphorylates Cx43 at one or multiple serines within a triplet of consensus sites at serines 325, 328 and 330 within its carboxy-terminal domain (Cooper and Lampe 2002; Lampe et al. 2006) (Figure 1). Inhibition of CK1 δ activity results in enhanced accumulation of Cx43 in non-junctional membrane at the expense of accumulation in gap junction plaques (Cooper and Lampe 2002). These data suggest that CK1 δ -dependent phosphorylation promotes the trafficking of newly synthesized Cx43 to junctional membrane, and as a corollary, a shift in the balance of CK1 δ and phosphatase activities resulting in hypophosphorylation of residues 325, 328 and 330 may play a mechanistic role in pathologic GJR.

To address whether hypophosphorylation of Cx43 at CK1 δ -dependent sites is associated with pathologic GJR *in vivo*, we examined several pro-arrhythmic murine disease models, including inherited and acquired syndromes. Oculodentodigital dysplasia (ODDD) is an autosomal dominant systemic disorder due to mutations in the GJA1 gene encoding Cx43 (Paznekas et al. 2003). To explore the molecular pathophysiology of this syndrome, we generated a mouse knock-in model of ODDD, by introducing the pathologic I130T missense mutation into the GJA1 gene encoding Cx43 (Kalcheva et al. 2007). Interestingly, these mice showed substantial reductions in total and phosphorylated cardiac Cx43 levels as well as diminished junctional Cx43. Moreover, using phospho-specific Cx43 antibodies, we demonstrated a profound decrease in phosphorylation at the CK1 δ -dependent target sites, suggesting that diseasecausing mutations could act by influencing post-translational modification and disrupting normal trafficking of connexons to the junctional membrane (Figure 2A). Importantly, hearts from these mice showed abnormal impulse propagation and increased arrhythmic susceptibility, suggesting that the pathologic GJR was accompanied by substantial functional sequelae (Figure 2B). Remarkably similar findings were observed in a murine model of pressure overload hypertrophy induced by constriction of the transverse aorta (TAC). Again, we observed progressive hypo-phosphorylation of Cx43 (Figure 2C), also associated with pathologic GJR and functional evidence of diminished gap junction function (Qu et al. 2009). Taken together, these data suggest that aberrant post-translational phosphorylation at CK1 δ -dependent target sites may represent a common molecular response to diverse pathologic stimuli that induce GJR.

CK1 δ dependent phosphorylation of Cx43 and gap junction formation

While the observations described above suggest an association between aberrant posttranslational phosphorylation of Cx43 and pathologic GJR, they do not establish causality. Accordingly, we recently generated genetically engineered mice in which the CK1 δ -dependent triplet of serines within the Cx43 carboxy-terminus were mutated to either negatively charged phosphomimetic glutamic acid residues (so-called S3E mice) or conversely, to non-phosphorylatable alanines (S3A mice) (Figure 3A). In agreement with prior *in vitro* work, the electrophoretic mobility of Cx43 in the hearts of S3A and S3E mutant mice was significantly altered compared to wild type controls (Figure 3B, upper). The S3A mutants were unable to form the slower migrating phosphorylated isoforms P2 and P3, while the S3E mutants demonstrated more of these isoforms, along with an absence of

the faster migrating isoforms P0 and P1 (Remo et al. 2011). Consistent with this observation, we found that the abundance of total and junctional Cx43 in the S3A mice was substantially reduced compared to the WT and S3E strains (Figure 3B, lower). Taken together, these findings are in agreement with prior *in vitro* data implicating the importance of CK1 δ dependent phosphorylation of Cx43 as a key regulator of Cx43 trafficking to the junctional membrane (Lampe et al. 2006).

CK1 δ dependent phosphorylation of Cx43 and structural gap junction remodeling

To determine whether manipulation of CK1 δ dependent phosphorylation of Cx43 influenced the response to pathologic stimuli, we tested the effects of TAC on Cx43 expression. Imposition of TAC resulted in a significant decline in junctional (Triton X-100 insoluble) Cx43 in both WT and S3A mutant mice, whereas the phosphomimetic S3E strain was largely protected from pathologic remodeling (Figure 3C). This resistance to GJR in S3E mutant mice was also evident by immunofluorescent analysis of cardiac gap junction expression (Figure 3D).

CK1 δ dependent phosphorylation of Cx43 and functional gap junction remodeling

Importantly, the relative resistance of S3E mutant mice to structural GJR was associated with preserved gap junction function, as assayed by optical mapping of isolated-perfused hearts using voltage-sensitive fluorescent dyes. Whereas both WT and S3A hearts showed significant slowing of both longitudinal and transverse conduction velocity 4 weeks after imposition of TAC, the S3E hearts were unaffected. Moreover, we and others have found that primary changes in gap junction function may induce secondarily electrical remodeling (Danik et al. 2008; Delmar and McKenna 2010; Leaf et al. 2008). In fact, the S3A mice displayed significantly greater action potential duration dispersion compared to WT or S3E mutants as well as a trend toward altered restitution properties, with a steeper relationship between coupling interval and APD values. Most significantly, these complex alterations in passive and active channel properties translated into significant differences in arrhythmogenicity. *In vivo* programmed electrical stimulation of S3A mutant mice revealed significantly greater susceptibility to inducible ventricular arrhythmias compared to S3E mice, which were largely resistant to both standard and highly provocative induction protocols. (Figure 3E).

Taken together, our data suggest that CK1 δ -dependent phosphorylation of Cx43 promotes normal trafficking of Cx43 to the junctional membrane and that pathologic stimuli inhibit this post-translational modification, resulting in diminished steady-state abundance of junctional Cx43 (as schematized in Figure 4). Whether or not the hypophosphorylation of Cx43 reflects diminished CK1 δ activity or an increase in phosphatase activity is uncertain, however protein phosphatases PP1 and PP2a are known to colocalize with Cx43 and are upregulated in heart failure models (Ai and Pogwizd 2005; Duthe et al. 2001), suggesting increased phosphatase activity is likely to play a role. Regardless, our results suggest that manipulations that normalize the balance between CK1 δ and phosphatase activity in the pathologically stressed heart may diminish the structural and deleterious functional manifestations of gap junction remodeling and provide some protection against life-threatening cardiac arrhythmias.

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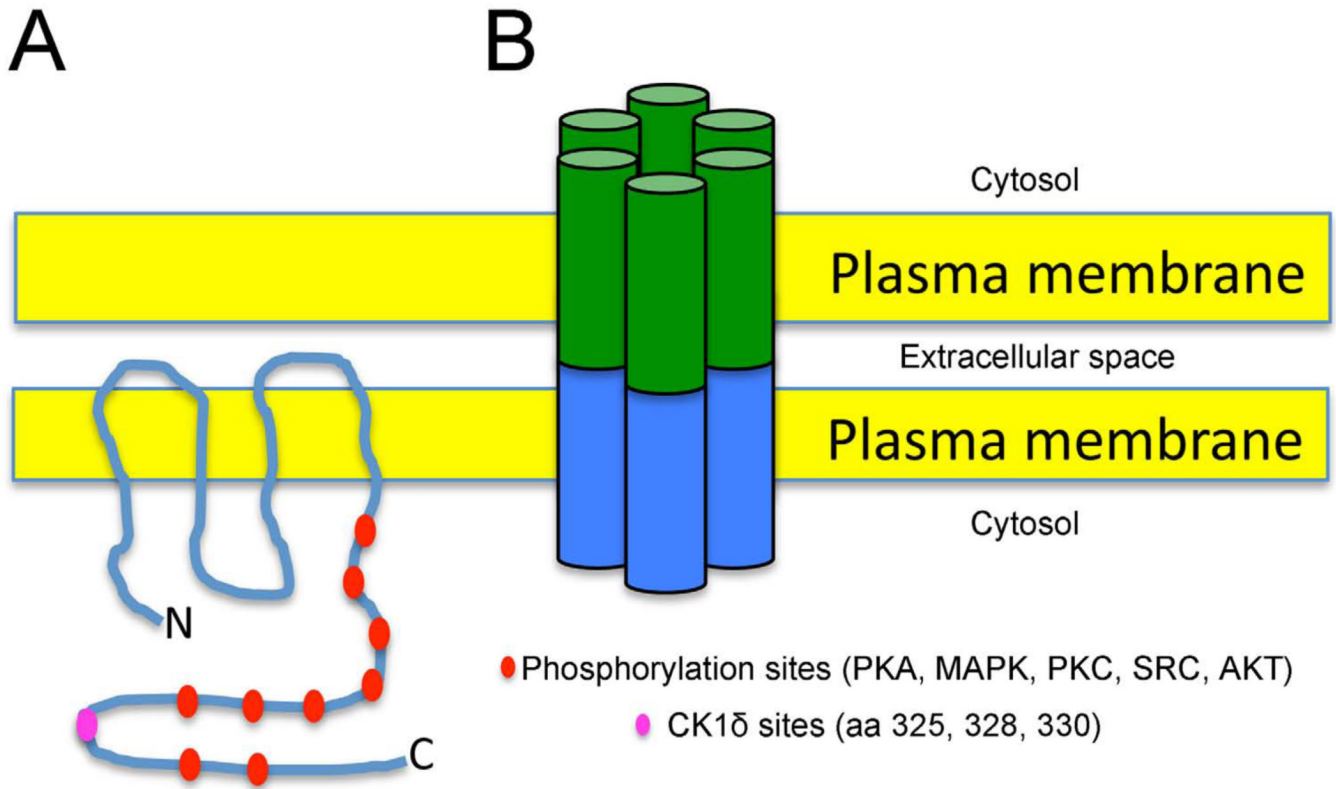


Figure 1. Connexin 43 and Cardiac Gap Junctions

A. Schematic representation of a Cx43 monomer embedded within the plasma membrane of a cardiomyocyte. The carboxy-terminal intracellular domain contains numerous predicted sites of phosphorylation by various kinases, including CK1δ. B. Intercellular channels are formed by the docking of two hemi-channels, each comprised of six connexin monomers.

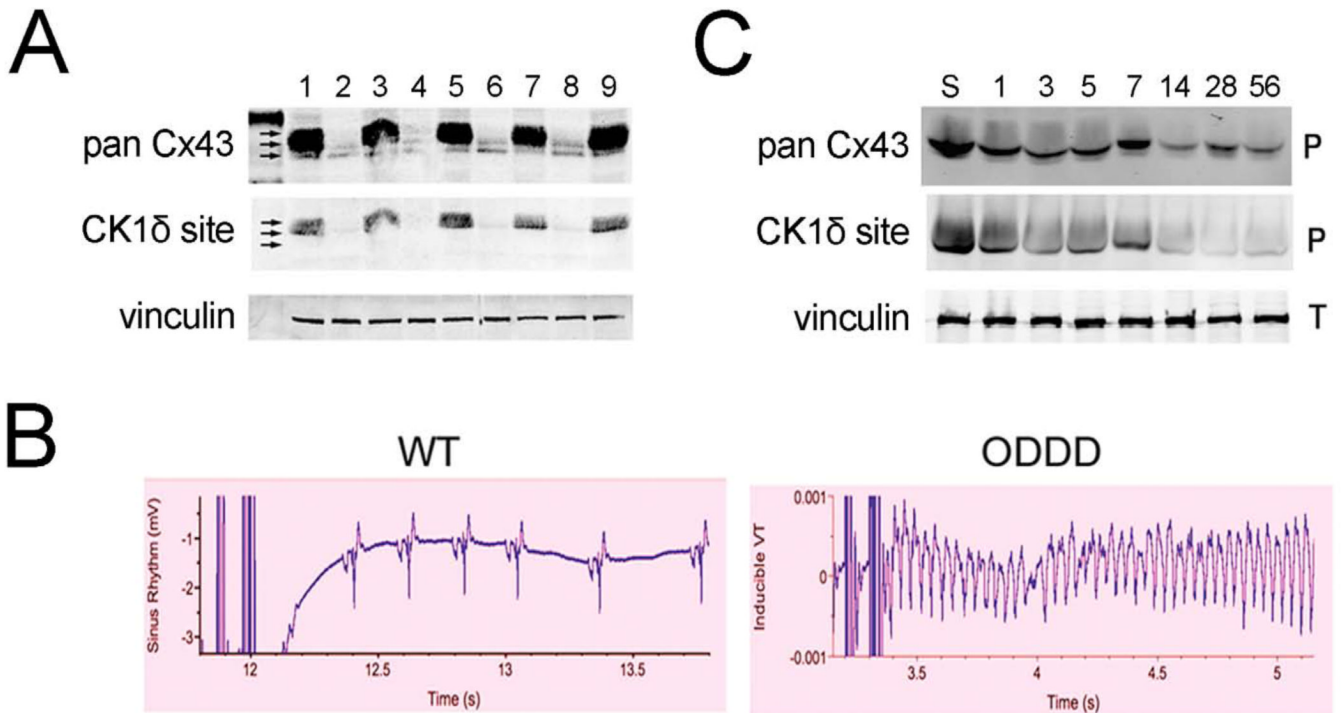


Figure 2. Gap junction remodeling and arrhythmogenesis

A. Western blot analysis of WT (1, 3, 5, 7, 9) and ODDD mutant (2, 4, 6, 8) hearts showing marked reduction in total Cx43 (pan Cx43) and p325/328/330 phosphoCx43 (CK1δ site). B. Programmed electrical stimulation showing resistance to inducible ventricular tachycardia in WT hearts and induction of sustained ventricular tachycardia in ODDD mutant hearts. C. Western blot analysis of WT hearts subjected to transverse aortic constriction showing progressive reduction in total Cx43 and p325,328/330-Cx43. S is a sham control and numbers refer to days after TAC. Figure adapted from (Kalcheva et al. 2007; Qu et al. 2009).

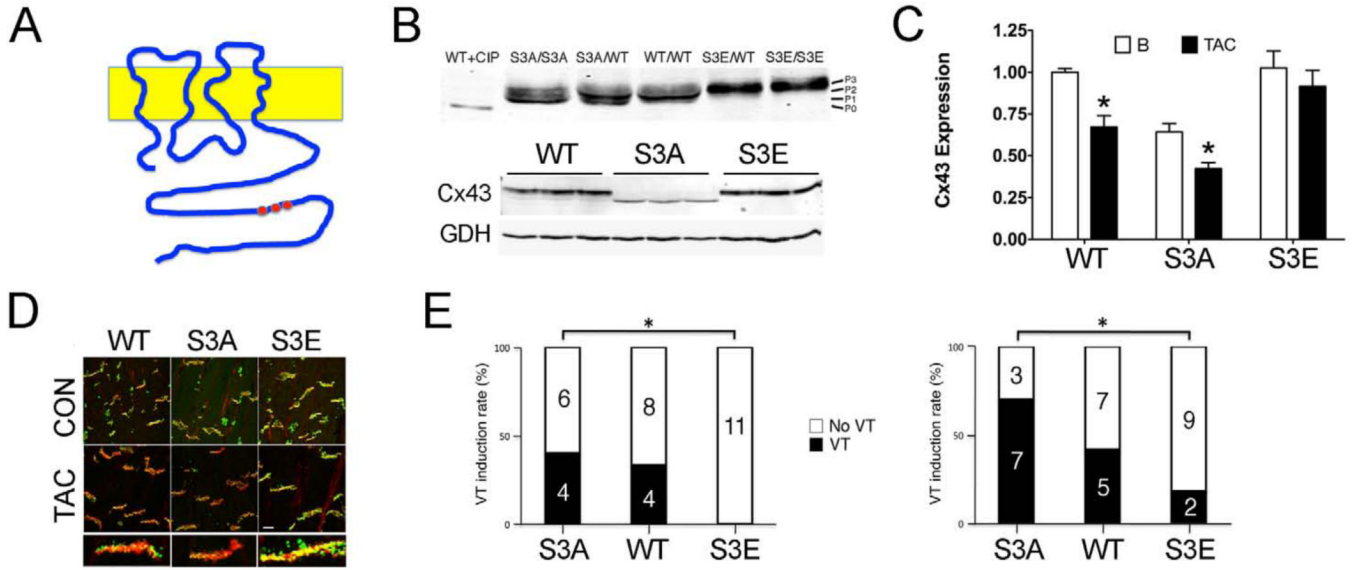


Figure 3. Designer connexins resistant to gap junction remodeling

A. Schematic showing location of serines residues that represent CK16-dependent phosphorylation sites in the carboxy terminus of Cx43. B. Western blot analysis of junctional membrane preparations (Triton-X insoluble pellets) from ventricles of mice with the indicated genotypes, probed with polyclonal panCx43 antisera. WT Cx43 lysate treated with calf intestine phosphatase (CIP) migrates at P0 and is shown for comparison with various major phosphorylated forms of Cx43 (P1, P2, P3). C. Quantification of Cx43 expression in junctional membrane preparations for each genotype at baseline and 4 weeks after TAC. D. Confocal immunofluorescent staining for Cx43 (green) and N-cadherin (red), showing loss of Cx43 at the intercalated discs in WT and Cx43-S3A mutant mice in response to transverse aortic constriction, but preserved expression in Cx43-S3E mutant mice. E. Programmed electrical stimulation with either premature stimuli (left) or burst pacing (right) showing enhanced susceptibility to ventricular tachycardia in the Cx43-S3A mice and protection from inducible arrhythmias in Cx43-S3E mice. Figure adapted from (Remo et al. 2011).

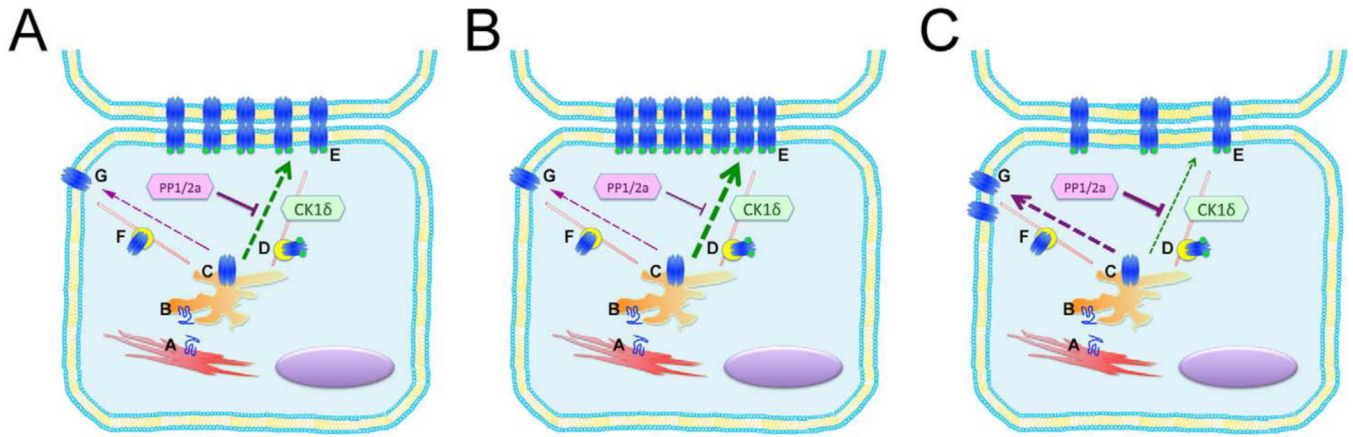


Figure 4. Potential Model of Kinase Dependent Regulation of Cx43 Targeting

Cx43 is synthesized in the rough endoplasmic reticulum (A), transported to the Golgi where monomers (B) oligomerize into connexons or hemi-channels (C), and then targeted to the intercalated disc through a mechanism that is thought to utilize microtubules and actin (D). Panel A. Under normal physiological conditions, the balance of activity between CK1 δ and PP1 and PP2A favors directed transport of phosphorylated connexons (D) to gap junction plaques within junctional membrane (E), rather than targeting of non-phosphorylated connexons (F) to non-junctional membrane (G). Panel B. Under conditions where CK1 δ activity greatly exceeds phosphatase activity, or is mimicked by pseudo-phosphorylation of Cx43 at CK1 δ consensus sites (Cx43-S3E), targeting to junctional membrane is enhanced. Panel C. Under conditions where phosphatase activity exceeds kinase activity, or is mimicked by introduction of non-phosphorylatable residues at CK1 δ consensus sites (Cx43-S3A), connexons are no longer preferentially targeted to junctional membrane, resulting in lateralization and early degradation.