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Structure, Function, and Regulation of the Junctophilin Family

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Abstract

In both excitable and nonexcitable cells, diverse physiological processes are linked to different calcium microdomains within nanoscale junctions that form between the plasma membrane and endo-sarcoplasmic reticula. It is now appreciated that the junctophilin protein family is responsible for establishing, maintaining, and modulating the structure and function of these junctions. We review foundational findings from more than two decades of research that have uncovered how junctophilin-organized ultrastructural domains regulate evolutionarily conserved biological processes. We discuss what is known about the junctophilin family of proteins. Our goal is to summarize the current knowledge of junctophilin domain structure, function, and regulation and to highlight emerging avenues of research that help our understanding of the transcriptional, translational, and post-translational regulation of this gene family and its roles in health and during disease.

INTRODUCTION

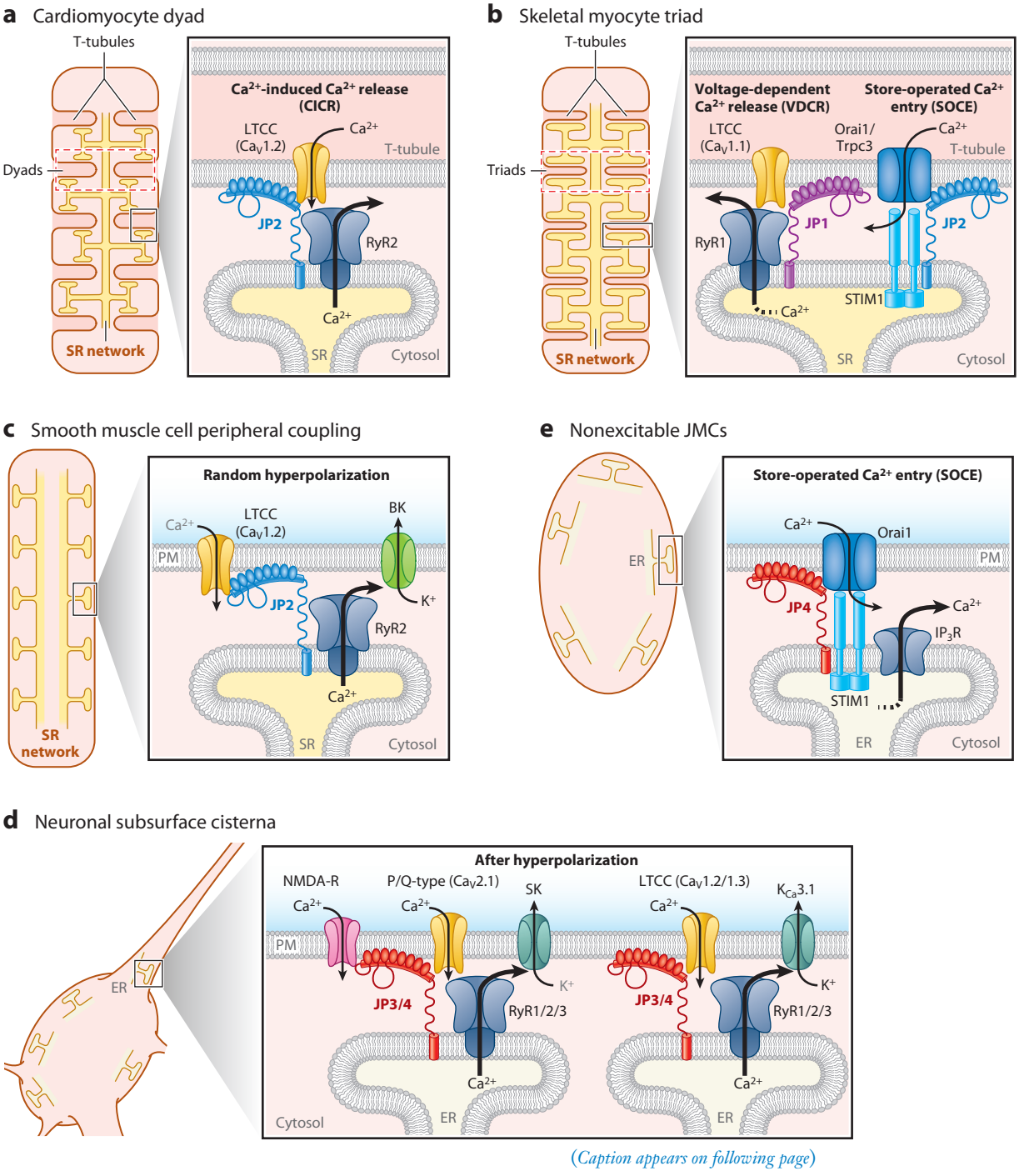
The plasma membrane (PM) bidirectionally communicates with endo-sarcoplasmic reticula to mediate essential physiological processes such as muscle contraction and brain activity. Such events are typically initiated by the conversion of PM depolarization into Ca^{2+} release from nearby endoplasmic reticulum (ER) or sarcoplasmic reticulum (SR) Ca^{2+} stores (1–3). Early microscopy studies revealed that the PM of many cell types is frequently aligned within 10–30 nm of SR/ER membrane patches (4–9). The physical nature of these junctional membrane contacts varies by tissue, and these contacts are called ER/PM junctions in nonexcitable cells, subsurface cisternae in neurons, and peripheral couplings in smooth and striated muscle (7, 10) (**Figure 1*a–e***). Striated myocytes have additional junctional structures known as dyads and triads that associate with specialized interiorly projecting extensions of the PM, i.e., transverse (T-) tubules. Dyads are the major junction of cardiomyocytes in which single SR cisternae make contact with a T-tubule. Skeletal myocytes have both dyad and triad junctions along relatively thinner T-tubules. Triads are named after the appearance of SR terminal cisternae making extensive contacts on opposite sides of a given T-tubule. Each myocyte possesses thousands of T-tubules arranged in a highly organized network (11, 12). Dyad and triad junctions within this network are responsible for synchronous SR Ca^{2+} release and uniform myofilament contraction in response to PM depolarization (13–15).

Electron-dense material was revealed by original studies to concentrate within junctional PM–ER/SR clefts (4–6) and was later recognized to be composed of proteins that enable PM–ER/SR communication (7–9). The junctions and their constituent proteins are broadly referred to as junctional membrane complexes or JMCs (10). JMCs incorporate surface Ca^{2+} (and other) ion channels that partner with ER/SR-specific Ca^{2+} channels and sensors. Although the precise combination of JMC proteins varies by cell type to mediate specific physiological outcomes, all JMCs require at least one member of a fascinating family of proteins known as junctophilins. Junctophilins have a unique multivalent capacity to bind to PM phospholipids, PM ion channels, the ER/SR membrane, ER/SR ion channels, and their associated regulatory molecules. It has therefore been proposed that junctophilins provide a physiological tether or glue in stabilizing the JMC (16). Furthermore, junctophilins can functionally modify the biophysical properties of the ion channels with which they interact.

This review aims to provide the reader with a greater appreciation of junctophilins in health and disease. We survey the physiological functions of junctophilin family members in different cell types and discuss how their domain structure, post-translational modifications, disease-associated mutations, and transcriptional regulation impact their overall function. Throughout, we discuss current challenges being addressed by the field including possible junctophilin-focused therapies to treat diseases associated with JMC dysfunction.

JUNCTOPHILINS STABILIZE PLASMA MEMBRANE–ENDO-SARCOPLASMIC RETICULUM COMMUNICATION

The four mammalian junctophilin paralogs were identified as JP1–JP4 by Takeshima and colleagues more than 20 years ago (17, 18). Current nomenclature, however, now designates junctophilins with JPH or Jph prefixes (see the sidebar titled Junctophilin Diversification). As both naming conventions are still widely used, we attribute *JPH/Jph* to junctophilin genes and RNAs and JP to their protein counterparts. Mammalian *Jph1–4* genes are transcribed in spatially unique patterns for performing cell type–specific functions. *Jph1* is most abundantly expressed in skeletal muscle (19–21). *Jph2* is also expressed in skeletal muscle and is the primary junctophilin of the heart and smooth muscle (17, 22–26). *Jph3* and *Jph4* are produced throughout the brain (18, 27) and in endo-/exocrine glands (28). *Jph3* is also reported throughout the gastrointestinal tract



(Caption appears on following page)

Figure 1 (*Figure appears on preceding page*)

Junctophilin-mediated JMCs. Cellular JMCs (*black boxes*) have different names in different cell types and are responsible for different physiological responses. JMCs are commonly stabilized by junctophilin proteins for localizing ion channels on the PM with Ca^{2+}-permeable channels and sensors on the SR/ER membrane. (*a*) In cardiomyocytes, the dyad (*red dashed box*) formed by T-tubular PM–SR network contacts facilitates CICR. (*b*) Skeletal muscle triads (two junctional SR cisternae flanking one T-tubule; *red dashed box*) are responsible for voltage-dependent Ca^{2+} release in parallel with SOCE. (*c*) PM–SR peripheral couplings in smooth muscle cells determine random hyperpolarization. (*d*) Neurons have subsurface cisternae for mediating afterdepolarization phenomena. (*e*) SOCE is the primary function of nonexcitable cell JMCs and is likely present in most excitable cells. Abbreviations: BK, big-conductance K^+; Ca_V, voltage-dependent Ca^{2+} channel; CICR, Ca^{2+}-induced Ca^{2+} release; ER, endoplasmic reticulum; IP_3R, inositol 1,4,5-triphosphate receptor; JMC, junctional membrane complex; JP, junctophilin; K_{Ca}, Ca^{2+}-activated K^+ channel; LTCC, L-type Ca^{2+} channel; NMDA-R, *N*-methyl-D-aspartate receptor; Orai1, *Orai1*-encoded Ca^{2+} release-activated Ca^{2+} modulator protein 1; PM, plasma membrane; P/Q, Ca^{2+} channel responsible for P- and Q-type currents in cerebellar Purkinje and granule neurons, respectively; RyR2, type 2 ryanodine receptor; SK, small-conductance K^+ channel; SOCE, store-operated Ca^{2+} entry; SR, sarcoplasmic reticulum; STIM1, stromal interaction molecule 1; Trpc3, transient receptor potential cation channel 3.

JUNCTOPHILIN DIVERSIFICATION

Genome databases have increasingly indicated that *Jph* genes are conserved across phyla, including invertebrates and sponges (75, 142). Although a single junctophilin gene is present in most lower organisms, repeated duplication events in the vertebrate lineage suggest multiple paralogs have arisen to perform more nuanced functions. Most vertebrates have three *Jph* loci (*Jph1–3*), while *Jph4* is found primarily in mammals. Instead of a distinct *Jph4* gene, some ray-finned fish have two homologous *Jph1* genes. An elegant study in *Drosophila melanogaster* demonstrates that cardiac-, muscle-, and neuronal-specific knockout (KO) of its lone junctophilin gene (*jp*) generates phenotypes that largely parallel the phenotypes of mammalian *Jph1*, *Jph2*, and *Jph3*+*Jph4* KO models, respectively (57).

and reproductive organs (29). Vertebrate JP proteins typically range in size from 600 to 800 amino acids in length, but shorter alternatively spliced isoforms are predicted in humans. The function of these shorter proteins remains to be defined. The four junctophilin paralogs have distinct functions in the tissues in which they are expressed, but they are homologous to each other; thus, what we have learned about one may be generally applied to the others. The most investigated is JP2, and studies related to its structure and function have led to robust insights into how junctophilins maintain JMC integrity and mediate PM–ER/SR communication.

Junctophilin-2 Is Essential for Cardiomyocyte Dyad Function

The primary role of JP2 in cardiomyocytes is to establish, maintain, and regulate dyad junctions. The importance of an intact T-tubule/SR dyadic system was inferred from observations that cardiomyocytes from failing hearts of patients (30, 31) and in animal models of heart failure exhibit disrupted T-tubule and dyad ultrastructure that correlate with defects in Ca^{2+} handling (31–40). Mice with global KO of *Jph2* have a weak and irregular heart rhythm in utero, with half of homozygous animals dying by embryonic day 10.5 from cardiac arrest (17). Lethality may be due to a drastic reduction in the frequency of immature dyad junctions and abnormal Ca^{2+} transients. This notion was validated a decade later in studies showing that cardiomyocyte expression of a *Jph2* small hairpin RNA (shRNA) causes cardiac dilatation, acute heart failure, pulmonary congestion, and mortality within one week in the absence of heart failure–related hypertrophic remodeling (41). Similar studies in isolated adult rat cardiomyocytes showed that *Jph2* knockdown reduces the frequency and length of dyad junctions correlating with lower cytosolic Ca^{2+} transient amplitudes and slower rise times (37). These results demonstrated that JP2 plays an essential role in dyad stability by mediating phenomena in cardiomyocytes called

CICR, VDCR, AND E-C COUPLING

CICR is the process in cardiomyocytes by which modest Ca^{2+} influx through T-tubule-localized L-type Ca^{2+} channels (LTCCs, specifically $Ca_V1.2$) induce efficient and massive Ca^{2+} efflux from nearby SR terminal cisterna expressing type 2 ryanodine receptor (RyR2) channels. E-C coupling converts membrane depolarizations into CICR-induced spikes of cytoplasmic Ca^{2+} that trigger myofilament contraction. Cytosolic Ca^{2+} levels then return to baseline through Ca^{2+} uptake into the SR via SR Ca^{2+}-ATPase (SERCA2a) and Ca^{2+} extrusion by PM Na^+/Ca^{2+} exchangers, allowing for myofilament relaxation. Skeletal myocytes depend less on CICR and instead rely on a similar phenomenon known as voltage-dependent Ca^{2+} release (VDCR) in which T-tubule-localized $Ca_V1.1$ LTCCs activate skeletal myocyte–enriched RyR1 channels on the SR membrane via a direct voltage-dependent confirmational change.

Ca^{2+}-induced Ca^{2+} release (CICR) and excitation-contraction (E-C) coupling (**Figure 1*a*,*b***) (see the sidebar titled CICR, VDCR, and E-C Coupling).

JP2 protein expression in the mouse heart is detectable at embryonic day 9.5 (17) and gradually increases throughout cardiac development (42, 43), particularly in ventricles (44, 45). Cardiomyocyte PM structure in newborn rodents is rather unremarkable (46) but begins to display invaginating T-tubules within 10 days of postnatal life (42, 43). Dyad structure and function become fully mature around three weeks of age (42, 46, 47). JP2 is first documented at the PM of nontubulated postnatal mouse myocytes (48) before concentrating along early invaginating T-tubules (42, 43, 46). A correlation then develops between JP2 colocalization with RyR2 Ca^{2+} channels already present on the SR membranes and a significant increase in E-C coupling (46). Proper JP2 trafficking to the dyad requires microtubule dynamics. Increasing microtubule density, as occurs during heart failure, relocalizes JP2 away from T-tubules (49–51). Conversely, depolymerizing the microtubules in failing hearts preserves JP2 distribution in dyads, attenuates T-tubule degeneration, and normalizes contractile dysfunction (49).

Jph2 overexpression in the mammalian heart, in contrast to *Jph2* loss of function, is well tolerated and has no effect on cardiac function or viability (52). Postnatal JP2 overexpression accelerates the development of T-tubules (43) and increases SR/T-tubule contact length and number (52). Approximately 10% of dyads in these hearts exhibit extended and complex T-tubules with multiple SR contacts, consistent with the role of JP2 in initiating and stabilizing JMCs. Despite such changes, overexpressing hearts do not have changes to cardiac ejection fraction, left ventricular mass, or Ca^{2+} handling function at baseline but do confer protection against pathological insult. Overexpressing mice have increased survival, attenuated cardiac hypertrophy, and sustained T-tubule integrity after pressure overload stress relative to wild-type mice (52).

Studies using superresolution microscopy techniques, primarily from Soeller and colleagues (53, 54), have shed light on how JP2 and RyR2 channels are arranged within cardiomyocyte dyads and at peripheral coupling sites located within and outside of T-tubules, respectively. JP2 molecules intersperse among RyR2 channels in both junction types, with dyads having more than four times as many RyR2 channels on average than peripheral couplings (53–55). RyR2 clustering is induced by *Jph2* overexpression but is not affected by *Jph2* knockdown despite a loss of T-tubules and E-C coupling efficiency (56). Both *Jph2* overexpression and knockdown approaches increase the proportion of longitudinal tubules that interconnect T-tubules, but why this occurs is not understood.

Taken together, the loss of JP2 is recognized as a major factor in stress-induced T-tubule disorganization and degeneration, inefficient CICR, and maladaptive cardiac remodeling. Studies

too numerous to list here have monitored JP2 protein levels as an early and reliable indicator of heart failure from most etiologies, including inherited and acquired cardiomyopathies, ischemia/reperfusion injury, arrhythmias, and pressure overload. Similarly, studies testing the efficacy of heart failure interventions now commonly assess JP2 levels, its localization, and its correlation with improved T-tubule structure, E-C coupling efficiency, and cardiac function. The precise function of peripheral couplings and longitudinal tubules, however, is not nearly as well understood as T-tubular-associated dyads. Novel studies designed to differentiate the role of JP2 at different junctional sites are likely to unveil the unique contribution and composition of specific JMCs in cardiac pathophysiology.

Skeletal Myocyte Junctions Incorporate Junctophilin-1 and Junctophilin-2

Mouse JP2 is first expressed in embryonic skeletal myocytes and correlates with the formation of dyad structures. JP1 is then induced prior to birth when triad structures are observed (see **Figure 1*a*,*b***). Although mice with specific depletion of *Jph2* in mammalian skeletal myocytes have yet to be developed, global *Jph1* KO mice have muscle weakness and die perinatally, likely from an inability to suckle (21). Skeletal myocytes in *Jph1* KO mice consist of swollen, irregular, and partially vacuolated SR features similar to *jp* KO in *D. melanogaster* (57) and exhibit a specific reduction in muscle triad junctions. These observations suggest that JP1 and JP2 support different JMC structures and/or functions (20). Both JP1 and JP2 are capable of interacting with the C terminus of skeletal myocyte–expressed $Ca_V1.1$ LTCCs (58), but only JP1 copurifies with skeletal myocyte–expressed RyR1 (59). Similar voltage-dependent SR Ca^{2+} release phenomena can be replicated in heterologous cells when RyR1, $Ca_V1.1$, and Stac3 are coexpressed with either JP1 or JP2 as well as with neuronal JP3 (60, 61).

Early studies examining junctophilin function in adult mouse skeletal muscle involved inducible and simultaneous knockdown of both *Jph1* and *Jph2* (26, 62). Dual silencing results in the formation of irregular triad and JMC structures with misaligned or missing SR/T-tubule contacts. Furthermore, voltage-induced Ca^{2+} release is attenuated, and store-operated Ca^{2+} entry (SOCE)-dependent mechanisms, activated by SR Ca^{2+} depletion, become uncoupled. Both studies show that reversal of *Jph1* and *Jph2* shRNA expression restored myocyte ultrastructure, SR Ca^{2+} release, and SOCE coupling (26, 62). Specific loss of JP1 appears to be sufficient to explain many of the phenotypes caused by double *Jph1* and *Jph2* silencing as *Jph1* KO in mouse myotubes impairs SOCE through a reduction and/or displacement of its associated *Orai1*-encoded Ca^{2+} release-activated Ca^{2+} channel protein 1 (Orai1), stromal interaction molecule 1 (STIM1), and transient receptor potential C1/C3 (Trpc1/3) channels despite no change in JP2 expression (63). The full function of JP2 in skeletal myocytes has yet to be defined, but a differential role for JP1 and JP2 in anterograde versus retrograde PM-SR mechanisms likely exists (64, 65).

Although the consequences of increased expression of JP1 in mammalian skeletal muscle have not been reported, transgenic overexpression of *Jph1* in the murine heart results in increased dyad contacts but not triads. Similar to *Jph2* overexpression (52), cardiac *Jph1* expression facilitates extended, thin, and convoluted SR membrane structures that are able to surround T-tubules (66). These findings, combined with the developmental JP1 versus JP2 studies described above, suggest that skeletal myocytes possess a yet to be characterized factor that assists JP1-dependent triad formation that is not present in cardiomyocytes.

Junctophilin Function in Other Cell Types

Jph2 is the primary junctophilin in smooth muscle peripheral coupling junctions (**Figure 1*c***) as validated for cerebral and mesenteric arteries (24, 25) and bladder (23). *Jph2* silencing in smooth

muscle cells does not affect spontaneous SR Ca^{2+} release events (also known as Ca^{2+} sparks), total SR Ca^{2+} uptake, or SR Ca^{2+} load as it does in cardiomyocytes, but it uncouples RyR2 from PM Ca^{2+}-activated big-conductance K^+ (BK) channels (24). BK channels are localized near RyR2 channels and within caveolar microdomains that contain JP2. Physiologically, BK channels are responsible for membrane hyperpolarization that controls the magnitude and duration of smooth muscle constriction in response to elevated hemodynamic pressure (25).

The mammalian brain primarily expresses *Jph3* and *Jph4* for regulating subsurface cisternae junctions (**Figure 1*d***). In *D. melanogaster*, neuronal-specific *jp* overexpression or silencing reduces survival, with overexpressing flies showing a more dramatic phenotype (57). Adult *Jph4* KO mice are phenotypically normal (67), and *Jph3* disruption leads to modest age- and sex-dependent coordination and balance deficits (27, 68). Double *Jph3*/*Jph4* knockout (DKO) animals have a more dramatic phenotype in displaying severe neuromuscular defects, growth retardation, short- and long-term memory deficits, and lethality by one month of age (67, 69). Hippocampal N-methyl-D-aspartate (NMDA) receptors become uncoupled from both RyR-dependent ER Ca^{2+} release and Ca^{2+}-dependent activation of hyperpolarizing small-conductance K^+ (SK) channels (69). RyR–SK channel cross talk is also affected in DKO Purkinje neurons and does not properly respond to $Ca_V2.1$ Ca^{2+} channel activation. $Ca_V2.1$ channels are known as P/Q-type channels as they are responsible for P- and Q-type Ca^{2+} currents in cerebellar Purkinje and granule neurons, respectively. Furthermore, there is a requirement for JP3 and JP4 in the hippocampus to stabilize a tripartite $Ca_V1.3$ (or $Ca_V1.2$) LTCC–RyR2–$K_{Ca}3.1$ channel complex responsible for slow afterhyperpolarization (70). JP3 and JP4 are not entirely functionally redundant as their regulation of neuronal voltage-dependent Ca^{2+} channels and their coupling with different RyR isoforms are different (61, 71).

Emerging evidence points to a greater expression pattern for junctophilins in tissues beyond muscle and brain. For example, *Jph3* knockdown in pancreatic beta islet cells attenuates glucose-stimulated insulin secretion (28). JP4 expression is enriched in dorsal root ganglia, and injection of *Jph4* small interfering RNAs (siRNAs) into the lumbar spine of rats blunts bradykinin-induced hind limb pain responses (72). In nonexcitable T cells, SOCE depends on STIM1 channel recruitment to ER/PM junctions via a direct interaction with the JP4 C terminus (**Figure 1*e***). Silencing of JP4 blocks STIM1 recruitment and prevents T cell activation (73).

JUNCTOPHILIN STRUCTURE AND FUNCTION

Junctophilin Domain Structure

Genetic gain-of-function and loss-of-function approaches clearly establish an essential role for junctophilins in JMC organization, but how these proteins stabilize PM–ER/SR contacts for efficient Ca^{2+} handling processes cannot be understood without further molecular dissection. A tandem array of glycine-rich membrane occupation and recognition nexus (MORN) motifs was first identified in the junctophilin family (17) and is the consensus feature of all junctophilin paralogs and predicted spliced isoforms. Full-length isoforms have eight N-terminal MORN motifs split into a 6 + 2 arrangement separated by a serine-rich joining or linker region that is followed by pseudo-MORN, alanine-rich α-helical, proline-rich, and hydrophobic transmembrane (TM) helical domain sequences (**Figure 2*a*,*b***). Human JP paralogs share 42–52% identity, with JP4 being the most dissimilar (**Figure 2*c***). MORN motifs are the most conserved of the domains, while the proline-rich region has the lowest degree of homology and is aptly referred to as the divergent region. There is generally a high degree of species-to-species conservation for each paralog except within the proline-rich divergent region.

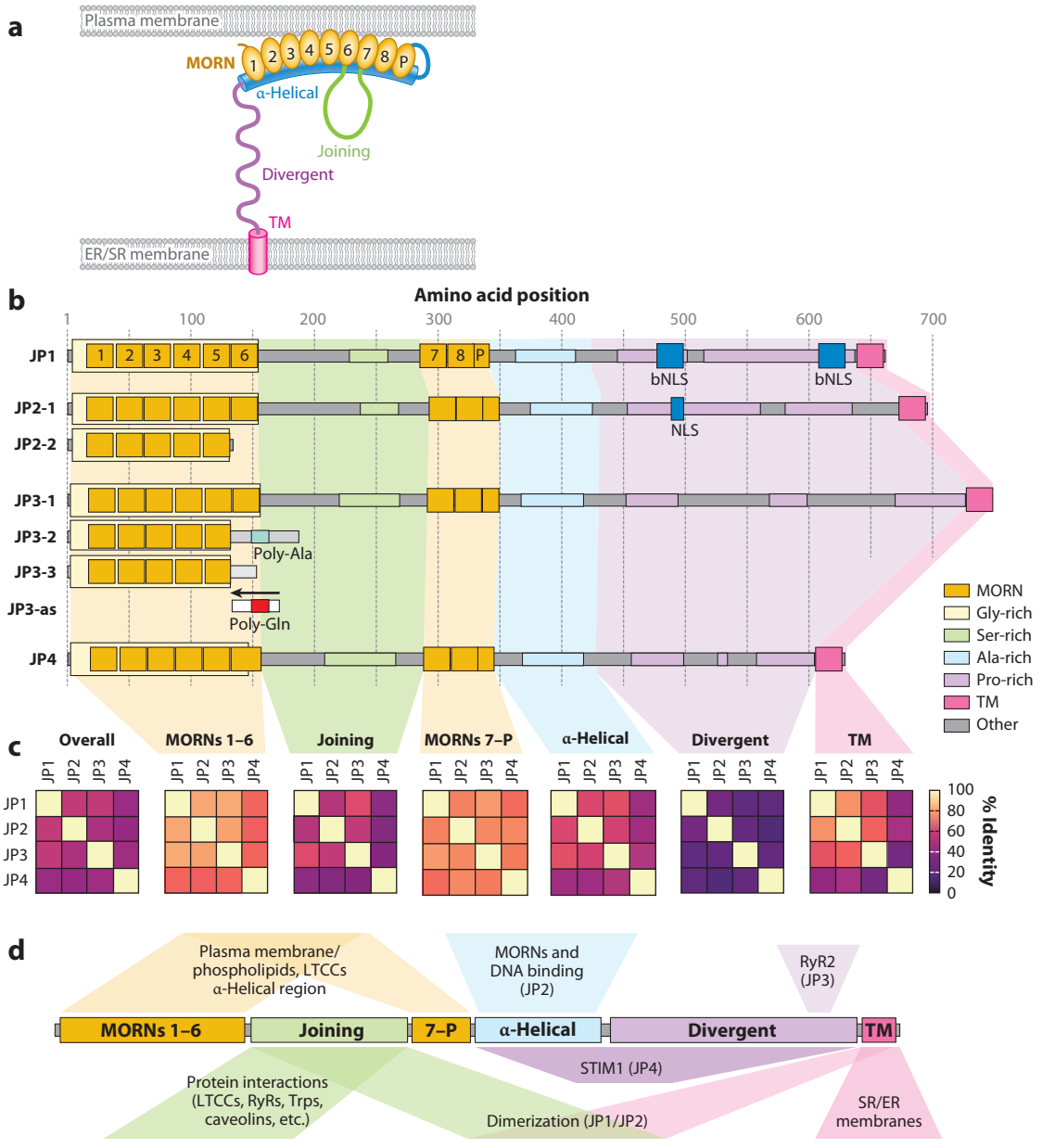


Figure 2

Sequence and structural similarity among junctophilin family members. (*a*) Schematic representing our current understanding of junctophilin structure within a typical junctional membrane complex. For JP2, the N-terminal MORN motifs are reported to bind plasma membrane phospholipids (81) and interact with the α-helical region (78). The C-terminal TM tethers junctophilins to the ER/SR membrane. (*b*) Protein alignments of human JP1–4 isoforms highlighting their functional domains and regions enriched for specific amino acids. (*c*) Heatmap representations of percent human JP1–4 protein sequence identity across full-length (overall) and individual domain regions, as indicated in panel *b*. (*d*) Summary of reported intra- and interprotein junctophilin interactions. Abbreviations: Ala, alanine; bNLS, bipartite NLS; ER, endoplasmic reticulum; Gln, glutamine; Gly, glycine; JP, junctophilin; JP3-as, antisense JP3 protein implicated in Huntington's disease–like 2 syndrome; LTCC, L-type Ca^{2+} channel; MORN, membrane occupation and recognition nexus; NLS, nuclear localization signal; P, pseudo-MORN; Pro, proline; RyR, ryanodine receptor; Ser, serine; SR, sarcoplasmic reticulum; STIM1, stromal interaction molecule 1; TM, transmembrane; Trps, transient receptor potential channels.

Function of Junctophilin Domains

The MORN, joining, α-helical, divergent, and TM domains serve specialized roles within the larger junctional membrane complex and beyond. Here, we summarize how specific domains contribute to junctophilin structure and function.

MORN motifs mediate plasma membrane ion channel and phospholipid interactions. Beyond junctophilins, MORN motifs have been identified in a handful of other protein classes in plants and animals (74). MORN motifs are typically found as tandem arrays in proteins known to be involved in membrane junctions and fission events. Each motif has a core fourteen-residue consensus sequence (Y-X-G-X-W/F-X_2-G-X_3-G-X-G; W/F represents either a tryptophan or phenylamine in the fifth position, and X_n is any amino acid sequence of length n) (17, 75). The MORN consensus motif may stretch to 23 residues, considering other conserved glycine and hydrophobic residues (74). Structural studies show that these motifs fold into a repetitive antiparallel beta hairpin configuration that collectively forms a concave binding pocket for peptide-substrate interactions (76, 77). LTCCs and SK channels are now reported to interact with the MORN motifs of JP1 and JP2 (78, 79). The Van Petegem group (78) recently solved the crystal structures of N-terminal JP1 and JP2 fragments, showing that all eight MORN motifs fold into a single twisting concave configuration. Interestingly, a pseudo-MORN segment just after MORN8 was found to continue the beta hairpin fold despite lacking a MORN consensus sequence. This study went on to show that a highly conserved LTCC C-terminal peptide derived from $Ca_V1.1$ binds within the concave groove formed by the first three MORN motifs of JP2.

Although absent in crystal structures, prior evidence suggests that the junctophilins and their MORN motifs have an affinity for phospholipids, particularly phosphatidylinositol phosphates (PIPs). Overlay of an array of spotted lipids with recombinant JP1 lacking its C-terminal TM domain (80) or a $JP2^{1–452}$ fragment (81) shows interaction with PIP, PIP_2, and PIP_3. The N-terminal JP2 fragment also simultaneously binds phosphatidyl serine for regulating JP2 flexibility. It is noteworthy that full-length JP2 has an increased affinity for phospholipids relative to a truncated $JP2^{1–452}$ fragment, suggesting that C-terminal regions also contribute (81). An elegant study by Rossi et al. (82) demonstrated that the PM localization of green fluorescent protein (GFP)-tagged JP1 and JP2 in HeLa cells is sensitive to PIP_2 levels. Redistribution of GFP fluorescence away from the PM was triggered by phospholipase C activation and readily reversed by inhibition with atropine. This phenomenon likely occurs in cardiomyocytes, as cardiac KO of phosphoinositide-3-kinase p100α and p110β catalytic subunits promotes JP2 translocation away from dyad sites and induces a lethal heart failure phenotype reminiscent of *Jph2* shRNA-expressing hearts (36). Similarly, transfection of skeletal muscle fibers with mutant JP1 devoid of its MORN1–6 or MORN7–8 motifs along with the joining region increases JP1 mobility away from triads (82). The possible requirement for junctophilin MORN motif interactions with specific phospholipids at dyad and triad junctions is intriguing and in need of further investigation. It would be important for future studies to resolve the ongoing issue of whether MORN motifs directly bind phospholipids (e.g., 78) and whether phospholipids alter junctophilin interactions with their ion channel substrates. Determining the physiological role of predicted JP2 and JP3 short isoforms (**Figure 2*b***) may be helpful in this respect.

The serine-rich joining region supports multiple protein interactions. Emerging literature supports the notion that the serine-rich joining domain between MORN1–6 and MORN7–8 is responsible for self-dimerization (82) and mediating multivalent protein–protein interactions within the JMC (**Figure 2*d***). In addition to MORN motif binding to the proximal C terminus of striated muscle LTCCs (58, 78), $JP1^{230–369}$ and $JP2^{216–399}$ fragments that encompass the second half of the joining region through most of the alanine-rich region also interact with LTCCs (83).

The Houser group (84) pinpointed the functional interaction responsible for stabilizing cardiac T-tubules and dyad assembly to seven conserved residues in the human JP2 joining region. Viral infection of feline cardiomyocytes with JP2 mutated at these sites increases T-tubule degeneration, reduces LTCC association, and leads to arrhythmogenic Ca^{2+} handling dysfunction in response to the catecholamine isoproterenol. The interaction between JP2 and Trpc3 also maps to the analogous sequence in mouse and specifically to E^{227} (E^{234} in human) (65). JP1 lacks homology with this segment and fails to coimmunoprecipitate with Trpc3, thus providing an explanation for how JP2 functions apart from JP1–RyR1 complexes in skeletal myocytes.

In cardiomyocytes, *Jph2* knockdown is known to increase RyR2 open probability in SR lipid bilayer assays and increase RyR2 activities, e.g., more frequent spontaneous Ca^{2+} sparks, compared to wild-type samples (85). The stabilizing effect of JP2 on RyR2 gating is thought to be mediated by the proximal joining region of JP2. The JP2 $E^{169}K$ mutation associated with cardiac hypertrophy and arrhythmia reduces the coimmunoprecipitation efficiency between JP2 and RyR2. Addition of a 25-residue wild-type peptide spanning E^{169} attenuates RyR2 opening when added to SR lipid bilayers and reduces Ca^{2+} spark frequency when incubated with atrial myocytes (86). RyR2 stabilization by JP2, however, remains controversial, as JP2 overexpression was unable to suppress RyR2 gating properties in transgenic hearts (52), and JP2 silencing did not alter RyR2-mediated Ca^{2+} activities in pressurized cerebral arteries (24). Finally, the joining region may be important for assembling JP2 and JMCs into lipid rafts (83, 87). *Jph2* overexpression promotes LTCC and caveolin-3 recruitment to cardiomyocyte T-tubules in a cholesterol-dependent manner (88). JP2 also interacts with caveolin-1 in mouse mesenteric artery smooth muscle cells with joining residues $T^{286}TTET^{290}$ lying just upstream of MORN7 (25). This interaction may explain why knockdown of *Jph2* uncouples caveolar BK channels from RyRs and results in suppressed spontaneous BK channel transient outward currents and vascular hypercontractility (24, 89).

The alanine-rich α-helical and proline-rich divergent domains. Experimental evidence shows that the central alanine-rich region of JP1 and JP2 forms a single ~45-Å α-helix that folds back onto the similarly sized antiparallel β-sheet array formed by the MORN motifs and adjacent sequences (78) (see **Figure 2*a***). The intermolecular interactions between the MORNs and α-helical domains as revealed by the crystal structure are potentially plastic, as discussed below with respect to the cytosolic versus nuclear function of JP2 (see the section titled Transcriptional Regulation by Junctophilin Proteolytic Fragments). The divergent region, as highlighted above, also contributes to JP3- and JP4-mediated regulation of SOCE and differential RyR isoform binding (71, 73). It is postulated that the divergent region may be involved in other unknown functions to mediate tissue-specific physiological responses and warrants further investigation.

The C-terminal transmembrane helical domain anchors junctophilin to the endo-sarcoplasmic reticulum membrane. Each of the four full-length junctophilin paralogs end in a hydrophobic α-helix that tethers it to the ER/SR membrane (**Figure 2**). Except for a terminal threonine residue in JP1, JP2, and JP4, no amino acids are thought to project into the SR lumen. Injection of newt embryos with wild-type *Jph1* complementary RNA (cRNA) promotes JMC formation, while cRNAs lacking TM codons do not (17). Without a TM segment, JP1 localization becomes diffusely distributed throughout the PM instead of being restricted to nearby ER. Reciprocally, electroporation of muscle fibers with constructs expressing a GFP-fused JP1 or JP2 TM domain shows a clear subsarcolemmal RyR2-like localization pattern (82). One final intriguing finding by Rossi et al. (82) is that JP1 and JP2 can form homo- and heterodimers in nonmuscle cells through either their TM or joining region domains. Parallel experiments with JP4 were unsuccessful and underscore that inherent functional differences exist between muscle and nonmuscle junctophilin TM motifs.

Other Interactions

JP2 forms stable interactions with several proteins involved in intracellular Ca^{2+} handling. For example, JP2 immune complexes isolated from canine hearts were found to be enriched with SR TM-spanning triadin and calnexin proteins and PM-localized $Ca_V1.2$ LTCCs and KCNQ1 voltage-gated potassium channels (90). JP2 binding appears to have opposite effects on $Ca_V1.2$ and KCNQ1. Heterologous expression of *Jph2* reduces the surface biotinylation of $Ca_V1.2$ channels but increases LTCC current amplitude, which is somewhat different from cardiomyocytes where JP2 overexpression increases both $Ca_V1.2$ expression and current density at the membrane (88). Conversely, JP2 increases KCNQ1 surface expression but attenuates K^+ current amplitude in COS7 cells by shifting its voltage-dependent activation in the positive direction (90). These findings could explain how JP2 promotes CICR by enhancing Ca^{2+} currents through LTCCs while suppressing inward K^+ currents responsible for action potential repolarization.

Mass spectrometry analyses of affinity complexes have found that JP2 interacts with a wide spectrum of cardiac proteins (91–95). SPEG (striated muscle preferentially expressed protein kinase), a serine/threonine-directed kinase, was separately identified in two studies using coimmunoprecipitation and proximity biotinylation purification methods (94, 95). As with *Jph2* silencing, conditional cardiac KO of SPEG results in T-tubule degeneration, abnormal SR Ca^{2+} release, and cardiac failure (94). SPEG appears to phosphorylate JP2 (94), but the consequences of this event remain unclear. There are many other JP2-interacting proteins that are awaiting functional characterization, and it is our hope that similar studies will be designed to identify and characterize novel partners of JP1, JP3, and JP4.

POST-TRANSLATIONAL MODIFICATIONS

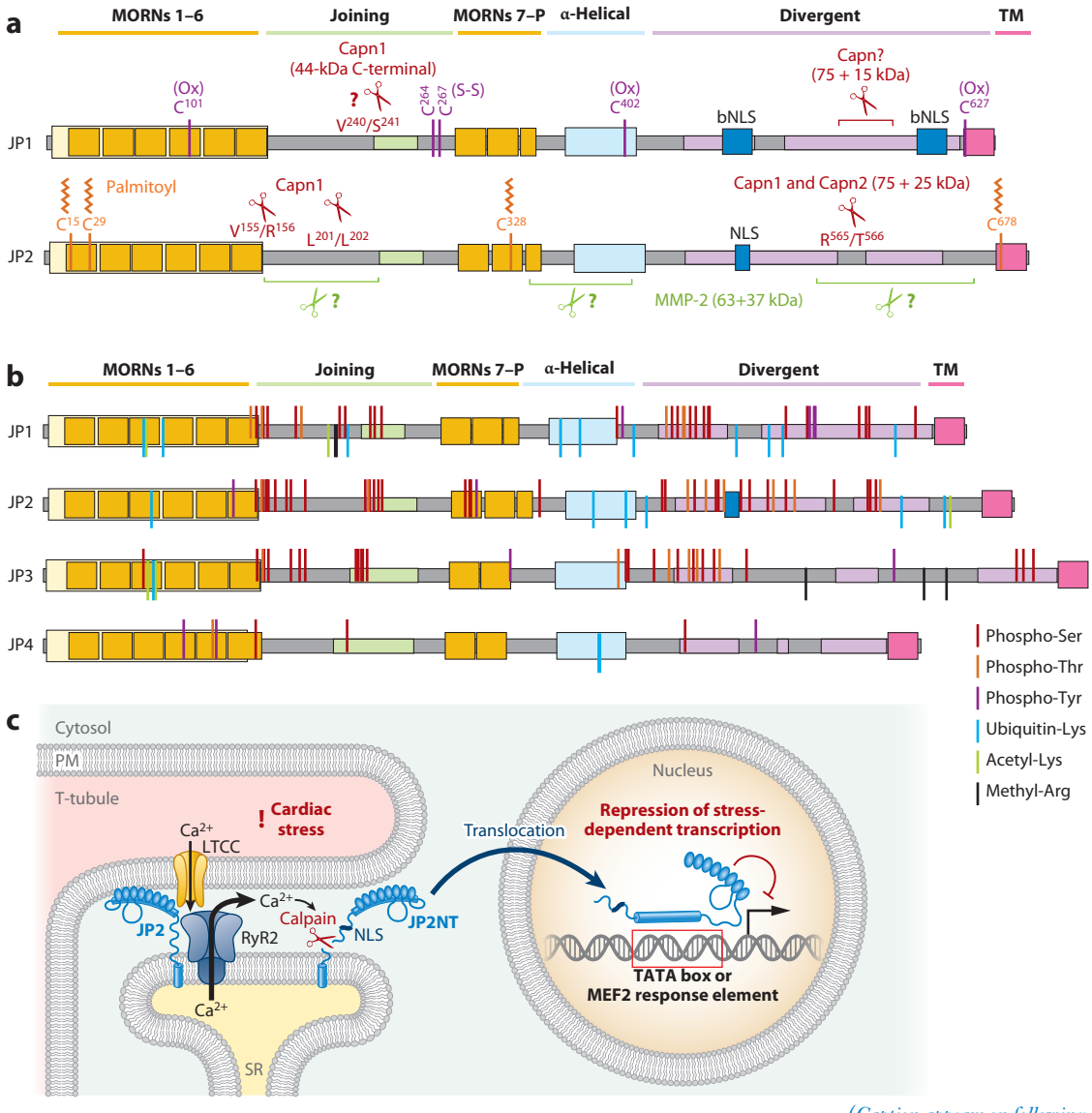
Full-length junctophilins have predicted molecular masses between 65 kDa (JP4) and 82 kDa (JP3). Published immunoblot results, however, are variable and frequently report apparent masses 15–25 kDa larger than expected (27, 59, 72, 81, 96–100). The reason for this discrepancy remains incompletely resolved. Purified bacterially expressed JP2 migrates at its expected size of 75 kDa (81). Alternative transcripts are also insufficient to account for these differences. Here, we discuss the events and factors that may contribute to differences in apparent mass and highlight those that are likely to generate important insight into junctophilin function.

Calpain Proteolysis

Pathological increases in cytosolic Ca^{2+} in cardiac and skeletal myocytes disrupt E-C coupling in part through proteolytic cleavage of JP1 and JP2 (100, 101). Evidence shows that cleavage primarily occurs through Ca^{2+}-activated calpain proteases (102, 103) but matrix metalloproteinase-2 (MMP2) may also contribute (104). Calpain catalytic subunits μ-calpain (Capn1) and m-calpain (Capn2) are ubiquitously expressed and activated by autolysis in response to μM and mM Ca^{2+}, respectively. Proteolysis of JP1 occurs at submicromolar Ca^{2+} levels in skeletal muscle, coinciding with Capn1 autocleavage at two possible sites (100, 105). Likewise, JP2 cleavage correlates with increased calpain activity, cardiac functional decline, T-tubule degeneration, and Ca^{2+} handling dysfunction (101) and is largely prevented with in vivo calpain inhibition (103). Conversely, *Jph2* overexpression partially overcomes the dysfunction in cardiac contractility, Ca^{2+} handling, and T-tubule integrity caused by *Capn1* transgenic overexpression (103), supporting JP2 as a major calpain substrate.

Understanding the mechanisms by which calpains recognize and cleave junctophilins in disease could have far-reaching therapeutic implications, but characterizing cleavage events, defining cleavage site(s), and identifying calpain isoform(s) involved in JP1 and JP2 proteolysis

have produced disparate results. In skeletal muscle, for example, JP1 has been reported to be expressed as a 90-kDa protein that is cleaved into N-terminal cytosolic 75-kDa and C-terminal SR-bound 15-kDa species (100), or as a 72-kDa protein that yields a 44-kDa C-terminal nuclear fragment (105) (**Figure 3*a***). JP2 is typically observed as a ~100-kDa protein and is readily cleaved into 75- and 25-kDa fragments in stressed or failing hearts (96, 97, 100, 101, 106). Our group was the first to map a primary calpain cleavage event to a conserved site in the divergent region (R^{565}/T^{566} in mouse JP2 and R^{572}/T^{573} in human) that is sensitive to Capn1 under mM Ca^{2+} conditions (96, 106). Wehrens and colleagues (98), however, reasoned that JP2 remains largely intact in unstressed hearts during normal Ca^{2+} cycling conditions sufficient for Capn1 activation but becomes preferentially cleaved by Capn2 during pathological stress when cytosolic Ca^{2+}



(*Caption appears on following page*)

Figure 3 (*Figure appears on preceding page*)

Junctophilin post-translational modifications. (*a*) Residues in JP1 and JP2 implicated in oxidation, palmitoylation and proteolysis. Cysteine residues in JP1 are oxidized (Ox) with a disulfide bond between C^{264} and C^{267} (S-S) (59). JP1 and JP2 are proteolytically cleaved by calpains (96, 100, 105, 106) and MMP2 (104). The precise calpain cleavage site of JP1 is not known but may occur in its divergent region to generate N-terminal cytosolic 75-kDa and C-terminal SR-bound 15-kDa species (100), or possibly in the joining region to yield a 44-kDa C-terminal fragment (105). The sites responsible for MMP2 cleavage of JP2 are also not known. JP2 is also palmitoylated on cysteine residues (99). (*b*) JP1–4 proteins are remarkably phosphorylated, ubiquitinated, acetylated, and methylated. Curated proteomics data for human, rat, and mouse JP1–4 were obtained from **https://www.phosphosite.org**. Modifications are shown relative to human protein sequences. Modifications identified in rat or mouse are included if the amino acid is conserved in human. The physiological relevance for the vast majority of these modifications is unknown. (*c*) Stress events promote the generation of the cardioprotective JP2NT fragment (97). Excessive SR Ca^{2+} release and cytosolic Ca^{2+} overload activate calpain, which proteolyzes JP2 downstream of its NLS. JP2NT translocates into the nucleus to attenuate maladaptive gene expression by binding to promoter TATA boxes and MEF2 response elements. Abbreviations: Arg, arginine; bNLS, bipartite NLS; JP, junctophilin; JP2NT, N-terminal cleavage product of JP2; LTCC, L-type Ca^{2+} channel; Lys, lysine; MEF2, myocyte enhancer factor 2; MMP2, matrix metalloproteinase 2; MORN, membrane occupation and recognition nexus; NLS, nuclear localization signal; PM, plasma membrane; RyR, ryanodine receptor; Ser, serine; SR, sarcoplasmic reticulum; TATA box, specific thymine- and adenine-enriched DNA sequence in many proximal gene promoters; Thr, threonine; TM, transmembrane; Tyr, tyrosine.

reaches mM levels. Based on this reasoning, the authors reported that Capn2 cleavage of JP2 at G^{482}/T^{483} produces similar 75-kDa N-terminal and 25-kDa C-terminal cleavage products (98) as those from R^{565}/T^{566} cleavage (96). But how similar fragments arise from cleavage sites 83 amino acids apart was not resolved at the time. Two independent studies have since systematically compared the two sites in human and mouse JP2 that demonstrate a clear requirement for R^{565}/T^{566}, but not G^{482}/T^{483}, in mediating the generation of the 75- and 25-kDa proteolytic products by both Capn1 and Capn2 (106, 107). Furthermore, immunoblots indicate that in vivo produced N- and C-terminal JP2 fragments migrate at the same molecular mass as heterologously expressed $JP2^{1–565}$ and $JP2^{566–696}$ peptides, respectively, but not those of $JP2^{1–482}$ and $JP2^{483–696}$ (106), indicating that cleavage of JP2 in vivo does not involve the G^{482}/T^{483} site.

Cysteine Palmitoylation and Oxidation

JP2 lipidation on conserved cysteine residues tethers JP2 to JMCs and facilitates its incorporation into caveolae as ER-localized puncta (99) (**Figure 3*a***). The majority of nonpalmitoylated JP2 remains ER-localized but more distant from the PM. Palmitoylated JP2 is detected only at 100 kDa, and a quadruple mCherry-JP2 $C^{15}/C^{29}/C^{328}/C^{678}$ mutant migrates faster than its wild-type counterpart (99). Whether dynamic JP2 palmitoylation occurs in cardiomyocytes is less clear, as it is inefficiently palmitoylated and only at the PM outside of T-tubules (99). JP2 crystal structures suggest that C^{15} and C^{328} are inaccessible and C^{29} is buried when bound to LTCCs (78). Three of these cysteine residues are conserved in other junctophilins, suggesting their JMC localization may also be regulated through lipidation. Protein palmitoylation is specifically regulated by the zDHHC family of *S*-acyltransferases that are named according to their conserved zinc finger domain and aspartate-histidine-histidine-cysteine tetrapeptide sequences. The Brody group (108) confirmed that JP2 is palmitoylated in the heart but not further palmitoylated in zDHHC9 transgenic mice, implicating that these modifications are probably meditated by other *S*-acyltransferase family members.

Other cysteines in JP1 act as redox sensors for modulating Ca^{2+} dynamics during oxidative stress conditions. RyR1 open conditions promote disulfide bond formation between JP1 C^{264} and C^{267}, which increases its association with RyR1 (59). Similarly, the reactivity of JP1 C^{101}, C^{402}, and C^{627} are reduced under conditions favoring RyR1 open versus closed states. To what extent modification of junctophilins by palmitoylation or oxidation contributes to their function remains to be investigated and could be achieved by knock-in in vivo models.

Phosphorylation and Other Modifications

Several kinases are predicted to phosphorylate junctophilins (75), but only protein kinase C (PKC) has been shown to mediate a specific phosphorylation event. The JP2 Ser^{165}Phe mutation is associated with hypertrophic cardiomyopathy (HCM) (109), and its expression in skeletal myotubes reduces PKC-dependent JP2 phospho-serine levels by ~60% (110). The mutation promotes skeletal myocyte hypertrophy, inhibits RyR1 channel activity, and interferes with JP2–Trpc3 interactions. KO of the SPEG kinase reduces JP2 serine phosphorylation by half and leads to the disorganization of T-tubules and E-C uncoupling (94), but which JP2 residue is responsive to SPEG remains unknown. Curated proteomics data sets (e.g., **https://www.phosphosite.org**) reveal that JP1–4 proteins are endogenously and extensively phosphorylated on serine, threonine, and tyrosine residues, especially within their joining and divergent regions (**Figure 3*b***), highlighting our lack of knowledge about the post-translational regulation of junctophilins. Furthermore, several ubiquitinated and acetylated lysine and methylated arginine residues are present. The field is therefore ready for determining how these modifications contribute to junctophilin function.

TRANSCRIPTIONAL REGULATION BY JUNCTOPHILIN PROTEOLYTIC FRAGMENTS

Investigation of JP2 proteolysis by calpain led to the unexpected discovery that the 75-kDa JP2 N-terminal cleavage product (JP2NT) acts as a cardioprotective transcription factor that reports E-C coupling dysfunction through a direct excitation-transcription coupling mechanism (97, 111). JP2NT localizes exclusively to the nucleus due to a nuclear localization signal (NLS) within the divergent region lying upstream of the mouse R^{565}/T^{566} or human R^{572}/T^{573} calpain cleavage site (**Figure 3*a*,*c***). Hearts expressing JP2NT via transgene or viral gene therapy approaches are protected against pathological cardiac remodeling, while those with an NLS deletion knock-in have exacerbated pressure overload-induced cardiac dysfunction (97, 112). JP2NT enriches at transcriptional start sites and represses stress-inducible transcripts involved in cardiac hypertrophy, fibrosis, and inflammation. JP2NT directly binds to specific thymine- and adenine-enriched DNA sequences such as proximal gene promoter TATA boxes and myocyte enhancer factor 2 (MEF2) response elements.

Interestingly, JP2NT DNA binding activity (97) maps to the central pseudo-MORN plus alanine-rich "backbone" α-helical sequences found to interact with the larger MORN array in the crystal structure (78). How such a structure allows for DNA binding is not readily predicted and will require additional structural studies. It is possible that the central JP2 region adopts one conformation at junctional membranes and another in nuclei. Similarly, JP1 proteolysis promotes nuclear enrichment of a 44-kDa C-terminal fragment that includes two potential bipartite NLSs (bNLS) (105). Although this fragment requires more thorough investigation, its heterologous expression represses genes involved in PI3K-Akt signaling and glycogenolysis. Whether other junctophilins (JP3, JP4) have a role in regulating gene transcriptions after pathological cleavage is worth further investigation.

PATHOLOGICAL GENE MUTATIONS AND ALLELES

JPH2 Mutations and Cardiac Disease

As proposed in the original manuscript describing JP2, JP2 mutations were already hypothesized to be a risk factor for heart disease based on the similar calcium handling defects observed between embryonic *Jph2* KO mouse myocytes and hypertrophic and failing hearts (17). Rare *JPH2* mutations have since been identified in patients with HCM, dilated cardiomyopathy, atrial fibrillation, and other cardiac conditions (see also 113–115) (**Figure 4*a***). Dozens of other unvalidated

a

Protein	Mutation	Domain	Disease	ClinVar pathogenicity
JP1	$R^{213}P$	Joining	CMT2K	Benign
JP1	$T^{520}M$	Divergent	CMT2K	NA
JP2	$W^{64}*$	MORN3	HCM	Likely pathogenic
JP2	$E^{85}K$	MORN4	DCM, LVNC	NA
JP2	$S^{101}R$	MORN4	HCM	Pathogenic
JP2	$Y^{141}H$	MORN6	HCM	Conflicting evidence
JP2	$T^{161}K$	Joining	HCM, AF, VT	Conflicting evidence
JP2	$S^{165}F$	Joining	HCM	Pathogenic
JP2	$E^{169}K$	Joining	HCM, AF	NA
JP2	$A^{189}T$	Joining	HCM, SD	Conflicting evidence
JP2	$L^{204}R$	Joining	HCM, stillbirth	Uncertain significance
JP2	$T^{237}A$ + $I^{414}L$	Joining, α-helical	DCM	NA
JP2	$E^{338}G$	α-helical	HCM, SD	NA
JP2	$R^{363}L$	α-helical	Stillbirth	NA
JP2	$E^{402}K$	α-helical	Stillbirth	NA
JP2	$A^{405}S$	α-helical	HCM	Conflicting evidence
JP2	$A^{405}T$	α-helical	HCM	Conflicting evidence
JP2	$R^{436}C$	Divergent	HCM	Benign
JP2	$R^{467}C$	Divergent	Stillbirth	NA
JP2	$G^{505}S$	Divergent	HCM	Benign
JP2	$R^{522}W$	Divergent	HCM, DCM, stillbirth	Uncertain significance
JP2	$T^{618}A$	Divergent	HCM	Conflicting evidence
JP2	$E^{641}*$	Divergent	DCM	NA
JP3	CAG/CTG expansion	Exon 2A, antisense RNA	HDL2	Pathogenic
JP3	$S^{209}T$	Joining	HDL2	Conflicting evidence
JP3	$R^{319}W$	MORN8	HDL2	Uncertain significance
JP4	-- No reported or annotated mutations --			

d **Consensus deleterious mutations predicted by HumDiv, PROVEAN, and SIFT**

JP1

$R^{213}P$

JP2

$G^{3}E$	$P^{158}L$	$E^{315}K$
$G^{31}D$	$L^{159}Q$	$R^{323}H$
$E^{47}A$	**$T^{161}K$**	$T^{329}N$
$P^{55}L$	**$S^{165}F$**	**$E^{338}G$**
$G^{57}R$	$L^{166}R$	$G^{339}S$
$T^{76}A$	$S^{168}R$	$A^{394}T$
$E^{85}K$	**$E^{169}K$**	**$E^{402}K$**
$R^{93}H$	$G^{173}R$	**$A^{405}T$**
$R^{97}Q$	$R^{197}L$	$R^{416}C$
$R^{97}W$	$R^{213}W$	$P^{424}L$
$E^{121}K$	$G^{219}C$	$R^{436}C$
$G^{126}R$	$F^{221}L$	$R^{572}C$
$Y^{129}D$	$R^{232}C$	
$G^{136}S$	$S^{255}L$	
$R^{138}S$	$S^{265}R$	
$Y^{141}H$	$M^{292}T$	
$S^{146}G$	$K^{296}T$	
$S^{146}R$	$R^{300}P$	
$P^{148}L$	$E^{307}K$	
$G^{150}R$	$E^{315}G$	

JP3

$A^{370}T$	**$R^{319}W$**	$R^{656}W$

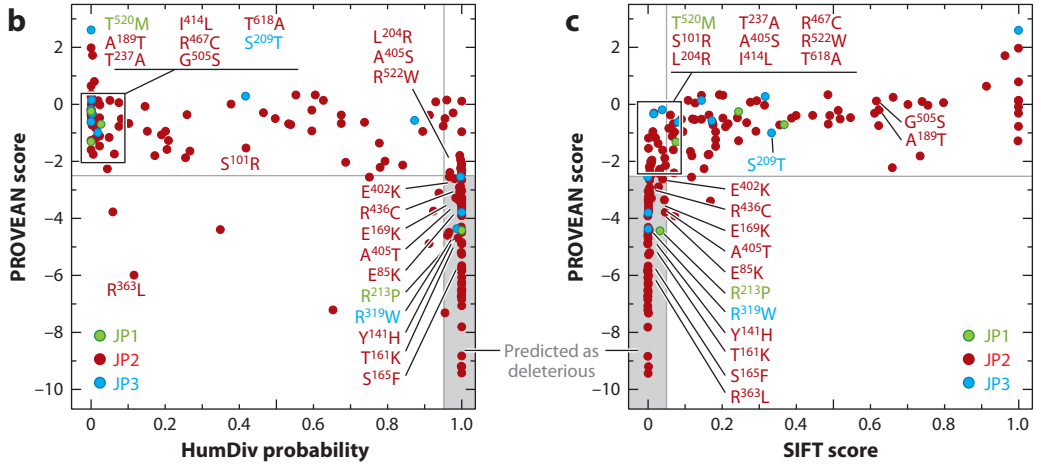


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Figure 4 (*Figure appears on preceding page*)

Pathogenic probability of junctophilin missense mutations. (*a*) Junctophilin mutations that are clinically associated with disease as annotated in the ClinVar database and/or have been published. Reported human variants include JP1 $R^{213}P$ (116); JP1 $T^{520}M$ (125); JP2 $E^{85}K$ (126); JP2 $S^{101}R$, $Y^{141}H$, and $S^{165}F$ (81, 109); JP2 $T^{161}K$ (127); JP2 $E^{169}K$ (86); JP2 $A^{189}T$ (128); JP2 $L^{204}R$, $R^{363}L$, $E^{402}K$, $R^{467}C$, and $R^{522}W$ (129); and JP2 $T^{237}A + I^{414}L$ (130); JP2 $E^{338}G$ (131); JP2 $A^{405}S$ (132); JP2 $A^{405}T$ (133); JP2 $R^{436}C$ and $G^{505}S$ (134); JP2 $E^{641}*$ (135); and JP3 CAG/CTG repeat expansion (117, 136–138). JP2 $W^{64}*$, JP2 $T^{618}A$, JP3 $S^{209}T$, and JP3 $R^{319}W$ have not been published but are annotated as human variants in the ClinVar database. (*b*,*c*) Predicted consequences of missense mutations on junctophilin function. Mutations predicted to be deleterious by both algorithms in each plot are shaded by gray boxes. HumDiv probability calculated by PolyPhen2 is based on the degree of all human disease variants relative to sequence divergence (139). Probable damaging missense mutations have values >0.95. The PROVEAN score is derived from alignment-based correlations and are considered deleterious when scores are <−2.50 (140). SIFT predicts whether a mutation affects protein function based on sequence homology and the physical properties of the amino acid using a score cutoff <0.05 (141). (*d*) List of missense mutations predicted by all three algorithms to be deleterious. Specific mutations in panels *b*,*c* are those listed in panel *a* and indicated in bold in panel *d*. Asterisk denotes a nonsense mutation. Abbreviations: AF, atrial fibrillation; CMT2K, Charcot-Marie-Tooth disease type 2K; DCM, dilated cardiomyopathy; HCM, hypertrophic cardiomyopathy; HDL2, Huntington's disease-like 2; JP, junctophilin; LVNC, left ventricular non-compaction; NA, not applicable; SD, sudden death; VT, ventricular tachycardia.

JPH2 mutations are annotated as likely or possibly pathogenic in the ClinVar database, as are infrequent mutations in *JPH1* and *JPH3* associated with Charcot-Marie-Tooth disease (116) and Huntington's disease-like 2 (68, 117), respectively. Mutations in *JPH4* have yet to be reported. Computational algorithms that can predict how damaging a given mutation is reveal there are likely numerous deleterious alleles for JP1, JP2, and JP3 (**Figure 4*b–d***) which, if pursued, could provide novel mechanistic insights.

Future efforts will be important for validating the pathogenicity of mutations identified in patients. Approaches for functionally characterizing *JPH2* mutations include introduction into human embryonic stem cells (118), *Jph2* genetic editing in the adult mouse heart through cardiotropic adeno-associated virus (AAV2/9) delivery of CRISPR/Cas9 and *Jph2* guide RNAs (gRNAs) (119), and viral delivery of *Jph2* transgenes (112, 120). For example, injection of mice after pressure overload surgery with AAV2/9 particles expressing intact JP2 or nuclear localizing JP2NT blunts T-tubule disorganization, Ca^{2+} handling dysfunction, cardiac remodeling processes, and hypertrophic gene expression (112, 120).

TRANSCRIPTIONAL REGULATION OF JUNCTOPHILINS

The mechanisms underlying junctophilin gene transcription remain more enigmatic than their post-translational modifications. Only two transcription factors have been identified to directly regulate *Jph2* expression. Cardiac contraction in hibernating ground squirrels was found to be stronger than in nonhibernating animals due to a compensatory reduction in LTCC current density and Ca^{2+} influx combined with a near doubling in EC-coupling gain. This is achieved through increased dyad contact surface area and decreased junctional gap distance. Not surprisingly, JP2 expression was increased along with the cardiac transcription factor myocardin (121). *Jph2* expression was found to require promoter CArG motifs known to bind serum response factor (SRF)/myocardin dimers and depend on the synergistic actions of SRF and myocardin.

Non-coding RNAs help shape the transcriptome during growth, differentiation, and adaptation processes. Of these, a handful of microRNAs (miRNAs) have been experimentally shown to regulate junctophilin miRNA levels. MiR-24, miR-34, and miR-331 are induced in different forms of heart disease, and increasing their expression or abundance in cardiomyocytes leads to *Jph2* downregulation, E-C coupling dysfunction, and the initiation of heart failure (38, 122, 123). Mutation of two conserved miR-24 binding sites within the *Jph2* 3′ untranslated region prevents regulation by miR-24 (38). Injection of either miR-24 or miR-34 antagomirs in preclinical mouse

models restores *Jph2* levels and attenuates cardiac dysfunction following cardiac stress (122, 124). These encouraging preclinical studies demonstrate that targeting the miRs that downregulate *Jph2* expression is theoretically possible and of translational value.

CONCLUDING REMARKS

After extensive research over the last 20 years, junctophilins are now widely acknowledged to play an essential role in mediating cellular Ca^{2+} homeostasis within the narrow interface that lies between the PM and ER/SR terminal cisternae. Junctophilins have gone from an obscure family of conserved molecules into a fascinating quartet of multipurpose proteins with physiologically relevant functions. It will be interesting to see how our knowledge of junctophilin function expands over the next 10–20 years, especially if the questions listed below are effectively addressed.

SUMMARY POINTS

1. Junctophilins organize plasma membrane endoplasmic reticulum/sarcoplasmic reticulum (ER/SR) junctional contacts involved in physiologically important processes in excitable and nonexcitable cells.
2. Junctophilins bind to plasma membrane phospholipids, membrane ion channels, ER/SR-localized ion channels, the ER/SR membrane, and nuclear DNA through their membrane occupation and recognition nexus (MORN), joining, divergent, transmembrane, and α-helical domains.
3. The essential requirement of *JPH2* in the heart has provided great insights into the overall function of the junctophilin gene family.
4. JP2 is a dual-function protein for maintaining junctional membrane complex integrity and E-C coupling at baseline and for regulating gene transcription and protecting against pathological remodeling under stress conditions.
5. Identification of disease-associated mutations has provided important insights into the tissue-specific functions of individual junctophilin genes.
6. Many unknowns remain regarding junctophilins, including fine details underlying their transcription and the purpose of their post-translational modifications.

FUTURE ISSUES

1. What is the function of the smaller, alternatively spliced junctophilin isoforms containing only the N-terminal MORN domains, and do they act separate from or interfere with the function of full-length junctophilin proteins?
2. Why do *JPH2* gene mutations associate with cardiac disease but not with skeletal muscle defects?
3. How do MORN domains exactly bind to or associate with the plasma membrane? Do membranous phospholipids compete with and/or modulate junctophilin interactions with other proteins?
4. Do specific junctophilin domain(s) determine the 12–15 nm physical gap distance of junctophilin-organized PM–ER/SR junctions?

5. What is the function of the C-terminal divergent region in the intact and cleaved junctophilin proteins?
6. Does JP3 or JP4 possess nuclear translocating and DNA-binding activity similar to JP1 and JP2 proteolytic fragments and, if so, can such fragments be leveraged as gene therapy agents?
7. How do the many endogenous post-translational modifications and interacting partners regulate junctophilin function?
8. What are the mechanisms underlying *JPH* gene transcription beyond the few that have been reported thus far?

DISCLOSURE STATEMENT

L.-S.S. is an inventor on a patent regarding the use of junctophilin-2 fragments for the treatment of heart failure and other disease (WO2017214296A1; US 11,351,270 B2). The other authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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