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Identification of a New Modulator of the Intercalated Disc in a Zebrafish Model of Arrhythmogenic Cardiomyopathy

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Abstract

Arrhythmogenic cardiomyopathy (ACM) is characterized by frequent cardiac arrhythmias. To elucidate the underlying mechanisms and discover potential chemical modifiers, we created a zebrafish model of ACM with cardiac myocyte-specific expression of the human 2057del2 mutation in the gene encoding plakoglobin. A high-throughput screen identified SB216763 as a suppressor of the disease phenotype. Early SB216763 therapy prevented heart failure and reduced

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Competing interests: A patent application has been filed for the use of SB216763 in ACM and related arrhythmia syndromes.

SUPPLEMENTARY MATERIALS

www.sciencetranslationalmedicine.org/cgi/content/full/6/240/240ra74/DC1 Materials and Methods

References (36, 37)

mortality in the fish model. Zebrafish ventricular myocytes that expressed 2057del2 plakoglobin exhibited 70 to 80% reductions in I_{Na} and I_{K1} current densities, which were normalized by SB216763. Neonatal rat ventricular myocytes that expressed 2057del2 plakoglobin recapitulated pathobiological features seen in patients with ACM, all of which were reversed or prevented by SB216763. The reverse remodeling observed with SB216763 involved marked subcellular redistribution of plakoglobin, connexin 43, and Nav1.5, but without changes in their total cellular content, implicating a defect in protein trafficking to intercalated discs. In further support of this mechanism, we observed SB216763-reversible, abnormal subcellular distribution of SAP97 (a protein known to mediate forward trafficking of Nav1.5 and Kir2.1) in rat cardiac myocytes expressing 2057del2 plakoglobin and in cardiac myocytes derived from induced pluripotent stem cells from two ACM probands with plakophilin-2 mutations. These observations pinpoint aberrant trafficking of intercalated disc proteins as a central mechanism in ACM myocyte injury and electrical abnormalities.

INTRODUCTION

Arrhythmogenic cardiomyopathy (ACM) is a highly arrhythmic form of human heart disease and a significant cause of sudden death in the young (1, 2). First described as an isolated right ventricular disease (arrhythmogenic right ventricular cardiomyopathy), ACM is now recognized to include biventricular and left dominant forms, which may present as dilated cardiomyopathy or myocarditis (3). Degeneration of cardiac myocytes and replacement by fibrofatty scar tissue develop with disease progression, but arrhythmias are often the earliest feature of ACM, and typically precede structural remodeling of the myocardium (1, 2).

ACM is caused by mutations in genes that encode proteins in the desmosome, a specialized intercellular-junction complex (1, 2). Desmosomes reside within the intercalated disc, along with fascia adherens junctions and gap junctions, which connect cardiac myocytes and synchronize mechanical and electrical functions in the heart. Abnormal localization of intercalated disc proteins including the desmosomal protein plakoglobin (γ -catenin), the gap junction protein connexin 43 (Cx43), and the sodium channel protein Nav1.5 has been observed in patients with ACM and in experimental disease models (4–8). It has been proposed that ACM develops as a consequence of altered canonical Wnt signaling mediated by the mutant desmosomal proteins (9), but the extent to which ACM pathways perturb the constitution of junctional macromolecular assemblies as well as the downstream mechanisms of myocyte injury and arrhythmogenesis remain obscure.

Homozygous null mutations in the genes that cause ACM are embryonic lethal in mice (10–12), and heterozygous null alleles for plakoglobin or plakophilin-2 cause little if any phenotype (10, 12). Although heterozygous cardiac-restricted deficiency of desmoplakin expression causes ACM-like features (9), the molecular mechanisms by which human germline mutant alleles cause ACM are unknown for all of the ACM-related genes described to date. In previous work, we transiently modeled specific splice mutants using morpholinos in the zebrafish and demonstrated the ability to identify developmental regulators and recapitulate the pathobiology of heart disease (13, 14). Now, to efficiently

explore ACM pathophysiology, we developed a stable inducible transgenic zebrafish model capable of propagation through multiple generations and amenable to high-throughput genetic or chemical screening. We used the GAL4/UAS transactivation system to drive cardiac myocyte-specific expression of the human 2057del2 (2-base pair deletion) mutation in the gene encoding the desmosomal protein plakoglobin (γ -catenin). This mutation causes Naxos disease in patients, a highly penetrant form of ACM also associated with changes in hair and skin (15, 16). Transgenic fish expressing this mutation develop abnormal cardiac physiology within 48 hours of fertilization with subsequent progression, within 4 to 6 weeks, to a fully penetrant cardiomyopathy characterized by cardiomegaly, cachexia, peripheral edema, and, eventually, death as a result of heart failure or arrhythmia.

To optimize this zebrafish line for high-throughput screening, we introduced a previously described natriuretic peptide b (nppb)::luciferase reporter line (17) onto the ACM mutant background. We screened a library of bioactive compounds for disease modifiers and identified three compounds that suppressed the disease phenotype. One such suppressor, SB216763, previously annotated as an activator of canonical Wnt signaling (18), reduced nppb expression, prevented bradycardia and contractility defects, and increased survival in mutant fish. Characterization of the electrophysiology of zebrafish ventricular myocytes and the pathobiology of neonatal rat ventricular myocytes that expressed 2057del2 plakoglobin revealed that arrhythmias and myocyte injury in ACM are related to a common mechanism involving abnormal trafficking of key proteins to the intercalated disc mediated by the PDZ domain-containing protein SAP97. Multiple, complex features of the disease phenotype in both fish and rat myocytes were rapidly corrected by SB216763. Similar findings were observed in cardiac myocytes derived from human induced pluripotent stem cells (hiPSCs) from two ACM disease probands with mutations in the gene encoding plakophilin-2. These observations suggest that it will be possible to define ACM pathophysiology and develop new disease-modifying agents using SB216763 and its derivatives.

RESULTS

Generating a screenable zebrafish model of ACM

To study the effects of desmosomal mutations in a screenable system, we used the GAL4/UAS transactivation system to create zebrafish with cardiac myocyte-specific expression of the 2057del2 mutation in the human gene encoding plakoglobin (fig. S1A). Heart enlargement (cardiomegaly) with marked thinning of atrial and ventricular walls, cachexia (wasting), and peripheral edema were evident by early adulthood (4 to 6 weeks of age) (Fig. 1, A and B). Mutant fish exhibited substantial mortality (45% survival in mutants versus 77% in controls; $n = 125$; $P < 0.0001$, Mantel-Cox test) (Fig. 1C). The heart weight-to-body length ratio was increased at 3 months (Fig. 1D). Transmission electron microscopy (EM) showed interruptions in cell boundaries and structural disarray in mutant hearts compared to control siblings (fig. S1, B and C). An increase in glycogen granules in zebrafish cardiac myocytes was seen with EM, and there was a significant increase in total myocardial glycogen content measured in the 2057del2 plakoglobin zebrafish model ($n = 5$; $P < 0.05$, unpaired t test) (fig. S1D).

To determine the feasibility of high-throughput screening for a phenotype in 96-well plates at an early embryonic stage, we explored the phenotypes in larval mutant fish. By 48 hours after fertilization (late larval stage), mutant embryos exhibited a clear phenotype with mild bradycardia (144.2 ± 10.8 beats/min in control versus 120.8 ± 12.6 beats/min in mutant; $n = 50$; $P < 0.05$, unpaired t test), decreased stroke volume (0.31 ± 0.06 nl in control versus 0.17 ± 0.05 nl in mutant; $n = 8$; $P < 0.05$), and reduced cardiac output (42.8 ± 8.6 nl/min in control versus 20.5 ± 6.3 nl/min in mutant; $n = 12$; $P < 0.05$, unpaired t test) (Fig. 1, E to G).

Identification of disease modifiers in zebrafish via high-throughput chemical screening

To optimize this zebrafish line for high-throughput screening, we introduced a previously described *nppb::luciferase* reporter line (17) onto the ACM mutant background (fig. S1E). Using qRT-PCR, we first demonstrated that the ACM mutant fish exhibited a significant induction (about twofold; $n = 3$; $P < 0.01$, unpaired t test) of native *nppb* transcription at 48 hours after fertilization (Fig. 1H). We confirmed that the *nppb::luciferase* reporter was also induced on the ACM background when crossed with fish that expressed the *cmhc2::GAL4* driver construct (201.2 ± 14.4 luciferase units per ACM mutant fish versus 117.8 ± 11.9 luciferase units per wild-type fish; $n = 30$ fish in each group; $P < 0.01$, unpaired t test) (Fig. 1I).

Once we had defined the baseline for the larval model of ACM, we began to screen a chemical library for modifiers of the *nppb::luciferase* phenotype (fig. S1F). We anticipated that toxic compounds would lead to very high or very low levels of *nppb::luciferase* activity (stress or death, respectively) depending on the relative timing of the drug's effect with respect to the assay schedule. Therefore, we designed our screen to identify compounds that normalize *nppb::luciferase* activity with tandem secondary screens that confirmed the effects of potential rescue compounds on cardiac physiology directly and also assessed more subtle forms of toxicity (14). To minimize false positives, we prespecified assays in duplicate, and only those compounds in which *nppb::luciferase* activity was within 1 SD of the normal range in both instances were considered potential positives. This approach identified more than 50 first-round "hits" in a screen of 4200 small molecules, all of which were followed up with additional testing in large numbers of embryos ($n > 50$) for confirmation. Subsequent retesting and secondary assays restricted the initial number to three compounds of which SB216763 has the largest body of extant data (18). The other two compounds were given lower priority because of inadequate data on their use in mammals. SB216763 at 3 μ M in the well between 48 and 72 hours after fertilization normalized *nppb::luciferase* activity at 72 hours after fertilization ($n = 36$; $P < 0.001$, unpaired t test) (Fig. 1J), and longer-term treatment of larval fish (7 days) led to substantially increased survival at 3 months ($n = 300$; $P < 0.01$, unpaired t test) (Fig. 1K).

Cellular electrophysiology of 2057del2 plakoglobin zebrafish ventricular myocytes

We next assessed the effects of SB216763 on cellular electrophysiology in zebrafish myocytes that expressed 2057del2 plakoglobin. Marked changes in action potential morphology were observed in myocytes obtained from mutant fish at 5 to 7 weeks after fertilization compared with myocytes from control fish (either wild type or those expressing the mutant 2057del2 construct but without the *GAL4* driver to elicit transgene expression)

(Fig. 2, A and B, and Table 1). Resting membrane potential (RMP) was significantly depolarized in cells expressing 2057del2 plakoglobin compared with controls (-69 ± 1 mV versus -79 ± 1 mV, respectively; $n = 11$; $P < 0.001$, unpaired t test). The maximum rate of rise in voltage during phase 0 of the action potential, $dV/dt_{\max}$, was markedly blunted (18 ± 2 V/s versus 63 ± 11 V/s; $n=10$; $P < 0.001$, unpaired t test), and action potential duration at 80% completion of repolarization ($APD_{80\%}$) was prolonged (319 ± 44 ms versus 240 ± 24 ms; $n=11$; $P < 0.05$, unpaired t test). Nearly identical changes in action potential morphology were observed in myocytes isolated from fish 3 to 4 weeks after fertilization, indicating no major progression in the action potential phenotype between 3 and 7 weeks of age (Table 1). Furthermore, virtually identical changes in action potential morphology (positive shift in RMP, reduction of $dV/dt_{\max}$, and prolongation of $APD_{80\%}$) were observed in neonatal rat ventricular myocytes expressing 2057del2 plakoglobin (Fig. 2C). These observations suggest that the action potential phenotype observed in the fish model reflects critical features of disease exhibited by mammalian ventricular myocytes expressing a known human ACM disease gene and, presumably, by ventricular myocytes in patients with ACM.

The marked reduction in $dV/dt_{\max}$ in phase 0 of the action potential prompted us to characterize potential changes in the sodium current, I_{Na} , in zebrafish ventricular myocytes expressing 2057del2 plakoglobin. Using whole-cell voltage-clamp assays and starting from a holding potential of -80 mV, we observed a large reduction in I_{Na} current density across a broad range of membrane potentials (Fig. 3, A and B, and Table 2). Peak I_{Na} current density was reduced by 84%, from 196 ± 22 pA/pF in control cells ($n = 18$) to 31 ± 3 pA/pF ($n = 16$; $P < 0.001$, unpaired t test) in myocytes expressing 2057del2 plakoglobin isolated from fish at 5 to 7 weeks of age. Similar reductions (76%) were observed in myocytes isolated from fish at 3 to 4 weeks of age (Table 2). No significant shift was seen in steady-state activation or inactivation of I_{Na} curves (Fig. 3C). Thus, the marked reduction in I_{Na} current density in fish myocytes expressing 2057del2 plakoglobin appeared to result solely from a reduction in the number of functional channels at the sarcolemma.

The lower depolarized RMP observed in myocytes that expressed mutant plakoglobin relative to controls raised the possibility of alterations in the inward rectifier current, I_{K1} , known to be important in maintaining normal resting potential. Indeed, we observed a similar marked decrease (71%) in I_{K1} current density at -100 mV (from 17.0 ± 2.7 to 5 ± 0.8 pA/pF; $n = 8$ and 13 , respectively; $P < 0.001$, unpaired t test) in cells expressing 2057del2 plakoglobin (Fig. 3, D to F, and Table 2). However, no difference was observed between control myocytes and myocytes expressing 2057del2 plakoglobin in the density of I_{Kr} , a major current responsible for phase 3 repolarization of the action potential (fig. S2 and Table 2). Thus, some, but not all, ionic currents are reduced in zebrafish ventricular myocytes that express 2057del2 plakoglobin.

Reversal of electrophysiological derangements by SB216763 in cells expressing 2057del2 plakoglobin

To determine whether SB216763 affects the action potential remodeling and changes in I_{Na} and I_{K1} observed in 2057del2 plakoglobin zebrafish ventricular myocytes, we characterized

the cellular electrophysiology in isolated myocytes exposed to SB216763 at two different developmental stages. In these experiments, we used a concentration of SB216763 (5 μ M) similar to that found previously in the chemical screen to reverse the disease phenotype. Vehicle controls (DMSO) were also analyzed and showed no effect. In the first protocol, zebrafish embryos at 1 day after fertilization were treated with SB216763 for 6 days and then maintained under normal conditions (in the absence of SB216763) until 5 to 7 weeks after fertilization. At this time, ventricular myocytes were isolated and studied by current-clamp and whole-cell voltage-clamp methods. SB216763 did not affect action potentials in myocytes from control fish, whereas it normalized RMP and $dV/dt_{\max}$, and actually shortened APD_{80%} beyond control levels in fish expressing mutant plakoglobin. Similarly, early exposure to SB216763 had no effect on I_{Na} or I_{K1} current densities in control myocytes, but it almost completely normalized these currents in cells expressing 2057del2 plakoglobin (Table 2). Thus, transient exposure to SB216763 during embryonic and larval development durably prevents action potential remodeling in fish expressing mutant plakoglobin even as adults.

To determine whether SB216763 can reverse these changes once they occur, we performed a second protocol in which isolated ventricular myocytes were prepared from transgenic fish at 5 to 7 weeks after fertilization (when the action potential phenotype is fully developed) and then treated in culture with SB216763 for 36 hours before being analyzed. As shown in Fig. 4 and Tables 1 and 2, short-term exposure of isolated cells to SB216763 normalized action potential remodeling and reversed reductions in I_{Na} and I_{K1} current densities. The fact that these changes can be normalized in isolated cells *in vitro* indicates that the marked cardiac myocyte electrical phenotype produced by expression of an ACM disease gene is cell-autonomous. Moreover, the electrical phenotype can apparently be prevented from occurring in adult fish when early embryos are transiently exposed to SB216763. Once the phenotype becomes fully developed in adult fish, it can be reversed by briefly exposing cells to SB216763.

Recapitulation of ACM cellular features in an *in vitro* mammalian model

Previous studies of human myocardium have identified four features that appear to play a role in the pathogenesis of ACM in patients: (i) decreased immunoreactive signal for plakoglobin at cell-cell junctions (4); (ii) gap junction remodeling indicated by decreased immunoreactive signal for the major ventricular gap junction protein, Cx43, at cell-cell junctions (4); (iii) myocardial apoptosis (19); and (iv) high circulating levels of proinflammatory cytokines and expression of cytokines by cardiac myocytes (20). To gain insights into mechanisms responsible for these features of ACM in patients, we developed an *in vitro* model in which normal neonatal rat ventricular myocytes were transfected with adenovirus to express 2057del2 plakoglobin containing a V5 epitope tag. We identified conditions yielding >90% transfection [assessed using adenovirus containing green fluorescent protein (GFP)] and expression of 2057del2 plakoglobin at levels roughly equivalent to that of the endogenous normal protein (Fig. 5, A and B). We then assessed the effects of transgene expression on the development of features identified in the human disease.

As illustrated in Fig. 5 (C to E), expression of 2057del2 plakoglobin for 24 hours led to (i) a marked change in the distribution of immunoreactive signal for plakoglobin with diminished signal at cell-cell junctions and abundant signals in cell nuclei; (ii) greatly reduced immunoreactive signal for Cx43 at cell-cell junctions; and (iii) increased myocyte apoptosis, as indicated by greater numbers of TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling)-positive nuclei and increased expression of caspase-3. Apoptosis in cells expressing 2057del2 plakoglobin could be prevented by treatment of cells with pifithrin-A (5 μ M), which inhibits p53 transcriptional activity, or greatly increased by subjecting cells to brief intervals of uniaxial cyclical stretch (Fig. 5F). Finally, cells transfected to express 2057del2 plakoglobin for 24 hours secreted various inflammatory mediators into the culture medium including IL-6, TNF- α , MIP-1 α , and the chemokine RANTES (Fig. 5G), several of which have been identified in the blood or myocardium of patients with ACM (20). Together, these results show that expression of 2057del2 plakoglobin by neonatal rat ventricular myocytes recapitulates features consistently identified in the hearts of patients with ACM.

Reversal of disease features by SB216763 in mammalian myocytes expressing 2057del2 plakoglobin

To determine whether SB216763 is capable of mitigating the effects of 2057del2 plakoglobin expression in neonatal rat ventricular myocytes, we transfected cells and then, 24 hours later, exposed them to SB216763. As illustrated in Fig. 6A, SB216763 prevented the marked change in subcellular distribution of plakoglobin and the accumulation of nuclear signal seen in cells expressing mutant plakoglobin. The drug also prevented adverse remodeling of gap junctions as indicated by the presence of control levels of junctional Cx43 signals in treated cells, and it increased the amount of Cx43 signal at cell-cell junctions in control (nontransfected) cultures (Fig. 6A). Most of the Cx43 signals in control cells and in SB216763-treated cells expressing 2057del2 plakoglobin were localized at the cell surface as shown by double-label (Cx43 and N-cadherin) immunohistochemistry (fig. S3). The marked changes in the distribution of immunoreactive signals for plakoglobin and Cx43 and the nearly complete reversal of these changes in cells expressing 2057del2 plakoglobin occurred with no apparent change in the total cellular content of plakoglobin and a modest increase in the total cellular content of Cx43 (in both control and transfected myocytes) as assessed by immunoblotting (Fig. 6B).

These observations, coupled with the temporal course with which these proteins changed their subcellular distribution, suggest that 2057del2 disturbs the normal trafficking of plakoglobin and Cx43 to the cell surface, and that SB216763 corrects this defect. SB216763 also markedly reduced the number of TUNEL-positive nuclei in myocytes expressing mutant plakoglobin (Fig. 6C). Finally, there was a clear reduction in cytokine levels in media recovered from cells exposed to 5 μ M SB216763 for 48 hours (Fig. 6D). An even greater diversity of cytokines was identified in the culture medium from cells expressing 2057del2 plakoglobin for 48 hours versus 24 hours (compare Figs. 5G and 6D), but in each case, these were all reduced by SB216763. Together, these observations indicate that changes in the distribution of critical junctional proteins, expression of inflammatory

markers of cell injury, and apoptosis of cardiac myocytes are all mediated by a common disease pathway that can be ameliorated by SB216763.

To determine whether the cellular electrophysiology phenotype seen in zebrafish myocytes expressing 2057del2 plakoglobin also occurs in mammalian myocytes expressing the same mutant protein, we measured I_{Na} and I_{K1} current density across a broad range of membrane potentials in isolated neonatal rat ventricular myocytes. As shown in fig. S4, I_{Na} and I_{K1} current densities were reduced in myocytes expressing 2057del2 plakoglobin compared to control cells. SB216763 also normalized the reduced current densities in cells expressing mutant plakoglobin (fig. S4).

Effects of 2057del2 plakoglobin on expression and distribution of SAP97 and Nav1.5

PDZ domains secure signaling complexes and anchor membrane proteins to the cell cytoskeleton. The PDZ domain-containing protein synapse-associated protein-97 (SAP97) has been shown to regulate normal targeting, to the cell surface, of Nav1.5 and Kir2.1 (21), the major protein subunits responsible for I_{Na} and I_{K1} , respectively. To determine whether derangements in this trafficking pathway might account for the rapidly reversible reductions in I_{Na} and I_{K1} observed in myocytes expressing mutant plakoglobin, we used immunohistochemistry and immunoblotting to characterize the distribution and total cellular content of SAP97 in neonatal rat ventricular myocytes transfected to express 2057del2 plakoglobin. As shown in Fig. 7, immunoreactive signals for SAP97 were concentrated at the cell surface in control myocytes, similar to the previously reported localization of this protein to intercalated discs in adult rat ventricular myocardium (22). However, there was a marked decrease in the amount of SAP97 signal at the cell surface in myocytes expressing 2057del2 plakoglobin, which was fully reversed by exposing myocytes to SB216763 for 24 hours (Fig. 7). These rapidly reversible changes in subcellular distribution of SAP97 immunoreactive signal occurred without apparent changes in the total amount of SAP97 within the myocytes as shown by Western blotting (Fig. 7). Together with the previous electrophysiological studies, these results suggest that defective trafficking of ion channel proteins to the cell membrane underlies action potential remodeling and reduced altered I_{Na} and I_{K1} current densities in ventricular myocytes that express 2057del2 plakoglobin.

To provide independent evidence in support of this mechanism, we characterized the distribution and total cellular content of Nav1.5 in neonatal rat myocytes. As shown in Fig. 7, there was a marked reduction in immunoreactive signal for Nav1.5 in myocytes expressing mutant plakoglobin, and this was reversed in cells exposed to SB216763 for 24 hours. Nav1.5 signals appeared to be distributed both intracellularly and at the cell surface in control neonatal rat myocytes and in SB216763-treated myocytes expressing 2057del2 plakoglobin. Attempts to quantify the amount of Nav1.5 signal at the cell surface using double-label immunohistochemistry were technically difficult because of high background signal. However, the marked reduction in I_{Na} in both zebrafish and rat myocytes expressing mutant plakoglobin and its rapid, near-total recovery after exposure to SB216763 suggest a similar reversible loss of cell surface Nav1.5 in mammalian myocytes expressing 2057del2 plakoglobin.

Despite the marked changes in the apparent distribution of Nav1.5 immunoreactive signal in neonatal rat ventricular myocytes, there was no difference in the total content of Nav1.5 protein, as assessed by immunoblotting in control cells or cells expressing mutant plakoglobin (Fig. 7). These observations provide additional independent evidence that changes in cellular electrophysiology in ACM are related to defective forward trafficking of key ion channel proteins rather than to insufficient channel protein production. They also suggest that a common disease pathway, sensitive to the mitigating effects of SB216763, underlies both myocyte injury and apoptosis as well as arrhythmias in ACM.

To further investigate the role of SAP97 in ACM, we used short hairpin RNA (shRNA) to knock down SAP97 expression in normal neonatal rat ventricular myocytes and characterized the effects on the distribution of key intercalated disc proteins. As shown in Fig. 8, knockdown of SAP97 expression (demonstrated by greatly reduced SAP97 immunosignal in treated cells) led to marked reduction in immunosignal not only for Nav1.5, as previously shown (21), but also for plakoglobin. There was no effect, however, on the distribution of signals for other desmosomal proteins such as desmoplakin or plakophilin-2 (Fig. 8). This suggests a unique relationship between plakoglobin and SAP97 not shared by other desmosomal linker proteins. Finally, knockdown of SAP97 expression had no effect on the distribution of Cx43, whose trafficking is known to involve a different transport mechanism (22).

Abnormal distribution of SAP97 in myocardium from patients with ACM

To determine whether insights into disease mechanisms gained from analysis of zebrafish myocytes and confirmed in mammalian myocytes *in vitro* apply to human cells affected by the disease, we immunostained sections of formalin-fixed, paraffin-embedded myocardium from patients with ACM using anti-SAP97 antibodies. We analyzed tissue from four control subjects (noncardiac deaths) and eight patients with documented ACM including two each with mutations in plakophilin-2 or desmoplakin, one each with mutations in plakoglobin or desmoglein2, and two patients with clinical expression of disease but no apparent desmosomal gene mutation. Control human myocardium showed immunoreactive signals concentrated at intercalated discs and in a sarcomeric pattern, identical to those shown previously in adult rat ventricular myocardium (representative samples shown in fig. S5) (21). By contrast, a marked reduction in SAP97 signal was seen in the ventricular myocardium of patients with ACM independent of the specific mutation involved in causing their disease. Attempts to analyze the distribution of Nav1.5 in patient material were unsuccessful because available antibodies did not work in formalin-fixed, paraffin-embedded tissues. To determine whether the reduced SAP97 signal was specific for ACM, we stained 15 myocardial samples from patients with end-stage ischemic, dilated, or hypertrophic cardiomyopathy. The myocardium in these other forms of human heart disease showed near-control levels of SAP97 signals in a sarcomeric distribution, although there did seem to be modest relative loss of signals at intercalated discs (representative samples are shown in fig. S5). Thus, SAP97 immunoreactive signals appear to be preferentially decreased in the hearts of patients with ACM compared to other forms of heart disease.

Recognizing the limitations of immunofluorescence in formalin-fixed, paraffin-embedded sections of human myocardium, we performed additional studies in cardiac myocytes derived from hiPSCs generated from peripheral blood mononuclear cells obtained from two ACM probands, each with distinct truncating mutations in plakophilin-2 (Q617X and 2013delC). Cardiac myocytes derived from hiPSCs of unaffected (mutation-negative) siblings of each proband were used as controls. Methods of blood cell reprogramming and cardiac myocyte differentiation are included in the Supplementary Materials and depicted in fig. S6. As shown in Fig. 9, cardiac myocytes from probands in both families exhibited marked reduction in immunoreactive signals for plakoglobin, Cx43, SAP97, and Nav1.5 when compared to cardiac myocytes from unaffected siblings. These abnormalities were reversed in both proband myocyte lines after exposing cells to SB216763 for 24 hours. Together with observations in patient myocardium (fig. S5), these results further implicate aberrant SAP97 distribution in ACM, and validate our observations in both fish and neonatal rat models of ACM.

DISCUSSION

ACM is a deadly disease for which no mechanism-based therapies exist (1, 2). To understand why it is so arrhythmogenic and to identify potential drug candidates, we developed a screenable model of ACM in zebrafish and identified a small molecule that rescues the disease phenotype and reduces mortality. We also identified marked abnormalities in action potentials and ionic currents in fish ventricular myocytes that likely contribute to arrhythmogenesis in ACM. Armed with information from studies in fish, we then confirmed and extended these observations in studies of mammalian ventricular myocytes to show that myocyte injury and arrhythmogenesis are linked to a common disease pathway. Insights gained from studies in fish and rat myocytes ultimately led to the identification of SAP97 as a central component of the ACM disease mechanism. These results provide the foundation for future chemical biology efforts focused on preventing or reversing aberrations in this disease mechanism to limit degenerative changes in the myocardium and reduce the risk of sudden death.

The genetics of ACM are complex. Most cases are autosomal dominant, and many are a consequence of mutations in desmosomal protein genes (1–3). However, penetrance is usually highly variable, and disease expression can vary widely even within individual families. Recessive forms of ACM exist for some disease genes. In such cases, the phenotype may be more severe and more fully penetrant, and include hair and skin components. True null alleles in desmosomal protein genes have not been shown to cause ACM in patients. Available evidence indicates that at least some mutant proteins are expressed in patients with ACM, and multiple murine models show that homozygous null alleles in these same genes are embryonic lethal (10–12). This is certainly the case in Naxos disease in which the mutant gene product is abundantly expressed in the myocardium (23). To allow in vivo screening at scale, we chose to model a bona fide human allele in transgenic form. This sensitized system not only faithfully recapitulates key features of the human disease in both the zebrafish and mammalian cells but also offers proof of concept for chemical suppression of a structurally abnormal junctional protein.

Normal neonatal rat ventricular myocytes respond to brief intervals of cyclical stretch by rapidly increasing levels of immunoreactive signals for proteins at cell-cell junctions including plakoglobin and Cx43 (24). By contrast, myocytes expressing 2057del2 plakoglobin fail to increase cell-cell junction proteins in response to stretch. Rather, they exhibit greatly increased rates of apoptosis after being stretched, whereas control cells show no appreciable apoptosis. These observations are consistent with clinical studies showing that exercise may increase age-related penetrance and arrhythmic risk in ACM patients (25). They also suggest that zebrafish and mammalian myocyte models involving transgenic expression of 2057del2 plakoglobin may be useful in future studies to define mechanisms responsible for exercise-induced disease exacerbations in ACM patients.

Reduced I_{Na} current density has been observed previously in neonatal rat myocytes after knockdown of plakophilin-2 (6) and in a transgenic mouse model with cardiac myocyte expression of mutant desmoglein-2 (7). Our observations provide evidence in both fish and neonatal rat myocyte models that reduced I_{Na} current density is attributable largely, if not entirely, to a reduced number of functional channels at the cell surface rather than to changes in the activation and inactivation kinetics. This conclusion is supported by reduced immunosignals for Nav1.5 in neonatal rat myocytes expressing mutant plakoglobin, an observation we have also reported in patients with ACM (8). Our finding of reduced I_{K1} current density implicates another potentially highly arrhythmogenic electrophysiological defect in ACM and led to discovery of aberrant SAP97 distribution in mutant plakoglobin-expressing myocytes and in ACM patients whose disease is linked to one of several mutations in desmosomal genes. This observation is intriguing because SAP97 associates with Nav1.5 (26) and regulates forward trafficking of Nav1.5 and Kir2.1 to the cell surface (21). These proteins form channels responsible for I_{Na} and I_{K1} , respectively, both of which we found were markedly reduced in fish and neonatal rat myocytes expressing 2057del2 plakoglobin. However, we observed no such reduction in I_{Kr} current density, which is formed by a channel whose trafficking is not associated with SAP97 (27). An unanticipated observation was abnormal distribution of plakoglobin (but not other desmosomal linker proteins, N-cadherin, or Cx43) in normal neonatal rat myocytes after knockdown of SAP97 expression. Together, these observations strongly implicate altered trafficking of Nav1.5, Kir2.1, and plakoglobin as a critical component of the ACM disease mechanism.

SB216763 exhibits a remarkable ability to prevent or reverse features of the disease phenotype in zebrafish and rat models of ACM induced by expression of 2057del2 plakoglobin, and also in human cardiac myocytes derived from iPSCs in two probands with ACM due to mutations in the gene encoding plakophilin-2. SB216763 is thought to activate the canonical Wnt signaling pathway by inhibiting GSK-3 β (glycogen synthase kinase-3 β) (18), although this effect may not be entirely responsible for rescuing the disease phenotype. Increasing evidence from mouse models of ACM has implicated changes in Wnt signaling in disease pathogenesis (9), but the underlying mechanisms are poorly understood and there has been little, if any, direct evidence to prove a causal relationship. We observed increased Cx43 expression in both control myocytes and 2057del2 plakoglobin-expressing cells exposed to SB216763, consistent with previous studies showing that Cx43 gene expression can be up-regulated by activation of the canonical Wnt signaling pathway (28). Our

observations provide strong evidence for a unifying trafficking abnormality in ACM that can account for diverse features of the disease including loss of plakoglobin from junctions, remodeling of gap junctions, myocyte injury and apoptosis, and action potential remodeling.

Transient exposure to SB216763 of early zebrafish embryos undergoing cardiogenesis produced lasting effects that prevent action potential remodeling. Although we have not defined the precise mechanism, we note that excessive mortality in fish with cardiac myocyte-specific expression of 2057del2 plakoglobin occurs within the first 10 days of development and subsequently levels off thereafter to control rates (Fig. 1C). These observations imply that blocking of an early effect of the disease pathway during heart maturation might prevent subsequent action potential remodeling. Yet, even after becoming fully manifest, action potential remodeling was rescued by SB216763 in both zebrafish and rat myocytes, indicating an ongoing reversibility of the disease.

MATERIALS AND METHODS

Rationale and study design

The overall objective of this study was to create a model of ACM in zebrafish to facilitate drug discovery via high-throughput chemical screening. After identifying a small molecule that rescues the phenotype in fish, we characterized electrophysiological and cell injury phenotypes in zebrafish and neonatal rat ventricular myocyte models and showed, first, that they exhibit key features of the disease seen in patients and, second, that both the electrophysiological and cell injury phenotypes can be reversed by the drug candidate identified in the chemical screen. Finally, we applied insights gained through this approach to studies of diseased myocardium and iPSC-derived cardiac myocytes from ACM patients to implicate aberrant trafficking of critical intercalated disc proteins as a central mechanism responsible for myocyte injury and electrical derangements in ACM.

Methods used in this study that have been published previously in detail are included in the Supplementary Materials. At least 25 fish were analyzed in each experiment to characterize the phenotype in the ACM model. In electrophysiology experiments, at least five cells were analyzed in at least three separate cultures in studies of action potential morphology and individual ion current measurements. In studies involving neonatal rat ventricular myocytes, cells in at least five separate culture preparations were analyzed. In studies of patient myocardial samples, multiple sections were analyzed in eight patients with documented ACM (involving five different desmosomal gene mutations) and compared with sections of five patients each with end-stage ischemic, dilated, or hypertrophic cardiomyopathy, and sections from four control myocardial samples. All patient myocardial samples came from our paraffin block tissue archives. Experiments involving hiPSC-derived cardiac myocytes were performed in triplicate cultures of both control and patient cells.

Zebrafish care and embryo collection

All experiments were performed in Tuebingen AB zebrafish or Danio fish obtained from EkkWill Waterlife Resources. Zebrafish were bred and maintained at 28.5°C with 14-hour light and 10-hour dark exposure (day/night cycle) according to protocols approved by the

Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee and the Harvard Subcommittee for Animal Research. The stages (hours after fertilization) described in this report are based on the developmental stages of normal zebrafish embryos at 28.5°C (29).

Cloning and transgenic fish generation

The UAS/2057del2 plakoglobin responder construct was created using the Gateway cloning system (Invitrogen) and the Michael L. Nonet laboratory (Washington University, St. Louis, MO) pBH (bleeding heart) UAS vector (fig. S1A). The destination vector was modified by removing the yellow fluorescent protein sequence and replacing the cmlc2 promoter with the *Xenopus* crystalline promoter, which drives expression in zebrafish lens, allowing efficient identification of the transgenic line in subsequential generations. Gateway recombination reactions were performed according to the manufacturer's instructions (Invitrogen). Single-cell embryos were injected with destination vector DNA (15 ng/nl) and Tol2 transposase RNA (15 ng/nl). Stable transgenics were selected and outbred for multiple generations.

Zebrafish cardiac physiology

Images of live zebrafish hearts were acquired on an Axioplan (Zeiss) upright microscope with a 5× objective lens using integrated incandescent illumination and a FastCam-PCI high-speed digital camera (Photron) with 512 × 480 pixel gray-scale image sensor. Images were obtained sequentially at 250 frames per second, with 1088 frames (about eight cardiac cycles) acquired per condition. In-house software (implemented in MATLAB) was used to determine heart rate, whereas measurements of ventricular long and short axes in both diastole and systole were obtained using ImageJ (<http://rsbweb.nih.gov/ij/>). Cardiac output was measured as diastolic minus systolic ventricular volume multiplied by heart rate, as outlined by Shin *et al.* (30). Fractional shortening was calculated as end-diastolic diameter minus end-systolic diameter divided by end-diastolic diameter.

Chemical screening

Chemicals were dissolved in DMSO (Sigma-Aldrich). Embryos (double homozygous for the 2057del2 plakoglobin mutation and the nppb:: luciferase reporter) were arrayed in 96-well plates in Hepes-buffered E3 medium, and small molecules were pin-transferred (200 nl) from arrayed chemical libraries. Positive (untreated wild-type nppb::luciferase embryos) and negative controls (untreated 2057del2 plakoglobin/nppb:: luciferase fish) were included in each plate. Compounds were added at 24 hours after fertilization, and assays were performed at 72 hours after fertilization (fig. S1F). Each set of compounds was tested in duplicate to improve the discriminatory power of the assay. After being evaluated for viability, an equal volume of long half-life luciferase reagent (Promega Steady-Glo) was added to each well. The plate was then incubated in the dark for 60 min, and activities were measured with a Victor 3 luminometer (PerkinElmer). Each measurement was performed in duplicate, and results were normalized to the number of fish in the well. Follow-up chemical treatments were performed in Hepes-buffered E3 medium in petri dishes.

Survival studies

Embryos were kept in petri dishes until 7 days of age and then transferred to nursery tanks with larval food. Observations for phenotype and survival were recorded for several weeks. Data are presented as a Kaplan-Meier curve using GraphPad software.

Zebrafish ventricular myocyte isolation and culture

Hearts were extracted from anesthetized fish at 3 to 4 or 5 to 7 weeks after fertilization using microdissecting tweezers (Roboz Surgical Instruments), washed vigorously in culture medium (M199, Primocin, Hepes, L-glutamine, and 10% fetal bovine serum), and placed in fresh medium. Ventricles were separated at the atrioventricular junction, pooled in a tube containing enzyme solution (calcium/magnesium-free Hanks' balanced salt solution containing trypsin, penicillin/streptomycin, and Hepes) at 37°C, and agitated in a thermomixer at 1000 to 1200 rpm. Dissociated cells were collected every 10 min from supernatants and transferred to tubes containing culture medium and 10% fetal bovine serum to halt trypsinization. Cells were then pelleted by centrifugation, resuspended in fresh culture medium, and seeded on sterile cover slips coated with laminin (20 µg/ml) at 28.5°C in a 5% CO₂ incubator. After 36 hours, culture medium was changed and unattached cells were removed.

Cellular electrophysiology methods

Current-clamp experiments in isolated neonatal rat and zebrafish ventricular myocytes were carried out using standard protocols (31). Micropipettes had a tip resistance of 2 to 5 megohms. After opening the gigaseal, the microelectrode amplifier (EP-10, HEKA) was set to zero holding current, and cells were stimulated at a frequency of 0.5 to 1 Hz. Recordings were corrected for a junction potential of 12 mV (32, 33). The chemical composition of the external and pipette solutions is described in the Supplementary Materials.

I_{Na} activation and inactivation were measured by whole-cell voltage-clamp according to standard protocols (32, 33). Cells were held at a membrane potential of -80 mV, after which pulses between -120 and +50 mV were applied in 5-mV steps each with a duration of 250 ms. Inactivation was measured at membrane potentials between -120 and +50 mV at maximal activation (26, 33). In view of the small size of zebrafish myocytes (cell capacitance of 6.7 ± 2.6 pF; $n = 47$), it was not necessary to reduce extracellular $[Na^+]$ to achieve voltage control; extracellular $[Na^+]$ was reduced to 50 mM in studies of neonatal rat ventricular myocytes. Access resistance of the patch electrodes was <7 megohms. Measurements showing incomplete control of membrane potential were eliminated from analysis. The chemical composition of the external and pipette solutions is described in the Supplementary Materials.

The inward rectifier current I_{K1} was measured using the protocol described by Dobrev *et al.* (34) and Nemtsas *et al.* (35). Ramp clamps of 1400-ms duration were applied between -120 and +20 mV. After each clamp, external solution containing Ba²⁺ (1 mM) was applied via a large pore pipette placed adjacent to the clamped cell. I_{K1} was defined as the total current minus the Ba²⁺-insensitive current (33). Subtraction of the two currents was performed

using a MATLAB script. The chemical composition of the external and pipette solutions is described in the Supplementary Materials.

The outward K^+ current I_{K_r} was measured according to the protocol of Dobrev *et al.* (34) using identical extracellular and pipette solutions (see Supplementary Materials). In brief, I_{K_r} was activated by clamping the membrane potential from a holding potential of -50 mV for 1000 ms to different levels in 5-mV steps. I_{K_r} was obtained as the tail current upon repolarization to -50 mV after the 1000-ms conditioning steps. Interfering $I_{Ca,L}$ was inhibited by nisoldipine ($1 \mu\text{M}$). Addition of the specific I_{K_r} blocker E4031 identified the tail current as I_{K_r} (35).

Cytokine expression

Conditioned culture media (after 24 or 48 hours) from cells that expressed 2057del2 plakoglobin in the presence or absence of SB216763 ($5 \mu\text{M}$, 24 hours) were collected, mixed with a cocktail of biotinylated detection antibodies, and incubated with nitrocellulose membranes spotted in duplicate with control and capture antibodies (R&D Systems). Chemiluminescent signal produced at each spot corresponded to the amount of cytokine bound. Conditioned media from nontransfected cultures were assayed for cytokine expression as controls.

Knockdown of SAP97 expression in neonatal rat ventricular myocytes

Control (nontransfected) neonatal rat ventricular myocytes were isolated and plated on collagen-coated chamber slides for 24 hours before being infected with anti-SAP97 shRNA lentiviral particles ($7 \mu\text{g/ml}$, Santa Cruz Biotechnology) in a polybrene mixture ($5 \mu\text{g/ml}$) (12 hours, 37°C). At 72 hours after transduction, cultures were fixed in 4% paraformaldehyde and analyzed by immunofluorescence (as described in Supplementary Materials and Methods). Nonspecific shRNA (Santa Cruz Biotechnology) was used as a negative control.

Culture and analysis of iPSC-derived cardiac myocytes

iPSC-derived cardiac myocyte cultures were grown in RPMI + B27 medium for 5 to 14 days, rinsed in serum-free medium, fixed in 4% paraformaldehyde, and immunostained. Antibodies included mouse monoclonal anti-plakoglobin (Sigma), anti-Cx43 (Millipore), and anti-SAP97 (Santa Cruz Biotechnology) and rabbit polyclonal anti-Nav1.5 (provided by H. Abriel, University of Bern, Switzerland). Specific immunoreactive signal was detected by laser scanning confocal microscopy. Selected cultures were treated with SB216763 ($5 \mu\text{M}$) for 24 hours before fixation. iPSC-derived cardiac myocytes derived from unaffected nonmutation carrier siblings of the two probands were subjected to the same protocol and used as controls.

Statistical analysis

Data from zebrafish studies were expressed as means $\pm$ SEM and were analyzed by unpaired *t* test or Mantel-Cox tests using GraphPad software. Data from electrophysiology studies in zebrafish and neonatal rat ventricular myocytes were expressed as means $\pm$ SEM. Groups

were compared using analysis of variance or unpaired *t* tests, as appropriate (SigmaStat, Systat Software).

Data from neonatal rat ventricular myocyte studies were expressed as means $\pm$ SEM and analyzed by two-tailed Student's *t* tests.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Zebrafish model of ACM and chemical screen

(A and B) Representative images of a 5-week-old control sibling (A) and 2057del2 plakoglobin (PG) zebrafish (B) (scale bars, 1 mm), dissected hearts [control sibling (A') and 2057del2 plakoglobin (B'); scale bars, 200 μm; OFT, outflow tract; a, atrium; v, ventricle], and hematoxylin and eosin–stained sections [control sibling (A'') and 2057del2 plakoglobin mutant fish (B''); scale bars, 200 μm] showing cardiomegaly, wall thinning, and chamber dilatation in early adulthood. (C) Survival curves for control and 2057del2 plakoglobin mutant fish. Data were pooled from three independent experiments and presented as total percentage fish survival as a function of time ($n = 125$; $P < 0.0001$, Mantel-Cox test). WT, wild type. (D) Ventricle/body size ratios in control sibling versus 2057del2 plakoglobin mutants at 5 weeks of age. (E to G) Heart rate [beats per minute (Bpm); $n = 50$; $P < 0.05$, unpaired t test] (E), stroke volume [diastolic volume minus systolic volume (nl); $n = 8$; $P < 0.05$, unpaired t tests] (F), and cardiac output [stroke volume $\times$ heart rate (nl/min)] (G) in control sibling versus 2057del2 plakoglobin fish measured in 48-hour post-fertilization larvae ($n = 12$; $P < 0.05$, unpaired t test). (H) Quantitative reverse transcription polymerase chain reaction (qRT-PCR) showing twofold induction of the cardiac natriuretic peptide BNP in Naxos embryos compared to control siblings at 72 hours after fertilization ($n = 3$; $*P < 0.01$, unpaired t tests). (I) Luciferase-based reporter for cardiac natriuretic peptide expression in control siblings (BNP-LUC) and 2057del2 plakoglobin (BNP-LUC 2057del2 PG) embryos at 72 hours after fertilization ($n = 30$; $*P < 0.01$, unpaired t tests). (J) BNP luciferase activity of BNP-LUC 2057del2 PG embryos (72 hours after fertilization) after

dimethyl sulfoxide (DMSO) and SB216763 (SB2) treatment ($n = 36$; $*P < 0.001$, unpaired t test). (**K**) Percent survival of untreated 2057del2 plakoglobin fish and 2057del2 plakoglobin fish treated with SB216763. SB216763 was added to the water at 24 hours after fertilization and washed out at 6 days. Fish were put on regular flow for an additional 4 weeks, and survival was counted. Data were pooled from three independent experiments ($n = 300$; $*P < 0.01$, unpaired t test).

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Fig. 2. Cellular electrophysiology in zebrafish ventricular myocytes

(A) Representative action potential tracings from a zebrafish ventricular myocyte that expressed 2057del2 plakoglobin (red) or a control fish myocyte (black) measured at 5 weeks after fertilization. (B) Action potential upstrokes and first-time derivatives (dV/dt) in zebrafish myocytes that expressed 2057del2 plakoglobin (red) versus control myocytes (black) at enlarged time scale. (C) Representative action potential tracings in a neonatal rat ventricular myocyte that expressed 2057del2 plakoglobin (red) versus a control myocyte (black) showing a positive shift in resting potential, and action potential prolongation. Action potential $dV/dt_{\max}$ decreased from 75 ± 20 V/s in control neonatal rat ventricular myocytes to 6 to 32 ± 7 V/s in myocytes that expressed 2057del2 plakoglobin ($n = 12$ for each; $P < 0.01$, unpaired t test). Note virtually identical changes to those seen in zebrafish myocyte action potentials.

Fig. 3. Decreased I_{Na} and I_{K1} current density in mutant zebrafish myocytes

(A to C) Changes in I_{Na} current density in zebrafish myocytes that expressed 2057del2 plakoglobin. (A) Original traces. (B) Dependence of I_{Na} on membrane potential (red, 2057del2 plakoglobin; black, control). (C) Steady-state activation (squares) and inactivation (triangles) curves (closed symbols, 2057del2 plakoglobin; open symbols, controls). The Boltzmann fit to individual experiments used to calculate the $V_{0.5}$ values for steady-state activation and inactivation (mean $\pm$ SEM; red symbols) showed no significant effect of 2057del2 plakoglobin expression. (D to F) Changes in I_{K1} current density in zebrafish

myocytes that expressed 2057del2 plakoglobin. (D) Original traces obtained after subtraction of the Ba^{2+} -insensitive component. (E) I_{K1} current at -100 mV. (F) I_{K1} slope of linear portion between -100 and -60 mV. Numbers above bars in graphs indicate n for each condition. $*P < 0.001$ versus control, unpaired t test.

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Fig. 4. Reversal of electrophysiological defects in mutant zebrafish myocytes by SB216763
 (A to C) Effects of SB216763 on action potential parameters. (D and E) Effects of SB216763 on I_{Na} and I_{K1} current densities in control zebrafish ventricular myocytes and myocytes that expressed 2057del2 plakoglobin. Black, controls; red, myocytes expressing 2057del2 plakoglobin; blue, myocytes expressing 2057del2 plakoglobin and treated with SB216763. Numbers above bars in graphs indicate n for each condition. * $P < 0.001$ versus 2057del2 plakoglobin for (A), (B), and (E); ** $P < 0.002$ versus 2057del2 plakoglobin for (C), unpaired t tests.

Fig. 5. Modeling ACM in neonatal rat ventricular myocytes in vitro

(A) Representative image showing >90% transfection efficiency in neonatal rat ventricular myocytes transfected with a GFP-expressing adenoviral construct. (B) Western immunoblots showing equivalent levels of 2057del2 plakoglobin and endogenous plakoglobin in transfected cultures. 2057del2 plakoglobin migrates at a lower molecular weight than does the wild-type protein. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. (C and D) Representative confocal immunofluorescence images showing plakoglobin and Cx43 immunoreactive signal distribution in control cells and cells transfected with 2057del2 plakoglobin. (E) TUNEL labeling and caspase-3 activity in control myocytes and 2057del2 plakoglobin-expressing myocytes under rest conditions and after 4 hours of uniaxial cyclical stretch. White arrows point to apoptotic nuclei. Graphs show % TUNEL-stained nuclei in five microscopic fields. (* $P < 0.01$ versus resting controls; ** $P < 0.01$ versus resting cells that expressed 2057del2 plakoglobin, two-tailed Student's t tests), and caspase-3 assays were done in five cultures for each condition (* $P < 0.05$ versus resting controls; ** $P < 0.05$ versus resting cells that expressed 2057del2 plakoglobin, two-tailed Student's t tests). (F) TUNEL labeling of control cells and cells expressing 2057del2 plakoglobin under resting conditions and after 4 hours of stretch in the presence or absence of pifithrin-A. Graphs show % TUNEL-stained nuclei in five microscopic fields ($n = 6$; * $P < 0.001$ versus resting controls; ** $P < 0.001$ versus resting cells that expressed 2057del2 plakoglobin; # $P < 0.001$ versus stretched cells that expressed 2057del2 plakoglobin, unpaired Student's t tests), and caspase-3 assays were done in five cultures for each condition (* $P < 0.05$ versus resting controls; ** $P < 0.05$ versus resting cells that expressed 2057del2 plakoglobin; # $P < 0.001$ versus stretched cells

that expressed 2057del2 plakoglobin, unpaired Student's *t* tests). **(G)** Cytokine expression profiles showing increased secretion of interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α), macrophage inflammatory protein-1 α (MIP-1 α), and the chemokine RANTES (regulated on activation, normal T cell expressed and secreted) by neonatal rat ventricular myocytes that expressed 2057del2 plakoglobin compared to control cells.

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Fig. 6. Reversal of the disease phenotype in neonatal rat ventricular myocytes by SB216763
(A) Representative confocal immunofluorescence images showing normalization of plakoglobin and Cx43 immunoreactive signal distribution in neonatal rat ventricular myocytes treated with SB216763 (SB2). Images of untreated cells are the same as those shown in Fig. 5C because they were obtained from the same experiment. **(B)** Western immunoblots showing the total cellular content of plakoglobin and Cx43 in myocytes in the presence or absence of SB216763 (SB2) in control cells and cells that expressed 2057del2 plakoglobin. GAPDH was used as a loading control. **(C)** Effects of SB216763 (SB2) on TUNEL labeling in control myocytes and cells expressing 2057del2 plakoglobin. White arrows point to apoptotic nuclei. Graph shows %TUNEL-stained nuclei in 5 microscopic fields; $n=6$, $*P<0.001$ versus controls; $**P<0.001$ versus untreated cells that expressed 2057del2 plakoglobin; 2-tailed Student's t -test. **(D)** Effects of SB216763 (SB2) treatment for 24 or 48 hours (hrs) on cytokine expression profiles in control cells and cells that expressed 2057del2 plakoglobin (IFN γ , interferon- γ ; MIP-1 γ , macrophage inflammatory protein-1 γ ; IP-10, interferon- γ -induced protein-10; IL-1ra, interleukin-1 receptor- α).

Fig. 7. Abnormal distribution of SAP97 and Nav1.5 and its reversal by SB216763

(**A**) Representative confocal immunofluorescence images showing the distribution of SAP97 and Nav1.5 in control neonatal rat ventricular myocytes and myocytes that expressed 2057del2 plakoglobin in the presence or absence of SB216763. Arrows point to cell borders where differences in immunoreactive signals are most apparent. (**B**) Western immunoblots showing the total cellular content of SAP97 and Nav1.5 in control myocytes and myocytes that expressed 2057del2 plakoglobin in the presence or absence of SB216763. GAPDH was used as a loading control.

Fig. 8. Effects of knockdown of SAP97 on the distribution of intercalated disc proteins in normal neonatal rat ventricular myocytes

Representative confocal immunofluorescence images showing the distribution of SAP97, Nav1.5, plakoglobin, Cx43, N-cadherin, plakophilin-2 (PKP2), and desmoplakin in control cells and neonatal rat ventricular myocytes infected with anti-SAP97 shRNA for 72 hours. Arrows point to cell borders where differences in immunoreactive signals are most apparent. Cultures treated with nonspecific shRNA were used as negative controls. shRNA treatment led to significant loss of SAP97 expression as shown by the marked reduction of SAP97 signals. Knockdown of SAP97 expression also significantly reduced signals for Nav1.5 and plakoglobin but not desmoplakin, plakophilin-2, N-cadherin, or Cx43.

Fig. 9. Disease features in cardiac myocytes from iPSCs from two ACM probands with plakophilin-2 mutations

Representative confocal immunofluorescence images showing the distribution of plakoglobin, Cx43, Nav1.5, and SAP97 in iPSC-derived cardiac myocytes obtained from two ACM probands bearing mutations in the plakophilin-2 gene (family A: Q617X; family B: 2013delC) in the presence or absence of SB216763. Arrows point to cell borders where differences in immunoreactive signals are most apparent. iPSC-derived cardiac myocytes from unaffected nonmutation carrier siblings of the two AC probands were subjected to the same protocol and used for control purposes. Localization of all four proteins examined was disrupted in the ACM iPSC-derived cardiac myocytes but restored after treatment with SB216763 for 24 hours.

Table 1

Action potential parameters in 2057del2 plakoglobin–in zebrafish ventricular myocytes that expressed 2057del2 plakoglobin, and expressing zebrafish myocytes and effects of SB216763

Shown are changes in RMP, maximal upstroke velocity, and action potential duration reversal of phenotypes by SB216763 administered at two different ages (weeks after fertilization). Data are expressed as means $\pm$ SEM (*n*).

	Time of cell isolation (weeks of age)	Control ventricular myocytes	Experimental ventricular myocytes (with 2057del2 plakoglobin)	Control + SB216763	Experimental + SB216763
Resting membrane potential (mV)	3–4 [*]	-78 ± 1 (8)	-67 ± 2 (8) [†]	-79 ± 1 (10)	-75 ± 1 (7)
	5–7 [‡]	-79 ± 1 (11)	-69 ± 1 (12) [†]	-77 ± 1 (11)	-78 ± 1 (11)
Maximal upstroke velocity (V/s)	3–4 [*]	37 ± 8 (8)	8 ± 1 (8) [§]	35 ± 8 (7)	22 ± 4 (5)
	5–7 [‡]	63 ± 11 (10)	18 ± 2 (12) [†]	53 ± 11 (7)	66 ± 15 (8)
Action potential duration (ms)	3–4 [*]	190 ± 7 (8)	380 ± 38 (8) [†]	173 ± 8 (7)	175 ± 17 (5)
	5–7 [‡]	240 ± 24 (11)	319 ± 44 (12) [§]	195 ± 17 (11)	154 ± 18 (11)

^{*} Ventricular myocytes were prepared from transgenic fish at 3 to 4 weeks of age and then treated in culture with SB216763 for 36 hours before action potentials were analyzed.

[†] $P < 0.001$, unpaired *t* test, significantly different from control.

[‡] Zebrafish embryos at 1 day after fertilization were treated with SB216763 for 6 days (3.0 μ M) and then maintained under normal conditions (in the absence of SB216763) until 5 to 7 weeks of age. At this time, ventricular myocytes were isolated and characterized.

[§] $P < 0.05$, unpaired *t* test, significantly different from control.

Table 2

Current measurements

Shown are changes in I_{Na} , I_{K1} , and I_{Kr} in zebrafish ventricular myocytes expressing 2057del2 plakoglobin and reversal by SB216763 at different ages (weeks after fertilization). Data are expressed as means $\pm$ SEM (n).

	Time of cell isolation (weeks of age)	Control ventricular myocytes	Experimental ventricular myocytes (with 2057del2 plakoglobin)	Control + SB216763	Experimental + SB216763
I_{Na} : peak current density (pA/pF)	3–4 [*]	-212 $\pm$ 19 (7)	-51 $\pm$ 5 (17) [†]	-230 $\pm$ 27 (10)	-163 $\pm$ 58 (7)
	7–8 [‡]	-196 $\pm$ 22 (18)	-31 $\pm$ 3 (17) [†]	-168 $\pm$ 39 (7)	-172 $\pm$ 31 (8)
I_{K1} : current density at -100 mV (pA/pF)	3–4 [*]	-17 $\pm$ 2.7 (8)	-5 $\pm$ 0.8 (13) [†]	-18 $\pm$ 3.5 (8)	-15 $\pm$ 3.0 (8)
I_{K1} : slope between -90 and -15 mV (pA/pF at 100 mV)	3–4 [*]	-27 $\pm$ 4.3 (8)	-8 $\pm$ 1.0 (11) [†]	-27 $\pm$ 5.0 (8)	-21 $\pm$ 5.0 (6)
I_{Kr} : density of tail currents at after activation at +30 mV (pA/pF)	3–4 [*]	5.4 $\pm$ 0.8 (9)	5.6 $\pm$ 0.7 (10)	—	—

^{*} Isolated ventricular myocytes were prepared from transgenic fish at 3 to 4 weeks of age and then treated in culture with SB216763 for 36 hours before currents were analyzed.

[†] $P < 0.001$, unpaired t test, significantly different from control.

[‡] Zebrafish embryos at 1 day after fertilization were treated with SB216763 for 6 days (3.0 μ M) and then maintained under normal conditions (in the absence of SB216763) until 7 to 8 weeks of age. At this time, ventricular myocytes were isolated and characterized.