SAP97 and Dystrophin Macromolecular Complexes Determine Two Pools of Cardiac Sodium Channels Na\(_{\alpha,1.5}\) in Cardiomyocytes

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**Rationale:** The cardiac sodium channel Na\(_{\alpha,1.5}\) plays a key role in excitability and conduction. The last 3 residues of Na\(_{\alpha,1.5}\) (Ser-Ile-Val) constitute a PDZ-domain binding motif that interacts with the syntrophin–dystrophin complex. As dystrophin is absent at the intercalated discs, Na\(_{\alpha,1.5}\) could potentially interact with other, yet unknown, proteins at this site.

**Objective:** The aim of this study was to determine whether Na\(_{\alpha,1.5}\) is part of distinct regulatory complexes at lateral membranes and intercalated discs.

**Methods and Results:** Immunostaining experiments demonstrated that Na\(_{\alpha,1.5}\) localizes at lateral membranes of cardiomyocytes with dystrophin and syntrophin. Optical measurements on isolated dystrophin-deficient mdx hearts revealed significantly reduced conduction velocity, accompanied by strong reduction of Na\(_{\alpha,1.5}\) at lateral membranes of mdx cardiomyocytes. Pull-down experiments revealed that the MAGUK protein SAP97 also interacts with the SIV motif of Na\(_{\alpha,1.5}\), an interaction specific for SAP97 as no pull-down could be detected with other cardiac MAGUK proteins (PSD95 or ZO-1). Furthermore, immunostainings showed that Na\(_{\alpha,1.5}\) and SAP97 are both localized at intercalated discs. Silencing of SAP97 expression in HEK293 and rat cardiomyocytes resulted in reduced sodium current (\(I_{\text{Na}}\)) measured by patch-clamp. The \(I_{\text{Na}}\) generated by Na\(_{\alpha,1.5}\) channels lacking the SIV motif was also reduced. Finally, surface expression of Na\(_{\alpha,1.5}\) was decreased in silenced cells, as well as in cells transfected with SIV-truncated channels.

**Conclusions:** These data support a model with at least 2 coexisting pools of Na\(_{\alpha,1.5}\) channels in cardiomyocytes: one targeted at lateral membranes by the syntrophin-dystrophin complex, and one at intercalated discs by SAP97. (Circ Res. 2011;108:294-304.)

**Key Words:** sodium channel Na\(_{\alpha,1.5}\) MAGUK proteins SAP97 dystrophin

The cardiac sodium channel Na\(_{\alpha,1.5}\) initiates the cardiac action potential, thus playing a key role in cardiac excitability and impulse propagation. The physiological importance of this channel is illustrated by numerous cardiac pathologies caused by hundreds of mutations identified in SCN5A, the gene encoding Na\(_{\alpha,1.5}\).\(^1\) The Na\(_{\alpha,1.5}\) channel is composed of one 220-kDa \(\alpha\)-subunit that constitutes a functional channel, and 30-kDa \(\beta\)-subunits. In addition to these accessory \(\beta\)-subunits, several proteins have been shown to regulate and interact with Na\(_{\alpha,1.5}\).\(^1,2\) In most cases, the physiological relevance of these interactions is poorly understood, mainly because of a lack of appropriate animal models. Many of the interacting proteins bind to the C terminus of Na\(_{\alpha,1.5}\), where several protein–protein interaction motifs are located.\(^1,2\) We have shown that the ubiquitin–protein ligase Nedd4-2 binds the PY motif of Na\(_{\alpha,1.5}\) and reduces the sodium current (\(I_{\text{Na}}\)) in HEK293 cells by promoting its internalization.\(^3\) We have also demonstrated that Na\(_{\alpha,1.5}\) associates with the dystrophin–syntrophin multiprotein complex (DMC) in cardiac cells.\(^4\) In dystrophin-deficient mice (mdx\(^{5cv}\)), \(I_{\text{Na}}\), and total Na\(_{\alpha,1.5}\) protein expression are reduced. The association between Na\(_{\alpha,1.5}\) and dystrophin occurs via...
the syntrophin family of adaptor proteins, 3 of which are expressed in the heart.4 The PDZ domain of syntrophin binds to the last 3 C-terminal residues of Na1.5 (Ser-Ile-Val, SIV), a PDZ-domain binding motif that mediates the interaction with dystrophin and other proteins of the DMC.2 Interestingly, dystrophin has been shown to be absent from the intercalated discs of human and rat cardiomyocytes, suggesting that the Na1.5 channels present at these locations may interact with other regulatory or anchoring proteins.

Proteins of the membrane associated guanylate kinase (MAGUK) family are characterized by numerous protein–protein interaction domains, including PDZ domains.8 They are involved in the function and localization of many ion channels in neurons and epithelial cells, mostly at cell to cell junctions, but little is known about their function in the heart.9 SAP97 (synapse-associated protein) and ZO-1 (zonula occludens) are the main MAGUK proteins expressed in cardiomyocytes.9 PSD95 (postsynaptic density) is also expressed in the human heart, but not in the mouse heart.9,10 SAP97 regulates the targeting and localization of cardiac potassium channels, such as Kir2.x11 and K,1.5 in myocytes.10 El-Haou et al.12 recently demonstrated that SAP97 interacts with K,4.2 and K,4.3 channels via their PDZ-domain binding motif. They showed that suppression of SAP97 expression in rat myocytes decreases the K,4.x-mediated current, whereas its overexpression increases it. In neurons, PSD95, which is closely related to SAP97, plays a role in clustering and anchoring ion channels to the postsynaptic plasma membrane.13 Because PSD95 is also present in the human heart and SAP97 has been shown to interact with potassium channels in cardiac cells, we hypothesized that one of these cardiac MAGUK proteins may also associate with Na1.5.

In this study, we provide evidence for the coexistence of at least 2 pools of Na1.5 channels in cardiomyocytes, one located at the lateral membrane with the DMC, and the other localizing with SAP97 at the intercalated discs. In sections and isolated cardiomyocytes of dystrophin-deficient mice, we observed a drastic reduction of the pool of Na1.5 channels located at the lateral membrane. This loss could underline the significant reduction of conduction velocity observed in these dystrophin-deficient hearts using optical measurements. Finally, we also found that the surface expression of Na1.5 in HEK293 cells is regulated by its interaction with SAP97.

Methods
An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Cell Preparation, Transfection, and Infection
HEK293 and Sk-Hep cells were cultured as previously described.3

Silencing and Lentiviruses
HEK293 cells stably expressing Na1.5 were transfected using Lipofectamine (Invitrogen) as detailed in the Online Data Supplement. HEK293 cells were infected with VSV-G pseudo-typed lentiviruses.14 Adult rat cardiomyocytes were transfected with silencing plasmids, as previously published.12

Transfection
For patch-clamp experiments, HEK293 cells were transiently transfected using calcium phosphate with the different constructs mentioned in the text. Details are given in the Online Data Supplement.

Non-standard Abbreviations and Acronyms

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<th>Acronym</th>
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<tr>
<td>Cx</td>
<td>connexin</td>
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<td>CV</td>
<td>conduction velocity</td>
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<td>DMC</td>
<td>dystrophin multiprotein complex</td>
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<tr>
<td>MAGUK</td>
<td>membrane-associated guanylate kinase</td>
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<td>PSD95</td>
<td>postsynaptic density 95</td>
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<td>SAP97</td>
<td>synapse-associated protein 97</td>
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<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
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<td>WT</td>
<td>wild type</td>
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Protein Extraction, Pull-Down, and Western Blot
These procedures were performed as previously reported with HEK293 and Sk-Hep cells.3 See Online Data Supplement for other tissues.

Biotinylation Assay
Transfected HEK293 cells were biotinylated as detailed in the Online Data Supplement. Biotinylated proteins were then analyzed by Western blot.

Immunohistochemistry of Rat and Mouse Ventricular Sections
Immunostainings were performed on heart cryosections as described in the Online Data Supplement.

Isolation and Immunocytochemistry of Mouse Ventricular Myocytes
See the Online Data Supplement for a detailed description.

Electrophysiology
Patch-clamp recordings were carried out in the whole-cell configuration at room temperature using solutions previously described.4 See the Online Data Supplement for details.

Conduction Velocity Measurements on Isolated Mouse Hearts
Optical measurements of conduction velocity (CV) were performed on isolated wild-type and dystrophin-deficient mdx mouse hearts as described in detail in the Online Data Supplement.

Statistical Analysis
Data are represented as mean values±SEM, unless otherwise indicated.

Results
Na1.5 Is Localized With Dystrophin and Syntrophins Only at the Lateral Membrane
We recently demonstrated that Na1.5 associates with the DMC in cardiac cells.3 We also observed that sodium current (I Na) and Na1.5 protein expression is reduced in dystrophin-deficient (mdx5cv) mice compared to wild type (WT).4 To further study the importance of this multiprotein complex, we analyzed the distribution of Na1.5 channels, as well as that of dystrophin and syntrophin in rat ventricular sections (Figure 1A and 1B). Immunostaining experiments showed that Na1.5 is distributed along the entire membrane of rat cardiomyocytes (Figure 1A and 1B, left). In contrast, we
observed that Na\textsubscript{v}1.5 and dystrophin are in the same membrane compartment exclusively at the lateral membrane (Figure 1A, right), and not at the intercalated discs, where dystrophin is absent (white arrowheads, Figure 1A, middle and right). Similar results were obtained using an antibody that recognized all syntrophin isoforms (Figure 1B). Syntrophins are similarly in close proximity to Na\textsubscript{v}1.5 exclusively at the lateral membrane (Figure 1B, white arrowheads). In mouse ventricular sections, similar findings were obtained with Na\textsubscript{v}1.5, dystrophin, and syntrophin (Online Figure I). These results suggest that Na\textsubscript{v}1.5 could be part of at least 2 different protein complexes: with dystrophin at the lateral membrane and with another partner at the intercalated discs.

**Na\textsubscript{v}1.5 Is Decreased at the Lateral Membranes of Dystrophin-Deficient Myocytes**

Immunohistochemistry experiments were performed using WT and mdx\textsuperscript{5cv} mouse ventricular sections (Figure 2A). A strong reduction of Na\textsubscript{v}1.5 staining was observed at the lateral membrane of mdx\textsuperscript{5cv} mouse cardiomyocytes (Figure 2A, bottom) compared to WT mice (Figure 2A, top). Similarly, immunocytochemistry performed on isolated mouse cardiomyocytes confirmed that Na\textsubscript{v}1.5 is distributed along the entire cell membrane in these cells, and that lateral Na\textsubscript{v}1.5 staining in dystrophin-deficient cardiomyocytes is strongly reduced when compared to WT (Figure 2B). These results suggest that the decrease in Na\textsubscript{v}1.5 protein and $I_{Na}$ previously seen in dystrophin-deficient mice\textsuperscript{4} is the consequence of a loss of Na\textsubscript{v}1.5 channels at the lateral membrane of cardiomyocytes.

**Impulse Propagation Is Significantly Slowed in Hearts of mdx Mice**

Reduced $I_{Na}$ and Na\textsubscript{v}1.5 protein in dystrophin-deficient hearts may contribute to the previously observed prolongation of the QRS complex duration observed in ECG recordings of mdx\textsuperscript{5cv} mice.\textsuperscript{4} To investigate whether conduction velocity (CV) is reduced in dystrophin-deficient hearts, we performed optical measurements in isolated Langendorff-perfused WT and mdx hearts stained with the fluorescent voltage-reporter dye Di-4-ANNEPS. Conduction spread of the paced (cycle length 100 ms) left ventricle free wall was recorded following equilibration and confirmation of physiological spontaneous heart rates (500 to 600/min, by volumetric ECG). Maximal CV occurred in the same direction in WT and mdx hearts along the longitudinal (apico-aortal) heart axis. Isochronic maps from WT hearts showed regular anisotropic conduction spread around a point-like pacing stimulus originating from the free LV wall center (Figure 3A, left). Accordingly, CV analysis by ellipsis fitting confirmed longitudinal and transversal velocities consistent with rapid ellipsoid conduction spread and physiological anisotropy (Figure 3A, right) (see online supplemental methods for detailed description). However, conduction spread of mdx hearts resulted in tighter isochrone maps indicating conduction slowing in all directions (Figure 3B, left). In agreement with this observation ellipsis fitting analysis confirmed slowing of conduction spread in the transversal axis resulting in reduced conduction velocities (Figure 3B, right). Figure 3C shows that in contrast to WT hearts, dystrophin-deficient hearts showed on average a reduction in transversal CV by 22.2% from 0.45±0.08 m/sec to 0.35±0.07 m/sec (WT n=5, mdx n=8; $P<0.05$) and in longitudinal CV by 12.7% from 0.71±0.14 m/sec to 0.62±0.11 m/sec (WT n=5, mdx n=8; $P=NS$).

Additionally, CV has been analyzed by a second method using averaged CV vectors along the apparent longitudinal and transversal heart axis (see online supplementary methods for detailed description). Figure 3D confirms that in contrast to WT hearts, dystrophin-deficient hearts showed a longitudinal 28.4% reduction in CV (WT n=5, mdx n=8; $P<0.005$). In addition, transversal CV was significantly reduced by 22.4% in mdx compared to WT hearts (WT n=5, mdx n=8; $P<0.05$; Figure 3D). The combined reduction of transversal and longitudinal CVs resulted in a 7.3% reduction in the anisotropic ratio (WT n=5, mdx n=8; $P=NS$; Figure 3D) when analyzed by averaged CV vectors and by 10.7%...
when analyzed by the ellips fitting method (WT n=5, mdx n=8; P=NS; Figure 3C). Thus, under quasi-physiological conditions mimicking the resting mouse heart rate of 600/min the mdx left ventricle showed significantly slower excitation spread both in the longitudinal and transversal directions, confirming the slowed impulse propagation suggested by the widening of QRS complex. As distribution and expression of the main ventricular connexin, Cx43, is unaltered in mdx hearts (see Online Figure II and elsewhere4), this may suggest a selective role of Nav1.5 channels located at the lateral membrane for impulse propagation in cardiac muscle.

The Carboxyl Terminus of Na\textsubscript{1.5} Interacts With SAP97
To identify proteins that could specifically interact with the fractions of Na\textsubscript{1.5} at the intercalated discs, we studied proteins of the MAGUK family. We performed pull-down experiments using GST-fusion proteins comprising the last 66 amino acids of the C terminus (C-ter) of Na\textsubscript{1.5}, as well as fusion proteins lacking the 3 last SIV residues (ΔSIV, Figure 4A). Western blot experiments performed using an anti-PSD95 family antibody, known to recognize several cardiac MAGUKs (Figure 4B, Online Figure III), revealed that Na\textsubscript{1.5} C-ter WT precipitated proteins containing PDZ domains (2 bands at ≈140 kDa, the molecular weight of SAP97) from mouse ventricles and human atrium. This interaction is dependent on the PDZ-domain binding motif of Na\textsubscript{1.5}, as no protein was observed with the truncated construct. Similar results were obtained using a SAP97 specific antibody (Figure 4C, Online Figure III). The similarity between these 2 results (Figure 4B and 4C) suggests that SAP97 is the predominant MAGUK interacting with Na\textsubscript{1.5}. To assess if this interaction was specific for SAP97, we tested a specific PSD95 antibody (Figure 4D). No interaction was seen with either fusion protein (Na\textsubscript{1.5} C-ter WT or ΔSIV). We also verified whether Na\textsubscript{1.5} C termsis could interact with ZO-1, a cardiac MAGUK protein known to interact with connexins. We generated a fusion protein containing the last amino acids of connexin-45 (Cx45) (Figure 4A), which has previously been shown to bind ZO-1.15 Pull-down experiments performed using SK-Hep cell lysates, which express ZO-1 endogenously, confirmed that Cx45 C-ter precipitased ZO-1, but that Na\textsubscript{1.5} C-ter (Figure 4E) did not. Combined, these results support a specific interaction between the PDZ-domain binding motif of Na\textsubscript{1.5} and SAP97 in mouse ventricles and human atria.

Figure 2. Loss of Na\textsubscript{1.5} lateral membrane staining in dystrophin-deficient cardiomyocytes. A, Na\textsubscript{1.5} (green) in WT (top) and mdx\textsuperscript{5cv} (bottom) ventricular sections. Note the loss of lateral membrane Na\textsubscript{1.5} staining in the mdx\textsuperscript{5cv} sections; intercalated disc staining remains almost unaffected. B, Cardiomyocytes labeled for Na\textsubscript{1.5} (green, left) and dystrophin (red, right). Note the absence of dystrophin staining at the intercalated discs in WT cardiomyocytes (cf. insets with magnifications). Mdx cardiomyocytes were identified by the negative dystrophin staining (bottom, right). In isolated mdx cardiomyocytes, Na\textsubscript{1.5} staining was similarly markedly reduced at the lateral membrane.

Localization of SAP97, Na\textsubscript{1.5}, Cx43, and Syntrophin in Rat Cardiomyocytes
To analyze the distribution of Na\textsubscript{1.5} and SAP97 in native cardiac tissue, we performed immunostainings using Na\textsubscript{1.5}, SAP97 and Cx43 antibodies on rat heart sections. As present-
Silencing of SAP97 in HEK293 Cells, Cardiomyocytes, and Na,1.5 Channels Lacking the SIV-Domain Reduces \( I_{Na} \)

The results presented in Figure 4 indicate that Na,1.5 and SAP97 interact via their SIV-motif and PDZ domain, respectively. Electrophysiological studies were performed to characterize the role of this interaction. First, patch-clamp experiments using HEK293 cells transiently transfected with Na,1.5 and SAP97 were carried out. Unexpectedly, expression of SAP97 did not modify \( I_{Na} \) (not shown). This result could be an indication that endogenous SAP97 is expressed at a saturating level in HEK293 cells. We subsequently reduced the expression of SAP97 using short hairpin (sh)RNA. When HEK293 cells expressing Na,1.5 (HEK293-Na,1.5) were transfected with SAP97 silencing plasmids, \( I_{Na} \) was reduced by 55±4% (Figure 6B). However, the transfection technique was not efficient enough to silence SAP97 expression in the majority of the cells. Therefore, we developed a lentivirus-based strategy to deliver shRNA. Western-blot performed with HEK293 cells infected with shRNA-containing lentiviruses revealed that SAP97 expression was drastically reduced (~83%, Figure 6A) when compared to nonsilenced cells infected with scrambled shRNA. In HEK293-Na,1.5 cells silenced with lentiviruses, we observed a reduction of \( I_{Na} \) that was comparable to the results obtained when using the silencing plasmid (not shown). Next, we studied the consequence of silencing SAP97 on endogenous \( I_{Na} \) in rat atrial myocytes maintained in short term culture. This procedure has been previously shown to efficiently suppress endogenous cardiac SAP97.\(^{12}\) After 3 to 4 days of culture, current recordings at -20 mV using green-fluorescent atrial myocytes (~20% of the cells) revealed a much smaller \( I_{Na} \) in myocytes transfected with shSAP97 than those with scrambled shRNA (~66±8%, Figure 6C). SAP97-silenced or scrambled HEK293 cells were then transiently transfected with Na,1.5 WT or ΔSIV. The current generated by Na,1.5 ΔSIV channels was reduced by 57±9% compared to WT channels in control cells expressing the scrambled shRNA (Figure 6D). In silenced cells (shSAP97), WT and ΔSIV \( I_{Na} \) were comparable (49±11% and 57±7% of control values, respectively, Figure 6D). Voltage dependence of activation and inactivation was not significantly different between the four conditions (see Online Figure V). Because \( \beta 1 \) subunits are known to associate with Nav1.5,\(^{16}\) we performed similar experiments using HEK293 cells transfected with Na,1.5 WT or ΔSIV and \( \beta 1 \). The results were similar whether the \( \beta 1 \) subunit was present or not (Online Figure VIA), without affecting the voltage dependence of activation and inactivation (Online Figure VIB). We finally investigated whether the reduced \( I_{Na} \) could be rescued by another protein bearing a PDZ binding domain. As shown in Online Figure VII, overexpression of \( \alpha 1 \)-syntrophin (previously shown to interact with Na,1.5\(^{12}\)) in silenced cells did not increase \( I_{Na} \).

Silencing of SAP97 in Cultured Rat Atrial Myocytes Reorganizes Na,1.5 Expression

We also investigated whether silencing of SAP97 (Ad shSAP97) alters the organization of Na,1.5 in cardiomyocytes, using a model of cultured atrial myocytes at confluence.
stage when cells reestablish highly organized cell-cell contacts as previously shown. Immunostainings on short-term cultured rat atrial myocytes indicate that the total expression of Na$_v$1.5 channels in myocytes transduced with shSAP97 is reduced when compared to cells overexpressing SAP97 (Ad SAP97), and that the localization of the channels at the plasma membrane is reorganized (Online Figure IVA). Costainings of Na$_v$1.5 and syntrophin on rat cardiomyocytes confirm that these 2 proteins are both localized at the cell surface but not at cell-cell contacts where syntrophin is absent.

Figure 5. Localization of Na$_v$1.5, SAP97, and Cx43 in rat heart sections. A, Left, Na$_v$1.5 (green) both at the lateral membranes and the intercalated discs; middle, SAP97 (red) only at the intercalated discs. Right, Merge of the 2 images showing close localization of Na$_v$1.5 and SAP97 at the intercalated discs (white arrowheads, and magnification in the inset). B, Left, Cx43 (green) only at the intercalated discs. Middle, SAP97 (red) only at the intercalated discs. Right, Merge of the 2 images showing punctuate staining of Cx43 and SAP97 at the intercalated discs (white arrowheads and magnification in the inset).
In SAP97 silenced myocytes, syntrophin expression remains unchanged at the level of non–cell-to-cell contacts (Online Figure IVB, bottom).

**Expression of Na\(_{\text{v1.5}}\) Channels Lacking the SIV-Domain Is Reduced at the Cell Surface of HEK293 Cells and SAP97-Silenced Cells**

Electrophysiological data indicate that 
\(I_{\text{Na}}\) is dependent on the presence of SAP97 as well as on the SIV motif of Na\(_{\text{v1.5}}\). We examined whether the reduced current amplitude was attributable to decreased expression of the channel at the cell surface. Surface biotinylation assays performed on HEK293 cells that had been transiently transfected with Na\(_{\text{v1.5}}\) WT or ∆SIV (Figure 7A and 7B) revealed that cell membrane expression of truncated channels was reduced by 27±3% compared to WT channels, without affecting total Na\(_{\text{v1.5}}\) protein expression. Similar experiments using scramble-infected HEK293 cells yielded comparable results (35±6% reduction; Figure 7C and 7D). When SAP97-silenced HEK293 cells were transfected with WT or ∆SIV channels, the number of channels expressed at the cell membrane was further reduced (56±5% and 56±6%; Figure 7C and 7D).

These results suggest that the decreased \(I_{\text{Na}}\) measured in SAP97-silenced cells or those with truncated channels may be attributable to reduced cell surface expression of Na\(_{\text{v1.5}}\), supporting the idea that SAP97 regulates cell membrane density of Na\(_{\text{v1.5}}\). Data presented in Online Figure VIII show that this regulation is independent of the ubiquitin-protein ligase Nedd4-2, which we have previously shown to regulate the number of Na\(_{\text{v1.5}}\) channels at the cell surface by interacting with a PY-motif found at the C terminus of the channel.3

**Discussion**

The major findings of the present study are: (1) Na\(_{\text{v1.5}}\) is part of at least 2 distinct multiprotein complexes in cardiomyocytes, one localized at the lateral membrane with dystrophin and syntrophin proteins, and the other involving the MAGUK protein SAP97 at the intercalated discs; (2) specific regulation of lateral membrane localization and density of Na\(_{\text{v1.5}}\) is dependent on dystrophin expression, the absence of which may underlie conduction slowing observed in dystrophin-deficient hearts; and (3) the absence of the SIV motif of Na\(_{\text{v1.5}}\) or depletion of SAP97 results in reduced channel expression of Na\(_{\text{v1.5}}\).
expression at the cell surface, and a subsequent decrease of $I_{Na}$ in HEK293 cells and adult cardiac myocytes.

**Distribution of Na$_v$1.5 Channels in Cardiomyocytes**

Studies investigating the localization of Na$_v$1.5 at the cell membrane of cardiac cells have revealed conflicting results. Although many articles have described Na$_v$1.5 stainings at the intercalated discs and lateral membrane of rat, mouse, dog, and human cardiomycocytes, other studies have shown almost exclusive expression of the channel at the intercalated discs. These discrepancies may be attributable to the alternative use of either heart sections or isolated myocytes, where a reorganization of proteins at the plasma membrane can occur. In a recent study, macropatch recordings of the 2 subcellular domains were performed, and it was concluded that the Na$_v$1.5-mediated current is almost nonexistent at the lateral membrane. The expression, or lack thereof, of Na$_v$1.5 channels at the lateral membranes likely has functional consequences on impulse propagation, as suggested by modeling studies. In the present work, we demonstrate that Na$_v$1.5 channels are present all along the plasma membrane of both rat and mouse cardiomyocytes. This finding is supported by the reduction of lateral Na$_v$1.5 expression in dystrophin-deficient myocytes, with no significant alteration in the expression at the intercalated discs. This observation is consistent with our previous study, in which we observed a $\approx$50% decrease of total Na$_v$1.5 protein expression in the absence of dystrophin, with a concomitant $I_{Na}$ reduction. Taken together, these observations strongly support the notion that a significant fraction of functional Na$_v$1.5 channels are located outside of the intercalated discs. These lateral membrane channels are likely to play a role in conduction, because their reduction alters ventricular conduction, which is reflected in a widening of the QRS complex in ECG recordings from mdx mice, where the hearts are otherwise free from structural remodeling. Confirming this concept, we show here for the first time, using noninvasive optical measurements of conduction velocity from the left ventricle free wall, and by 2 different analysis methods, that impulse propagation is significantly slowed in the transversal direction in dystrophin-deficient hearts that have reduced expression Na$_v$1.5 at the lateral membrane. These findings are in line with those of Baba and coworkers, who showed that dog cardiomyocytes isolated from peri-infarcted zones displayed reduced $I_{Na}$ density, associated with specific loss of the Na$_v$1.5 staining at the lateral membranes only. As the Na$_v$1.5 staining remained unchanged at intercalated discs, the reduction of $I_{Na}$ was proposed to be the cause of altered impulse propagation in the diseased tissue. These observations...
tions are also in agreement with those of a recent study reporting reduced excitability, slowed conduction velocity, and altered Na$\sub{1.5}$ expression in hearts of mdx mice. However, these investigators partly attributed these results to a reorganization of Cx43. Similarly to Sanford and coworkers, we could not confirm this finding in the present study, and found no remodeling of Cx43.28

Interaction Between Na$\sub{1.5}$ and SAP97 at Intercalated Discs

The carboxyl terminus of Na$\sub{1.5}$ has several protein–protein interaction domains that associate with numerous partners to regulate channel activity.2 As aforementioned, we have reported that the last 3 residues of Na$\sub{1.5}$ (SIV) interacts with the DMC, as well as with the protein tyrosine phosphatase PTPH1.29 Other groups have reported that ion channels bearing a PDZ-domain binding motif interact with members of the MAGUK family in various tissues.8 Several potassium channels of the K$\text{ir}$ and K$\text{s}$ families display PDZ-domain binding motifs that interact with the MAGUK proteins PSD95 or SAP97.8,12 Our pull-down experiments strongly support the existence of an interaction between Na$\sub{1.5}$ and SAP97 in cardiac tissue via the Na$\sub{1.5}$ SIV motif. These results are supported by a recent study,30 that suggests that Na$\sub{1.5}$ binds the first PDZ domain of SAP97. In our study, we also describe a close localization of SAP97 and Na$\sub{1.5}$ at intercalated discs. Immunostainings confirm data from Godreau et al who found an enrichment of SAP97 at this subcellular site. These results suggest that SAP97 is involved in targeting or anchoring of Na$\sub{1.5}$ channels to the intercalated discs of cardiomyocytes. Furthermore, SAP97 expression is unaltered in hearts of mdx mice (see Online Figure IX), indicating that the pool of Na$\sub{1.5}$ channels regulated at their PDZ domain-binding motif in the intercalated discs could be independent of the one complexed to syntrophin and DMC at lateral membranes of cardiomyocytes.

Physiological Relevance of the Two Regulating Mechanisms

In addition to the DMC, ankyrin-G has also been shown to interact with and regulate the trafficking of Na$\sub{1.5}$ at the cell membrane of cardiomyocytes.31,32 Ankyrin-G is primarily expressed at the intercalated discs, with another population at T-tubules; it is therefore unlikely that it is responsible for the specific targeting of Na$\sub{1.5}$ to the 2 compartments observed in our study. Whether the roles of syntrophin-dystrophin and ankyrin-G in trafficking, targeting, and anchoring of Na$\sub{1.5}$ to the lateral membrane are complementary or partly redundant remains to be elucidated. It may be speculated that ankyrin-G is involved in forward trafficking and correct targeting of the channel, and that PDZ-domain proteins anchor it in specific membrane compartments. The exact molecular mechanisms underlying the loss of Na$\sub{1.5}$ expression at the lateral membrane in the absence of dystrophin remains still unclear. Cheng and coworkers described several mutations in the gene encoding α1-syntrophin in patients with sudden infant death syndrome. Despite these results, clearly demonstrating the importance of α1-syntrophin in cardiomyocytes function, our data suggest that this protein complex is restricted to the lateral membrane. Sato and coworkers recently demonstrated that $I_{Na}$ is regulated by the protein plakophilin-2, which is enriched at the intercalated discs.35 Because plakophilin-2 does not contain a PDZ domain, this suggests that Na$\sub{1.5}$ could be differentially regulated by multiple proteins at the intercalated discs including SAP97 and plakophilin-2, similarly to the lateral membrane where it interacts with the DMC and ankyrin-G, within complexes that may or may not be distinct (Figure 8). Deciphering the specific and distinct roles of the different proteins in trafficking and/or anchoring of cardiac ion channels including Na$\sub{1.5}$ to different cellular compartments will be the goal of future experiments.

Limitations of the Study

This study demonstrates the presence of at least 2 different populations of Na$\sub{1.5}$ channels in cardiac cells. However the use of the mdx mouse as a disease model does not allow final conclusions about the physiological function of the 2 different Na$\sub{1.5}$ compartments. The optical mapping data indicate conduction slowing in all directions, and because the exact fiber orientation of the given cardiac substrate is not known, we assume that fiber orientation is similar in WT and mdx hearts at young ages in agreement with previous studies. In addition, our data do not show significant reduction of Na$\sub{1.5}$ expression at the intercalated disc in mdx hearts based on confocal immunofluorescence data. Nevertheless, potential changes of the mdx membrane ultrastructure at this complex site could underlie alterations in intercalated disc excitability.
that could not be established specifically here because of resolution limitations of confocal light microscopy. Furthermore, subtle, as yet unrecognized intercellular histological changes in mdx hearts could also contribute to the conduction slowing observed in optical mapping experiments and are subject to the same microscopy diffraction limitations. On the other hand, the present data suggests that SAP97 is predominantly localized in intercalated discs, and that SAP97 protein expression is not different in mdx mouse hearts. However, because immunostaining of SAP97 in mouse heart failed to produce specific staining despite repeated attempts with different antibodies and tissue fixations, possible reorganization of SAP97 cannot be excluded. Finally, potential upregulation or remodeling of connexins other than Cx43 was not addressed in this study.

Conclusions
The present study provides evidence that a fraction of Na\(_{1.5}\) channels is regulated by the DMC at the lateral myocyte membrane, and a different pool interacts with SAP97 at the intercalated discs of cardiomyocytes. Although the functional relevance of these distinct localizations of the main cardiac sodium channel has yet to be clarified, this study establishes that lateral sodium channels have a role in cardiac impulse propagation. Generation of a cardiac-specific SAP97 knock-out mouse models will likely elucidate the specific roles of this protein in cardiac function. Finally, similar to previous examples of proteins interacting with Na\(_{1.5}\) genetic variants of SAP97 may be linked to pathological cardiac phenotypes.

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Disclosures
None.

References
The scaffolding protein SAP97, which is predominantly found at the lateral membrane with the dystrophin/syntrophin complex and one at the intercalated discs, interacts with Na\textsubscript{v}1.5 and regulates its membrane density, hence forming another macromolecular complex.

Previously, we have demonstrated that Na\textsubscript{v}1.5 forms a macromolecular complex with dystrophin and syntrophin proteins. However because dystrophin is absent from the intercalated discs, we investigated the composition of the complex in this compartment. We found that Na\textsubscript{v}1.5 channels are parts of distinct macromolecular complexes in cardiomyocytes, one localized at the lateral membrane with the dystrophin/syntrophin complex, and the other involving the scaffolding protein SAP97 at the intercalated discs and that specific regulation of lateral membrane localization and density of Na\textsubscript{v}1.5 is dependent on dystrophin, the absence of which underlie conduction slowing observed in dystrophin-deficient hearts. We report that depletion of SAP97 reduces channel expression at the cell surface and increases sodium current and slower conduction velocity in cultured cardiac myocytes.

What New Information Does This Article Contribute?

- Cardiac sodium channels are parts of at least 2 distinct macromolecular complexes in cardiac cells: one localized at lateral membranes with the dystrophin complex and one at the intercalated discs.
- Absence of dystrophin leads to a specific downregulation of lateral Na\textsubscript{v}1.5 channels and impulse propagation slowing.
- The scaffolding protein SAP97, which is predominantly found at the intercalated discs, interacts with Na\textsubscript{v}1.5 and regulates its membrane density, hence forming another macromolecular complex.

Novelty and Significance

Previously, we have demonstrated that Na\textsubscript{v}1.5 forms a macromolecular complex with dystrophin and syntrophin proteins. However because dystrophin is absent from the intercalated discs, we investigated the composition of the complex in this compartment. We found that Na\textsubscript{v}1.5 channels are parts of distinct macromolecular complexes in cardiomyocytes, one localized at the lateral membrane with the dystrophin/syntrophin complex, and the other involving the scaffolding protein SAP97 at the intercalated discs and that specific regulation of lateral membrane localization and density of Na\textsubscript{v}1.5 is dependent on dystrophin, the absence of which underlie conduction slowing observed in dystrophin-deficient hearts. We report that depletion of SAP97 reduces channel expression at the cell surface and increases sodium current and slower conduction velocity in cultured cardiac myocytes. This indicates that there is not "one" sodium channel in cardiac cells, but multiple Na\textsubscript{v}1.5 channels that are differentially regulated in the different membrane compartments. These differences may underlie the variability of the cardiac phenotypes observed with genetic dysfunction of the cardiac sodium channel and could impart specific roles to different macromolecular complexes in normal and diseased heart.
SAP97 and Dystrophin Macromolecular Complexes Determine Two Pools of Cardiac Sodium Channels Na\textsuperscript{v1.5} in Cardiomyocytes

Séverine Petitprez, Anne-Flore Zmoos, Jakob Ögrodnik, Elise Balse, Nour Raad, Said El-Haou, Maxime Albesa, Philip Bittihn, Stefan Luther, Stephan E. Lehnart, Stéphane N. Hatem, Alain Coulombe and Hugues Abriel

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Supplement Material

SAP97 and dystrophin macromolecular complexes determine two pools of cardiac sodium channels \(\text{Na}_v1.5\) in cardiomyocytes

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Petitprez - Two pools of \(\text{Na}_v1.5\) channels

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Two pools of Na,1.5 channels

Supplement Material

Methods

Cardiac tissue samples and myocyte isolation

The experimental procedures were approved by the ethical commission on clinical research of the faculty of medicine of the University of Lausanne. Human right atrial samples were collected from patients in normal sinus rhythm undergoing coronary bypass surgery. The tissue was placed in ice cold PBS for transportation before being processed in our laboratory. C57BL/6 mice purchased at Janvier (Le Genest St Isle, France), and mdx<sup>5cv</sup> mice were anesthetized by intraperitoneal injection of 100 µl of pentobarbital (50 mg/ml) and 100 µl of heparin (Liquemin 5000 U.I./ml, Roche, Basel, Switzerland). Male Sprague rats (kind gift of Dr. D.Diviani, University of Lausanne) were anesthetized with 1.5L O<sub>2</sub>/ 5% isoflurane. A thoracotomy was performed and the cardiac ventricles were excised, rinsed with ice cold PBS and either frozen in liquid nitrogen or directly used for an experiment.

For patch-clamp experiments, Wistar rats (handled in accordance with guidelines for the care and use of laboratory animals published by the US National Institutes of Health) were anesthetized by intraperitoneal injection of heparin (0.1 ml/100 g of body weight) and pentobarbital (0.15 ml/100 g of body weight). Atria were dissected out and chopped into small pieces in Tyrodebicarbonate solution containing (mM): 120 NaCl, 5.6 KCl, 0.6 NaH<sub>2</sub>PO<sub>4</sub>, 1.1 MgCl<sub>2</sub>, 11.1 glucose and 24 NaHCO<sub>3</sub>, pH adjusted to 7.6 by bubbling with carbogen gas (95% O<sub>2</sub>, 5% CO<sub>2</sub>), and supplemented with 30 mM BDM, 10 mM taurine and 0.5 mM EGTA. Tissue fragments were then transferred into a Cell-Stir and predigested for 15 minutes with a mixture of type XXIV protease (20 U/ml, Sigma) and type V collagenase (10 U/ml, Sigma) diluted in calcium-free Krebs solution containing (mM): 10 HEPES, 35 NaCl, 10 glucose, 134 sucrose, 16 Na<sub>2</sub>HPO<sub>4</sub>, 25 NaHCO<sub>3</sub>, 4.75 KCl, 1.2 H<sub>2</sub>PO<sub>4</sub> and 1% BSA. The supernatant was discarded and tissue pieces were then subjected to four or five successive baths of 10 minutes each with type V collagenase (685 U/ml). Each supernatant was centrifuged to remove the enzyme and the pellets were resuspended in calcium-free Tyrode buffer containing (mM): 135 NaCl, 20 glucose, 10 HEPES, 4 KCl, 1 Na<sub>2</sub>HPO<sub>4</sub>, 2 MgCl<sub>2</sub> and supplemented with 10 mM BDM and 1% BSA. At the end of the digestion, resuspended pellets were pooled, centrifuged and resuspended in 3 ml of Tyrode solution containing 0.2 mM calcium for 5 minutes. To gradually increase the calcium concentration, the same volume of culture medium was added and the cells were incubated for another 5 minutes. After a final centrifugation step, the pellets were resuspended in M199 medium (Gibco) with 100 IU/ml penicillin and 100 µg/ml streptomycin, supplemented with 1% FBS (Gibco) and 1/1000 ITS (Sigma). Isolated cells were cultured under standard conditions (37°C, 5% CO<sub>2</sub>) until patch-clamp recordings were performed.

Isolation of mouse ventricular myocytes. Single cardiomyocytes were isolated according to established enzymatic procedures as previously described<sup>1</sup> from 3 month old WT (C57BL/6) and mdx (C57BL/10ScSn-Dmd<sup>mdx</sup>) mice (Jackson Laboratories, Bar Harbor, ME, USA). Briefly, mice were euthanized by cervical dislocation. Following intraventricular administration of heparin (500 U through the apex; Biochrom, Berlin, Germany), hearts were rapidly excised, cannulated and mounted on a Langendorff column for retrograde perfusion at 37°C. Hearts were rinsed free of blood with nominally Ca<sup>2+</sup>-free solution containing (in mmol/L): 135 NaCl, 4 KCl, 1.2 MgCl<sub>2</sub>, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 10 HEPES, 11 glucose, pH 7.4 (NaOH adjusted), and subsequently digested by a solution supplemented with 50 µM Ca<sup>2+</sup> and collagenase type II (0.6 mg/mL, Worthington, Switzerland) for 15 minutes. Following digestion, the atria were removed and the ventricles transferred to phosphate buffered saline, PBS (pH 7.4, Gibco, Basel, Switzerland), where they were minced into small pieces. Cardiomyocytes were liberated by gentle trituration of the digested ventricular tissue, filtered through a 200 µm nylon mesh and washed twice in PBS.
Cell preparation, transfection and infection
Human Embryonic Kidney (HEK293) and Sk-Hep cells were cultured in DMEM (Gibco) supplemented with 10% FBS, 0.2% glutamine and gentamicin (20 mg/mL) or geneticin (400 μg/mL), at 37°C in a 5% CO₂ incubator.

Silencing and lentiviruses
For preliminary silencing studies, HEK293 cells stably expressing Na,1.5 channels were transfected using lipofectamin™ (Invitrogen) with 0.6 µg EBO-pCD-Leu2-CD8 cDNA encoding CD8 antigen as a reporter gene and 1.8 µg of either SAP97 silencing oligos (cloned in pSUPER) or a scramble sequence of these oligos or pSUPER (empty vector). The following sequence was used to silence SAP97: SAP97-shRNA: 5’-GATATCCAGGAACATAAAT-3’ Scramble-shRNA: 5’-CCATAATACAGGTATAA-3’ (sequences from Gardner and co-workers). Then, VSV-G pseudotyped lentiviruses were produced by co-transfecting HEK293-T cells with 20 µg of the pSD28-GFP or pAB286.1 vectors containing the SAP97 shRNA cassette, 15 µg of pCMVDR8.91, and 5 µg of pMD2.VSVG by using the calcium phosphate method (empty plasmids: kind gift of D.Diviani group). Culture medium was replaced by serum-free DMEM 12h after transfection. Cell supernatants were collected 48h later and filtered through a 0.45-µm filter unit. HEK293 cells and HEK293 cells stably expressing Na,1.5 channels were infected with lentiviruses containing shRNA against SAP97, the scramble sequence or the empty vector. Two days after infection, puromycin was added to the culture medium at a final concentration of 4 µg/ml. After 4 days of selection, puromycin-resistant cells were collected and amplified in selective medium containing puromycin at a final concentration of 4 µg/ml.

To silence rat cardiomyocytes the following shRNA sequences were used: ShSAP97: 5’-CCCAAATCCATGGAAAATA-3’ (conserved sequence between SAP97 isoforms), or scramble: 5’-GTATAATACACCGCGCTAC-3’. These oligos were cloned into the psiSTRIKES™ hMGFP vector which renders transfected myocyte a green fluorescent cells (Promega, Fr). Following the dissociation, adult cardiomyocytes were cultured for 1 night in a 1% CO₂ incubator before transfection with either scrambled or SAP97 shRNA using a liposomal approach (Lipofectamine 2000, Invitrogen).

Transfection
For patch clamp experiments, HEK293 cells were transiently transfected using calcium phosphate with 0.3 µg of GFP-tagged Na,1.5 (kind gift from Dr. T. Zimmer, University of Jena, Jena, Germany) or GFP-tagged Na,1.5ΔSIV. For experiments addressing the roles of Nedd4-2 or Syntrophin, 1.4 µg of Nedd4-2 was added to 0.3 µg of Na,1.5 WT or YA and 1.4 µg of Syntrophin or empty pcDNA3.1 plasmid were added to 0.3 µg of GFP-tagged Na,1.5. 0.6 µg of pIRESH-hβ1-CD8 cDNAs encoding hβ1 subunit (CD8 antigen as a reporter gene) or 0.6 µg EBO-pCD-Leu2-CD8 cDNA were used.

Protein extraction
Frozen human atrial appendages or fresh mouse ventricles were transferred into Lysis Buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF and Complete® protease inhibitor cocktail from Roche). Tissues were then homogenized using a Polytron. Triton Tx-100 was added to achieve a final concentration of 1% and solubilisation occurred by rotating for 1h30 at 4°C. HEK293 or Sk-Hep cells were washed with Phosphate Buffer Saline (PBS, Invitrogen) and lysed in Lysis Buffer containing 1% Tx-100 lysed for 45 minutes at 4°C on a wheel. The soluble fractions from a subsequent 15-min centrifugation at 13,000 g (4°C) were then used for the experiments. In order to load each lane of the SDS-page with equivalent amounts of total
protein, the protein concentration of each lysate was measured in triplicate by Bradford assay using a BSA standard curve.

**Pull-down assays**
cDNAs encoding the last 66 amino acids of Na\(_{1.5}\) WT and S2014Stop were cloned into pGEX-4T1 (Amersham Bioscience, Piscataway, USA). Expression of GST-fusion proteins in E.Coli K12 cells was induced with 0.5 mM IPTG for 2 h at 30°C. Cells were harvested by centrifugation and re-suspended in lysis buffer (20 mM Tris pH 7.5, 250 mM NaCl, 0.5% NP40, 1 mM EDTA, 1 mM PMSF, 0.2 mg/ml DNAse I (Roche) and 0.2 mg/ml Lysozyme (Roche)). After bacterial lysis, fusion proteins were purified on Glutathione-Sepharose beads (Amersham Bioscience). The soluble fractions obtained after applying the protein extraction protocol (see above) were incubated with GSH-sepharose beads containing either GST or the GST fusion protein off the beads. After four washes in Lysis Buffer, samples were re-suspended with 2.5x Sample Buffer containing 60 mM 1,4-Dithiothreitol (Uptima) and boiled five minutes at 95°C. Beads were removed from the samples and the resulting pulled-down proteins were analyzed by Western blot as described below.

**Biotinylation Assay**
HEK293 cells silenced for SAP97 as well as control cells (scrambled) were transfected either with the WT or truncated Na\(_{1.5}\) channels. 48h after transfection, cells were washed twice with PBS and biotinylated for 30 minutes at 4°C using PBS containing 2.5mg of EZ Link Sulfo-NHS-SS-Biotin. Plates were washed twice with PBS-200mM Glycine (to quench unlinked biotin) and twice with PBS. Cells were then lysed for one hour at 4°C with Biotinylation Lysis Buffer (50 mM Hepes pH7.4; 150 mM NaCl; 1.5 mM MgCl\(_2\); 1 mM EGTA; 10% Glycerol; 1% Triton X-100; 1.2mg/ml N-Ethyl-maleimide and Complete® protease inhibitor cocktail from Roche ). After centrifugation, supernatants were incubated with 30 µl of Streptavidin-Sepharose beads (GE healthcare, Waukesha, Wisconsin, USA) for 2 hours at 4°C and pelleted by centrifugation. After 5 washes with Biotinylation Lysis Buffer containing 100 mM PMSF the samples were warmed for 30 minutes at 37°C in 2.5x Sample Buffer (containing 60 mM DTT). Biotinylated proteins were then analysed by Western Blot as described below.

**Western blot**
Proteins were separated on a 5-15% acrylamide SDS-PAGE, then transferred to a nitrocellulose membrane and incubated with primary antibodies followed by infrared IRDye\({\text{TM}}\) secondary antibodies. Proteins were detected using the Odyssey\textsuperscript{®} Infrared Imaging System (Bad Homburg).

**Immunohistochemistry on rat and mouse heart sections**
Hearts from C57BL/6 male mice at 10 weeks of age or Sprague male rats at 7 weeks of age were longitudinally cut and embedded in Tissue-Tek\textsuperscript{®} O.C.T\textsuperscript{TM} Compound (Sakura Finetek, Zoeterwoude, Netherland). Frozen sections of 5 µm thickness were stored at -80°C until further use. Cryosections were let to dry at room temperature (RT) for about 15 minutes before starting the immunostainings. Except when indicated, all further manipulations were performed at room temperature (~25°C). Tissue sections were rehydrated in PBS before being fixed using either 2% paraformaldehyde or cold aceton for 10 minutes depending on the primary antibody. Cryosections were rinsed in PBS and then blocked for one hour in PBS containing 0.5% Triton X-100, 1% BSA and 10% goat serum. Frozen heart slices were incubated with primary antibodies diluted in PBS containing 0.5% Triton X-100, 1% BSA and 3% goat serum for 2h. Tissues slices were rinsed with PBS and then incubated for 1 hour with secondary antibodies (diluted in the same solution as the primary antibody). After washes in PBS, Fluorsave (Merck)
was added to the cryosections. Frozen heart slices were then covered with a glass coverslip and kept at 4°C till further analysis. For control experiments, primary antibodies were omitted. Immunostainings were analyzed using a confocal microscope (Leica SP5 AOB).

**Immunocytochemistry of isolated mouse ventricular myocytes.** Cell suspensions containing WT and *mdx* cardiomyocytes respectively were mixed, and cells were plated on laminin-coated glass cover slips and allowed to adhere for 1h. Cardiomyocytes were fixed for 10 min with 4% paraformaldehyde in PBS, and free aldehyde groups were quenched with 150 mM glycine for 10 min. Cells were washed in PBS (2 × 10 min) and subsequently permeabilized with 0.5% Tween 20 for 30 min. After the permeabilization solution had been washed out with PBS (3 × 10 min), WT and *mdx* cardiomyocytes were labelled with antibodies directed against rabbit Na,1.5 and mouse dystrophin by incubation overnight at 4°C in primary antibody buffer (1% BSA, 5% goat serum in PBS). Following washout of excess primary antibodies (3 × 10 min PBS), cells were incubated with secondary antibodies for 1h and washed with PBS (3 × 10 min) before the cover slips were mounted onto frosted slides in a solution composed of 90% FluorSave Reagent (Calbiochem, La Jolla, CA, USA) and 10% 10X PBS.

**Immunocytochemistry and 3D-microscopy of cultured atrial myocytes.** Adult atrial myocytes were isolated from rat atria as described previously and transduced the day after with adenoviruses in order to either over-express (Ad SAP97, personal production) or silence SAP97 (Ad shSAP97). Transduction with an Ad shß-Gal was used as control. Three days later, cells were fixed for 10 minutes with acetone. Coverslips were incubated for one hour in blocking buffer (PBS, 1% BSA, 0.5% Triton X100, 10% goat serum and 10% chicken serum) before overnight incubation with primary antibodies (diluted in blocking buffer: PBS, 1% BSA, 0.5% Triton X100 and 3% goat serum and 10% chicken serum). Indirect immunofluorescence was performed using secondary antibodies while nuclei were stained with the DAPI dye. Images were acquired with an Olympus epifluorescent microscope (60X, UPlanSApo, 0.17) equipped with a cooled CoolSnap camera (Roper-Scientific). Sections of 0.2 μm thickness were collected using a piezoelectric system driven by the Metamorph software (Molecular Devices) and supplemented with the 3D-deconvolution module. For each sample, 3 consecutive z-images were thresholded to similar levels and z-projections were carried on using the ImageJ software. Brightfield images were also acquired to provide information on cell shape and cell membranes.

**Antibodies**

The following antibodies were used: Polyclonal anti-actin (A 2066) monoclonal anti-dystrophin (clone MANDYS8) obtained from SIGMA (Buchs, Switzerland). Polyclonal antibody recognizing human/rat Na,1.5 was purchased from Alomone labs (ASC-005; Jerusalem, Israel). Monoclonal anti-PSD-95 family (recognizing PDZ domains of PSD95, chapsin110 and SAP97) used for biochemical assays were from United States Biological (clone 0.T.124; Swampscott, Massachusetts, USA), and those used for immunohistochemistry were from UC Davis/NIH NeuroMab Facility. Polyclonal anti-PSD95 was from Abcam (ab12093; Cambridge, England). Monoclonal anti-SAP97, used for biochemistry and immunohistochemistry on isolated rat myocytes, was obtained from StressGen (VAM-PS005; Ann Arbor, Michigan, USA) and that used for immunohistochemistry was obtained from UC Davis/NIH NeuroMab Facility (antibodies were developed at NeuroMab, supported by NIH grant U24NS050606 and maintained by the Department of Neurobiology, Physiology and Behavior, College of Biological Sciences, University of California, Davis, CA 95616). Polyclonal anti-ZO-1 was purchased from ZYMED Laboratories (ZMD.436; Carlsbad, California, USA). Monoclonal pan-syntrophin (recognizing
two pools of Na,1,5 channels

alpha 1, beta 1 and beta 2 subunits of syntrophin) was obtained from Affinity Bioreagents (Thermo Fisher Scientific, Rockford, Illinois, USA). A polyclonal anti-Connexin-43 was from Abcam (ab66151) and a monoclonal form from Chemicon (Mab3068; Millipore; Billerica, Massachusetts, USA).

In Western blot experiments, infrared IRDye™ (680 or 800 CW) secondary antibodies were purchased from LI-COR Biosciences (Bad Homburg, Germany). Secondary antibodies used for immunohistostainings were the following: Fluorescein (FITC)-conjugated anti-rabbit (711-095-152) from Jackson Immunoresearch laboratories (West Grove, Pennsylvania, USA); and Alexa Fluor® 647 anti-mouse (A-31571), goat anti-mouse-Alexa 488 and chicken anti-rabbit-Alexa 594 from Invitrogen (Basel, Switzerland).

**Electrophysiology**

For patch-clamp recordings, pClamp software, version 8 (Axon Instruments, Union City, CA) and a VE-2 (Alembic Instruments, Montreal, QC, Canada) were used. Data were analyzed using pClamp software, version 8 (Axon Instruments) and KaleidaGraph (Synergy Software, Reading, PA). Patch-clamp recordings of HEK cells were carried-out using an internal solution containing (mmol/L) CsCl 60; Cs aspartate 70; EGTA 11; MgCl₂ 1; CaCl₂ 1; HEPES 10; and Na₂-ATP 5, pH 7.2 with CsOH and external solution NaCl 50; n-methyl-D-glutamine 80; CaCl₂ 2; MgCl₂ 1.2; CsCl 5; HEPES 10; and glucose 5, pH 7.4 with CsOH. Measurements were done in the whole-cell configuration. Using these solutions, 5 min after rupturing the membrane, no significant alterations of the availability curve nor the peak current were observed. Holding potentials were -100 mV and no leak subtraction was performed. The resistance of the pipettes was in the range of 1.9–2.9 MΩ. We used only data from cells where the access resistance remained stable over the entire duration of the experiment. Cells for which signs of poor voltage-clamp control such as delayed inflections of the current or discontinuities in the peak Iₙa vs. Vm curve were not analyzed. Measurements were carried out at room temperature (~25°C).

Peak currents were measured at -20mV during a current-voltage protocol and Iₙa densities (pA/pF) were obtained by dividing the peak Iₙa by the cell capacitance obtained from the pClamp function. Activation properties were determined from I/V relationships (see inserted protocols in figures) by normalizing peak Iₙa to driving force and maximal Iₙa, and plotting normalized conductance vs Vm. Voltage-dependence of steady-state inactivation was obtained by plotting the normalized peak current (25-ms test pulse to -20 mV after a 500-ms conditioning pulse) vs Vm. For the voltage-dependence of steady-state activation and inactivation (fast and slow) curves, the data from individual cells were fitted with Boltzmann relationship, y(Vm) = 1/(1+exp[(Vm-V₁/₂)/K]), in which y is the normalized current or conductance, Vm is the membrane potential, V₁/₂ is the voltage at which half of the channels are activated or inactivated, and K is the slope factor.

Isolated rat adult atrial cardiomyocytes were patched with an internal solution containing (mM) NaCl 10, CsCl 130, CaCl₂ 1, EGTA 10, HEPES 10, MgATP 5 and D-glucose 10, pH 7.2 with CsOH, and an external perfusion solution containing NaCl 80, CsCl 50, CoCl₂ 2.5, CaCl₂ 2, 4-AP 5, HEPES 10 et glucose 10; pH 7.4 with CsOH. During the recordings, myocytes were incubated in a Tyrode solution containing (mM): NaCl 135, KCl 4, MgCl₂ 2, CaCl₂ 2, HEPES 10, NaH₂PO₄ 1, Na-pyruvate 2.5, and D-glucose 20, pH 7.4 with NaOH. Ionic currents were recorded with a patch-clamp amplifier (Axopatch 200A, Molecular Devices). Patch pipettes (Corning Kovar Sealing code 7052, WPI) had resistances of 1.5 to 2 MΩ. Holding potentials were -140mV and currents were recorded every 5 or 10mV from -90mV to 60mV at 0.2Hz and digitized with a DigiData 1200 (Molecular Devices). Data were acquired and analyzed with Acquis-1 software (G. Sadoc, CNRS, Gif/Yvette, France).
Optical mapping of the mouse left ventricle

Data were obtained from young adult (14-16 weeks) male wild-type (C57BL/10ScSn; n=6) and mdx (C57BL/10ScSn-Dmd<sup>mdx</sup>/J; n=8) mouse hearts. All animal care conformed with institutional guidelines and German laws for the Care and Use of Laboratory Animals. Mice were obtained from The Jackson Laboratory (Bar Harbor, MN).

High-resolution optical mapping studies were performed in Langendorff perfused mouse hearts extracted from euthanized, heparinized wild-type and mdx mice as indicated. The aorta of the intact hearts was carefully cannulated using a 22 G cannula and connected to a custom-made perfusion setup. The heart was perfused at 2-3 ml/min with O<sub>2</sub>-saturated Tyrode solution containing (mmol/L): NaCl 130, NaHCO<sub>3</sub> 24, KH<sub>2</sub>PO<sub>4</sub> 1.2, MgCl<sub>2</sub> 1, glucose 5.6, KCl 4, CaCl<sub>2</sub> 1.8, 37°C, pH 7.4; as well as insulin (5 IU/L), blebbistatin (5 μmol/L), and albumin (1%). Only hearts with a physiological spontaneous beating rate of 500/min or higher were used for imaging to ensure consistent perfusion rates and electrical heart activity. The LV free wall was projected with the maximal cross-diameter in the objective light path for consistent imaging analysis of conduction spread. Following 10 min equilibration the heart was stained with a voltage-sensitive reporter dye (di-4-ANNEPS, Molecular Probes, Portland, OR) by injecting a 2.0 mL bolus containing 80 nmol/L into a 10 ml compliance chamber proximal to the heart aorta. When fluorescence intensity and heart rate reached steady-state, imaging was begun (typically after 8-10 mins) using an upright macro-zoom epifluorescence microscope (MVX10; Olympus) equipped with a high-speed CMOS (complementary metal oxide semiconductor) camera (Ultima-L; SciMedia), as reported previously.<sup>4</sup> Excitation light from a 100 W mercury arc lamp (Olympus) was passed through an interference filter (520 ± 30 nm) and a dichroic mirror (565 nm) to the heart. The emitted fluorescent light was collected with a long-pass filter (>590 nm) and projected onto the camera. The CMOS recording array was 100 x 100 pixels, and data were acquired at 2,000 frames/s with 14-bit resolution. Imaging was carried out using a 1x objective (N.A. 0.25; Olympus) at 4x zoom magnification of the left ventricular free wall.

Pacing and volumetric ECG recording

The tip of a bipolar mouse heart pacing electrode mounted on a micromanipulator device was gently positioned on the surface center of the LV free wall. Pacing capture was confirmed at 2 ms pulse length at CL 100 ms by a specialized cardiac stimulating electrode (50-100 kΩ, FHC, USA). Two custom-made Ag-AgCl electrodes were placed near the septum plane to record volume-conducted electrocardiograms (vECGs). Recordings of vECGs were amplified and low-pass filtered at 200 Hz using an electronic amplifier (HSE, Germany) and digitally sampled at 1 kHz with 16 bit resolution using the NI USB-6216 BNC (National Instruments) digitizer and custom-made software written in Labview (National Instruments). Continuous vECG recordings were obtained throughout the experiment and stored for off-line analysis. Recordings were electronically tagged to indicate the exact time of optical recording. Intermittent pacing capture was confirmed by vECG signal analysis and by optical signal analysis.

Conduction velocity measurements

The center of the LV free wall was paced at a cycle length of 100 ms by a bipolar pacing electrode (see above) to study conduction spread at a constant mouse physiological heart rate of 600 min<sup>-1</sup>. All data were analyzed offline using a custom-made open source software platform written in Java and optimized for mouse heart analysis. The image sequence was filtered in space using a 5x5 uniform kernel. For each pixel, the signal was filtered in time using a moving average filter (5 frames), normalized to [0,1] and inverted. Activation time maps were obtained by measuring the activation times for each pixel. Activation times were estimated by the time (i.e. frame number) at which the fluorescence signal exceeded a given threshold (0.5). Sub-
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Frame accuracy was achieved by linear interpolation of the activation times. Isochrones were computed from interpolated activation time maps using a contour isochrone plot routine (Matlab, The MathWorks). The local conduction velocity was measured by two independent methods. 1) An ellipsis fitting algorithm (investigator independent) was used which approximates individual isochrones by least squares fitting and thereby determines the major (longitudinal) and minor (transversal) electrical conduction vectors from each successive isochrone within a given conduction map. The conduction maps used for ellipsis fitting were averaged from 10 consecutive cardiac cycles in order to exclude beat-to-beat variability (representative examples are shown in figure 3). Bias of the isochrone data by optical distortions due to the pacing electrode is indicated by a small symbol in each map to facilitate data review. After completion of ellipsis fitting to each thresholded and averaged data set, longitudinal and transversal isochrone data points were plotted in a distance-time graph and linearity of conduction established by linear regression. The successive data points representing a linear relation where used to determine the slope from the linear regression to estimate conduction velocity. On the other hand, data points which significantly deviated from linear behavior due to pace site proximity or organ border aberrations were excluded from the CV analysis. In addition 2) the isochrone gradient of the activation map was analyzed along the longitudinal and transversal axis as determined by the analyzing investigator. The velocity field was regularized by removing outliers, i.e. velocity vectors that deviated more than 15% in velocity or 20% in angle with respect to the corresponding average value of the nearest neighbors. Average LV conduction velocity measurements were analyzed in the major heart axis (apex to aorta) referred to as longitudinal and perpendicular referred to as transversal conduction velocity. The main vector average CV is defined as the average spatial-temporal conduction spread along the given axis and includes a homogenous data section containing conduction data within a ±15° triangle. The regions immediately surrounding the pacing electrode (a known source of stimulus CV artifact) as well as the tangentially projected heart borders or sides (a known source of ellipsoid object side border CV artifact) were excluded from the analyzed sector region used for signal analysis.

**Statistical analysis**

Data are represented as mean values +/- SEM except for figure 3 data which are represented as mean values +/- SD. Two-tailed Student t-test was used to compare means of two groups of samples and for more groups, an ANOVA test with a Bonferroni post-test was applied. Statistical significance was set at P<0.05. For conduction velocity measurements, data are represented as mean values +/- SD. In these data, t-test was applied for comparison of independent groups and p < 0.05 was accepted as significant.
References for the supplementary material


Two pools of Na,1.5 channels

**Online figures**
Online Fig. I. Localization of Na\textsubscript{v}1.5, dystrophin and syntrophin in mouse heart ventricular sections
(A) Left: Na\textsubscript{v}1.5 (green) both at the lateral membranes and the intercalated discs; middle: dystrophin (red) only at the lateral membranes; right: merge of the two images showing close localization of Na\textsubscript{v}1.5 and dystrophin at the lateral membranes. The white arrow heads show the absence of dystrophin at the intercalated discs. Inset: magnification of a cell (B) Left: Na\textsubscript{v}1.5 (green) both at the lateral membranes and intercalated discs; middle: syntrophin (red) only at the lateral membranes; right: merge of the two images showing close localization of Na\textsubscript{v}1.5 and syntrophin at the lateral membranes. The white arrow heads show the absence of syntrophin at the intercalated discs, Inset: magnification of a portion of cell.
Online Fig. II. Localization of Cx43 in ventricular sections of WT and mdx mice
(A) Representative images of ventricular sections immunolabeled for Cx43 from three different WT mice, showing predominant Cx43 localization at intercalated discs of mouse cardiomyocytes. (B) In ventricular sections from mdx mice, Cx43 is similarly organized and found mainly at intercalated discs. The images are from three different mdx mouse hearts.
Online Fig. III.
Full size images of western blots presented in Fig. 4B and C.
Online Fig. IV. Na\textsubscript{v}1.5 co-staining with SAP97 and syntrophin in adult-cultured myocytes

(A) Na\textsubscript{v}1.5 and SAP97 co-staining. a-c: SAP97-transduced myocytes, d-f: SAP97-silenced myocytes. After silencing, the expression of both SAP97 and Na\textsubscript{v}1.5 channels is reduced. Note that the Na\textsubscript{v}1.5 channel expression at the plasma membrane without neighboring cells (non-contact areas) is drastically inhibited. c' and c'' are enlargements of two representative areas of c showing the high density staining for SAP97 at the level of the cell-cell contact (c', arrow heads) and the expression of the SAP97 protein at a non-contact site (c'', arrows). f' and f'' are magnifications of the SAP97-silenced myocytes in f illustrating the reduction of Na\textsubscript{v}1.5 staining at the cell membrane.
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(B) Nav1.5 and syntrophin co-staining. a-c: SAP97-transduced myocytes, d-f: SAP97-silenced myocytes. The magnification image $c'$ shows that some Nav1.5 channels are associated with syntrophin at the level of cell border; Nav1.5 staining is also observed at the level of cell-cell contacts where syntrophin staining is absent ($c''$). As in the panel A, Nav1.5 channel expression is reduced after SAP97-silencing, whereas syntrophin staining remains exclusively located at non-contact areas ($f$ and $f''$).
Online Fig. V. Voltage-dependence of inactivation and activation.

(A) Inactivation curves of scrambled (squares) and shSAP97 (circles) cells transiently transfected with WT (empty) or ΔSIV (black) Na\textsubscript{v}1.5. Voltage-dependence of steady-state inactivation was obtained after applying a 25-ms test pulse to -20 mV after a 500-ms conditioning pulse (see inserted protocol). (B) Steady-state activation of scrambled (squares) and shSAP97 (triangles) cells transiently transfected with WT (empty) or ΔSIV (black) Na\textsubscript{v}1.5. Activation properties were determined from current/voltage relationships obtained after applying the inserted step protocol. See also Material and Methods.
Online Fig. VI. Effect of SAP97 silencing and Beta1 on \( I_{Na} \) in HEK293 cells.

(A) \( I_{Na} \) density of scrambled HEK293 cells, transiently transfected with WT or \( \Delta SIV \) Nav1.5 channels with or without Beta1 subunits (CD8: reporter gene), mean values in pA/pF: Scrambled+WT+CD8: 214±62, Scrambled+WT+Beta1: 280±42, Scrambled+\( \Delta SIV \)+CD8: 104±20, Scrambled+\( \Delta SIV \)+Beta1: 114±13. **p<0.01 compared to Scrambled+WT+Beta1 condition. (B) Inactivation (left) and steady-state activation (right) curves obtained after applying the inserted protocols on the corresponding cells. See also Material and Methods.
Online Fig. VII. Effect of SAP97 silencing and Syntrophin on $I_{\text{Na}}$ in HEK293 cells.
$I_{\text{Na}}$ density of scrambled or silenced (ShSAP97) HEK293 cells, transiently transfected with WT Nav1.5 channels with or without syntrophin (inset: representative currents) (scrambled+WT+Ø: 111.9±13.7, scrambled+WT+Syntrophin: 107.4±21.3, shSAP97+WT+Ø: 47.3±9.7, shSAP97+WT+Syntrophin: 31.35±7.9 pA/pF), *p<0.05, **p<0.01 (one way ANOVA and Bonferroni post-test). The number of cells is written in the corresponding columns.
Online Fig. VIII. Investigation of the role of Nedd4-2 and PY motif of Na\textsubscript{v}1.5

(A) I\textsub{Na} density of scrambled or SAP97-silenced (shSAP97) HEK293 cells expressing Na\textsub{v}1.5 WT or \textit{ΔSIV} with empty plasmid (Ø, black columns) or Nedd4-2 (red columns), *p<0.05, **p<0.01, ***p<0.001 (one way ANOVA and Bonferroni post-test, compared to the "scrambled + WT + Ø" condition). (B) I\textsub{Na} density of scrambled or silenced (ShSAP97) HEK293 cells, transiently transfected with Na\textsub{v}1.5 WT or YA *p<0.05, **p<0.01; ns: not significant (one way ANOVA and Bonferroni post-test compared to the "scrambled + WT" condition). Number of cells is written in the corresponding columns.
Online Fig. IX. SAP97 protein level is comparable in hearts of mdx5cv mice compared to WT.

For comparison of the amounts of SAP97 present in the hearts of control and mdx5cv mice, 40 μg of ventricular lysates were loaded on SDS-page gels. The protein concentration of each lysate was measured in triplicate by Bradford assays to guarantee equivalent loading and confirmed by Ponceau coloration. (A) Representative Western blots performed with the indicated antibodies. (B) The amount of SAP97 examined in four hearts for each genotype was assessed by fluorescence intensity quantification with LICOR Odyssey software. Statistical significance was tested with a two-tailed Student’s t-test.