# Animal models of arrhythmogenic right ventricular cardiomyopathy: what have we learned and where do we go? Insight for therapeutics.

**Authors**: Laura Padrón-Barthe, PhD[1,#], Fernando Domínguez, MD[1,2,#], Pablo Garcia-Pavia, MD, PhD[1,2,3*] and Enrique Lara-Pezzi, PhD[1,4,5*]

**Affiliations:**

[1]CIBER Cardiovascular Diseases (CIBERCV), Madrid, Spain;

[2]Heart Failure and Inherited Cardiac Diseases Unit. Department of Cardiology. Hospital Universitario Puerta de Hierro Majadahonda, Madrid, Spain;

[3]Francisco de Vitoria University, Madrid, Spain;

[4]Centro Nacional de Investigaciones Cardiovasculares Carlos III, Madrid, Spain;

[5]Faculty of Medicine, National Heart & Lung Institute, Imperial College, London, UK.

**[#] L P-B and FD contributed equally to this work**

*** PG-P and E L-P are co-correspondig authors.**

***Correspondence**:

Enrique Lara-Pezzi, PhD, Myocardial Pathophysiology Area, Centro Nacional de Investigaciones Cardiovasculares Carlos III, Melchor Fernandez Almagro, 3, 28029 Madrid, Spain, elara@cnic.es.

Pablo Garcia-Pavia, MD, PhD, Inherited Cardiac Diseases Unit. Department of Cardiology. Hospital Universitario Puerta de Hierro, Manuel de Falla, 2. Majadahonda, Madrid, 28222, Spain, pablogpavia@yahoo.es

**Funding:** This work was supported in part by the Instituto de Salud Carlos III (ISCIII) [grants PI14/0967, and RD012/0042/0066], the Todos somos Raros program [grant to PG-P], and the Spanish Society of Cardiology [2014 Basic Research Grant to PG-P]. ISCIII grants are supported by the Plan Estatal de I+D+I 2013-2016 – European Regional Development Fund (FEDER) “A way of making Europe”.

Keywords: ARVC, animal models, exercise, therapies, desmosomes

## ABSTRACT

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a rare genetically-determined cardiac heart muscle disorder characterized by fibro-fatty replacement of the myocardium that results in heart failure and sudden cardiac death (SCD), predominantly in young males. The disease is often caused by mutations in genes encoding proteins of the desmosomal complex, with a significant minority caused by mutations in non-desmosomal proteins. Existing treatment options are based on SCD prevention with the implantable cardioverter defibrillator, antiarrhythmic drugs and anti-heart failure medication. Heart transplantation may also be required and there is currently no cure. Several genetically modified animal models have been developed to characterize the disease, assess its progression and determine the influence of potential environmental factors. These models have also been very valuable for translational therapeutic approaches, to screen new treatment options that prevent and/or reverse the disease. Here we review the available ARVC animal models reported to date, highlighting the most important pathophysiological findings and discussing the effect of treatments tested so far in this setting. We also describe gaps in our knowledge of the disease, with the goal of stimulating research and improving patient outcomes.

## INTRODUCTION

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is an inherited heart muscle disease characterized by the progressive degeneration of cardiac myocytes, which are replaced by fibro-fatty tissue [44, 61]. While the disease was initially named because of right ventricle (RV) involvement [43], it can also affect the left ventricle, in which case it is frequently termed arrhythmogenic cardiomyopathy (AC) [25, 69]. Patients with right ventricular arrhythmias and left ventricular involvement have a worse prognosis [56]. ARVC is distinguished from dilated cardiomyopathy by a greater propensity towards arrhythmia [62, 69]. However, current diagnostic ARVC criteria focus on the RV and non-classical disease patterns could still be underdiagnosed [44]. ARVC can be a devastating disease since the first symptom is often sudden cardiac death (SCD) [71]. Clinical manifestations, such as dizziness, heart palpitations and SCD appear in young men from an average age of 31 [56]. Early manifestations of ARVC are frequently subclinical and escape detection by conventional imaging and surface electrocardiogram (ECG). Thus, in the early “hidden phase” of the disease, patients are asymptomatic even if they are at high risk for ventricular arrhythmia and SCD. Pathologically, the disease is characterized by myocardial atrophy, fibro-fatty replacement and chamber dilation [3, 44].

ARVC is a significant cause of premature SCD in young people and athletes, accounting for more than 10% of overall cases in these individuals, a five-fold increased risk as compared with non-athletes [5, 15]. Indeed, restriction from strenuous exercise is recommended for affected individuals and healthy mutation carriers in order to delay the progression of the disease [45]. Current treatment options for ARVC include the implantable cardioverter defibrillator (ICD) and

ventricular arrhythmia catheter ablation, as well as antiarrhythmic and anti-heart failure drugs to treat ventricular dysfunction and heart failure. These measures, however, do not completely prevent ARVC and treatment focuses on slowing disease progression by decreasing the burden of arrhythmias with anti-arrhythmic drugs and preventing SCD with ICDs. Heart transplantation is often considered in the case of end-stage heart failure or an arrhythmia storm. Because the early molecular and cellular events underlying ARVC onset as well as its progression remain largely unknown, the current therapeutic modalities are mostly palliative (Figure 1). For these reasons, animal models are invaluable to help gain a deeper understanding of the molecular pathophysiological mechanisms of ARVC and to assist in the development of potential therapies that could ultimately be translated into clinical practice.

Up to 60% of ARVC cases are attributed to mutations in genes encoding proteins of the desmosomal complex [34], an anchoring structure crucial for cell-to-cell adhesion, with the remaining cases caused by mutations in proteins beyond the desmosome. Desmosomes are complex structures that function to connect neighbouring cells to each other. In the heart, desmosomes are composed of five proteins (Figure 2) and are indispensable for electrical conduction and mechanical contraction in myocardial cells [58]. To date, human genetic studies of ARVC have identified 15 independent loci and 13 dominant ARVC-causing genes [17, 38, 63]. Different genotypes of ARVC are classified into several groups based on the causative mutated gene involved (Table 1) [58].

This review focuses on the currently available animal, mostly mouse, models employed to study ARVC, and the pathophysiological mechanisms that have been reported to date. Based on the results obtained using a variety of

approaches, we discuss the utility and suitability of different models of ARVC in translational studies, including the role of exercise and new therapeutic targets.

## Desmosomal ARVC models

The genetic basis of ARVC is largely attributed to mutations in the desmosomal complex. Desmosomes are multiprotein structures of the cell membrane that act as specialized cell-cell junctions, providing structural and functional integrity to adjacent cells. They are particularly abundant in epithelial cells and cardiomyocytes [27]. Desmosomes are composed of transmembrane desmosomal cadherins (desmocollin 2 and desmoglein 2), armadillo proteins (junctional plakoglobin [γ-catenin] and plakophilin 2) and the centralized plakin protein, desmoplakin, which links the junctional complex to the intermediate filament, desmin, maintaining the mechanical integrity of cardiac muscle [58] (Figure 2). They differ from other intercellular junctions since they resist mechanical stress, adopting a strongly adhesive state. In desmosomal ARVC, mechanical stress in contracting cardiomyocytes results in desmosomal instability, ultimately leading to cardiac dysfunction and cell death.

The main function of desmosomes is thus to mechanically link tissues that experience mechanical stress. However, in many diseases related to the desmosome it is often difficult to ascribe all aspects of the phenotype to defective cell adhesion. Indeed, because desmosomes also participate in intracellular signaling networks, it is frequently not clear whether the symptoms that occur in human diseases that target desmosomes arise primarily from loss of adhesion or from modulation of desmosomal signaling pathways [26]. Desmosomal cadherins are involved in regulating crucial cellular processes such as proliferation,

differentiation and morphogenesis. While the mechanisms remain elusive, they may achieve this by regulating the availability of signaling molecules [58]. As we will discuss later, plakoglobin is closely related to β-catenin and has a role in Wnt/β-catenin signaling [24] (Figure 2). Desmosomes have also a critical role in embryonic development. Accordingly, it has been observed that homozygous null mutations in the genes encoding the desmosomal complex are embryonic lethal in mice [23, 24, 30, 66], whereas heterozygous null alleles for plakoglobin or plakophilin 2 cause little, if any, phenotypic changes without further stressors like ageing or exercise [24, 66, 75]. Further research is needed to define the signaling capacity of desmosomes and to specify the downstream consequences of their signaling activity.

Desmoplakin

Desmoplakin is the most abundant component of the desmosome [55]. It is encoded by the DSP gene, a member of the plakin family of coil-coiled proteins that lacks a transmembrane domain and resides in the cytoplasmic surface of the desmosome. Desmoplakin facilitates the association of the desmosome with intermediate filaments for desmosome assembly, cytoskeletal linkage and embryonic development [23]. Mutations in DSP can lead to ARVC in an autosomal dominant manner or to a more complex syndrome, called Carvajal syndrome, when inherited in an autosomal recessive manner. Carvajal patients present dilated cardiomyopathy, woolly hair and palmoplantar keratoderma [8]. No animal model is currently available for this syndrome.

It has been described that systemic null mutations in DSP in mouse embryos lead to early lethality at embryonic day 6.5 [23]. In heterozygous mice expressing a

point mutation or a disrupted form of DSP [24, 75], plakoglobin is mislocalized to the nucleus, where it suppresses Wnt/β-catenin canonical signaling. Inhibition of this pathway, a key regulator of the transcriptional switch from myogenesis to adipogenesis, leads to enhanced adipogenesis, fibrogenesis, and myocyte apoptosis, all of which are characteristic features of human ARVC. Moreover, reduced expression of DSP has been shown to cause mislocalization of the gap junctional protein connexin 43 in a cardiomyocyte-specific DSP haploinsufficiency mouse model [28], which presents significant alterations in conduction-repolarization kinetics that precedes morphological changes detectable on conventional cardiac imaging. In the cardiomyocyte-specific DSP-knockout mouse (DSP-cKO) [41], which models a biventricular form of human ARVC, mutant hearts harbor defects in desmosomal structural integrity, a primary causes of dysfunction in this model. Furthermore, connexin 40 expression is dramatically downregulated in cardiomyocytes of the ventricular conduction system in DSP-cKO hearts, illustrating the importance for anchoring desmosomes in the cardiac conduction system. Moreover, the Wnt/β-catenin signaling pathway is unimpaired and the mechanism by which the adipogenesis switch occurs is different to that of the models described above. The authors of this study posit that a dose-dependent loss of DSP would lead to downregulation of connexin 43, resulting in conduction abnormalities prior to the molecular dissociation of the mechanical junction complex, and preceding the fibro-fatty manifestation observed in ARVC.

These models have assigned a central role for DSP in the desmosomal structure, and while it lacks a transmembrane domain, it is essential to anchor desmosomal proteins and to provide structural integrity to the cell membrane

Plakophilin 2

Plakophilin 2 (PKP2) is an armadillo-related protein located in the outer dense plaque of desmosomes that links desmosomal cadherins with DSP and intermediate filaments. PKP2 facilitates lateral interaction of the desmosomal cadherins desmocollin and desmoglein using its N-terminal domain [26]. In humans, autosomal dominant mutations in PKP2 are the most common genetic cause of ARVC, contributing to approximately 70% of familial ARVC cases [72]. Germline PKP2 deficiency in mice results in heart rupture during cardiac development [30]. It has been described that PKP2 deficiency provokes a reduction in the association of the cardiac junctional plaques with DSP, plakoglobin and desmosomal cadherins, which is consistent with the reduced architectural stability of the intercalated discs (IDs) in ARVC9. Nevertheless, some elements of the ID remain intact, for example, several armadillo proteins such as β-catenin and plakoglobin remain in their correct locations, indicating that their binding to other plaque components is sufficient for their junctional integration. As the developing embryonic heart is particularly vulnerable to diverse forms of weakening of cardiomyocyte adhesion, these findings lead to the conclusion that PKP2 is a key organizer of cardiac architecture during embryogenesis. That PKP2 is the only plakophilin present in cardiac adherens junctions may explain go some way to this extraordinary sensitivity in mutant hearts.

Further studies performed on PKP2 heterozygous ($PKP2^{+/-}$) mice revealed myocyte sodium current defects, suggesting cross-talk between the desmosome and sodium channel complex, and pointing to the potential contribution of sodium

current dysfunction to arrhythmias. In contrast to other animal models of desmosomal deficiency, $PKP2^{+/-}$ mice do not seem to develop overt structural disease, as $PKP2^{+/-}$ hearts show ultrastructural, but not histological or gross anatomical differences when compared with wild-type (WT) littermates [11]. Although this could be considered a weakness of the model, it nonetheless enables the study of arrhythmogenic substrates in the absence of structural involvement.

Zebrafish models have also been used to study PKP2-related ARVC. PKP2 morphant embryos show a reduced number of ID and increased intracellular space, leading to an enlarged atrium and abnormal ventricular looping [54]. As in the mouse, PKP2 would have both structural and signaling roles in the developing zebrafish heart.

Desmoglein 2 and Desmocollin 2

The desmosomal cadherins –desmogleins and democollins– are single transmembrane glycoproteins that bridge the space between adjacent cardiac or epithelial cells. They mediate $Ca^{2+}$-dependent homophilic interactions between the extracellular regions of desmosomal cadherins, providing additional support for their adhesive functions [21]. The intracellular domain of desmosomal cadherins binds to two proteins of the armadillo family, plakoglobin and plakophilin, forming an indirect link to desmoplakin and desmin.

Inactivation of the DSG2 gene by homologous recombination in embryonic stem cells to generate a $DSG2^{-/-}$ knockout mouse model results in embryonic lethality [21], indicating that DSG2 has essential functions for early embryo development. To circumvent the embryonic lethality of $DSG2^{-/-}$ mice, which hinders the

characterization of the role of DSG2 in the post-natal heart, Pilichou *et al.* generated a transgenic mouse with cardiac overexpression of the N271S mutation of DSG2, equivalent to N266S in humans. This mutation recapitulates key clinical features of ARVC10 including spontaneous ventricular arrhythmias and sudden death, cardiac dysfunction, biventricular dilatation and ventricular aneurysms. In this model, myocyte necrosis was considered the central initiator of myocardial dystrophy, which would subsequently trigger an inflammatory response and massive calcification within the myocardium, followed by injury repair with replacement of dead cardiomyocytes by fibrous tissue [60]. The model does not exhibit fatty infiltration, which was traditionally a characteristic finding in human ARVC, although no longer a specific ARVC diagnostic criterion. Proposed explanations for the absence of fat deposition in mice transgenic for mutant DSG2, as well as in other models, include the difference between species and the fact that the mouse heart does not show evidence of epicardial fat. This feature is different from the human heart, where a physiological amount of fatty tissue can always be found in the subepicardium.

From further study of the N271S transgenic mouse, the same authors found an *in vivo* interaction between DSG2 and the Na(V)1.5 sodium channel protein, indicating that conduction disturbances and electrical instability would be early events of ARVC disease, before structural changes to the myocardium [64]. This analysis, along with the study of a cardiomyocyte-specific DSG2 knockout model [35], also showed intercellular space widening at the level of the ID (desmosomes/adherens junctions), indicating compromised adhesion of cardiomyocytes. These data strongly support an important adhesive role for DSG2.

In contrast to desmoglein 2, almost no functional knockout models are available for desmocollin 2 (DSC2). Nevertheless, a very recent study provided evidence that the microRNA, miR-130a, targets DSC2 [49]. Although this mouse model does not directly target DSC2, transgenic mice overexpressing miR-130a presented an 80% reduction in the protein expression of DSC2 in the myocardium together with right ventricular dilation. Moreover, surface ECG revealed spontaneous premature ventricular complexes, and histological examination showed fibrosis and lipid accumulation within both ventricles. Thus, miR-130a overexpression results in a disease phenotype characteristic of ARVC11. In another recent study, Brodehl *et al*. developed a transgenic mouse model overexpressing DSC2 specifically in cardiac myocytes to determine its potential contribution to cardiomyopathy and intercellular adhesion pathology [9]. Transgenic mice developed a severe cardiac biventricular dysfunction that was accompanied by necrosis, calcification and early up-regulation of inflammatory and fibrotic remodeling pathways. However, the mice did not present ventricular arrhythmias or SCD. Moreover, the expression of endogenous desmosomal proteins including DSG2, plakoglobin and DSP was downregulated in mutant mice only in fibrotic areas, indicating a local remodeling process due to the replacement of cardiomyocytes rather than a global downregulation of endogenous desmosomal genes. This remodeling would alter cell-to-cell adhesion, a process that must be fine-tuned in cardiomyocytes for their proper contraction.

Overall, results from these mouse models reveal that the cadherins desmoglein 2 and desmocollin 2 are not functionally interchangeable. Although desmosomal proteins remain efficiently recruited to and associated with the ID in the absence

of DSG2, the desmosomal structure *per se* is not maintained. Conversely, desmosomal proteins are downregulated in the absence of DSC2.

DSC2-related ARVC has also been studied in zebrafish. Morpholino knockdown of DSC2 in zebrafish embryos was shown to recapitulate the effects of a heterozygous mutation (c.631-2A/G) causing ARVC in humans [33]. Loss of DSC2 resulted in a reduction in the desmosomal plaque area, loss of desmosome extracellular electron-dense midlines and associated myocardial contractility defects, indicating that DSC2 is necessary for normal myocardial structure and function[33].

Plakoglobin

Plakoglobin (γ-catenin, JUP) is a member of the armadillo family of proteins, which is characterized by domains with tandem *arm* repeats that form a distinct three-dimensional structure, allowing them to interact with distinct partners. JUP can be found in the desmosomal plaque and in intermediate junctions, as well as freely diffusible in the cytosol. In desmosomes, JUP is involved in the anchorage of cytoskeletal filaments to specific cadherins. JUP is an essential component of cardiac but not of epithelial desmosomes during embryonic development, and mutations in the protein lead to a highly penetrant form of ARVC, ARVC12 [6]. JUP and the closely related homologous protein β-catenin share 88% amino acid identity and functionally complete for common protein binding partners [39]. It has been described that homozygous JUP mutant mice die from embryonic day 10.5 onwards due to severe heart defects [6, 66], although some can survive until late gestation and die around birth [6] likely due to partially compensatory mechanisms between JUP and β-catenin. Enhanced adipogenesis due to

suppression of Wnt/β-catenin signaling has been demonstrated in a mouse model of truncated JUP [13].

To overcome embryonic lethality in homozygous JUP mutant mice, Li *et al.* [39] generated a cardio-restricted conditional JUP knockout model using Cre/*loxP* technology. Knockout mice developed cardiomyopathy 5 months after recombination at 6–8 weeks of age, and presented progressive loss of cardiac myocytes, extensive inflammatory infiltration, fibrous tissue replacement and cardiac dysfunction, essentially mirroring the phenotype of ARVC patients. Nonetheless, no spontaneous or induced cardiac arrhythmias were detected in these mice, and more than 50% of JUP cardiac-deficient mice survived 17 months after recombination. Given that ARVC predominantly affects the young, ablating JUP at embryonic or early postnatal stages may be more physiologically informative.

To circumvent the embryonic lethality found in the mouse model and because zebrafish can survive a few days without a cardiovascular system, other authors have turned to this model system to examine JUP function. Morpholino-induced JUP knockdown resulted in a reduced number of adhesion junction proteins that could only be partially compensated by β-catenin [47]. Cardiac defects found in the JUP zebrafish model are similar to those observed after DSC2 knockdown in zebrafish [33] or mouse [6, 66].

Overall, these models indicate that the loss of JUP from the desmosome cannot be compensated by β-catenin, which leads to an altered desmosome structure. Moreover, JUP is able to antagonize β-catenin signaling in the heart.

While autosomal dominant is the most common pattern of inheritance in ARVC, a recessive form of JUP deficiency that presents with ARVC and a cutaneous

phenotype has also been reported. Naxos disease, named after the Greek island of Naxos where the first cases were described, is caused by a frameshift mutation in JUP, resulting in premature termination and expression of a truncated protein [50]. Phenotypically, Naxos disease is characterized by woolly hair, which appears from birth, palmoplantar keratoderma, which develops during the first year of life, and cardiomyopathy, which is clinically manifested by adolescence with 100% penetrance. Several mouse models have successfully reproduced Naxos disease, showing defects in embryonic skin architecture and extreme sensitivity to mechanical stress [6, 7, 77]. Unfortunately, mutant mice die during late embryogenesis or soon after birth, indicating that there could be differences in the mutant JUP expression levels between human patients and mouse models, or divergent requirements for the amount of JUP in the two species.

**Non-desmosomal ARVC models**

While the non-desmosomal ARVC subtype is less common, several animal models have been developed to reproduce different aspects of ARVC pathogenesis. Notwithstanding the fact that some of the mutations have not yet been reported in human patients with ARVC, the models recapitulate most of the features of the human disease and could improve our understanding of the mechanisms involved and also help in the development of new therapies.

Laminin receptor 1 (LAMR1)

Mutations in the nuclear protein laminin receptor 1 (LAMR1) have not been detected in humans; however, a serendipitous mouse model carrying a LAMR1 mutation induced by a transcribed intron-processed retroposon was discovered

to present an ARVC phenotype [1]. Phenotype penetrance was almost 100% and macroscopic examination of the heart at 8 weeks of age showed massive fibrosis limited to the RV. Histological analysis revealed macrophage infiltration between fibrotic and viable tissue and no fat infiltration was reported. Whereas electrocardiographic monitoring failed to reveal ventricular tachyarrhythmias, a prolonged QRS duration was detected by ECG. These findings were subsequently confirmed in two independent transgenic mouse expressing mutant LAMR1 [1].

Striatin

ARVC is the most prevalent primary myocardial disease in Boxer dogs. It was first described by Harpster in the 1980s [31]. These animals present a naturally occurring form of ARVC. The disease is familial and seems to be inherited as autosomal dominant with reduced penetrance [52]. In the Boxer dog, ARVC is characterized by fibro-fatty replacement of the left or right ventricle, as observed in humans, and symptoms include SCD, ventricular arrhythmias and syncope [4]. Striatin is a scaffold protein expressed in cardiomyocytes that associates with caveolin and calmodulin, which are key regulators of excitation-contraction coupling in the heart [52]. An eight base-pair deletion in the striatin gene (STRN) has been proposed as the causal mutation for ARVC in the Boxer dog [52]. More recently, a pedigree-based genetic study found that both affected and unaffected dogs presented the aforementioned variant, and that not all Boxer dogs with ARVC carried this mutation. Nevertheless, the STRN mutation is genetically linked to the responsible gene, located on chromosome 17, which was associated

with Boxer dog ARVC [10]. Further study of this locus is needed to clarify the role of striatin or other genes in this disease.

Ryanodine receptor 2 (RYR2)

Ryanodine receptor 2 (RYR2) is a $Ca^{2+}$ channel that mediates calcium release from the sarcoplasmic reticulum [51]. Patients with RYR2 pathogenic mutations normally present with structurally normal hearts and catecholaminergic polymorphic ventricular tachycardia (CPVT) [37]. Although an association with ARVC was proposed in one Italian family [73], most cardiologists believe that the RYR2 mutated phenotype should be labeled as CPVT and not be considered an ARVC-causing gene. However, a recent genetic screening of ARVC patients without desmosomal gene mutations revealed a 9% prevalence of rare missense RYR2 variants, which is higher than the estimated frequency in controls [65].

Boxer dogs also present a spontaneous form of ARVC associated with a deficiency in calstabin2, a RyR2 modulator [59]. In this animal model, RYR2 expression is decreased in all cardiac chambers as compared with controls. The predisposition to ARVC may reflect a lower baseline concentration of RYR2 protein caused by calstabin2 depletion.

Regulators of desmosomes.

It has been recently observed that deficiency of the inhibitor of apoptosis-stimulating protein p53 (iASPP) caused by spontaneous mutations is associated with a lethal autosomal recessive cardiomyopathy in cattle [70] and mice [32]. Similar to some human ARVC subtypes including Naxos disease, cattle showed woolly hair and mice presented skin abnormalities. iASPP is also a key regulator

of desmosomes and forms a ternary complex with desmoplakin and desmin. To explore why this defect caused cardiomyopathy, Notari *et al.* surveyed the distribution of iASPP in humans and mice [57], and detected expression at the cardiomyocyte ID in post-mortem human donors and in WT mice. They further showed that the presence of desmin, DSP, JUP and N-cadherin at the ID was strikingly decreased in iASPP-deficient mice when compared with WT specimens. However, the localization of β-catenin was preserved and co-precipitation with specific antibodies demonstrated that iASPP interacted more directly with desmin and DSP and had a minimal effect on the remaining desmosomal components. Cardiac magnetic resonance imaging in 12-week-old iASPP-deficient mice revealed biventricular dilation and systolic dysfunction, and histological analysis revealed lipid droplets at the sites of fibrosis, which is consistent with an ARVC phenotype. Moreover, iASPP-deficient mice presented spontaneous non-sustained ventricular tachycardia (VT) and a lower median survival than WT littermates [57]. Cardiomyocytes of humans with ARVC were subsequently analyzed and showed reduction or even loss of iASPP in most of the cases compared with controls. Given these findings, the authors suggested that mutations in the iASPP gene (*PPP1R13L*) may contribute to human ARVC; however, no pathogenic mutations have been reported to date in patients with ARVC. The main findings of each of the aforementioned ARVC mouse models are summarized in Table 2.

**Effects of exercise in ARVC animal models**

Competitive sports have been associated with a worse prognosis in patients with ARVC. When compared with inactive ARVC subjects or those who participate in

recreational sports, athletes with ARVC were found to have a two-fold increased risk for ventricular tachyarrhythmia and death [67]. Furthermore, frequent and endurance exercise has been related to an increased likelihood of an ARVC diagnosis, ventricular arrhythmias and heart failure in desmosomal mutation carriers [34]. The mechanisms by which physical activity promotes disease progression, however, remain elusive.

To investigate whether susceptibility to mechanical cardiac strain contributes to ARVC, Kirchhof *et al.* studied the effects of aging and exercise on heterozygous JUP-deficient mice [36]. At 10 months of age, all heterozygous mice had increased right ventricular volume, reduced right ventricular function, and increased spontaneous ventricular ectopy as compared with WT littermates. Moreover, a subgroup of mice subjected to endurance training (swimming and running on a floating wheel) presented premature right ventricular dilatation and dysfunction at 5 to 6 months of age, while no changes were observed in the trained WT subgroup [36]. Additionally, heterozygous JUP-deficient mice presented a higher incidence of VT than WT mice at the age of 10 months and this occurred 4 months earlier in trained specimens, indicating that endurance training accelerated the development of right ventricular dysfunction. As occurs in patients with ARVC, VT seems to originate from the RV in this model; however, unlike humans, no repolarization abnormalities were observed on ECG and no fibro-fatty replacement was found in the RV in these mice. Further analysis by the same group showed that myocardial concentration of connexin 43 was reduced in mutant mice, possibly explaining the conduction slowing and VT inducibility due to an increase in re-entry substrate [22].

Using a transgenic cardiac-specific overexpression model of a desmoplakin mutation (DSP R2834H), Matherus *et al*. also observed accelerated cardiac remodeling in response to endurance exercise (daily running) [46]. Whereas cardiac function was preserved in all mice, mutant animals presented RV dilation and focal fat infiltration. All sedentary mice with DSP overexpression, both mutant and WT, showed an up-regulation of β-catenin compared with non-transgenic littermates. Upon exercise, non-transgenic mice presented increased levels of nuclear and cytoplasmic β-catenin, whereas cytoplasmic levels decreased in mice overexpressing the WT form of DSP, and both nuclear and cytoplasmic levels decreased in mice overexpressing the R2834H DSP form. This latter group also presented patchy focal interstitial fibrosis in the RV. Furthermore, activation of GSK3-β, a β-catenin regulator, was seen only in mutant mice [46]. Overall, these results point to an impairment in Wnt/β-catenin signaling in exercised mice expressing mutant DSP.

DSP-cKO mice with specific homozygous loss of desmoplakin in cardiomyocytes, described earlier, recapitulate the postnatal disease features of a biventricular form of ARVC [41]. They also present increased susceptibility to exercise and catecholamine-induced arrhythmias, suggesting that exercise can exacerbate the ARVC phenotype. These findings are consistent with the aforementioned observations that ARVC is a common cause of sudden death, especially in young athletes.

The effect of endurance training on cardiac manifestations of ARVC has also been studied in a novel PKP2 R735X dominant-negative mouse model using adeno-associated virus gene delivery. Expression of the mutant protein was induced after a single adeno-associated virus type 9 injection [18], and

endurance exercise training (swimming) was initiated 2 weeks later. After 10 months, trained but not sedentary mice developed RV dysfunction. Moreover, connexin 43 presented an abnormal localization and a punctate distribution at the ID only in exercised-mutant mice.

These results collectively show that murine models reproduce the association between endurance training and ARVC observed in humans: it worsens prognosis by an increased incidence of ventricular arrhythmias and cardiac dysfunction and it accelerates disease progression in healthy mutation carriers.

**Effect of therapies**

Current ARVC treatment is fundamentally palliative and is based on the prevention of SCD by implanting an ICD [14, 16] and on management of heart failure, including heart transplant. The main goals in the evaluation and management of ARVC are early diagnosis, SCD risk stratification, minimizing ventricular arrhythmias, and delaying the progression of disease.

While ICD implantation represents a major advance in human ARVC treatment by preventing SCD [68], implants may cause clinical complications. Indeed, a study carried out on 610 ARVC patients with ICD showed that up to 20% of these individuals have complications, with the most frequent being the difficulty of electrode positioning (18.4%), followed by electrode dysfunction (9.8%), electrode shift (3.3%) and infection (1.4%) [68]. Furthermore, ICD focuses only on SCD prevention or treatment and does not consider the molecular mechanisms of the disease and its progression to heart failure. Thus, animal models represent an excellent tool to improve our understanding of the disease, to test existing therapies and to develop new pathophysiology-based therapies.

Along this line, a heterozygous JUP-deficient mouse model was used to test the usefulness of load-reducing therapy consisting of furosemide and nitrates [22]. Echocardiographic analysis revealed that therapy prevented the increase of the RV area and volume and normalized VT inducibility in JUP mutant mice, suggesting that load-reducing therapy prevents training-induced ARVC. Surprisingly, to the best of our knowledge, no other commonly used drug therapy such as ACE inhibitors or beta-blockers has been published in ARVC animal models thus far.

To generate a screenable model of AC, Asimaki *et al*. used the GAL4/UAS transactivation system to create a cardiac-specific transgenic zebrafish line overexpressing the human 2057del2 mutation in JUP [2]. Transgenic zebrafish developed mild bradycardia, a decreased stroke volume and a reduction in cardiac output within 2 days of fertilization, and cardiomyopathy was fully penetrant at 4–6 weeks, with cardiomegaly, cachexia and peripheral edema. Furthermore, mortality at 80 days was significantly higher in mutant zebrafish than in control animals (45% survival in mutants versus 77% in controls). The zebrafish model was subsequently used to test pharmacological suppressors of the disease phenotype by high-throughput chemical screening. This search identified a GSK3β inhibitor (SB216763) that prevented heart failure and reduced mortality in this model [2]. The same authors tested SB216763 in a DSG2 mutant mouse model and initiated therapy from 3 weeks of age, before the onset of the disease. By 8 weeks of age, treated mutant mice presented a significantly higher LV ejection fraction than untreated mutants, which was similar to values in WT controls [12]. SB216763 therapy also prevented cardiac dysfunction in response to exercise in the same model. Animals were trained to swim from 5 to 16 weeks

of age and treatment with SB216763 improved survival and LV function among mutants. In the same study, mice with a mutation in JUP were also treated with SB216763. Both DSG2 and JUP untreated mutants exhibited focal areas of fibrosis and inflammation that was absent in SB216763-treated equivalent mice. Furthermore, the abnormal localization of GSK3β at the ID in mutant mice was reversed by SB216763 treatment [12] and translocated back to the cytoplasm, its normal location. The same authors showed that neonatal rat ventricular myocytes expressing a JUP or a PKP2 mutation had a normal distribution of ID proteins when GSK3β was knocked down. Overall, these findings support the key role of aberrant GSK3β signaling in ARVC pathogenesis. Indeed, GSK3β inactivation has been shown to be beneficial in other cardiovascular pathological settings, including myocardial infarction [74], ischemia/reperfusion injury [53], and pressure overload [48]. That said, it has to be considered that GSK3β is involved in several cellular processes and that its inactivation affects numerous signaling pathways. GSK3β inhibition may also have beneficial effects in other settings, such as improving glycemic metabolism or inducing apoptosis in cancer [42]. Therefore, the lack of specificity GSK3β inhibitors could result in undesired side effects.

## Conclusions and future perspectives

It is evident that results in animal models, while direct and robust (Table 2), still require complementary studies in human patients with ARVC. In the interim, it is important to characterize the effects of genetic mutations and to attempt to establish associations with clinical phenotypes. In the early stages of the disease, the “hidden phase”, patients are asymptomatic even if they are at high risk for

ventricular arrhythmia and SCD. Thus, the identification of the initial molecular events triggering the disease must be a major aim of future studies.

Other inherited heart diseases, such as hypertrophic cardiomyopathy, have benefited from animal models to create new molecular therapies that are currently being tested in clinical trials [29]. Furthermore, viral-mediated gene therapy has also been proposed to treat other genetically-determined heart diseases. For example, calsequestrin-2 (CASQ2) overexpression in cardiomyocytes was found to improve cardiac function in a mouse model of CPVT [19, 20]. The same authors subsequently showed that the administration of CASQ2 restored the physiological function of calsequestrin-2 protein in human induced pluripotent stem cells derived from patients [40]. These results pave the way for individualized genetic therapies in cardiomyopathies, including ARVC.

To date, the GSK3β inhibitor SB216763 is the only molecule that has been shown to change the course of disease in murine models by enhancing β-catenin signaling. It was initially discovered via chemical drug screening in a zebrafish model, which may represent a powerful platform for future ARVC drug research and should be further explored. The Wnt/β-catenin pathway appears to have a central role in the pathogenesis of ARVC and is a key regulator of myogenesis versus adipogenesisis. As it is specifically blunted in ARVC patients and mouse models, reduced Wnt/β-catenin signaling leads to enhanced adipogenesis, a characteristic feature of ARVC. These emerging concepts need be explored in depth in future research. In this regard, recent data on microRNA profiling in ARVC using functional enrichment analysis have shown a correlation between miR-21-5p and miR-135b and the Wnt pathway [76]. Of interest, these

microRNAs also target Hippo signaling, which interacts with the Wnt/β-catenin pathway, and whose pathogenic activation leads to enhanced adipogenesis in ARVC [13]. These findings may guide the search for new microRNA-based therapeutic targets to modify Wnt/β-catenin and Hippo signaling to improve the outcome of ARVC.

Finally, while ARVC is mainly a disease of desmosomal proteins, future research should explore mechanisms beyond the desmosome that may lead to the identification of curative therapies that target specific pathogenic pathways. Although it is evident that certain aspects of human ARVC remain to be demonstrated in animal models (Figure 1), they represent the best available tools to improve our knowledge of the pathophysiology of human disease and to explore the best strategy for effective treatment. Additionally, animal models have shed light on some other relevant issues, such as the effect of physical activity on accelerating disease progression. These findings are very important for clinically unaffected mutation carriers, as they confirm what has already been observed in humans.

Future research should take into account that many animal models are currently available, and that they are reproducible and offer promising therapeutic solutions. An example of this are the β-catenin activators that have demonstrated a central role for GSK3β in ARVC and could shape future research in this area.

Acknowledgements: We gratefully acknowledge Kenneth McCreath, PhD, for English editing.

| ARVC type | Gene | Location | Recessive form | Model refs | Exercise refs | Treatment refs | Comments |
|---|---|---|---|---|---|---|---|
| ARVC1 | TGFβ3 | 14q24.3 | | | | | |
| ARVC2 | RYR2 | 1q43 | | [31] | | | Causes CPVT. No longer recognized as an ARVC-causing gene. |
| ARVC3 | Unknown | 14q12-q22 | | | | | |
| ARVC4 | TTN | 2q32.1-q32.3 | | | | | Causes DCM. Not uniformly recognized as an ARVC-causing gene |
| ARVC5 | TMEM43 | 3p25.1 | | | | | |
| ARVC6 | Unknown | 10p14-p12 | | | | | |
| ARVC7 | DES | 2q35 | | | | | Can cause DCM or RCM |
| ARVC8 | DSP | 6p24.3 | Carvajal syndrome | [13, 23, 24, 28, 41, 75] | [41, 46] | [57] | |
| ARVC9 | PKP2 | 12p11 | | [11, 30, 54] | [18] | | |
| ARVC10 | DSG2 | 18q12.1 | | [21, 35, 60, 64] | [12] | [12] | |
| ARVC11 | DSC2 | 18q12.1 | | [9, 33, 49] | | | |
| ARVC12 | JUP | 17q21.2 | Naxos disease | [6, 7, 13, 39, 47, 66, 77] | [22, 36] | [12, 22, 77] | |
| ARVC13 | CTNNA3 | 10q21.3 | | | | | |
| - | PLN | 6q22.31 | | | | | Causes DCM. Not uniformly recognized as an ARVC-causing gene |
| - | LMNA | 1q22 | | | | | Only DCM models. Not uniformly recognized as an ARVC-causing gene |

**Table 1. Type, causative mutated gene and gene location associated with human ARVC.** CTNNA3: Catenin Alpha 3; DES: desmin; DSC2: desmocollin 2; DSG2: desmoglein 2; DSP: desmoplakin; DCM: dilated cardiomyopathy; JUP: junction plakoglobin; LMNA: Lamin A/C; RCM: restrictive cardiomyopathy; RYR2: ryanodine receptor 2; TTN: titin; TGFβ3: transforming growth factor beta 3;

TMEM43: transmembrane protein 43; PKP2: plakophilin 2; PLN: Phospholamban. References are listed for the animal model as well as for the effect of exercise in the model and/or a treatment tested. There are no animal models for the ARVC types not having references listed.

| Protein | Gene mutation / defect | What have we learned? | Reference |
|---|---|---|---|
| **Desmoplakin** | DSP-null | • Early embryonic lethality | [23] |
| | $DSP^{R2834H}$ point mutation | • JUP is mislocalized to the nucleus<br>• Interruption of DSP-desmin interaction at intercalated discs<br>• Increased cardiomyocyte apoptosis, cardiac fibrosis, and lipid accumulation<br>• Ventricular enlargement and cardiac dysfunction in both ventricles | [75] |
| | $DSP^{+/-}$ | • Reduced expression of desmoplakin leading to connexin mislocalization<br>• Alterations in conduction-repolarization kinetics prior to morphological changes | [28] |
| | DSP cardiac-specific KO | • Downregulation of connexin 43<br>• Wnt/β-catenin signaling pathway is unimpaired<br>• Ventricular arrhythmias exacerbated by exercise and cathecolamines | [41] |
| | Exons 4-5 deletion | • Cardiomyopathy develops by 8 weeks of age<br>• Absence of DSG2 protein by 16 weeks of age<br>• Abnormal JUP and connexin 43 at myocyte junctions<br>• SB216763 prevents cardiac dysfunction and fibrosis | [12] |
| **Plakophilin 2** | PKP2 null | • Decreased association of cardiac junctional plaques with DSP, JUP and desmosomal cadherins<br>• β-catenin and JUP remain in their correct locations<br>• Heart rupture during cardiac development | [30] |
| | $PKP2^{+/-}$ | • Myocyte sodium current defects<br>• No histological or anatomical differences versus controls | [11] |
| | PKP2 morpholino KO | • Cardiac edema, blood pooling, heart looping failure and decreased heart rate<br>• Abnormal desmosomes | [54] |
| **Desmoglein 2** | DSG2 KO | • Embryonic lethal for all $DSG2^{-/-}$ and some $DSG2^{+/-}$ | [21] |
| | DSG2 overexpression | • Necrosis, inflammatory response and myocardial calcification<br>• No fatty infiltration<br>• Ventricular arrhythmias and sudden death<br>• Cardiac dysfunction, biventricular dilation | [60] |
| | DSG2 overexpression | • *In vivo* interaction between DSG2 and Na(V)1.5 | [64] |

| | | | |
|---|---|---|---|
| | | • Electrical instability before structural changes of the myocardium | |
| | DSG2 cardiac-specific KO | • Space widening in ID and reduction in action potential<br>• Compromised adhesion of cardiomyocytes | [35] |
| **Desmocollin 2** | miR-130a overexpression | • miR-130a represses 80% of DSC2 translation in the myocardium<br>• Biventricular fibrosis and lipid accumulation<br>• Right ventricular dilation, premature ventricular complexes | [49] |
| | DSC2 cardiac overexpression | • Biventricular dysfunction<br>• Cardiac fibrosis, necrosis and calcification<br>• Early inflammatory and fibrotic remodeling pathways up-regulated | [9] |
| | DSC2 (c. 631-2A/G) | • Heterozygous morpholino induced mutation in zebrafish<br>• Desmosome structure disruption<br>• Contraction defects | [33] |
| **Plakoglobin** | JUP null | • Embryonic lethality between days 12 and 16 | [66] |
| | JUP null | • Embryonic lethality from embryonic day 10.5 onward; some die around birth<br>• Partially compensatory mechanisms for JUP and β-catenin<br>• JUP translocates to the nucleus<br>• Suppression of the Wnt/β-catenin signaling pathway<br>• Enhanced adipogenesis | [6] |
| | JUP null | • beta-catenin becomes localized to desmosomes and associated with desmoglein | [7] |
| | JUP cardiomyocyte-specific KO | • Cardiomyopathy develops by 6–8 weeks of age<br>• Loss of cardiac myocytes, inflammatory infiltration, fibrous tissue replacement and cardiac dysfunction<br>• No spontaneous or induced arrhythmias | [39] |
| | Cardiac specific truncated JUP (Myh6:JUP) | • Suppression of the Wnt/β-catenin signaling pathway<br>• Pathogenic activation of the Hippo pathway<br>• Enhanced adipogenesis | [13] |
| | Truncated JUP: 2-base pair deletion (OriNax) or last 5 exons fused (FuseNax) | • Naxos disease models: OriNax and FuseNax<br>• OriNax: low levels of truncated JUP, perinatal lethality without arrhythmogenic cardiomyopathy. Skin and hair abnormalities<br>• FuseNax: WT levels of truncated JUP, no cardiac defect, no hair and skin abnormalities | [77] |
| | $JUP^{2157del2}$ mutation | • Focal areas of inflammation and increased cardiomyocyte apoptosis<br>• Reduced JUP signal at intercalated disks<br>• SB216763 prevents cardiac dysfunction and fibrosis | [12] |
| | JUP-1b morpholino KO | • JUP-1b is the zebrafish transcript variant of human JUP<br>• Cardiac defects similar to DSC2 knockdown in zebrafish or mouse | [47] |
| **Laminin receptor 1** | Lamr1-tp1 encoded retroposon | • Fibrosis limited to the right ventricle, no fat infiltration<br>• ARVC phenotype by 8 weeks of age (100% penetrance) | [1] |
| **Inhibitor of apoptosis-stimulating protein p53 (iASPP)** | iASPP deficiency | • iASPP interacts with DES and DSP, with minimal effects on other desmosomal components<br>• Preserved β-catenin localization<br>• Biventricular dilation and systolic dysfunction by 12 weeks of age | [57] |

**Table 2. Highlights from ARVC animal models published to date**

## Figures

**Figure 1**. Achievements to date in ARVC research and medical management (left) and what remains to be addressed to reach an effective therapy to cure the disease (right).

**Figure 2.** The desmosome (central part) and signaling pathways implicated to date in the progression of ARVC in WT (left) and pathological ARVC (right and shaded) conditions. Activating (black) and inactivating (red) phosphorylations. Upon exercise, JUP can translocate to the nucleus. When DSP is altered in ARVC, JUP is either translocated to the nucleus, inhibiting Wnt/β-catenin pathway, or sequestered at the cell membrane with DSP. Frz: Frizzled receptor of Wnt. Arrows, translocation to the nucleus; IS: intercellular space; PM: plasma membrane; β-cat: β-catenin; desmin (medium green); DSC2: desmocollin 2 (blue); DSG2: desmoglein 2 (red); DSP: desmoplakin (yellow); Frz: Frizzled receptor; JUP: junction plakoglobin (light grey); PKP2: plakophilin 2 (dark grey); TCF/LEF: T-cell factor/lymphoid enhancer factor.

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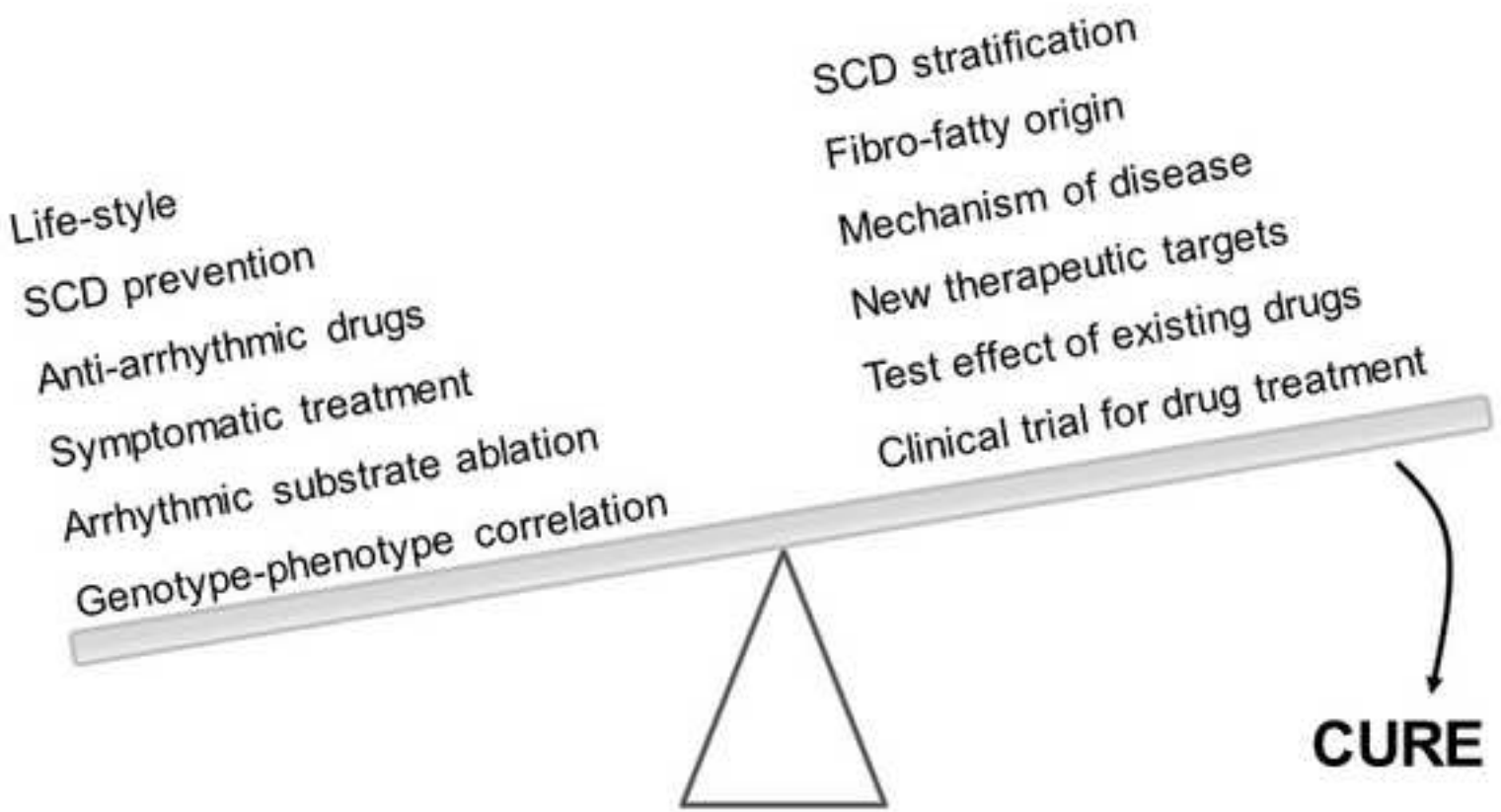


**Figure 1.**

Figure 2 revised

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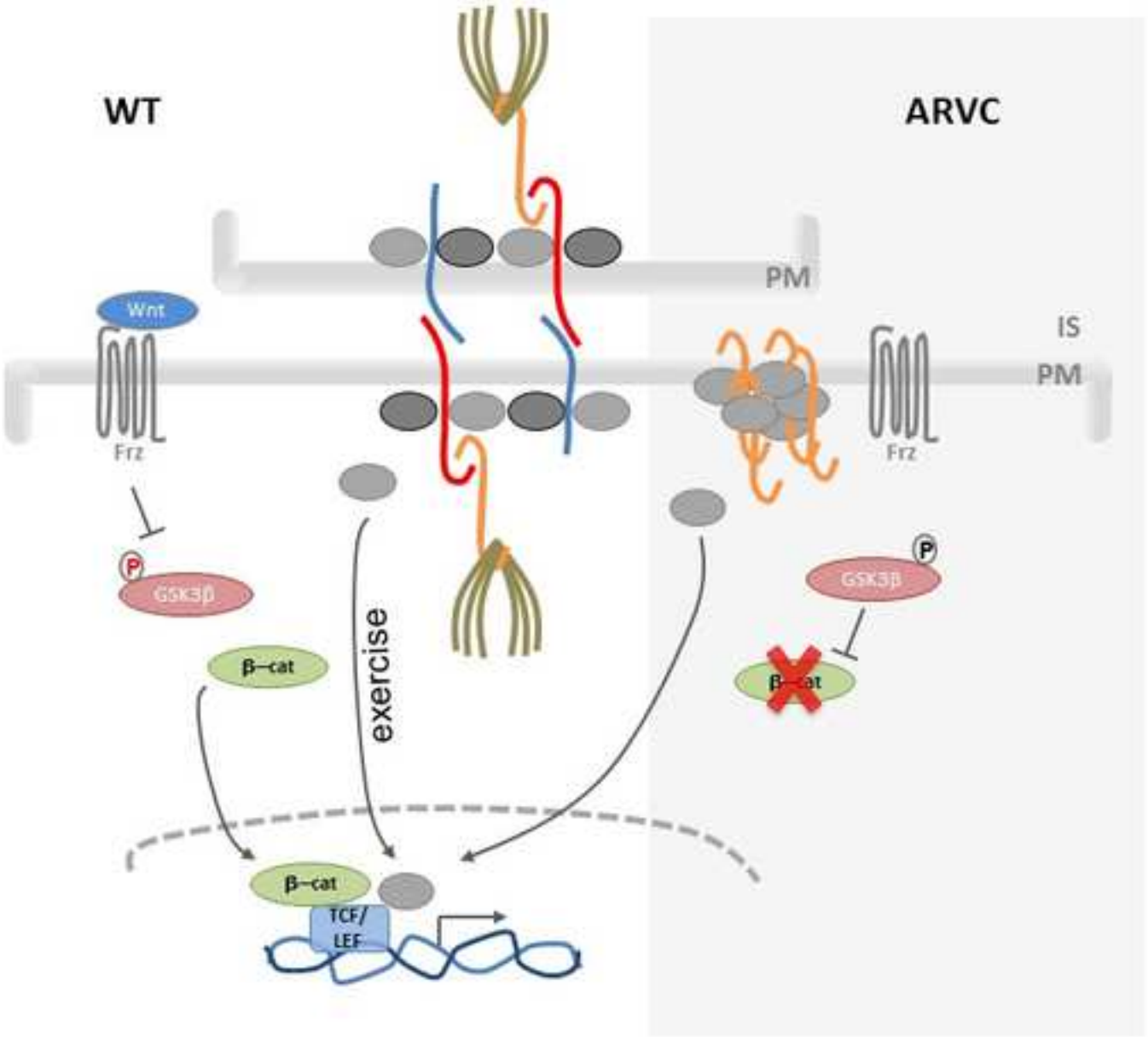


**Figure 2.** DSG2 DSP DSC2 Desmin PKP2 JUP