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REVIEW

Refining the molecular organization of the cardiac intercalated disc

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Abstract

This review presents an extensively integrated model of the cardiac intercalated disc (ID), a highly orchestrated structure that connects adjacent cardiomyocytes. Classically, three main structures are distinguished: gap junctions (GJs) metabolically and electrically connect cytoplasm of adjacent cardiomyocytes; adherens junctions (AJs) connect the actin cytoskeleton of adjacent cells; and desmosomes function as cell anchors and connect intermediate filaments. Furthermore, ion channels reside in the ID. Mutations in ID proteins have been associated with cardiac arrhythmias such as Brugada syndrome and arrhythmogenic cardiomyopathy. However, rather than being independent, all ID components work together intensively by multifunctional proteins such as ZO-1, Ankyrin G, and β-catenin, integrating mechanical and electrical functions. GJs form a plaque surrounded by the perinexus in which free connexons reside; the connexome integrates Na_V channels, the desmosome and GJs; and the area composita hosts AJs and desmosomes, also integrated as adhering junctions. Furthermore, the transitional junction connects sarcomeres to the plasma membrane. Lastly, this review integrates all these findings in comprehensible figures, illustrating the interdependencies of ID proteins.

Keywords Intercalated disc • Cardiac arrhythmia • Brugada syndrome • Arrhythmogenic cardiomyopathy • Wnt signaling

1. Introduction

The cardiac intercalated disc (ID) is a tightly regulated and complex structure joining together adjacent cardiomyocytes in the heart (*Figure 1*). It ensures fast propagation of the electrical signal that initiates contraction throughout the heart, and allows the cardiomyocytes to withstand the strong mechanical forces imposed by the beating of the heart (reviewed in e.g.[1]). It is because of the ID that cardiomyocytes collectively act as a functional syncytium, both electrically and mechanically. This makes the ID indispensable for the normal functioning of the heart. Moreover, the ID is closely connected to the cytoskeleton and plays a role in signaling cascades.

As opposed to the ID, the lateral membrane (LM) of cardiomyocytes has a different makeup. It hosts, among others, costamers and focal adhesions, linking sarcomeres to the extracellular matrix (ECM).[2] Although the ID and LM have several proteins in common, such as vinculin and α-actinin, and ion channels,[3,4] discussing the LM exceeds the scope of this review.

The classic definition of the ID includes three main structures: the desmosome, which functions as a cell anchor, the adherens junction (AJ), which provides cell strength, and the gap junction (GJ), which couples cells electrically and metabolically. AJs and desmosomes are tightly connected to the cytoskeleton (*Figure 1*). Furthermore, several proteins that are not involved in direct cell-cell contact reside in the ID, such as ion channels,[5] while some membrane areas do not carry proteins, mostly at the apex of a plasma membrane fold, where the transitional junction is located.[6]

Due to the major role of the ID, it is no surprise that mutations in ID proteins cause a range of diseases, including arrhythmogenic cardiomyopathy (AC; until recently known as arrhythmogenic right ventricular cardiomyopathy/dysplasia (ARVC/D)), Carvajal disease, Naxos disease and Brugada syndrome.[7]

The notion that the ID is one functional unit rather than a collection of individual structures has increasingly found its way into the common knowledge of ID researchers. Interacting ID components have been collectively renamed into adhering junction, area composita[8] or connexome,[9] which integrate electrical and mechanical functions.[10] Moreover, mutations in ID proteins and cardiac disease often lead to severe ID remodelling. Still, research articles have focused mainly on one component of the ID. An integrated approach concerning the interplay between all ID components was still lacking. This review aims to fill this gap.

Firstly, this review will discuss in detail the molecular composition of the ID and the interactions between its components. This includes both

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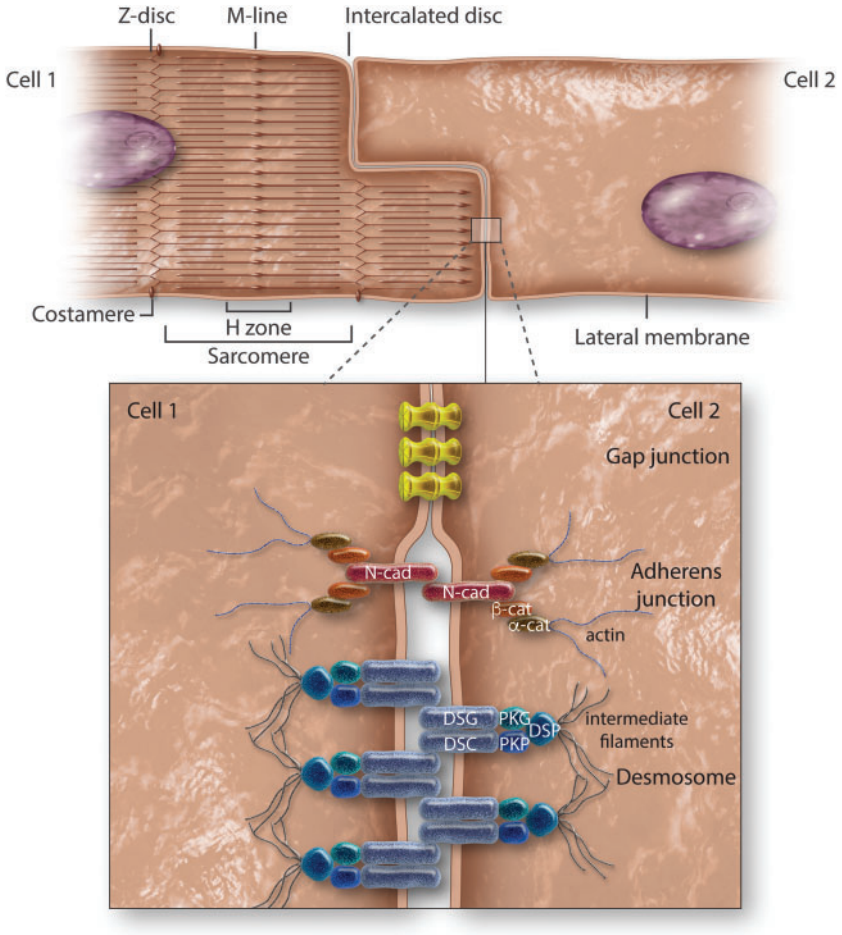

Figure 1 Old model of the cardiac intercalated disc, in which adherens junctions, desmosomes, and gap junctions are independent structures. Please note that these structures do not anchor the sarcomeres to the ID, although the steps of the ID correspond to the length of a sarcomere. At the lateral membrane, costameres ensure the anchoring of sarcomeric Z-discs to the extracellular matrix. Gap junctions bring the adjacent sarcolemmas close together, whereas desmosomes are visible at electron micrographs as relatively large electron-dense structures compared to adherens junctions.

its structural and its accessory proteins. Furthermore, mechanical and electrical insights will be integrated. Research in this field mainly concerns either mechanical or electrical changes caused by mutations in ID proteins as if these changes were independent; however, mutations in certain ID proteins can result in both mechanical and electrical changes. This indicates a cross-talk between mechanical and electrical players, possibly by common accessory proteins. Where possible, the relationship between mutations, the development of arrhythmias and ID remodelling will be made as well. Signaling pathways in which ID components are involved will be addressed. Finally, a comprehensive picture is given of the new insights in ID organization.

2. Adherens junction

Figure 2 illustrates the composition of the adherens junction (AJ), alternatively named fascia adherens. The AJ is the primary anchor for myofibrils and connects actin filaments from adjacent cells, which allows the cell to retain shape upon mechanical stress.[4] Furthermore, it transduces signals concerning the actin cytoskeleton and it senses mechanical forces on the cell. (reviewed in Ref. 3).

The transmembrane protein N-cadherin (N-Cad) is the main constituent of AJs. It homodimerizes with N-Cads from adjacent cells in the extracellular space, as an intercellular zipper. This provides tissue specificity during development, allowing cells to interact only with cells expressing the same cadherin. Calcium ions ensure the rod shape of N-Cad. The intracellular domain of N-Cad primarily binds β-cat.[1] N-Cad also possesses regulatory functions: see its role in mechanosensing later in this review.

β-catenin directly interacts with the C-terminal cytoplasmic domain of N-Cad. By associating with α-cat and Vcl, it connects AJs to the actin cytoskeleton. Also, β-cat plays a central role in cadherin-mediated signaling. (reviewed in Ref. 3) Moreover, β-cat can activate the canonical Wnt signaling pathway. It translocates to the nucleus when Wnt binds its Frizzled receptor, to initiate transcription of transcription factors of the TCF/LEF family. The canonical Wnt pathway is crucial in cardiac development but also has been proposed as the key mechanism in certain cardiomyopathies: activation induces cardiac hypertrophy. Therefore, N-Cad has been thought to sequester β-cat to prevent Wnt

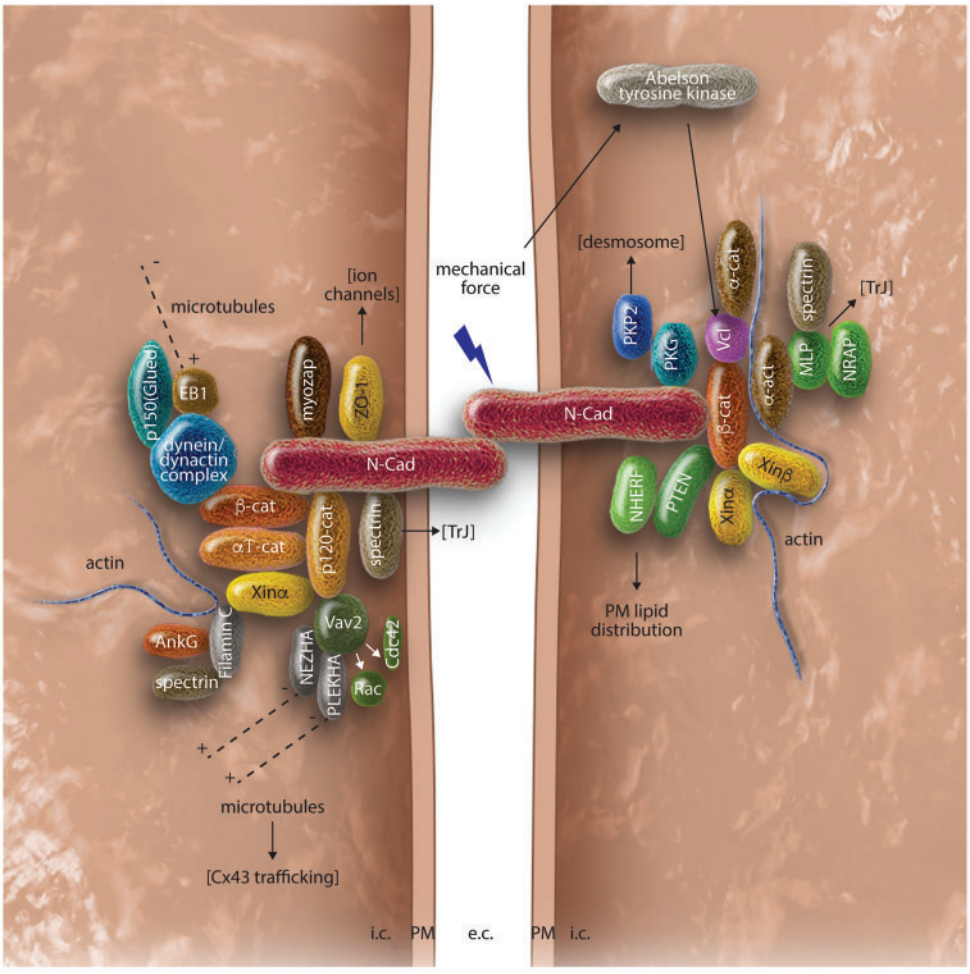

Figure 2 Structural and associated proteins of the adherens junction. Pathways are depicted with arrows. Connections to other ID components are shown in dotted lines and between brackets. Please note that exact interaction sites are not specified to prevent unnecessary complexity.

activation.[11] Interestingly, activation of the Wnt pathway increases expression of the GJ protein Cx43, and the C-terminus of Cx43 can interact with β-cat. Although increased Cx43 expression seems to contradict conduction slowing in cardiac disease, defective trafficking and the remodelling of GJs to the LM may explain this.[12] When Wnt is not present, cytoplasmic β-cat is targeted for degradation by the proteasome.[3,13,14] Moreover, instead of β-cat, α-actinin and plakoglobin (PKG)—alternatively named γ-catenin—can bridge N-Cad to actin.[15] Note that desmosomes also contain PKG, indicating interplay between AJ and desmosomes.[16] Interestingly, Garcia-Gras *et al.* proposed a model in which PKG, as a functional and structural β-cat homologue, can compete with β-cat at the APC/Axin/GST complex, which facilitates the degradation of cytoplasmic β-cat. The observation that DSP deficiency increases nuclear PKG concentration and the transcription of adipogenic genes underlines this notion.[17,18]

p120-catenin (p120-cat) binds the cytoplasmic domain of N-Cad close to the membrane. There, it regulates adhesion and cell shape by binding to the guanine nucleotide exchange factor Vav2, which activates the Rho family GTPases Rac1 and Cdc42.[3] Also, p120-cat binds to PLEKHA7 and Nezha, two proteins that mediate the binding of the minus end of microtubules, thereby ensuring the connection of AJ to microtubules.[15]

2.1 Associated proteins

Vinculin is spread evenly over the ID membrane and resides at the edge of the ID plaque proteins, in contrast to β-catenin that is found at the membrane.[6] Vcl links β-cat to actin via α-act, while β-cat primarily binds N-Cad. At the LM, Vcl links costameres to actin via integrins. Its crystal structure reveals a globular head, hinge region and a C-terminal flexible tail.[19] Vcl may however also insert into the plasma membrane in acidic phospholipid-rich areas where its tail associates with the acidic phospholipid PIP_2 (phosphatidylinositol-4,5-biphosphate), which recruits actin regulatory proteins. The tight interaction between Vcl and PIP_2 discloses a role of Vcl in pathways that are initiated by PI3Ks (class I phosphoinositide 3-kinases), a scaffolding adapter protein that phosphorylates PIP_2 and generates PIP_3, which traditionally leads to Akt phosphorylation. This pathway plays many roles, for instance in apoptosis and mechanosensing. Indeed, a small reduction in Vcl induces rapid cell death, although the pathway by which Vcl affects PI3K is unclear.[19]

Other important accessory AJ proteins are PTEN, spectrin and cortactin. PTEN (phosphatase and tensin homologue deleted on chromosome ten) associates with AJ-associated β-cat and with the sodium-proton exchanger regulatory factor NHERF. Hereby, PTEN contributes to the polarized distribution of lipids in the inner leaflet of

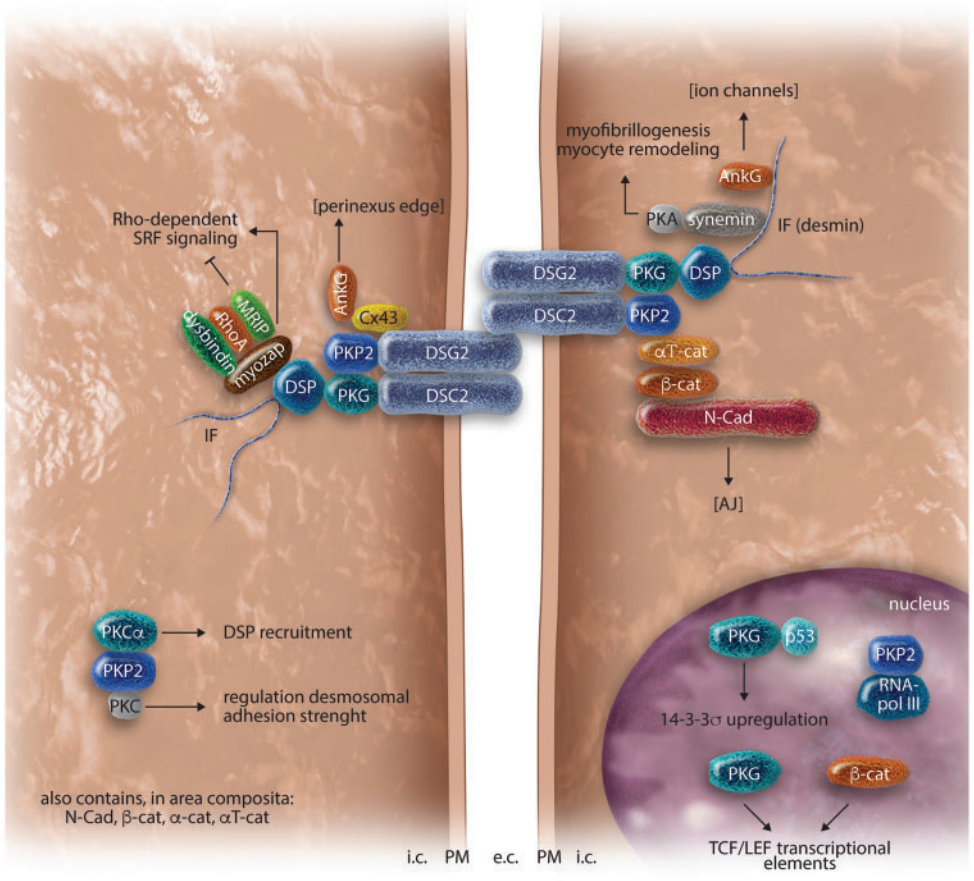

Figure 3 Structural and associated proteins of the desmosome. Interestingly, PKP2, β-cat, and PKG also play a role in the nucleus.

the plasma membrane.[20] Spectrin however binds N-Cad,[21] and its heterodimers form tetramers to bind actin filaments at their distal ends. At the ID, spectrin probably occurs in the most stable tetramer form $(\alpha II\beta II)_2$, whereas αII spectrin is found at the distal Z-discs of myofibrils, closest to the ID. Moreover, spectrin is predominantly found at the axial extremes of the ID, the transitional junction (see later chapter), whereas Vcl and β-catenin are spread more evenly over the ID surface.[6] Lastly, cortactin is an actin-binding protein that cooperates with N-Cad to regulate actin reorganization and adhesion strength. Its binding protein Arp2 (actin-related protein 2) ensures proper function, which, in turn, forms a complex with Arp3 and thereby regulates actin polymerization and the formation of actin branches.[22] hXinα and hXinβ are Xin proteins named after the Chinese word for heart, alternatively named cardiomyopathy-associated 1 resp. 3 (CMYA1 and -3). Their respective mouse orthologues mXinα and mXinβ have been studied extensively. They associate with AJs: the Xin repeats characteristically bind actin and they contain a β-cat binding domain, whereas mXinα also contains a p120-cat binding domain. Therefore, mXinα and mXinβ have been thought to form predominantly a stable link between AJs and actin. Also, mXinα interacts with filamin, the crosslinker protein of actin, and recruits p0071, a member of the p120-cat subfamily of armadillo proteins. Interactions between mXinα and p120-cat and p0071 might influence Vav2 and Ect2, regulators of the Rho family small GTPases Rac1 and Rho that play an important role in cardiac development. Lastly, GJ remodelling seen in mXinα deficient hearts might be explained by direct interactions of mXinα and ZO-1 and/or Cx43.[3,23] Interestingly, mXinβ is predominantly found in the left ventricle, interventricular septum and apex. Expression is especially strong at the base of the aorta and pulmonary artery, where the tissue undergoes relatively high stress. The distribution of mXinα is not known. Moreover, mXinβ deficient hearts lack mature IDs, suggesting that Xin proteins are involved in N-Cad-mediated signaling. Also, mXinβ is thought to function downstream of angiotensin II (AngII) signaling, modulating hypertrophic responses in disease (reviewed in Ref. 3).

Several proteins that are associated with AJs in other tissues, such as epithelia, are not yet investigated in the context of the heart. This might be an interesting venue for future research. For instance, Bitesize, a synaptotagmin-like protein, is involved in the stabilization of epithelial AJs via E-Cadherin.[24] Furthermore, the intercellular adhesion molecule ICAM-1 regulates N-Cad localization in endothelium through ERM (ezrin-radixin-moesin) proteins. Although ICAM-1 is expressed in the heart, its ID involvement is unclear.[25]

3. Desmosome

The desmosome, also known as macula adherens, is a robust, dense and symmetrical cell anchor that provides structural support to

cardiomyocytes and other tissues that are subjected to strong mechanical forces, such as epithelia. Its composition is illustrated in *Figure 3*. While AJs also transduce forces to the cytoskeleton, desmosomes are more robust, thanks to their connection to mechanically resilient IFs.[18] The intercellular part of the cardiac desmosome is built up by the cadherins desmoglein-2 (DSG2) and desmocollin-2 (DSC2), that bind in a heterologous way. DSG2 and DSC2 are, like other cadherins, single-pass transmembrane proteins. The armadillo proteins PKG and plakophilin-2 (PKP2), and desmoplakin (DSP), member of the plakin superfamily, connect desmin to the desmosome. The hyperadhesive state of the desmosome, when DSC2 and DSG2 are bound, depends on the presence of calcium ions. During wound healing and embryogenesis, desmosomes can adopt a lower-affinity state.[5] Also, desmosomal proteins can participate in signaling pathways, influencing the expression of genes involved in proliferation and differentiation, tissue morphogenesis and wound healing.[1]

Considering the major desmosomal proteins, firstly, plakophilin-2 is associated with GJs, (reviewed in Ref. 3) and is required for the organization of ID and desmosomal function (reviewed in Ref. 1). Together with PKG, PKP2 mediates attachment to IFs. PKP2 knockdown causes a decrease in conduction velocity and an increased propensity to develop re-entry arrhythmias,[5] while *PKP2* mutations are most common in hereditary AC. Moreover, like β-cat is a PKG homologue, p120-cat is a PKP2 homologue, indicating that PKP2 may also play a role in signaling.[3,5] Moreover, PKP2 has been associated with RNA polymerase III, suggesting a role in transcriptional regulation.[5,26] Secondly, plakoglobin is present in both desmosomes and AJs. As described in the AJ chapter, PKG plays an important role in Wnt signaling, indicating desmosome-to-nucleus crosstalk. Furthermore, nuclear PKG was shown to bind p53 and upregulate expression of the tumor suppressor 14-3-3σ by interacting with its promotor.[18] Thirdly, desmoplakin is characterized by its plakin domain: a globular head with many α-helices and multiple spectrin repeats that are separated by a Src-homology-3 domain. DSP connects the desmosomes to the type III IF protein desmin. It's a large protein: its N- and C-terminal domains and the α-helical domain in between are each almost 1000 amino acids long. Interaction with PKP2 occurs at their N-terminal domains.[1,5] Lastly, *DSG2* mutations are, like all other cardiac desmosomal proteins, associated with AC. In other tissues, knockout of DSG2 is often compensated by higher expression of DSG1. Although desmosomal integrity is thus restored, cellular signaling is significantly altered since different subtypes of DSG are involved in different pathways. However, since DSG2 is the only cardiac DSG, the lack of compensatory options may explain the AC phenotype.[18]

3.1 Associated proteins

Protein kinase Cα (PKCα) can bind PKP2. PKCα is thought to hereby regulate desmosome adhesion when dynamic cell-cell adhesion is required.[20,27] Moreover, PKCα recruits and phosphorylates DSP during desmosome genesis.[5] αT-catenin (αT-cat) is a member of the α-cat family that interacts with PKP2 and β-cat, thereby linking the desmosome and AJ. αT-catenin plays therefore an important role in the area composita, which is the junction in which desmosomes and AJs are tightly linked by sharing structural proteins (see later chapter).[8,28] Possibly, 120-catenin is involved in cardiac desmosomes as well, because it is an interaction partner of DSG1 and DSG3, linking AJs to desmosomes.[3,29] However, DSG1 and DSG3 are not present in cardiac desmosomes, and this interaction only occurs in high calcium concentrations. It is therefore questionable if p120-cat can also bind the cardiac variant DSG2.

3.2 Intermediate filaments

Several IF proteins reside in the heart, although desmin is the most prevalent, as a constituent of the sarcomeric Z line. Impairment of the PKG-desmin interaction in transgenic mice impairs delivery of Cx43, DSP, DSC, N-Cad and PKG to the ID, followed by conduction slowing.[1] Furthermore, synemin is clearly located at the ID and binds to protein kinase A (PKA), thereby regulating myofibrillogenesis and myocyte remodelling, although synemin is predominantly expressed during heart disease.[30] It is suspected to interact with desmin.[31] Please note that desmosome-associated desmin is not directly associated to Z-discs, since mature Z-discs are absent at the ID. Immature Z-discs, in turn, are connected to the transitional junction region of the ID (see chapter 6.3).

3.3 Adhesion junction–bringing the desmosome and adherens junction together

The classical definition of the desmosome is based on electron microscopy images that reveal the inner dense plaque (IDP), outer dense plaque (ODP) and electron dense midline (EDM) or membrane core. The EDM comprises of the interacting domains of DSG and DSC. The ODP contains the transmembrane proteins DSG, DSC and intracellular PKG and PKP. Furthermore, to the ODP, the intermediate filaments (IFs) are connected.[18] However, this a rather old-fashioned model. Rather, the AJ and desmosomes together are named 'adhesion junctions' or 'adhering junctions' because cadherins make up the intercellular contact proteins of both junctions, and AJs and desmosomes both connect cells to the cytoskeleton of adjacent cells, albeit through different cytoskeletal proteins.[13,32] PKG is thought to be the most important factor in the adhesion junction, since it is a structural element of both AJs and desmosomes.[16] Also, p120-cat is a common constituent,[3] and PKP2 interacts with multiple AJ proteins.[33]

Observations in PKG-deficient mice give another clear example of AJ and desmosome interdependency: they lack desmosomes and instead have extended AJs with desmosomal proteins. Their hearts have increased right ventricular volume and spontaneous right ventricular ectopic activity.[1] Furthermore, DSP-deficient mice have normal GJs and AJs but lack desmosomes, indicating that DSP is essential for desmosome function and development, proposedly because DSP is essential for desmin binding. DSP also regulates actin assembly and organization via RhoA signaling.[1,5] However, the concept of adhesion junction can be extended to include GJs and ion channels, in which case it is called area composita, which will be discussed in detail later.

4. Gap junction

The gap junction (GJ) is an agglomeration of multiple individual gap junction channels with associated proteins (*Figure 4*). The GJ enables electrometabolic coupling, allowing propagation of action potentials and passage of small solutes between cells.[1] In general, GJs are composed of twelve connexin molecules: six molecules form a connexon in the membrane and connect with a connexon of the opposing membrane.[1] In ventricular myocytes, connexin43 (Cx43) is the most prevalent. GJ development, regulation and degradation seems to be a highly orchestrated process with a fast turnover[1,2]: the half-life of Cx43 is as short as 1–3 h.[34]

Cx43 also has noncanonical roles, independent of connexons and GJs. It is necessary for sodium channel function for instance (see later

chapter), and loss of the GJ-independent functions of Cx43 causes severe arrhythmias and death. (reviewed in Ref. 9) Interestingly, Cx43 is also found in mitochondria, while ischaemic preconditioning increases mitochondrial Cx43 localization.[35] Moreover, GJs can have direct physical contact with mitochondria.[36] However, the functional consequences of these phenomena are still unknown. Moreover, GJs between myocytes and fibroblasts also exist, although they are very hard to find. A fibroblast-specific small GJ subtype that is normally found between fibroblasts may also occur in IDs. This field is however still in its infancy (reviewed in Ref. 37).

4.1 Associated proteins

Mena (mammalian Enabled) is a member of the Ena/VASP (Enabled/vasodilator-stimulated phosphoprotein) family, which interacts with Cx43 and Vcl via its EVH1 domain. It regulates the actin cytoskeleton and microfilaments, both at the ID as well as at focal adhesions. Mena and VASP also exert actin polymerase and anti-capping activities and thereby increase actin filament assembly.[38] Mena is proposedly an important cardioprotective protein: by binding Rac1, it prevents the activation of Rac1-associated pathways. In fact, Mena knockout cardiomyocytes show an increase in Rac1 activity, leading to GJ remodelling, cardiomyocyte apoptosis, cardiac dysfunction and ID destabilization, while Cx43 expression is increased.[39]

Caveolin-1 (Cav-1) colocalizes and co-immunoprecipitates with Cx43. Caveolins are cholesterol-binding integral membrane proteins that do not span the membrane. Together with cholesterol and sphingolipids, they are important constituents of caveolae. Caveolae are the best characterized type of lipid rafts and recognizable as flask-shaped invaginations of the plasma membrane of 60–80 nm with a 10–50 nm neck.[3] Furthermore, caveolae are involved in endocytosis, transcytosis and outside-in signaling.[40,41] The Cx43-Cav-1 interaction implies a relation between GJs and caveolae.[40] The experimental evidence for Cav localization at the ID is however thin. Undoubtedly, caveolins and caveolae play a role at the ID, but the exact role remains to be determined.

4.2 Linking adhering/adhesion junctions to gap junctions

In development, GJs only appear at the ID when the adhering junctions are already formed, or in other words, mechanical coupling precedes electrical coupling.[2,13,42] GJ stability depends on the 'strong state' of AJs.[19,20] This shows that mechanical and electrical coupling of cardiomyocytes are tightly linked. Cx43 connexons are transported in vesicles

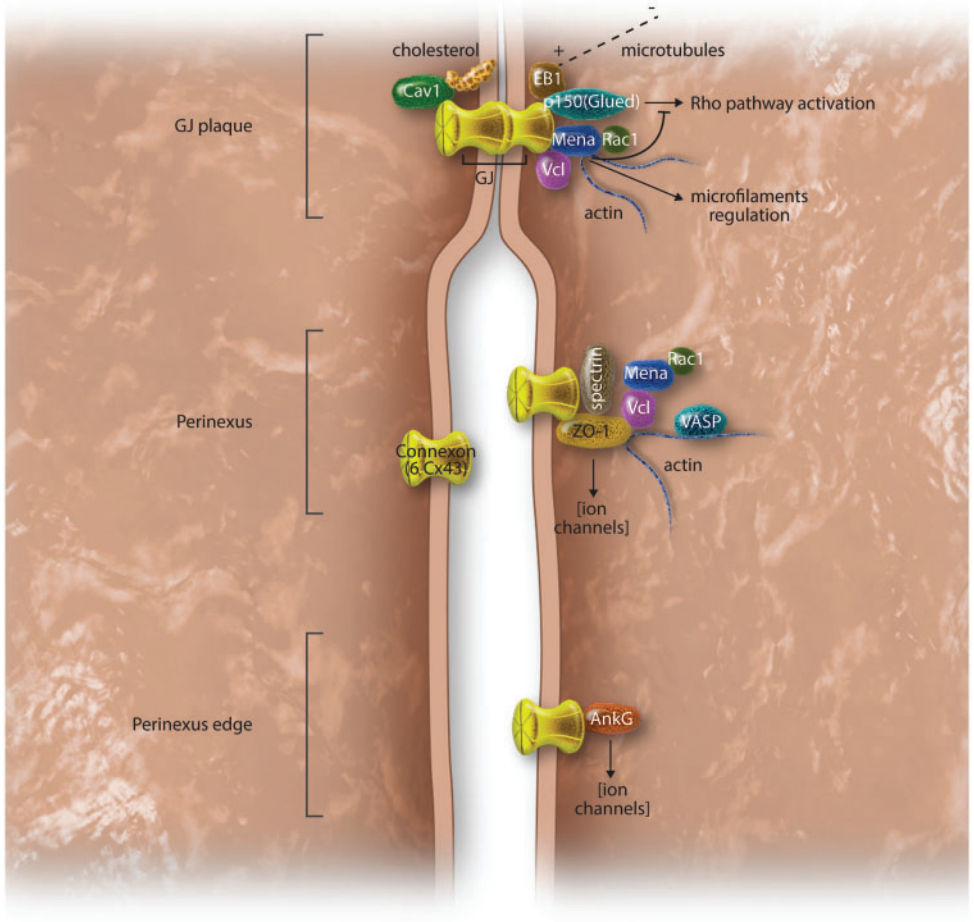

Figure 4 Structural and associated proteins of the gap junction. Please note that connexin43 binds different partners at the gap junction plaque, perinexus and perinexus edge.

from the Golgi system to the ID via microtubules that are tethered to AJs via N-Cad, β-cat and the dynein/dynactin complex. Hereby, Cx43 interacts with the +TIP EB1 and p150(Glued). Although EB1-capped microtubuli can also bind mature GJ plaques,[43] N-Cad-knockout mice are not able to form GJs, and blocking the intercellular binding of N-Cad inhibits GJ formation. Interestingly, this connexon delivery system assures that GJs only form between homologous cells, since cadherins only connect to the homologous cadherins.[43] When adhesion junctions function improperly, for instance due to PKP2 deficiency, GJs remodel: Cx43 lateralizes and its signal at the ID decreases. This may be an arrhythmogenic substrate.[1,13] Furthermore, GJ remodelling due to Xin deficiency is thought to occur through interaction with ZO-1 and/or Cx43. (reviewed in Ref. 3).

Since GJs are sensitive to mechanical stress, they locate in the vicinity of AJs and desmosomes. (reviewed in Ref. 2), while PKP2 and Cx43 also co-immunoprecipitate.[13] Desmosomal mutations that cause AC are associated with loss of GJ plaques at the ID, which illustrates the importance of this organization. However, a complete loss of PKP2 only causes a 50% reduction in functional GJs, which surprisingly does not affect conduction velocity.[9,44] Also in Carvajal and Naxos disease, the decrease of Cx43 at the ID is obvious. Conversely, it is disputed whether Cx43 is essential for AJ or desmosome functioning. In cell lines, loss of Cx43 expression preceded a loss of intercellular adhesion strength,[9] whereas other reports show the opposite.[1,2] Importantly, Cx43 expression is aberrant in several cardiac diseases: relocalization of dephosphorylated, unfunctional Cx43 to the LM is seen in atrial fibrillation (AF), overloaded human atria, and in CMC of a rat myocardial infarction (MI) model. This notably impairs ID coupling and may lead to conduction block.[45] In an aged mouse model, Cx43 expression was decreased, many IDs were disrupted, and the intercellular space was widened at AJ regions.[46] The phenomenon of ID remodelling is however, although interesting, too complex to be covered in this review as well; therefore, we refer to other reviews.[1,47] Interestingly, delocalized connexons are not phosphorylated, contrary to their ID counterparts, indicating that phosphorylation is important for the regulation of GJ function.[9] Lateralized connexons may however still be functional in action potential propagation.[48] However, it is clear that GJs are a vulnerable structure, which is illustrated by the observation that during cardiac disease, GJs are the first structures to delocalize from the ID. A cardiac specific N-Cad knockout mouse model showed for instance reduced levels of Cx43, which lead to a reduced conduction velocity and ventricular arrhythmias, although also α-, β- and p120-cat were reduced. This indicates that N-Cad and Cx43 are tightly related, probably via ZO-1.[1] CamKII-mediated signaling is probably involved in this Cx43 lateralization, illustrated by the observation that CaMKII inhibition enhances conduction and Cx43 localization at the ID at baseline, and preserves this localization under conditions of heart failure.[49]

The MAGUK ZO-1 is thought to connect AJs to GJs by means of its scaffolding properties. ZO-1 consist of three domains, the non-functional GUK domain, the SH3 domain that interacts with proline-rich PXXP sequences, and the PDZ domain. This PDZ domain interacts with N-Cad, which stabilizes both AJs and GJs, and with Cx43. Also, ZO-1 anchors actin to the cytoskeleton. Generally, MAGUKs localize close to the plasma membrane to regulate surface expression of certain specialized domains in a variety of tissues.[19,20] ZO-1 can also connect AJs to GJs by interacting with α-cat via its N-terminal fragment, and with actin via its C terminus (reviewed in Ref. 3). Furthermore, afadin might link actin and N-Cad in this interaction chain, since afadin bridges actin to E-cad in epithelia.[50] However, although afadin is present in cardiomyocytes,[51] we do not know its cardiac function.

In cardiomyocytes and cell lines without ZO-1, normal organization of Cx43 and N-Cad was lost, and GJs lateralized. This lateralization is thought to occur through microtubule-dependent mechanisms.[48] The effect of ZO-1 on GJ localization occurs however via the interaction between ZO-1 and AJ. This interaction ensures the strong state of the AJ, on which the GJ depends.[52] In cardiac disease, the function of ZO-1 in relation to AJs and GJs can change. In healthy hearts, ZO-1 colocalizes to a greater extent with N-Cad than with Cx43. In ventricles from patients with heart failure however, ZO-1 expression is increased, and Cx43 and ZO-1 colocalize stronger, which could imply that ZO-1 prevents the increase in functional GJs.[53] (reviewed in Ref. 3) Lastly, ZO-1 also links AJs to K_v1.5 ion channels, and interacts with PKG, APC (adenomatous polyposis coli), an important player in the Wnt pathway, and claudin, a protein generally associated with tight junctions, while its role at the ID is unknown.[19,20,54]

As described before, vinculin anchors actin filaments to the AJ via β-cat. Its head domain also interacts with the third PDZ domain of ZO-1, thereby stabilizing GJs, either directly or via α-cat. The notion that Cx43, ZO-1, and Vcl form a complex implies that Vcl is an important anchoring point between AJs and GJs. Indeed, GJs were destabilized when the interaction between Vcl and ZO-1 was impaired, and GJ function and even cardiomyocyte integrity was lost. Loss of GJ function was demonstrated in a Vcl-deficient cell line. Moreover, in a Vcl-deficient mouse model, Cx43 and ZO-1 signals were decreased at the ID. The hearts showed excessive replacement fibrosis and cardiomyocyte loss, which could be attributed to PI3K signaling. Additionally, Vcl plays an important role in the assembly and alignment of myofibrils and in regulation of cell shape.[19]

Muscle LIM protein (MLP) may also play a role at the intersection of GJ and AJ, while MLP mutations are a suggested cause of dilated cardiomyopathy (DCM). MLP is thought to form a ternary complex with the nebulin-related anchoring protein (NRAP) and α-actinin,[55] and also interacts with actin and spectrin. In DCM patients, *MLP* mutations inhibit the interactions with NRAP and α-actinin, and compromise its zinc finger domain. In MLP knockout mice, the expression of N-Cad, β-cat, PKG, α-catenin, Vcl and NRAP is upregulated, while Cx43 was downregulated. Furthermore, Vcl distribution at the ID was disturbed,[56] which might point to a structural disruption of the transitional junction, where sarcomeres are attached to the sarcolemma.[55]

4.3 Perinexus

The perinexus is a newly identified functional region in the ID (*Figure 5*), in which ZO-1 plays a crucial role.[34,57] The perinexus is defined as the area around the plaque of functional GJs, in which free connexons interact with ZO-1 via its PDZ-2 domain. Here, ZO-1 regulates size, number and localization of GJs: (reviewed in Ref. 3) first, new connexons appear at the periphery of the GJ aggregate, after which ZO-1 binds. Connexons are unable to associate with connexons of neighboring cells as long as ZO-1 is bound. Two ZO-1-free connexons are able to form a GJ, thereby increasing intercellular communication.[34] Indeed, in a ZO-1 knockout model, GJ plaques were larger.[52] Lastly, ZO-1 is thought to mediate Cx43 delivery from lipid raft domains to GJs in the ID.[19] Ankyrin G (AnkG) is also found at the perinexus; it binds the outer connexons and thereby probably determines the perinexus size, and the traffic between perinexus and GJ plaque (see *Figure 5*).[9] Concomitantly, the Cx43 plaque size and Cx43-mediated electrical coupling are inversely related.[58] Also, in super-resolution fluorescence microscopy, PKP2 is

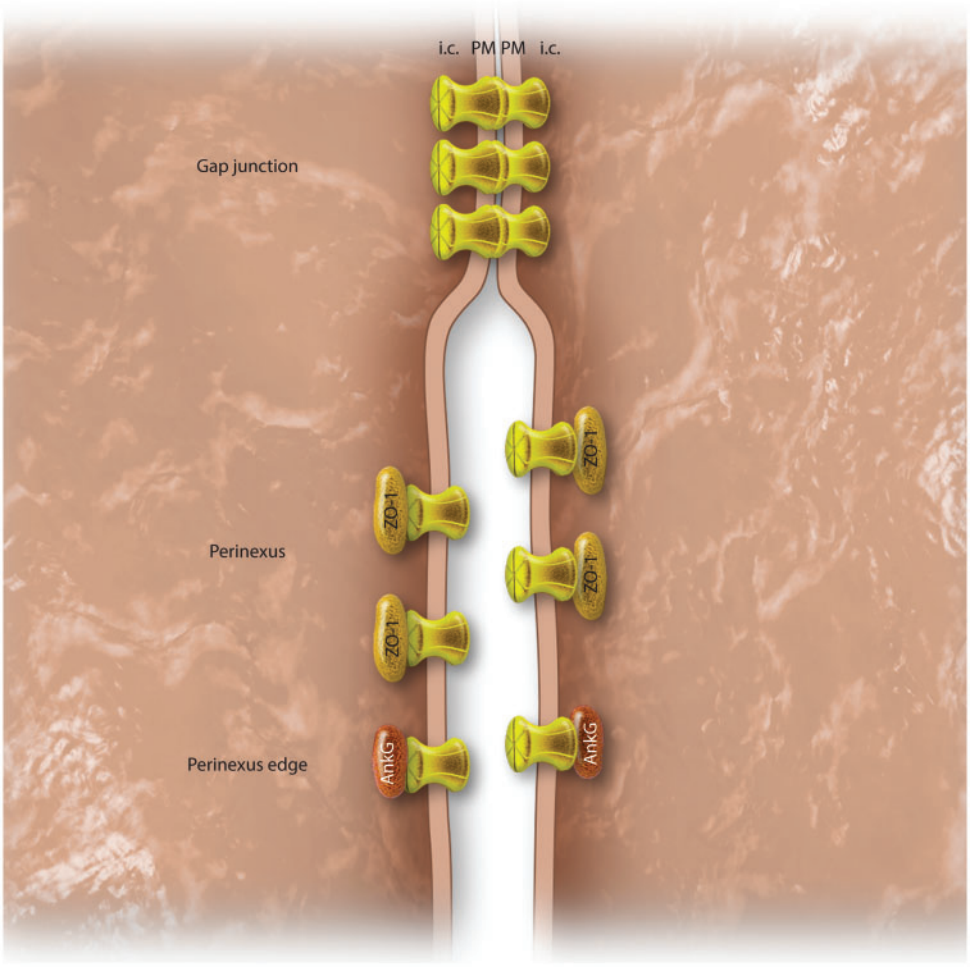

Figure 5 Model of the perinexus, in which ZO-1 binds connexons around the gap junction plaque. AnkG binding determines the perinexus edge.

seen at the edge of the perinexus.[9] AnkG is thought to scaffold the interaction between PKP2 and Cx43. When AnkG was silenced by means of siRNA interference, the GJ-PKP2 interacting areas increased in size, as did the GJ plaques, in accordance with the perinexus model as described before.[9] Other AnkG silencing effects include decreased reduced electrical coupling and intercellular adhesion strength in cardiomyocytes.

5. Ion channels

Ion channels in the sarcolemma are critical for the creation and propagation of action potentials throughout the heart and for excitation-contraction coupling. They are found at three main membrane areas of the cardiomyocyte: at the ID, in the LM, and in t-tubuli. The composition of ion channels is different in each area.[2,40] Ion channels at the ID (*Figure 6*) can be classified in three main categories: K_V channels (K_V1.4, –1.5, –4.2, –7.1, and 11.1), Kir channels (Kir2.1, –2.2, –2.3, –6.2, and SUR2a) and Na_V channels (Na_v1.5 and –1.3). Moreover, the stretch-activated channels TRPV2 and TREK-1 are expressed at the ID, which will be discussed in the mechanosensing section. Lastly, Na^+/K^+-ATPase is present at the ID.[2,59]

5.1 Potassium channels

Potassium channels control the resting membrane potential and the repolarization phase of the action potential. Although these channels are known to reside in the well-studied ID for a long time, their interaction partners remain underexposed.[60]

K_V channels are voltage-gated. Its α-subunits make up the actual channel, whereas accessory β-subunits ensure proper K_V function. K_V1.x channels, members of the shaker family, are essential for the repolarizing currents. K_V1.4 for instance underlies the rapidly activating, slowly inactivating 'slow transient' current, whereas K_V1.5 is responsible for a rapidly activating, very slowly inactivating current.[40,60] On the other hand, the Kir (inward rectifying) channels in the heart are ATP-dependent and generally regulate the action potential duration when heart rates rise. Furthermore, they have a stress-protective role. Kir2.x channels facilitate the primary inward-rectifying currents, also regulating the resting membrane potential.[40,60] Lastly, Kir6.2 ion channels are supported by SUR2A subunits, and the Na^+/K^+ pump interacts with K_{ATP} channels in the heart.[61]

5.1.1 Associated proteins

The actin cytoskeleton appears to associate with α- and β-subunits of K_V channels. The actin cytoskeleton probably determines ion channel

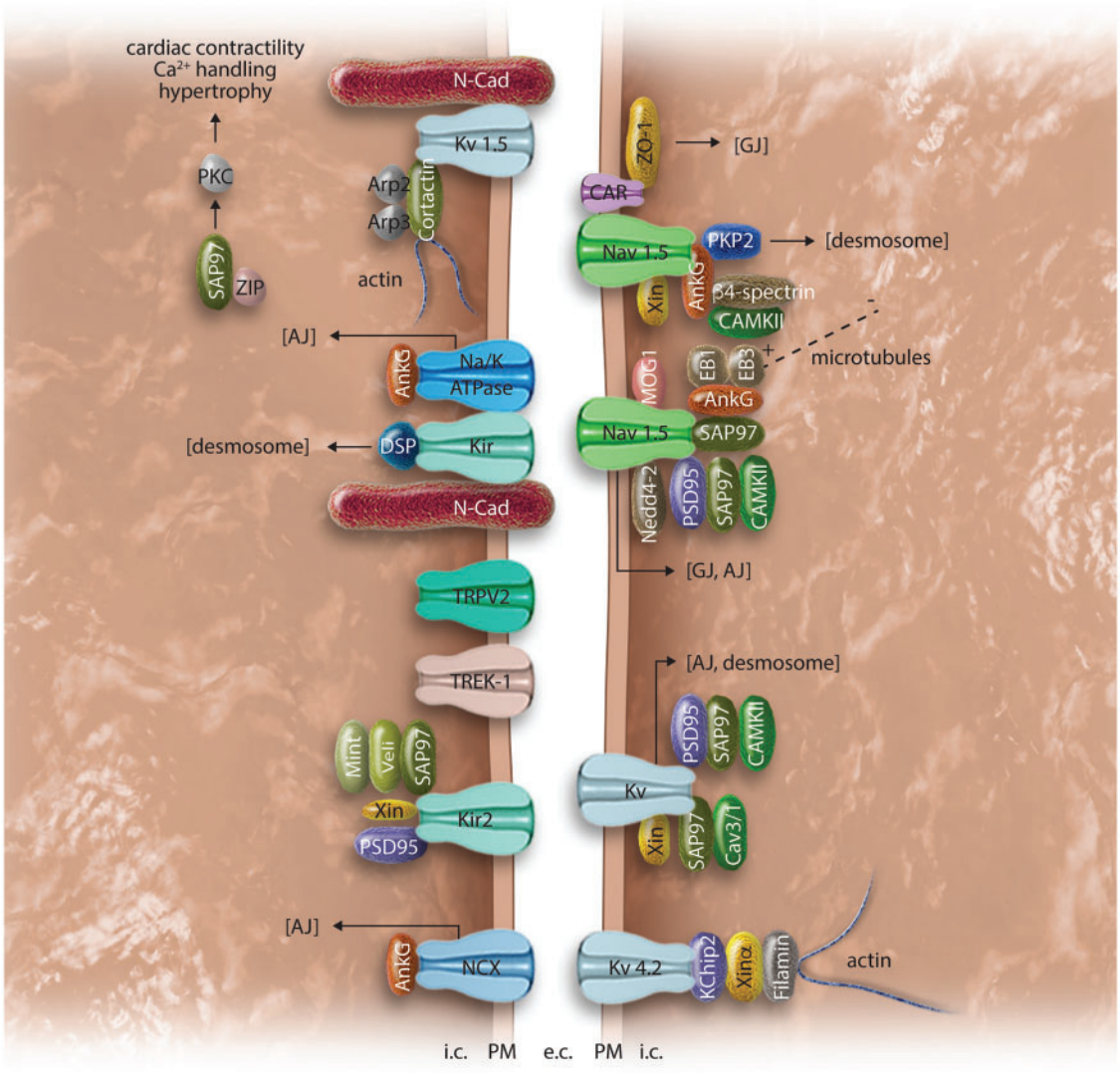

Figure 6 This model sums up the large variety of ion channels found at the ID, of which Nav1.5 is the most important, and their interaction partners for as far these are known.

stability and surface distribution, and has been associated with $K_V1.5$ channel endocytosis. Cortactin thereby links between actin to $K_V1.5$.[60] Moreover, PSD-95–post-synaptic density protein 95, alternatively named SAP90–is a member of the MAGUK family and associates with $K_V1.4$, $K_V1.5$, and Kir2.x, supposedly to ensure localization to lipid rafts. SAP97 is also present in lipid rafts to scaffold the interaction between $K_V1.5$ and its cytosolic subunit $K_V\beta$. Furthermore, SAP97 interacts with PDCζ-interacting protein (ZIP) to recruit PKC, which is involved in a variety of pathways, including the regulation of cardiac contractility, Ca^{2+} handling and hypertrophic response.[40,62] $K_V1.5$ and $Na_V1.5$ are also regulated by type-II Ca^{2+} activated calmodulin kinase (CAMKII), when it is bound to SAP97 and PSD95.[40] mXinα, the mouse homologue of hXinα that has been discussed in the AJ chapter, also regulates ion channels. mXinα-deficient mice presented with a prolonged AP due to delayed inward-rectifier K^+ currents. This, together with changes Ca^{2+} metabolism, increased the risk for arrhythmias. mXinα was shown to regulate the transient outward potassium (I_{To}) current by interacting with the $K_V4.2$ auxiliary subunit KChIP2 (K_V channel interacting protein 2) and the actin-crosslinking protein filamin.[63] However, the conclusions regarding conduction slowing and arrhythmias may be confounded since expression of Cx43 and N-Cad is also reduced in mXinα deficient mice (reviewed in Ref. 3). Because mXinα and mXinβ can interact with ion channels, actin, β-cat and p120-cat, these proteins may scaffold N-Cad-mediated adhesion and surface expression of ion channels, of which a recent study reported.[63] In fact, the role of mXinα in regulating function and expression of ion channels has been thought to be analogous to that of AnkG (reviewed in Ref. 3).

5.2 Sodium channels

Whereas both $Na_V1.3$ as $Na_V1.5$ localize at the ID,[2] most is known about $Na_V1.5$, while interaction partners of $Na_V1.3$ are still unknown. Furthermore, it is noteworthy that the β-subunits of Na_V channels are of unknown composition and function–although they participate in cell–cell adhesion in other cell systems than the heart.[5] The functional

complex of α- and β-subunits is called VGSC (voltage-gated sodium channel complex).

The cardiac voltage-gated sodium channel $Na_V1.5$ is responsible for the rapid upstroke of the cardiac action potential. It consists of one α-subunit of 220 kDa that forms the functional channel and β-subunits of 30 kDa each. Its essential function is illustrated by many pathologies associated with mutations in its gene *SCN5A*, such as Brugada syndrome, long-QT syndrome and familial ventricular fibrillation.[64] However, interestingly, the channel can function differently at different locations in the cells. At the ID, $Na_V1.5$ is more dependent on voltage to reach its steady-state inactivation than at the LM.[65,66]

Interestingly, $Na_V1.5$ is suggested to be enriched in the perinexus. AnkG might be a mediator between $Na_v1.5$ and Cx43.[57] The idea has emerged that GJs are not the only mediators of intercellular action potential propagation. At the perinexus, $Na_V1.5$ is potentially involved in ephaptic coupling. This term is derived from the neuronal synapse and is defined as cell-to-cell transfer of electrical activation via ion accumulation/depletion or transient extracellular electric fields within a confined interstitial space between closely apposed cells.[67] Interstitial oedema, which widens the cleft, would reduce conduction velocity, contrary to more common belief that interstitial oedema would increase conduction velocity due to a reduction in resistance. Indeed, inhibiting sodium channels with flecainide slowed conduction, but even more in an interstitial oedema model, which findings moreover follow the outcomes of a mathematical model.[67] Therefore, the interstitial volume could be an important determinant in arrhythmogenesis.[57] However, this mathematical model assumed all Nav1.5 at the perinexus, which is contrary to more recent findings which also describe Nav1.5 associated with N-Cad at excitation-excitability nodes.[68] A mathematical model considering these two pools of Nav1.5 is still awaited.

5.2.1 Associated proteins

$Na_V1.5$ has many cytoplasmic interacting proteins. Via its PDZ-domain-binding motif consisting of its three distal amino acids SIV, for instance, it binds the syntrophin-dystrophin complex. However, this interaction only occurs at the lateral sarcolemma. At the ID, SAP97 can bind the SIV motif of $Na_V1.5$, which implies a regulatory relation between the two.[64] SAP97 interacts also with Kir2.x, $K_V4.x$, and $K_V1.5$. Silencing of SAP97 has elucidated its essential role as an enhancer of the number of functional ion channels and potassium currents.[69] The underlying mechanism is however not yet understood. It is thought that SAP97 stabilizes the ion channel. Furthermore, SAP97 has the propensity to multimerize, and this SAP97 network may function as a scaffold for ion channels and accessory proteins. Regulation of multimerization is exerted by changing from its open to its closed state, the latter exposing protein binding sites.Regulation of multimerization is exerted by changing from its open to its closed state, the latter exposing protein binding sites (reviewed in Ref. 2).

In addition, MOG1 increases $Na_V1.5$ transport and thereby sodium-current density in ventricular myocytes,[70] and it interacts with Nav1.5 at the linker sequence between intramembrane region II and III. Immunostainings show that MOG1 mainly localizes at the ID[71]; however, these results require cautious interpretation, since a proper control for these stainings are missing. Thirdly, the α-subunit of the stimulatory heterotrimeric G-protein $G\alpha_s$ promotes the localization of $Na_V1.5$ at the sarcolemma. Hereby, β-adrenergic pathways indirectly control the number of $Na_V1.5$ channels and its current density.[40,72] Furthermore, Nedd4-2 (neural precursor cell expressed, developmentally down-regulated 4-2) is an E3 ubiquitin-protein ligase that binds $Na_V1.5$. This interaction promotes internalization of the channel, effectively decreasing the sodium current. Nedd4-2 is therefore an important player in the life cycle of $Na_V1.5$.[64] CAR (coxsackie and adenovirus receptor) is a single-pass transmembrane protein that interacts with Nav1.5 at the ID. CAR heterozygous myocytes showed reduced sodium current magnitude at the ID, which is associated with ventricular conduction slowing and earlier onset of ventricular arrhythmias during myocardial infarction.[73]

The most notable interaction partner for Nav1.5 however is Ankyrin G, whose members predominantly anchor ion transporters. AnkG resides at the ID as well as in at the LM (reviewed in Ref. 2). At the ID, AnkG primarily binds and regulates $Na_V1.5$.[74,75] Interaction partners of AnkG are the +TIPs EB1 and EB3, which link AnkG to microtubules. Knockdown of these proteins leads to a decrease of AnkG and $Na_V1.5$ channels.[65] AnkG also plays a role in the regulation of the $Na^{\pm}/K^{\pm}$-ATPase and the Na^{2+}/Ca^{2+} exchanger NCX1.[76] Furthermore, AnkG forms a 'signaling platform', linking CaMKIIδ to $Na_V1.5$.[75]

Interestingly, the spectrin network at the ID resembles the spectrin network in erythrocytes. Firstly, while AnkG anchors spectrin-actin complexes to the lipid bilayer and controls localization of ion channels, the cytoskeletal adaptor protein 4.1R does the same in the erythrocytes. However, not 4.1R but its family member 4.1N is present at the ID. Interestingly, Bennet *et al.* also found the anion exchanger Band 3 at the ID, which stabilizes the interaction between actin and spectrin in erythrocytes. However, no other source confirms the presence of this protein.[6,74]

6. Integrating ID components

Over the last years, the idea that the ID components AJs, GJs, desmosomes and ion channels are individual structures has been challenged. Components appear to work together and share many accessory proteins. This section aims to give a complete overview of the associations and interdependencies of the ID components AJ, GJ, desmosome and ion channels, and their related proteins. Furthermore, this chapter covers the transitional junction, junction-free stretches of the ID membrane as an anchor point of sarcomeres.

6.1 Area composita

6.1.1 Linking AJ to desmosome

The AJ and desmosome together are also named area composita, which definition has been expanded to include ion channels and GJs as well, to distinct the term from adhesion junction. Less common names for the area composita include hybrid adherens junction and composite junction.[77] The term area composita initially arose on the notion that in electron microscopy, AJs and desmosomes were detected as one electron-dense structure.[5] AJs contain desmosomal cadherins and cytoplasmic plaque proteins, whereas desmosomes also contain N-Cad, β-cat, and α-cat. An early analysis of the composition of the area composita listed the following cardiac proteins: cadherins, DSP, α- and β-cat, Vcl, p120-cat and ZO-1, and afadin, of which cardiac localization is still speculative.[51] Interestingly, mice hearts lose their regenerative capacity at 1 week of gestation, which exactly corresponds to the development of the area composita.[3] Moreover, in a ewe heart failure model, N-Cad/catenin complexes, desmin, and Cx43 were all upregulated, which suggests that HF affects the entire area composita. A 3D-reconstruction of the HF IDs revealed that both plicate and interplicate domains are more extended in

HF than in controls, more intercellular vacuoles are seen, and GJs are reduced in plicate regions.[78]

The important role of catenins in the area composita is illustrated by mutations in *CTNNA3* encoding αT-cat: these perturb the assembly and function of AJs and desmosomes. Interestingly, αT-cat is only found in desmosomes that are part of areae compositae.[8] Furthermore, the recently described protein myozap (myocardium-enriched zona occludens-1-associated protein) binds and colocalizes with β-cat, N-Cad, PKP2, DSP, and ZO-1.[11] It is involved in Rho-dependent SRF (serum response factor) signaling, which is antagonized by MRIP (myosin phosphatase-RhoA interacting protein), a binding partner of myozap. SRF-dependent genes include natriuretic peptides and c-fos.[79] Dysbindin is a binding partner of myozap that can also interact with RhoA.[11] The notion that myozap also influences the AJ is strengthened by the fact that myozap contains an ezrin, radixin, and moesin (ERM)-like domain, since ERM proteins are known to influence the function of AJs via binding and regulating the actin cytoskeleton.[79] In certain other tissues, ezrin, radixin, and moesin connect the actin cytoskeleton to the plasma membrane and regulate actin-related cell-cell junctions such as AJs.[24] However, ERM proteins have not yet been studied specifically in cardiomyocytes.

6.1.2 Linking GJ to ion channels

Later, the GJ and ion channels were included in the area composita model as well, although GJs are not continuous with the AJ and desmosome plaque.[32] Multifunctional proteins like AnkG, ZO-1 and SAP97, however, connect all four components, directly or indirectly (reviewed in Ref. 9). This implies that the ID is actually one functional unit in which all components depend on each other to ensure intercellular mechanical strength and communication. Furthermore, cell–cell junctions, such the ID, are proposed to form a 'protein-binding hotspot' to which modulators of gene transcription are sequestered, while also regulating contact inhibition. The observation that ZO-1 is found in the nucleus of non-contacted cells, but at the ID of contacted cells, and the notion that N-Cad sequesters β-cat, illustrate this.[20]

Considering the major multifunctional proteins, firstly, AnkG interacts with GJs, AJs and the VGSC complex. However, mutations in *ANK3* encoding AnkG seem to play a minor role in cardiac disease, although Brugada syndrome may be caused by mutations in the $Na_V1.5$ domain that interacts with AnkG (reviewed in Ref. 2). Brugada syndrome is a familial channelopathy characterized by life-threatening ventricular arrhythmias and a high risk on sudden cardiac death, but without structural cardiomyopathy.[80] Secondly, the PKG homologue β-cat is an important linker between AJs, GJs, and desmosomes. Its role in AJs and signaling pathways and interaction with Cx43 have been discussed previously. β-cat is however not essential for mechanical junction integrity, probably because PKG can compensate for β-cat deficiency. Conversely, PKG deficiency cannot be compensated by β-cat despite β-cat upregulation, leading to abnormal desmosome structure. Cardiac-specific double knockout mouse models for β-cat and PKG show disassembly of the ID structure, reduction of Cx43 phosphorylation and of functional GJs, and lethal arrhythmias. This phenotype is remarkably similar to N-Cad deficient mice, although replacement fibrosis was missing.[7]

Regarding less pivotal proteins, the transmembrane protein CAR that associates with $Na_V1.5$ seems also to be involved in GJ regulation, since cardiomyocytes of CAR knockout mice show reduced Cx43 expression and, surprisingly, increased dye coupling.[81] ZO-1 might modulate this, since it binds both Cx43 and CAR, although the ZO-1-CAR interaction only has been shown *in vitro*.[82,83] Moreover, the gene *Pitx2* (paired-like homeodomain 2) has been identified to play a role at the ID. Micro-array analysis revealed that many target genes of Pitx2 are involved in cell junction assembly, ion channels and transcriptional regulation. Target genes include genes encoding β-cat, DSP, Cx43, and Kir6.2. This diversity implies a role for Pitx2 on area composita level. Its precise function is unknown, but its chromosomal location is close to the 4q25 familial atrial fibrillation locus. Indeed, Pitx2 haploinsufficiency in mice show pacing-induced arrhythmias. Furthermore, the atrial IDs of mice that were conditionally deficient for atrial Pitx2 were remodelled, and spaces between cardiomyocytes were increased. Interestingly, mitochondria were swollen and dysfunctional. Identifying the function of the Pitx protein would therefore be very interesting.[84]

6.1.3 Linking ion channels to other ID components

Recently, super-resolution microscopy data revealed that Nav1.5 occurs in two distinct pools at the ID: one at the perinexus, which is described in a previous section, and one at AJs.[85] A connection between $Na_V1.5$ and the AJ at the ID is indicated by co-immunoprecipitation of N-Cad and $Na_V1.5$.[33] Moreover, a recent paper showed that clusters of $Na_V1.5$ and N-Cad preferentially localize in each other's close vicinity, illustrating that both proteins depend on each other, suggesting the presence of adhesion/excitability nodes rather than independent structures.[68] N-Cad is also related to potassium channels, since decreased N-Cad affected $K_V1.5$ channels.[86] Presumably, N-Cad is associated with $K_V1.5$ via cortactin, which specifically regulates the interactions between N-Cad and $K_V1.5$ and is required for proper $K_V1.5$ activity. However, N-Cad does not coprecipitate with $K_V1.5$. N-Cad knockout mice show a decrease in cortactin levels, actin skeleton disruption, and decreased expression of $K_V1.5$. This means that N-Cad deficiency leads to prolonged action potentials, conduction slowing, and an increase in arrhythmia incidence.[60]

K_{ATP} channel subunits Kir6.2 and SUR2A co-localize with PKP2 and PKG at the ID. Moreover, super-resolution microscopy shows that K_{ATP} channels are at interacting distance from the junctional proteins N-Cad, AnkG and DSP. Colocalization with Cx43 was weak, so a tight relationship with GJs is not expected.[61]

Desmosomal integrity is important to ensure sodium current, in which espec ially PKP2 plays a crucial role. Cardiomyocytes without PKP2 showed a decreased sodium current and reduced abundance of $Na_V1.5$ at the ID. This led to reentry activity and decreased conduction velocity. In a PKP2-haploinsufficient mouse model, desmosomal integrity was lost, sodium current amplitude decreased and conduction velocity reduced.[9,10,60] $Na_V1.5$ was remodelled, but unlike Cx43 remodelling, this process was not mediated by microtubules.[48] AnkG has been proposed to be an important modulator of the interactions between PKP2 and $Na_V1.5$, (reviewed in Ref. 9) also regarding the notion that PKP2 and $Na_V1.5$ occur in the same protein complex with β4-spectrin and CAMKIIδ. The desmosome is thought to anchor AnkG to the membrane, which stabilizes the spectrin-AnkG complex,[5,33] and desmosomal proteins might be involved in the targeting of Na channels to the ID. Indeed, *in vivo* AnkG loss results in PKP2 remodelling,[75] and PKP2 haploinsufficiency in mice reduced potassium current, but the mechanisms behind this reduction remain unclear.[61]

6.1.4 Noncanonical roles for Cx43

Classically, sodium channels are thought to be responsible for cell excitability, while GJs gate the transduction of charge between cells. However, noncanonical roles of Cx43 have emerged: Cx43 maintains the complex that generates the action potential, which means that Cx43

is needed for both charge transduction and cell excitability. (reviewed in Ref. 9) It is therefore noteworthy that Cx43 and $Na_V1.5$ co-immunoprecipitate[33] and Cx43 deficiency leads to a reduced $Na_V1.5$ expression and conduction delay. Cx43 insufficiency is deleterious in two ways: the actual GJ between cells is compromised, but also the charge generated by cells is diminished. Cx43 deficiency reduced the expression of $Na_V1.5$, leading to a reduced sodium current and increased vulnerability for arrhythmias.[87] Furthermore, truncating Cx43 by its five C-terminal amino acid residues confirms its noncanonical roles. Truncated Cx43 cannot bind ZO-1, while GJs remain functional. Mice expressing this truncated form die from ventricular arrhythmias, which were explained by decreased sodium and potassium current densities. The arrhythmias are therefore independent of GJ function. The changes in current density might however be not the only cause. Cx43 truncation may also affect the function of mitochondrial Cx43. Still, ZO-1 appears essential in linking GJ to ion channel function.[86] Depletion of AnkG also leads to a decrease in Cx43 abundance, GJ plaques, and intercellular conductance.[9,33] These effects are probably caused by a disruption of microtubule-mediated delivery of Cx43.[65]

6.2 Connexome

The VGSC, Cx43 and desmosomes–interestingly, without AJ–are together distinguished as one functional unit, named the connexome, regulating excitability, cell-cell adhesion, and intercellular contact.[80] Interestingly, the connexome is thought to include the outer connexons and Nav1.5 of the perinexus, and the adjacent PKP2.[58] How the perinexus, connexome and area composita relate to each other, is not described yet. However, it is probable that the connexome is a border zone between perinexus and area composita. According to the connexome hypothesis, Cx43 exerts GJ-independent functions in the connexome, ensuring normal sodium current density. Specifically, the C-terminus of Cx43 ensures functional surface expression of $Na_V1.5$ at the ID, probably by regulating microtubule-mediated vesicle delivery.[88] The VGSC is essential for proper cell-cell adhesion,[9,33] and desmosomal integrity is a requirement for proper VGSC function. In a mouse model with an AC-related mutation in *PKP2*, $Na_V1.5$ abundance was not affected, but sodium current amplitude was decreased, comparable to the results of PKP2 deficiency. PKP2 might be required to ensure proper microtubule capping that is needed for $Na_V1.5$ delivery at the ID. Brugada syndrome has been mentioned as a disease of the connexome, in which mutations in *PKP2* and the subsequent deficit in $Na_V1.5$ at the ID cause the arrhythmogenic phenotype and fibrosis, even in the absence of structural cardiomyopathy.[80]

AnkG is also thought to be crucial in the connexome, linking the VGSC complex, GJs, and desmosomes. It ensures for instance mechanical continuity and electrical coupling between cardiomyocytes, which makes it indispensable for action potential propagation. Moreover, AnkG integrates GJ and desmosome function. Also, it influences the subcellular localization of PKP2, but this association might be indirect. AnkG deficiency did not influence PKG or N-Cad distribution, which implies that the AJ and AnkG do not interact, although it might be a false-negative result.[33]

Recently, the ultrastructure of the connexome has been investigated. Lateral edges of GJs and desmosomes are shown to form a triad, with rough budding vesicles in between. Budding vesicles were also seen at the edge of the GJ plaque and in the intercellular space. The mechanisms behind these vesicles and its contents remain unclear.[36]

6.3 Transitional junction

The ID membrane is not completely studded with transmembrane proteins. The apex of membrane interdigitations appears to be junction-free, and there, sarcomeres are connected to the membrane. This region is called the transitional junction (TrJ).[6] Although typical Z-disc structures are not recognizable and actin filaments apparently seamlessly integrate into the membrane, typical Z-disc proteins such as α-actinin, titin, ZASP, non-muscle myosin IIB and NRAP (nebulin-related anchoring protein) are identified. Furthermore, the terminal thin filaments at the ID are composed of β-actin instead of the ID-specific α-actin (reviewed in Ref. 3). It has been proposed that α-actinin crosslinks sarcomeric α-actin, whereby Z-disc-associated protein ZASP supports α-act. Titin then binds to actin and α-actinin, linking the sarcomere to the ID.[6] The TrJ is the proposed site for sarcomerogenesis, where myofibrils are elongated and the cardiomyocyte grows longitudinally. In this respect, the TrJ is thought to function as a proto-Z-disc for sarcomeric addition. Interestingly, during cardiac growth, the membrane fold amplitude varies between 0.2 and 2 μm. The largest amplitude is equivalent to sarcomere length and at that stage, the TrJ hosts the development of the Z-disc. The TrJ is then an anchor for new filaments, incorporating titin and subsequently maturing into a Z-disc.[77]

Also, αII-Spectrin and Mena/VASP are crucial elements of the TrJ: in fact, their complexes protect the terminal sarcomeric filaments and connect them to the sarcolemma, thereby forming the TrJ. At the ID, the SH3*i* splice variant of αII-Spectrin is found, with an extra C-terminal 20 amino acid insertion in the SH3 domain.[38] Other spectrin-binding proteins at the TrJ include MLP and filamins.[56] The SH3 domain of spectrin is crucial for the direct interaction with Mena/VASP. This direct interaction ensures β-actin filament assembly and stabilization of intercellular contact. PKA-mediated VASP phosphorylation inhibits this binding. Also, Mena complexes with β-actin, sarcomeric α-actinin and Cx43. Although Mena and VASP both bind β-actin, it is unknown how they distinguish between different actin isoforms.[38] Mice deficient in αII-spectrin, and Mena/VASP double-knockout mice all suffer from severe cardiac abnormalities. These similarities indicate that both proteins are essential for connecting the sarcomeres to the ID, or in other words, for the transitional junctions.[38]

Furthermore, AnkG contains an obscurin/titin-binding-related domain (OTBD), which means that AnkG could also be involved in the transitional junction, since obscurin is a Z-disc-associated protein. The OTBD can also bind plectin and filamin C, factors that organize the cytoskeleton and regulate myogenesis.[76] The enormous protein plectin has not been reported in association with the ID before. It might be interesting to search for the role of plectin at the ID, since it anchors IFs to desmosomes in other tissues than the heart.[89] And as a last TrJ-related protein, filamin C is the cardiac-specific variant of filamin, which has been mentioned before in this review as an interaction partner of AnkG, spectrin, and mXinα. Mutations in *FLNC* encoding filamin *C* cause myopathy with cardiac involvement in humans. In medaka fish, the filamin C mutant *zac* caused similar symptoms. In the heart, this mutant protein localized at IDs, where myofibrils failed to attach to the sarcolemma, which made the hearts more vulnerable to mechanical stress. However, it remains to be seen if these results can be extrapolated to mice or humans.[90]

7. Role of focal adhesions

Although focal adhesions function primarily as cell-matrix junctions in the LM of cardiomyocytes, they also fulfill a role in mechanically

stabilizing IDs in development and disease. They stabilize cells in which IDs are assembled and which undergo myofibrillogenesis. When the ID is mature, the focal adhesions disassemble due to intercellular mechanical force transduction. In cardiac disease, fibrosis challenges cell-cell junctions because of increased mechanical forces. Then, focal adhesions are found around the ID again to ensure mechanical stability. Since GJs form relatively late in development when focal adhesions have already dissociated from the ID, it is interesting to note that remodelling of GJs during disease correlates with the renewed presence of focal adhesions of GJs. Unraveling the mechanism behind this observation might be crucial in understanding GJ remodelling during disease.[91]

8. ID mutations and disease

Mutations in desmosomal proteins, such as DSP and the cutaneous variant PKP1, have been associated with diverse diseases, including the blistering skin disease pemphigus, palmoplantar keratoderma, and inherited hypotrichosis. Desmosomal mutations may perturb cell-cell adhesion, IF adhesion, or both. These diseases illustrate the importance of desmosomes in other tissues than the heart, and are caused by mutations in or autoimmune reactions to non-cardiac homologues of desmosomal proteins. However, how *DSP* mutations can specifically cause a cutaneous phenotype while leaving the heart unaffected remains unclear.[5]

Cardiac desmosomal mutations are strongly associated with arrhythmogenic cardiomyopathy (AC), characterized by syncope and sudden death in young athletes or sustained ventricular tachycardia. *PKP2* mutations are most prevalent in AC patients, but mutations affecting the desmosomal cadherins, DSP, and PKG also have been associated with this disease.[1,5] These mutations distort proper desmosome structure, which might cause dissociation of PKG from the desmosome. PKG is then able to compete with β-cat at the AJ, which suppresses the Wnt pathway. This might lead to the fibrofatty replacement and apoptosis of the myocardium, characteristic of AC. Surprisingly, mutations affecting the AJ proteins N-Cad, α-cat, mXinα, and β-cat lead to dilated cardiomyopathy without myocyte loss and inflammation, unlike the AC phenotype. Therefore, desmosomes are thought to be involved in different signaling pathways than AJs.[3] Overall, a decrease in desmosomal protein expression has an effect on multiple levels. Firstly, it causes GJ remodelling. Secondly, nuclear PKG levels increase, the expression of Wnt/β-cat signaling genes c-Myc and Cyclin D1 decreases, and expression of several adipogenic genes increases. This might lead to a transdifferentiation of cardiomyocytes into adipocytes.[5]

Interestingly, cardiocutaneous diseases in which cardiac and skin abnormalities are combined are often caused by desmosomal mutations. Naxos disease is a type of AC combined with woolly hear and palmoplantar keratoderma, which is associated with mutations in *JUP* encoding PKG. Carvajal disease, symptomatically very similar to Naxos disease, is associated with *DSP* mutations. In AC, Naxos and Carvajal disease patients, GJs are also remodelled.[1,5]

Brugada syndrome, Carvajal disease and AC are also associated with mutations affecting the proteins desmin, titin, lamins A and C, transmembrane protein (TMEM) 43, striatin, and transforming growth factor β3. Desmin and titin play a role at the ID, but this is not the case for the other proteins listed here. Lamins for instance are a class of intermediate filaments at the nuclear membrane. Striatins have a caveolin-binding domain, implying a role at the ID.[92,93] Lastly, muations in *CTNNA3* are associated with AC. Although the mutated protein impairs the association with β-cat, this does not completely explain the perturbation of AJ and desmosome assembly.[8]

9. The ID as mechanosensor

Beyond mechanical and electrical coupling, the ID also exerts mechanosensing roles, which are realized via N-Cad, Cx43, and the ion channels TRPV2 and TREK-1. The roles of these elements only have been described individually, so a very interesting question is whether and how these mechanosensors influence each other. Considering the known connections between mechanics and electrics, this crosstalk very likely occurs.

9.1 Area composita

AJs don't just glue cells together. By means of mechanosensing cadherins, they affect the stiffness of neighboring CMCs. This is of great importance for the structural integrity of the heart: after all, a chain is as strong as its weakest link. Mechanical force on cadherins leads to changes in sarcomeric organization, cell shape, and stiffness. The force is transduced to α-actinin, which changes its conformation, thereby recruiting Vcl from costameric areas.[94] Another study showed that mechanical force on cadherins leads to activation of Abelson tyrosine kinase, which phosphorylates Vcl. This correlates with the finding that Vcl phosphorylation at position Y822 leads to higher Vcl concentrations at the AJ than at the costameres.[4] Further downstream, common global cytoskeleton regulators such as RhoA are probably responsible for the observed effects at the cytoskeleton level: changes in sarcomeric organization, cell shape, and stiffness.[95–97] Furthermore, mechanical stretch increases N-Cad protein expression.[98] Please note that integrins at the LM are also mechanosensitive, but they have different effects on the cytoskeleton.[95] Interestingly, stretch on ECM-integrin complexes induce upregulation of N-Cad, PKG, DSP, and Cx43 expression (reviewed in Ref. 99).

9.2 Gap junctions

GJs seem to be mechanodependent as well. Cyclic mechanical stress (CMS), representing the forces arising from cardiomyocyte contractions, increases GJ number and size,[98] as well as Cx43 protein and mRNA levels, which is similar to the effect of α- or β-adrenergic stimulation. Moreover, Cx43 phosphorylation is increased under CMS conditions.[100] These processes are probably mediated by vascular endothelial growth factor (reviewed in Ref. 99).

9.3 Ion channels

Mechanically gated channels (MGCs) are sensors and effectors in mechano-electric feedback (MEF), an excellent example of the integration of mechanical and electrical properties. The concept of MEF was coined after the discovery of the Bainbridge effect, which entails that stretch of the right atrium increases the spontaneous beating rate of the SA node. At the ID, two MGCs are described: transient receptor potential cation subfamily V member 2 (TRPV2) and the two-pore K^+ channel TREK-1. Firstly, TRPV2 is cell volume-activated, allowing K^+ efflux when cell volume increases. However, TRPV2 only translocates to the ID upon osmotic shock or insulin-like growth factor 1 (IGF-1) stimulation. TRPV2 also seems pivotal for ID integrity: cardiomyocytes of neonatal TRPV2 KO mice show impaired excitation-contraction coupling, and no ID formation. Concomitantly, induced cardiac-specific TRPV2 KO mice show disorganized ID structures, and impaired cardiac pump function. IGF-1 administration partly prevented chamber dilation and improved cardiac pump function.[101–103] Secondly, TREK-1 is a stretch-activated

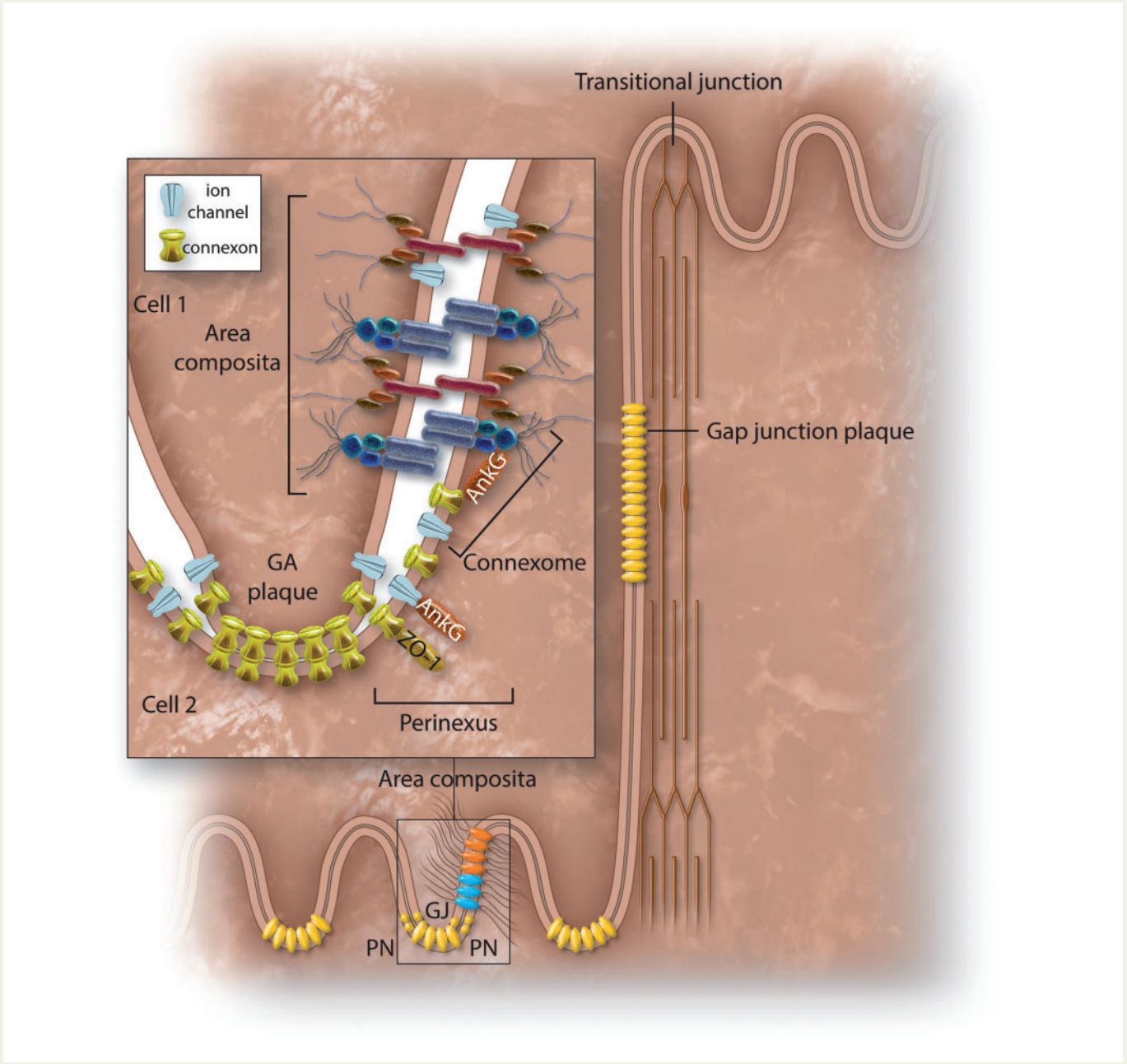

Figure 7 Spatial distribution of ID components. Large GJ plaques occur at the interplicate region, whereas smaller GJs are found in the apex of plicae of the plicate region. These GJs are surrounded by the perinexus and connexome. The connexome seems to be continuous with the area composita. Ion channels reside in the perinexus and area composita.

channel (SAC) that aids normal repolarization of the AP, while increased current shortens the AP. In mouse heart, TREK-1 interacts with βIV-spectrin, which ensures its membrane targeting and activity. However, it hasn't been found (yet) in human heart.[103,104] Lastly, it must be noted that Kir channels and Nav1.5 are, although mainly activated by other stimuli, also mechanically affected–at least in human embryonic kidney cells (reviewed in Ref. 103).

10. An integrated model of the ID

The data presented in this review together result in the detailed molecular models as expressed in the *Figures 3–6*, which summarize protein interaction data for all 'separate' ID structures. *Figure 7*, in turn, enhances our understanding by linking the molecular models of individual structures into a refined, spatially integrated model of the ID.

The ID can be viewed as a stair-like structure with several steps, with the 'tread' perpendicular to the longitudinal axis of the myocytes and the 'riser' in parallel. The membrane area in the longitudinal axis of the cell is called the interplicate region and is one or more sarcomere units long. Only GJs, and no other structures, have been reported in this part of the membrane.[68,105,106] The plicate region, perpendicular to the long axis of the cell, is heavily folded. TrJs are located at the transition between plicate and interplicate regions, gluing the sarcomere to the sarcolemma. Nesting on the innermost ends of these plicate folds are GJs[78]–but the

plaques are smaller than those in the interplicate region.[105] Although no report on the perinexus mentions whether it's located in either the plicate or interplicate region,[57] it resides most likely in the plicate regions. We deduce this from the findings that perinexi host sodium channels,[107] and are neighbored by desmosomes[34]–both structures are not found in interplicate regions.[68,105] This raises the question how GJs at interplicate regions look like, and if these also have perinexus-like structures. The outer connexons and sodium channels of the perinexus and the neighboring desmosomes together form the connexome, linked by AnkG.[36,58] These desmosomal proteins most probably continue into the area composita, which spans a large part of the plicate region.[78] In this relatively large area composita area, sodium channels–and likely potassium channels too–form adhesion/excitability nodes with N-Cad.[68] Whether there is a relationship between the area composita and TrJ remains however unknown.

11. Conclusion

AJs, desmosomes, GJs, and ion channels are not individually operating structures, but form one functional unit as the area composita; they work together in many different ways, for instance via scaffolding proteins such as ZO-1 and AnkG. Some structural components of cell-cell junctions can also interact with other ID proteins or function in signaling pathways, such as Cx43 and β-cat. GJs and ion channels together comprise the perinexus; VGSC complexes, GJs and desmosomes are together named the connexome; whereas AJs, desmosomes, GJs and ion channels together form the area composita. Moreover, not every stretch of ID membrane is covered with junctional proteins: certain parts of the membrane are involved in the connection of myofibrils to the membrane, which is called the transitional junction. Furthermore, GJs and ion channels likely create and propagate action potentials together. The model in which GJs solely transport charge and small solutes whereas ion channels only produce and propagate action potentials, appears to be outdated. Protein deficiencies may lastly lead to mechanical dysfunction of for instance the AJ, but also to arrhythmias, often via GJ remodelling. This illustrates the interdependency of the ID components and the coupling of mechanical and electrical elements.

11.1 Future perspectives

The introduction of the term area composita is an important step forward in ID research. However, the definition of the area composita is still unclear: do all AJs and desmosomes fuse into an adhering junction? To what extent are GJs involved? And does the area composita include ion channels? Furthermore, the relationship between the perinexus, the connexome, and the area composita remains to be determined, as well as the regulation, development and specific properties of these structures. Advances in mathematical modelling, and novel techniques, such as super-resolution microscopy, and scanning ion-conductance microscopy (SICM), should help to address these open questions in the near future.

To summarize, the ID is a very complex structure, which we are only starting to understand. New proteins and processes are continuously discovered, and they deserve to be investigated since their apparent roles in cardiac disease. The role of the ID far exceeds the ensuring of mechanical strength and electrical coupling; many components are involved in intracellular signaling pathways. The observation that mutations in one ID protein may have a wide range of effects in cardiac diseases like AC and Brugada syndrome illustrate that ID components strongly rely on each other for their proper function.

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