The Hippo Pathway Is Activated and Is a Causal Mechanism for Adipogenesis in Arrhythmogenic Cardiomyopathy

Suet Nee Chen,* Priyatansh Gurha,* Raffaella Lombardi,* Alessandra Ruggiero, James T. Willerson, A.J. Marian

Rationale: Mutations in the intercalated disc proteins, such as plakophilin 2 (PKP2), cause arrhythmogenic cardiomyopathy (AC). AC is characterized by the replacement of cardiac myocytes by fibro-adipocytes, cardiac dysfunction, arrhythmias, and sudden death.

Objective: To delineate the molecular pathogenesis of AC.

Methods and Results: Localization and levels of selected intercalated disc proteins, including signaling molecules, were markedly reduced in human hearts with AC. Altered protein constituents of intercalated discs were associated with activation of the upstream Hippo molecules in the human hearts, in Nkx2.5-Cre:Dsp+/− and Myh6:Jup mouse models of AC, and in the PKP2 knockdown HL-1 myocytes (HL-1PKP2-shRNA). Level of active protein kinase C-α isoform, which requires PKP2 for activity, was reduced. In contrast, neurofibromin 2 (or Merlin), a molecule upstream of the Hippo pathway and that is inactivated by protein kinase C-α isoform, was activated. Consequently, the downstream Hippo molecules mammalian STE20-like protein kinases 1/2 (MST1/2), large tumor suppressor kinases 1/2 (LATS1/2), and Yes-associated protein (YAP) (the latter is the effector of the pathway) were phosphorylated. Coimmunoprecipitation detected binding of phosphorylated YAP, phosphorylated β-catenin, and junction protein plakoglobin (the latter translocated from the junction). RNA sequencing, transcript quantitative polymerase chain reaction, and reporter assays showed suppressed activity of SV40 transcriptional enhancer factor domain (TEAD) and transcription factor 7-like 2 (TCF7L2), which are transcription factors of the Hippo and the canonical Wnt signaling, respectively. In contrast, adipogenesis was enhanced. Simultaneous knockdown of Lats1/2, molecules upstream to YAP, rescued inactivation of YAP and β-catenin and adipogenesis in the HL-1PKP2-shRNA myocytes.

Conclusions: Molecular remodeling of the intercalated discs leads to pathogenic activation of the Hippo pathway, suppression of the canonical Wnt signaling, and enhanced adipogenesis in AC. The findings offer novel mechanisms for the pathogenesis of AC. (Circ Res. 2014;114:454-468.)

Key Words: adipogenesis ■ cardiomyopathies ■ genetics ■ Wnt signaling pathway

A rrhythmogenic cardiomyopathy (AC), also known as arrhythmogenic right ventricular cardiomyopathy, is an enigmatic hereditary cardiomyopathy caused, in part, by mutations in genes encoding the desmosome proteins.1,2 AC commonly manifests with cardiac arrhythmias, sudden cardiac death, and heart failure.3,4 The pathological hallmark of AC is replacement of cardiac myocytes by fibro-adipocytes, predominantly in the right ventricle.5,6 Despite the grim prognosis of the affected individuals, there is no effective pharmacological or nonpharmacological therapy except for heart transplantation.

The discovery of junction protein plakoglobin (JUP) as a causal gene for Naxos disease led to partial elucidation of the causal genes for AC.7 Mutations in PKP2, DSG2, DSC2, and DSP, encoding desmosome proteins plakophilin 2 (PKP2), desmoglein 2 (DSG2), desmocollin 2 (DSC2), desmoplakin (DSP), and TMEM43, encoding transmembrane protein 43, account for ≈50% to 60% of the AC.1,2 PKP2 is the most common causal gene for familial AC, accounting for up to 40% of the cases.8,9 The majority of PKP2 mutations are frame-shift insertion/deletion mutations that result in or are expected to result in haploinsufficiency of the encoded protein.8,10

Desmosomes are multiprotein complexes that assemble at the cell membrane and together with the adherens junctions...
the gap junctions (GJs) form the intercalated discs (IDs). The molecular distinction between the components of the IDs in the heart is less evident because the protein constituents of the IDs assemble into a junctional area referred to as area composita. Consequently, the molecular effects of the mutant desmosome proteins in AC could extend to various components and functions of the IDs.

Conventionally, IDs are recognized as cell–cell adhesion structures responsible for maintaining the mechanical integrity of the heart. The IDs are also emerging as molecular hubs regulating signaling pathways involved in cell fate determination, differentiation, and proliferation. Notable among the signaling molecules that localize to the IDs is the β-catenin, the effector of the canonical Wnt pathway, which is inactivated on sequential phosphorylation by casein kinase 1 (CK1) at S45 and glycogen synthase kinase 3β (GSK-3β) on residues S33, S37, and T41. Likewise, protein kinase C-α isoform (PKC-α), which requires PKP2 as a scaffold protein at the junction, also localizes to the IDs. Another component of the IDs is neurofibromin 2 (NF2), also known as Merlin (moesin, ezrin, and radixin-like protein), which is an upstream molecule to the Hippo pathway. Thus, the canonical Wnt and the Hippo pathways are partly regulated at the cell membrane and likely through the IDs.

We postulate that mutations involving the ID proteins not only impair the structural integrity of the area composita but also disrupt signaling pathways that are regulated at the IDs. Accordingly, perturbed molecular changes in the IDs should affect the Hippo and the canonical Wnt signaling pathways, major regulators of cellular differentiation and proliferation. Activation of the Hippo pathway might also be a mechanism for suppression of the canonical Wnt signaling, which is implicated in the pathogenesis of AC.

### Human Samples

Human heart samples, without identifiers, from 4 patients with pathologically proven AC and from 2 to 4 normal individuals were used.

### Mouse Models

Cardiac-restricted heterozygous DSP-deficient (Nkx2.5-Cre:Dsp<sup>+</sup>) and cardiac-specific truncated JUP (Myh6:Jup<sup>+</sup>) mice have been described previously.

### HL-1<sup>PKP2:shRNA</sup> Myocytes Stable Cell Lines

Two independent, stable HL-1 myocyte lines were established using lentiviruses expressing short hairpin RNAs (shRNAs) that target the coding sequence of the <sup>Pkp2</sup> gene.

### Mobility Shift Detection of Phosphorylated Proteins (Phos-tag)

Mn<sup>2+</sup>-Phos-tag SDS-PAGE was prepared per another protocol. The membranes were probed with antibodies that detect both the total and the phosphorylated forms of the intended proteins.

### Treatment With PKC Activators PMA and OAG

Subconfluent HL-1 and HL-1<sup>PKP2:shRNA</sup> myocytes were treated with either 100 nmol/L of phorbol-12-myristate-13-acetate (PMA) or 50 μmol/L of 1-oleoyl-2-acetyl-sn-glycerol (OAG) for 15 or 30 minutes to activate PKC-α.

### Treatment With CK1, GSK-3β, and Proteasome Inhibitors

Subconfluent cells were treated with IC261, an inhibitor of CK1, or 6-bromoiindirubin-3′-oxime (BIO), an inhibitor of GSK-3β, or MG132 to block the ubiquitin proteasome system.

### RNA Sequencing and Ingenuity Pathway Analysis

DNAse-digested and rRNA-depleted RNAs were sequenced on an Illumina HiSeq instrument using sequencing reagents for a pair-end 100-basepair run. Library was prepared using the Illumina TruSeq

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**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AC</td>
<td>arrhythmogenic cardiomyopathy</td>
</tr>
<tr>
<td>BIO</td>
<td>6-bromoiindirubin-3′-oxime</td>
</tr>
<tr>
<td>CK1</td>
<td>casein kinase 1</td>
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<tr>
<td>DSC2</td>
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<tr>
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<td>DSP</td>
<td>desmoplakin</td>
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<td>GSK-3β</td>
<td>glycogen synthase kinase 3β</td>
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<tr>
<td>ID</td>
<td>intercalated disc</td>
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<tr>
<td>JUP</td>
<td>junction protein plakoglobin</td>
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<tr>
<td>LAT51/2</td>
<td>large tumor suppressor kinases 1/2</td>
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<tr>
<td>MST1/2</td>
<td>mammalian STE20-like protein kinases 1/2</td>
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<td>NF2</td>
<td>neurofibromin 2</td>
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<tr>
<td>OAG</td>
<td>1-oleoyl-2-acetyl-sn-glycerol</td>
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<td>PKC-α</td>
<td>protein kinase C-α isoform</td>
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<td>plakophilin 2</td>
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<td>PMA</td>
<td>phorbol-12-myristate-13-acetate</td>
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<td>pMST1/2</td>
<td>phosphorylated mammalian STE20-like protein kinases 1/2</td>
</tr>
<tr>
<td>pNF2</td>
<td>phosphorylated neurofibromin 2</td>
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<tr>
<td>pPKC-α</td>
<td>phosphorylated protein kinase C-α isoform</td>
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<td>pYAP</td>
<td>phosphorylated Yes-associated protein</td>
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<tr>
<td>pβ-catenin</td>
<td>phosphorylated β-catenin</td>
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<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
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<tr>
<td>TEAD</td>
<td>SV40 transcriptional enhancer factor domain</td>
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<td>YAP</td>
<td>Yes-associated protein</td>
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**Methods**

The Institutional Review Board at The University of Texas Health Science Center at Houston approved the protocol. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health and was approved by the Institutional Animal Care and Use Committee. A detailed Materials and Methods section is provided as Online Data Supplement.

**Human Samples**

Human heart samples, without identifiers, from 4 patients with pathologically proven AC and from 2 to 4 normal individuals were used.

**Mouse Models**

Cardiac-restricted heterozygous DSP-deficient (Nkx2.5-Cre:Dsp<sup>+</sup>) and cardiac-specific truncated JUP (Myh6:Jup<sup>+</sup>) mice have been described previously.

**HL-1<sup>PKP2:shRNA</sup> Myocytes Stable Cell Lines**

Two independent, stable HL-1 myocyte lines were established using lentiviruses expressing short hairpin RNAs (shRNAs) that target the coding sequence of the <sup>Pkp2</sup> gene.

**Histology**

Oil Red O, Masson trichrome, and immunofluorescence staining were performed as described previously.

**Molecular Biology**

Immunoblotting, coinmunoprecipitation, and quantitative polymerase chain reaction were performed per the conventional methods. Generation of recombinant lentiviruses and induction of adipogenesis were performed as described.

The HL-1 myocytes were transfected with the SV40 transcriptional enhancer factor domain (TEAD) and TCF7L2 reporter plasmids. The Hippo and the canonical Wnt signaling pathways were assessed by the ratio of Firefly to Renilla luciferase readouts. The list of antibodies, TaqMan probes, and the shRNA sequence is provided in Online Tables I, II, and III, respectively.

**Mobility Shift Detection of Phosphorylated Proteins (Phos-tag)**

Mn<sup>2+</sup>-Phos-tag SDS-PAGE was prepared per another protocol. The membranes were probed with antibodies that detect both the total and the phosphorylated forms of the intended proteins.
Figure 1. Molecular remodeling of intercalated discs (IDs) in human arrhythmogenic cardiomyopathy (AC). A, Immunofluorescence panels showing reduced levels and localization of selected ID proteins. B, Immunoblots showing reduced levels of selected ID proteins in the human AC. C, Bar graphs showing relative levels of the ID proteins. n=2 for normal hearts and n=4 for AC hearts. *P<0.05; **P<0.01; ***P<0.001. CDH2 indicates N-cadherin; DSC2, desmocollin 2; DSG2, desmoglein 2; DSP, desmoplakin; JUP, junction protein plakoglobin; MT, masson trichrome; and PKP2, plakophilin 2.
RNA library preparation kit. The CASAVA software was used for sequence readout.

Sequencing reads were mapped to the mouse reference genome build 9 and analyzed by TopHat. Differentially expressed genes were analyzed using the Cufflink package. The transcript counts were normalized and represented as fragments per kilobase of transcript per million mapped fragments. The data were also analyzed by de novo transcriptome assembly using RNA-Seq by Expectation Maximization software package, and the normalized data were represented as transcript per million. Heat maps were plotted using R (http://www.rstudio.com/), and Volcano plots were graphed using MATLAB and GraphPad Prism software. The threshold for a false discovery rate (q value) was set at 0.05.

Statistics

Differences in the means±SD of the continuous variables with a normal distribution pattern between the 2 groups were compared by the t test and among multiple groups by the ANOVA, followed by pairwise comparisons.36 Continuous variables that did not follow normality and the categorical variables were analyzed by the Kruskal–Wallis test or by the χ² test.

Results

Molecular Remodeling of IDs in the Human Hearts From Patients With AC

To determine the molecular changes in the ID proteins in AC, we obtained ventricular tissues from the explanted hearts of 4 anonymous patients with the clinical diagnosis and pathological diagnosis of advanced AC who were undergoing heart transplantation. Control hearts were donor hearts that were not used for transplantation. Histological examination of the ventricular tissues showed replacement of the myocytes by fibro-adipocytes in the AC hearts and was normal in the control hearts (Figure 1A). Expression levels and localization of selected ID proteins were significantly altered in the AC hearts. Junctional localizations of JUP, PKP2, DSC2, DSG2, and DSP were significantly reduced in the human hearts with AC (Figure 1A). In addition, localization of GJA1 (connexin 43), a GJ protein involved in electric conduction and arrhythmogenesis, was also perturbed. Immunoblotting showed marked reductions in the JUP, PKP2, DSC2, DSG2, and DSP, as well as in GJA1 levels in the hearts of patients with AC (Figure 1B and 1C). Localization of CDH2 (N-cadherin) to IDs was also reduced. However, a consistent change in CDH2 protein level was not detected (Figure 1A–1C).

Activation of the Hippo Pathway in AC

To test the hypothesis that the molecular remodeling of the IDs perturbs signaling pathways regulated at the junction, protein levels and localization of selected components of the Hippo pathway in the human hearts with AC were determined. NF2 and, to a lesser extent, phosphorylated NF2 (pNF2; S518) levels were increased significantly in the human hearts with AC (Figure 2A and 2B). pNF2 was predominantly localized to the IDs in the normal heart. In contrast, no discernible localization to the IDs was detected in the AC hearts (Figure 2C). The total NF2 was diffusely expressed in the myocardium, without a clear localization to IDs in the normal and AC hearts (Figure 2C). In conjunction with the increased NF2 levels, levels of phosphorylated Yes-associated protein (pYAP; S127), the downstream cascade target of NF2, were also increased in the human hearts with AC. pYAP was predominantly localized to junctions in the AC hearts (Figure 2C). There was no significant change in the phosphorylated WW domain containing transcription regulator 1, pWWTR1, a transducer of the Hippo pathway (Figure 2A). Changes in the expression levels and localization of pYAP and pNF2 were more prominent in the epicardial region (Figure 2D).

To corroborate the findings in the human hearts in the mouse models of AC, the Nkx2.5-Cre:Dspp/W and Myh6:Jun mice, which exhibit a phenotype partially resembling the human AC, were used.30,31 NF2 was markedly increased in the Nkx2.5-Cre:Dspp/W and Myh6:Jun mouse hearts (Figure 3A and 3B). In contrast, pNF2 (inactive) was reduced, suggesting an increase in the level of active (nonphosphorylated) NF2. In accord with increased NF2 levels, pYAP and, to a lesser degree, phosphorylated mammalian STE20-like protein kinase 1 (pMST1) levels were also increased in the hearts of the mouse models (Figure 3A and 3B). Increased levels of the phosphorylated Hippo components were consistent with changes in the levels and localization of the pNF2, pMST1/2, and pYAP, as detected by immunofluorescence staining of myocardial sections (Figure 3C and 3D). As observed in the human hearts, pNF2 and pYAP were predominantly localized to the cell membrane and prominently in the epicardial regions (Figure 3D).

To investigate mechanistic consequences of molecular remodeling of IDs and activation of the Hippo pathway, PKP2, the most common causal gene in AC,8,9 was knocked down in the HL-1 myocytes using recombinant lentiviruses expressing 2 independent sets of shRNAs against the Pkp2 gene. Knockdown of PKP2 in the HL-1 myocytes (HL-1PKP2:shRNA) was confirmed by quantitative polymerase chain reaction of the Pkp2 mRNA, immunofluorescence, and immunoblotting of the PKP2 protein (Figure 4A–4C). PKP2 protein was reduced by 66.6±17% (49%–88%) in the HL-1PKP2:shRNA myocytes (Figure 2C).

To identify changes in the transcriptome upon knockdown of the PKP2, whole-transcriptome sequencing (RNA sequencing) was performed. Ingenuity pathway analysis and upstream regulator analysis indicated activation of the Hippo pathway, as shown by reduced mRNA levels of genes positively regulated by the YAP/TEAD (the latter is a transcription factor of the Hippo pathway) in the HL-1PKP2:shRNA myocytes (Figure 4D–4G). A list of differentially expressed transcripts is provided in Online Table IV. Reduced transcript levels of the selected YAP–TEAD targets were confirmed by quantitative polymerase chain reaction (Figure 4H). To delineate the molecular changes responsible for activation of the Hippo pathway and suppression of gene expression through YAP–TEAD, protein constituents of the Hippo kinase cascade were analyzed by immunoblotting, Phos-tag blot, and immunofluorescence. Expression levels of several molecules of the Hippo pathway were increased markedly in the HL-1PKP2:shRNA myocytes (Figure 4I and 4J). The level of active NF2 was increased in conjunction with increased levels of phosphorylated forms of its downstream kinases pMST1/2, phosphorylated large tumor suppressor kinases 1/2 (pLATS1/2), and pYAP (the latter is transcriptionally inactive effector of the Hippo pathway) (Figure 4I and 4J). Increased pYAP levels were also confirmed by Phos-tag blotting (Figure 4K). To strengthen the evidence...
for activation of NF2, protein levels of pERK44/42 (mitogen activated protein kinase 1/3) and ras-related C3 botulinum toxin substrate 1, which are negatively regulated by active NF2,39–41 were determined. pERK44/42 and ras-related C3 botulinum toxin substrate 1 levels were significantly reduced in the HL-1PKP2:shRNA myocytes (Figure 4I). Finally, TEAD transcriptional activity, determined on transfection of the control HL-1 and HL-1PKP2:shRNA myocytes with a TEAD-luciferase reporter construct, was reduced by 55±16% in the HL-1PKP2:shRNA myocytes (Figure 4L). Collectively, the findings substantiate activation of the Hippo kinase pathway, inactivation of YAP, and suppression of gene expression through TEAD transcription factor in AC.

Inactivation of PKC-α

Active phosphorylated PKC-α (pPKC-α), which inactivates NF2 by phosphorylation,19,28 requires PKP2 as a scaffold protein for localization to the junction.18 pPKC-α was partially localized to the IDs in the normal hearts in a pattern similar to that of PKP2 (Figure 5A). In contrast, no discernible localization of pPKC-α (T696) to IDs was detected in the human hearts with AC (Figure 5A). Likewise, pPKC-α protein levels were also reduced in the human hearts with AC (Figure 5B and Online Figure IA).

Corroborating the findings in the human hearts, pPKC-α was also localized to the IDs in the nontransgenic mouse heart, whereas its localization to the IDs was significantly reduced in the hearts of Nkx2.5-Cre:DspW/F and Myh6:Jup mice (Figure 5C). Likewise, pPKC-α levels were noticeably reduced in the mouse models of AC as compared with control nontransgenic mice (Figure 5D and Online Figure IB).

As observed in the in vivo studies, pPKC-α levels were significantly reduced in the HL-1PKP2:shRNA myocytes (Figure 5E). Likewise, immunofluorescence staining showed localization of the pPKC-α to the membrane, cytosol, as well as the nucleus in the control HL-1 myocytes. In contrast, pPKC-α was almost absent in the membrane in the HL-1PKP2:shRNA myocytes and was
predominantly in the nucleus (Figure 5F). To determine the effects of reduced pPKC-α on phosphorylation of NF2, control HL-1 and HL-1PKP2:shRNA myocytes were treated with 2 different general PKC (and PKD) activators, namely PMA and OAG. Despite treatment with PMA and OAG, membrane localization of pPKC-α remained reduced in the HL-1 PKP2:shRNA myocytes (Figure 5G and 5H). Treatment with PMA and OAG increased pPKC-α levels in the wild-type HL-1 myocytes but not in HL-1PKP2:shRNA myocytes (Figure 5I and 5J and Online Figure IC and ID). In conjunction with the changes in the pPKC-α levels, pNF2 levels were increased in the wild-type HL-1 but not in the HL-1PKP2:shRNA myocytes (Figure 5J).

Suppression of the Canonical Wnt Signaling

Because activation of the Hippo pathway is known to suppress the canonical Wnt signaling,26 which is implicated in the pathogenesis of AC,30,31 changes in the levels and localization of total and pβ-catenin in the human hearts with AC, mouse models, and the HL-1PKP2:shRNA myocytes were analyzed. Levels of pβ-catenin were significantly increased in the human hearts with AC (Figure 6A and 6B), whereas the total β-catenin levels were unchanged (Figure 6A and 6B). However, localization of the β-catenin was significantly altered, being localized to the IDs in the normal heart (Figure 6C) but not in the human hearts with AC (Figure 6C). As observed in the human hearts with AC, pβ-catenin and pNF2 were predominantly localized to cell membrane. Immunofluorescence staining of the epicardial region of the heart in mouse models of AC showing distinct localization of pNF2, pMST1, and pYAP to the cell junction. TUBA1B indicates tubulin alpha 1B.
Figure 4. Activation of the Hippo pathway in the HL-1 myocytes on knockdown of plakophilin 2 (PKP2). A to C, Quantitative polymerase chain reaction (qPCR), immunofluorescence, and immunoblotting data showing knockdown of PKP2 mRNA and protein in the HL-1 myocytes (HL-1^{PKP2:shRNA}). PKP2 mRNA and protein were reduced by 60% to 80% (n=6 for mRNA and n=4 for protein; *P<0.0001). D, Volcano plot of RNA sequencing (RNA-Seq) data, demarcated at q<0.05. E, Heat plot of Yes-associated protein (YAP)–SV40 transcriptional enhancer factor domain (TEAD) targets constructed from the RNA-Seq data and analyzed by the ingenuity pathway analysis (IPA; q<0.05). F and G, Quantitative data for selected low-abundance (F) and high-abundance (G) YAP–TEAD targets identified by RNA-Seq and represented as transcript per million reads (n=2; q<0.05). H, qPCR data showing reduced level of selected Hippo pathway downstream targets (n=19 for Ccnd1, n=12 for Ctgf, n=3–7 for other genes; *P<0.05; **P<0.005; ***P<0.0001). I, Immunoblotting analysis of Hippo pathway components. Top, Phos-tag blot for neurofibromin 2 (NF2) showing increased active (nonphosphorylated) NF2 level. Increased phosphorylated mammalian STE20-like protein kinase 1 (pMST1), phosphorylated large tumor suppressor kinases 1 (pLAT51), and phosphorylated Yes-associated protein (pYAP) and reduced ras-related C3 botulinum toxin substrate 1 (RAC1) and mitogen activated protein kinase 1/3 (pERK) levels (the latter 2 are negatively regulated by the Hippo pathway). J, Phos-tag blot showing increased pYAP levels in HL-1^{PKP2:shRNA} cells. K, Immunofluorescence panels largely corroborating the immunoblotting findings. L, TEAD-luciferase reporter assay showing decreased transcriptional activity of TEAD transcription factor (n=3; *P<0.005). HL-1^{WT} indicates wild-type HL-1 myocytes.
analyzed. Ingenuity pathway analysis suggested suppression of the canonical Wnt signaling in the HL-1PKP2:shRNA myocytes. Transcript copy number of the selected established canonical Wnt targets was reduced in the HL-1PKP2:shRNA myocytes (Figure 7A). Conversely, mRNA levels of selected genes negatively regulated by the canonical Wnt pathway were increased (Figure 7B). The RNA sequencing findings were corroborated by quantitative polymerase chain reaction for a selected number of the well-established canonical Wnt targets (Figure 7C).

The most marked reduction was in the Ccnd1 mRNA, which was reduced by 99±5% (n=19; P<0.0001). In conjunction with changes in the mRNA levels of Ccnd1, CCND1 protein was also significantly reduced in the HL-1PKP2:shRNA myocytes (Figure 7D and 7E). Moreover, pβ-catenin was significantly increased in the HL-1PKP2:shRNA myocytes, in accord with the findings in the human hearts with AC and the mouse models (Figure 7E). In addition, localization of the β-catenin to cell membrane was significantly reduced in the HL-1PKP2:shRNA myocytes, whereas pβ-catenin was diffusely distributed (Figure 7F and 7G). To determine whether inactivation of β-catenin by phosphorylation was associated with reduced canonical Wnt transcriptional activity, HL-1 and HL-1PKP2:shRNA myocytes were transfected with a transcription factor 7-like 2 (TCF7L2) reporter construct and luciferase activity was determined. The luciferase activity was reduced by 54±25% (n=9; P=0.0019) in the HL-1PKP2:shRNA myocytes (Figure 7H). In accord with suppressed canonical Wnt signaling, adipogenesis was enhanced in HL-1PKP2:shRNA myocytes, as detected by Oil

Figure 5. Inactivation of protein kinase C-α isoform (PKC-α) and activation of neurofibromin 2 (NF2) in arrhythmogenic cardiomyopathy (AC). A, Immunofluorescence panels showing colocalization of plakophilin 2 (PKP2) and phosphorylated PKC-α (pPKC-α) in the normal human heart sections to the intercalated discs (IDs), whereas localization of PKP2 and pPKC-α to IDs was reduced or absent in the human hearts with AC. B, Immunoblotting analysis of cardiac proteins showing reduced pPKC-α in the normal hearts with AC (61±16%; n=2 for normal hearts and n=4 for hearts with AC; P=0.007). C, Immunofluorescence panels showing localization of PKP2 and pPKC-α to the IDs in the nontransgenic (NTG) mouse hearts but substantially reduced localization of pPKC-α to the IDs in the mouse models of AC (Nkx2.5-Cre:DepoW/F: 57±5%; n=2; P=0.003; and Myh6:Jup: 70±5%; n=2; P=0.002). D, Immunoblotting of cardiac proteins showing reduced pPKC-α levels in the mouse models of AC as compared with NTG. E, Immunoblotting showing reduced PKP2 and pPKC-α levels in the HL-1PKP2:shRNA myocytes. F, Immunofluorescence panels showing reduced pPKCα levels in HL-1PKP2:shRNA myocytes at the baseline as compared with control HL-1 cells. G and H, Differential (reduced) activation of pPKC-α in the HL-1PKP2:shRNA myocytes on treatment of cells with 2 independent PKC activators phorbol myristate acetate (PMA) and 1-oleoyl-2-acetyl-sn-glycerol (OAG). I, Immunoblotting showing a differential increase in the pPKC-α level in the HL-1 and HL-1PKP2:shRNA myocyte on activation with PMA and OAG at 2 different time points. Dimethyl sulfoxide-treated samples were used as control for the solvent. Corresponding changes in the level of phosphorylated NF2 (pNF2) on activation of pPKC-α are also shown for each set of experiments. TUBA1B indicates tubulin alpha 1B.
Red O and immunofluorescence staining for peroxisome proliferator-activated receptor gamma, an adipogenic transcription factor (Figure 7I–7K).

**Binding of pYAP, pβ-Catenin, and JUP**

To determine a molecular link between the Hippo and the canonical Wnt pathways and the IDs, protein extracts from human hearts with AC and controls were analyzed by coimmunoprecipitation. YAP and pβ-catenin as well as YAP and JUP were coimmunoprecipitated in the human heart protein extracts (Figure 8A). The corresponding coimmunoprecipitated blots in HL-1 and HL-1PKP2:shRNA myocytes also corroborated coimmunoprecipitation of the β-catenin and pβ-catenin in the NTG, Nkx2.5-Cre:DspW/F, and Myh6:Jup mice. pCTNNB1 indicates phospho beta catenin.

Changes in the pβ-catenin correlated with similar changes in pYAP levels, which remained differentially increased in the HL-1PKP2:shRNA myocytes as compared with wild-type HL-1 myocytes (Figure 8C). Likewise, treatment with BIO did not normalize differentially increased pβ-catenin in the HL-1PKP2:shRNA myocytes (Figure 8D). Finally, because unbound pβ-catenin is subject to degradation by the proteasome system, HL-1 and HL-1PKP2:shRNA were treated with MG132, an inhibitor of the ubiquitin proteasome system. Treatment with MG132 increased pβ-catenin levels in the control HL-1 and the HL-1PKP2:shRNA myocytes without normalizing the differentially increased pβ-catenin levels in the HL-1PKP2:shRNA myocytes (Figure 8E).

**Rescue of Adipogenesis on Suppression of LATS1/2**

To determine the pathogenic role of activation of the Hippo pathway in AC, LATS1 and LATS2, key upstream molecules that
Figure 7. Suppression of the canonical Wnt signaling in HL-1\textsuperscript{pKp2}\textsuperscript{shRNA} myocytes. Heat maps of canonical Wnt target genes (A) and Wnt inhibitors (B) constructed from the RNA sequencing (RNA-Seq) data (q<0.05). C, Downregulation of selected canonical Wnt targets as detected by quantitative polymerase chain reaction (n=3–7; ***P<0.0001). D, Immunofluorescence staining showing reduced nuclear localization of the canonical Wnt target cyclin D1 (CCND1) in the HL-1\textsuperscript{pKp2}\textsuperscript{shRNA} myocytes. E, Immunoblotting analysis of the components of canonical Wnt signaling pathway showing increased phosphorylated β-catenin (pβ-catenin) and reduced CCND1 levels. Phos-tag blot (bottom) showing increased β-catenin levels. Immunofluorescence staining for β-catenin (F) and pβ-catenin (G) showing reduced membrane localization of β-catenin in the HL-1\textsuperscript{pKp2}\textsuperscript{shRNA} myocytes. H, Transcription factor-like 2 (TCF7L2)–luciferase reporter assay showing reduced TCF7L2 activity by ≈55% in the HL-1\textsuperscript{pKp2}\textsuperscript{shRNA} myocytes (n=8; *P<0.05) I, Representative Oil Red O (ORO)-stained and peroxisome proliferator-activated receptor gamma (PPARG)-stained panels showing increased number of cells containing fat droplets and cells expressing PPARG, respectively, in the HL-1\textsuperscript{pKp2}\textsuperscript{shRNA}, as compared with control cells. J and K, Corresponding quantitative data for adipogenesis (1000–1250 cells per group; **P<0.005). HL-1\textsuperscript{WT} indicates wild-type HL-1 myocytes. TUBA1B indicates tubulin alpha 1B.
inactivate YAP by phosphorylation, were knocked down in the control HL-1 and HL-1PKP2:shRNA myocytes. Recombinant lentiviruses expressing 4 independent sets of shRNAs were analyzed to identify the most effective shRNA targeting Lats1 and Lats2, independently, followed by pairwise testing against both genes (Online Figure IIA–IID and Figure 9A–9C). Selected pairs of shRNAs suppressed mRNA levels of Lats1 and Lats2 by 75±10% (Figure 9A and 9B). Likewise, LATS1 protein was markedly reduced with combined pairs of shRNAs in the HL-1 as well as in the HL-1PKP2:shRNA myocytes (Figure 9C). Knockdown of LATS1/2 was associated with reduced levels of pYAP, which were more pronounced in the HL-1PKP2:shRNA myocytes (Figure 9C). Notably, the β-catenin, which also localizes to the IDs, was partially inactivated by phosphorylation at the CK1 and GSK-3β phosphorylation sites. In addition, junctional localization of pPKC-α, which requires PKP2,18 and its levels were reduced. In conjunction with partial inactivation of pPKC-α, NF2 or Merlin, which is inactivated on phosphorylation by pPKC-α, was activated in AC.19,28 Activation of NF2 was associated with cascade phosphorylation of the Hippo kinases, including MST1/2, LATS1/2, and YAP (the latter is the effector of the Hippo pathway, which is inactivated on phosphorylation).20

Discussion

The findings in the human hearts with AC, mouse models, and HL-1 myocytes collectively indicate activation of the Hippo pathway in AC, which contributes to the pathogenesis of adipogenesis in AC. The trigger for the activation of the Hippo pathway is presumed to be the molecular changes at the IDs in human patients with advanced AC. Accordingly, levels of several ID proteins were markedly reduced, a finding that is in accord with the previous data showing reduced junctional localizations of JUP in AC.42–44 The findings, in view of monogenic pathogenesis of AC, suggest that the assembly of the IDs requires coordinated interactions between its protein constituents.45 Impaired ID assembly not only is expected to affect the mechanical integrity of myocyte–myocyte attachment but also instigates a series of signaling events that are regulated at the IDs. Notably, the β-catenin, which also localizes to the IDs, was partially inactivated by phosphorylation at the CK1 and GSK-3β phosphorylation sites. In addition, junctional localization of pPKC-α, which requires PKP2,18 and its levels were reduced. In conjunction with partial inactivation of pPKC-α, NF2 or Merlin, which is inactivated on phosphorylation by pPKC-α, was activated in AC.19,28 Activation of NF2 was associated with cascade phosphorylation of the Hippo kinases, including MST1/2, LATS1/2, and YAP (the latter is the effector of the Hippo pathway, which is inactivated on phosphorylation).20

Figure 8. Binding of phosphorylated Yes-associated protein (pYAP), phosphorylated β-catenin (pβ-catenin), and junction protein plakoglobin (JUP) and the effects of inhibition of casein kinase 1 (CK1) and glycogen synthase kinase 3β (GSK-3β). A, Coimmunoprecipitation of human cardiac proteins with an anti-YAP antibody and detection of β-catenin (CTNNB1) or JUP by immunoblotting (IB) showing binding of CTNNB1 with YAP as well as binding of YAP with JUP. B, Corresponding coimmunoprecipitations on cell lysates obtained from the HL-1 and HL-1PKP2:shRNA myocytes showing binding of YAP with β-catenin and with JUP. Effects of treatment with CK1 inhibitor IC261 (C) or GSK-3β inhibitor 6-bromoindirubin-3′-oxime (BIO; D) and ubiquitin proteasome inhibitor MG132 (E) on levels of pβ-catenin and pYAP, showing differentially increased pβ-catenin and pYAP levels in the HL-1PKP2:shRNA myocytes. ARVC indicates arrhythmogenic right ventricular cardiomyopathy; and WT, wild type.
Figure 9. Rescue of adipogenesis by knockdown of large tumor suppressor kinase 1 (LATS1) and LATS2. A and B, Suppression of Lats1 (A) and Lats2 (B) mRNAs in wild-type HL-1 (HL-1WT) and HL-1Pkp2:shRNA myocytes on simultaneous targeting of LATS1 and LATS2 by short hairpin RNA (shRNA) pairs. C, Knockdown of LATS1 protein after LATS1 and LATS2 knockdown by 4 shRNA pairs. The antibody against LATS2 protein did not detect a specific band. D, Increased peroxisome proliferator-activated receptor gamma (PPARG) levels after 1 week of adipogenic induction in the HL-1Pkp2:shRNA myocytes and normalization on knockdown of LATS1/2. E, Rescue of adipogenesis on knockdown of LATS1/2, as detected by Oil Red O (ORO) and immunofluorescence staining for PPARG, corroborating the findings of increased PPARG protein detected by immunoblotting. F and G, Quantitative data showing increased number of PPARG-positive and ORO-positive cells in the HL-1Pkp2:shRNA myocytes and their normalization on knockdown of LATS1/2 (n=500 cells per group; 1-way ANOVA P<0.0001, Tukey post hoc test for pairwise comparison; *P<0.05; **P<0.01; ***P<0.001; ###P<0.0005). CTNNB1 indicates β-catenin; pYAP, phosphorylated Yes-association protein; and TUBA1B, tubulin alpha 1B.
Consequently, gene expression through YAP–TEAD was suppressed. Moreover, coprecipitation of YAP, β-catenin, and JUP was associated with increased membrane localization of YAP and β-catenin, which might contribute to suppression of gene expression through the Hippo and the canonical Wnt pathways, respectively. These molecular changes were associated with enhanced adipogenesis in AC models, which was rescued on knockdown of LATS1/2 or expression of active YAP. Collectively, the findings in the human hearts, mouse models, and the HL-1 myocytes indicate activation of the Hippo pathway through the IDs and provide for a novel mechanism for the pathogenesis of enhanced adipogenesis in AC.

The extent of the molecular changes observed in the human hearts with AC is remarkable and seemingly in discord with the monogenic nature of AC, wherein the primary deficit is in a single protein. This finding likely reflects the necessity of coordinated stoichiometric assembly of protein constituents of the IDs. In the human AC, a considerable number of the causal mutations result in or are expected to result in haploinsufficiency. The changes are also in accord with the current view on the ID structure in the heart lacking distinct components, as opposed to the epithelial cells, which have distinct substructures. The observed molecular changes also extend beyond proteins that are conventionally known as the desmosome proteins and involve others, such as the GJ protein GJA1. The diversity of the molecular changes, such as impaired GJA1 and SCN5A, a subunit of the sodium channel protein, localization, and function might partially explain the phenotypic complexity of the AC.

IDs, conventionally recognized as the structures responsible for the mechanical integrity of myocyte–myocyte attachment, are emerging as signaling hubs regulating contact-mediated pathways involved in cellular differentiation and proliferation. NF2 (Merlin), an upstream molecule of the Hippo pathway, which localizes to the IDs and is known to interact with β-catenin, PKC-α, and cadherins, is activated in AC. Reduced PKP2 resulting in impaired junctional localization of pPKC-α might provide a potential instigator for activation of NF2 in AC. Activation of NF2 was corroborated at multiple levels, albeit indirectly, by showing increased levels of the nonphosphorylated NF2, increased levels of its downstream target pMST1/2, and reduced levels of pERK1/2 and ras-related C3 botulinum toxin substrate 1, which are negatively regulated by the active NF2. Collectively, the findings point to activation of NF2 as a link between the IDs and downstream molecules of the Hippo pathway. Nevertheless, the Hippo pathway in the mammals is not fully understood and the pathway seems to be much more complicated than the initially envisioned linear cascade (Online Figure III). Alternative upstream proteins either directly or indirectly might link impaired assembly of the IDs to activation of the Hippo kinases in AC. Notably, the WW domain protein KIBRA in D. Melanogaster is upstream of NF2 and, along with Angiomotin, coregulates the Hippo pathway. In addition, salvador homolog 1, a coactivator of the Hippo pathway, was recently shown to localize to cell membranes. The Hippo pathway might also be activated through mechanisms other than those directly regulated at the IDs, such as disruption of the actin cytoskeleton. Consistent with this notion, the HL-1 PKP2-shRNA myocytes were smaller, differed in gross morphology, and had perturbed filamentous actin organization (Online Figure V). The role of these changes in inactivation of the Hippo pathway was not characterized in the present study. Finally, the Hippo pathway molecules are typically tumor suppressor multifunctional proteins with diverse phenotypic effects. Thus, it would not be surprising if alternative and complementary mechanisms activate the Hippo pathway in AC.

The pathogenic role of activation of the Hippo pathway in adipogenesis might be related to its suppressive effects on the canonical Wnt signaling. YAP binds to β-catenin and JUP (Figure 8A), which might result in membrane localization of pYAP, as observed in the human AC. These molecular interactions could sequester β-catenin and YAP and suppress the canonical Wnt and Hippo pathways, shifting the cell fate toward an adipogenic lineage. Activation of the Hippo pathway and inactivation of YAP are also implicated in the PKA-induced adipogenesis. YAP has been shown to inactivate phosphatase and tensin homolog, which is known to block the insulin-like growth factor I/mechanistic target of rapamycin 1 pathway and adipogenesis. The cardiac cell type targeted by the Hippo pathway for differentiation to adipocytes in AC remains to be identified. Moreover, the Hippo pathway has been shown to regulate cardiac myocyte size and proliferation. It is intriguing to postulate that activation of the Hippo pathway might also be a mechanism for myocyte atrophy, which are typically observed in the human AC. Finally, subtle differences in the pNF2 (inactive) level and membrane localization of pβ-catenin between the human heart tissues with AC and the cardiac tissues from the mouse models were noted, which might reflect differences among the species, tissue prepreparations (formalin-fixed human tissues versus fresh-frozen mouse heart tissues), or the affinity of the antibodies for the mouse and human proteins.

To determine whether activation of the Hippo pathway and inactivation of the YAP contribute to other features of AC, such as cardiac dysfunction or the predominant involvement of the right ventricle, in vivo rescue experiments are required, but they are currently challenging, partly because of the inadequately defined Hippo pathway in the mammalian heart. Genetic manipulation of NF2 is likely to result in a compounded phenotype because of the functional diversity of NF2. Conditional knockout of Salv (salvador homolog 1) has been used to suppress the Hippo pathway. Salvador homolog 1 is a coactivator of the pathway, and its deletion is not expected to suppress the Hippo pathway activated through NF2, as observed in the present studies. Conditional deletion of both Lats1 and Lats2 would be required to inactivate the Hippo pathway and activate YAP. However, this is currently not feasible. Finally, cell-specific expression of an active form of YAP in vivo, as in the in vitro studies, might provide an opportunity to discern the phenotypic effects of inactivation of YAP from those imparted on activation of the upstream Hippo kinases in the pathogenesis of AC.

In conclusion, molecular remodeling of the IDs observed in the human hearts with AC activates NF2 (Merlin), which
results in cascade phosphorylation of the Hippo kinases, in- 
avivation of YAP, and enhanced adipogenesis (Online Figure IV). The findings corroborated in the human hearts, mouse models, and HL-1 myocytes indicate activation of the Hippo pathway as a novel mechanism in the pathogenesis of AC.

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Disclosures

None.

References


Novelty and Significance

What Is Known?

- Arrhythmogenic cardiomyopathy (AC) is a hereditary disease that clinically manifests with cardiac arrhythmias, heart failure, and sudden death.
- Pathological hallmark of AC is a gradual replacement of cardiac myocytes by fibro-adipocytes.
- Mutations in genes encoding protein constituents of the desmosomes, members of the intercalated discs, cause AC.
- The molecular mechanisms that link the mutations to the phenotype in AC are largely unknown.

What New Information Does This Article Contribute?

- Human hearts with AC exhibit extensive molecular remodeling of the intercalated discs that extend beyond the desmosome proteins and involves several signaling molecules, including phosphorylated protein kinase C-α isoform (active), β-catenin (the latter is the effector of the canonical Wnt signaling), and neurofibromin 2, an upstream molecule of the Hippo pathway.
- Impaired localization of phosphorylated protein kinase C-α isoform to the junction is associated with activation of neurofibromin 2 (also known as Merlin) and results in cascade phosphorylation of the Hippo kinases, mammalian STE20-like protein kinases 1/2, large tumor suppressor kinases 1/2, and Yes-associated protein (YAP); the latter is the effector of the Hippo pathway.
- Increased phosphorylation of YAP and β-catenin is associated with reduced signaling through YAP–SV40 transcriptional enhancer factor domain and β-catenin–transcription factor 7-like 2, the transcription factors of the Hippo and the canonical Wnt signaling pathways, respectively.
- Activation of the Hippo and suppression of the canonical Wnt are associated with enhanced adipogenesis.
- Knockdown of large tumor suppressor kinases 1/2, upstream kinases of YAP, and expression of active YAP rescues the suppressed YAP–SV40 transcriptional enhancer factor domain and β-catenin–transcription factor 7-like 2 transcriptional activities and enhanced adipogenesis.

AC is an enigmatic disease and a major cause of cardiac arrhythmias and sudden cardiac death in the young. The findings implicate activation of the Hippo pathway, instigated by extensive molecular remodeling of the intercalated discs, in the pathogenesis of AC.
The Hippo Pathway Is Activated and Is a Causal Mechanism for Adipogenesis in Arrhythmogenic Cardiomyopathy
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The Hippo Pathway Is Activated And Is a Causal Mechanism For Adipogenesis in Arrhythmogenic Cardiomyopathy

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MATERIALS AND METHODS

The Institutional Review Board of the University of Texas Health Science Center at Houston approved the studies in human heart tissue samples. Studies in the animal models conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and was approved by The Institutional Animal Care and Use Committee.

Human heart tissues: The human hearts samples were from anonymous individuals and hence, did not include identifiers or complete demographic characteristics. Myocardial samples were from patients with advanced AC who were undergoing heart transplantation (N=4). The control samples were from donor hearts from individuals with no apparent cardiovascular diseases that were not used for heart transplantation (N=2 to 4). All samples were re-examined by histology and the presence of fibro-adiposis was confirmed in the AC samples. The control samples with normal myocardial histology were used in the studies.

Mouse models of arrhythmogenic cardiomyopathy (AC): Cardiac-restricted Desmoplakin-deficient \((Nkx2.5-Cre:Dsp^{WF})\) and cardiac specific truncated JUP \((Myh6:Jup)\) mouse models have been published \(^1,^2\). Briefly, animals were anesthetized in a carbon dioxide chamber, and euthanized by cervical dislocation. Then, the heart of the animal was excised,
rinsed twice with ice-cold Phosphate Buffered Saline (PBS). Cardiac proteins were extracted for immunoblotting analysis.

**HL-1**PKP2:shRNA** myocytes stable lines:** Briefly, HL-1 myocytes were grown in the Claycomb medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 0.1 mM norepinephrine (Sigma-Aldrich), 2 mM l-glutamine (Sigma-Aldrich), and penicillin/streptomycin/amphotericin B combination (Invitrogen) in a humidified 5% CO2 incubator at 37°C. After several passages, cells were transduced with Lentiviral constructs expressing shRNAs against Pkp2. After the viral transduction, fresh growth media supplemented with 50 mg/ml Puromycin (Sigma) as the selection agent. The selection was allowed to proceed for 14 days, and the selected colonies were harvested and maintained in the selection media. Expression level of the PKP2 protein was detected and quantified by immunoblotting (IB) as well as immunofluorescence (IF), whereas the Pkp2 mRNA level was quantified by qPCR.

**IB:** Expression level of the selected proteins in the human heart samples, mouse heart samples and HL-1 myocytes were detected and quantified by IB. Briefly, tissues were harvested and lysed in a RIPA buffer (PIERCE, Rockford, IL) containing 25mM Tris-HCl pH7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1 %SDS, and protease inhibitor (Complete; Roche Molecular Biochemicals). The samples were homogenized using a hand-held homogenizer, then lysed on a rotator in the cold room for an hour. The cell debris was removed by centrifugation at 13,000 rpm for 15 min at 4°C. Protein concentration was measured by colorimetric RC-DC protein assay (Biorad, Hercules, CA). Aliquots of 30ug of protein extracts were then denatured in a 5X Laemmli Buffer by boiling at 95°C for 5 min. The denatured proteins were then separated by electrophoresis on a SDS/PAGE gel, transferred to nitrocellulose membranes, and immunoblotted using primary antibodies to detect the Hippo and the canonical Wnt signaling molecules, desmosome proteins and GAPDH or TUBA1B (tubulin
α), the latter two controls for the loading conditions (the list of the antibodies is reported in Online Supplementary Material - Table I). The membranes were incubated with the primary antibody with gentle agitation overnight at 4°C in 5% non-fat dry milk/0.1% Tris Buffered Saline-Tween 20 (TBS-T). Then, the membranes were rinsed vigorously 3 times for 5 min each in 0.1% TBS-T followed by incubation with a HRP-conjugated donkey anti-mouse antibody at 1:10,000 in 5% non-fat dry milk/0.1% TBS-T for 1 hour at room temperature with gentle agitation. Finally, the membrane were rinsed vigorously three times for 5 min each in 0.1% TBS-T, followed by incubation with chemiluminescence detection reagents (Amersham) from 1 to 10 minutes for chemiluminescence signal detection. Densitometric analysis was performed to quantify protein expression levels by using Image J (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2011.). The read outs were normalized to a control for loading conditions.

**IF:** Cells were fixed in 4% paraformaldehyde for 10 minutes, washed three times in PBS, each wash for 5 minutes, permeabilized and then blocked in 5% feral bovine serum/5% bovine serum albumin/0.1% PBS-Tween 20 (PBS-T) for 60 minute. Subsequently, cells were incubated overnight at 4°C with the specific primary antibody of interest in 5% fetal bovine serum/5% bovine serum albumin/0.1% PBS-T. Then, cells were rinsed 3 times with PBS for 5 minutes each and incubated with the corresponding secondary antibody conjugated with either Alexa fluor® 488 (Invitrogen, A21206) or Texas Red (Santa Cruz, sc-2785) at room temperature for an hour. Then, cells were washed 3 times with PBS for 5 min each and nuclei were counter-stained with DAPI (VectorshieldTM).

Paraffin-embedded thin human myocardial sections (4 Normal and 4 patients with AC) were deparaffinized in xylene bath for 15 minutes. After deparaffinization, the myocardial sections were rehydrated through a series of ethanol gradient: 100% ethanol (2 times), 95% ethanol (2 times), and 70% ethanol (1 time), each for 2 minutes, respectively. After rehydration,
myocardial sections were washed in PBS for 2 minutes before epitope unmasking. To unmask the antigens, myocardial sections were boiled in 10mM Sodium Citrate buffer with pH 6.0 for 15 minutes. Then the sections were removed from the buffer bath and allowed to reach room temperature, washed twice with PBS for 2 minutes each, and blocked using 10% normal serum from the same species as the secondary antibody for 1 hour at room temperature. Next, myocardial sections were incubated with the primary antibodies (listed in Table I) overnight at 4°C. The samples were washed 3 times in PBS for 5 minutes each and were incubated with the secondary antibody and mounted with a mounting media containing DAPI.

IF staining of mouse myocardial sections were performed on fresh frozen heart sections, prepared immediately after euthanasia in a carbon dioxide chamber followed by cervical dislocation. The heart was excised, rinsed twice with ice cold Phosphate Buffered Saline (PBS), immediately placed in the frozen tissue matrix (OCT® compound) and snap froze in cold 2-methybutane (isopentane). Then 5 µm thick cryosections were prepared in a Cryostat at -20F. The cryosections were allowed to equilibrate to room temperature, rinsed with PBS, before completing drying, and then fixed in 2% paraformaldehyde for 10 minutes. IF was carried out as described above.

**Histology:** Masson Trichrome staining was performed to assess and visualize the extent of fibro-adiposis in human heart samples, as a means of verification of the diagnosis of AC in the anonymous heart samples, per the conventional method. In brief, samples are deparaffinized, and thin sections were prepared and treated with Phosphotungstic/Phosphomolybdic Acid solution. The sections were stained sequentially with Weigert’s Iron Hematoxylin solutions, Biebrich Scarlet-Acid Fuchsins solution and aniline blue to stain the nuclei, cytoplasm and collagen, respectively. Following three washes, the sections were treated with 1% Glacial Acetic acid, dehydrated upon treatment with ethanol gradient and xylol. Then, the sections were mounted with xylene-based mounting media.
Co-IP (Co-Immunoprecipitation): HL-1 myocytes and the human heart tissue were homogenized and lysed in a lysis buffer (50mM Tris-HCl, 120mM NaCl, 1%NP40 and 2mM EDTA; pH=7.5). Protein concentrations were measured by RC-DC protein assay. Aliquots of 500ug of protein lysate from each sample was then pre-cleared with IgG corresponding to the host species of the primary antibody together with 20ul of Protein A/G agarose beads (sc-2003, Santa Cruz) for 1 hour at 4°C. Then, the beads were separated by centrifugation at 2,500 rpm for 5 min at 4°C. The supernatants were then transferred to new 1.5ml tubes. The pre-cleared protein lysates were then incubated with 4ug of Anti-YAP (Pierce, PA5-17609) for 1 hour at 4°C. An aliquot of 20ul of Protein A/G agarose beads was added to each sample and incubated overnight at 4°C. The immunoprecipitates were collected by centrifugation, the pellets were washed 4 times with ice-cold PBS and supernatants were discarded. Finally, the immunoprecipitates were resuspended and eluted by 20ul of Laemmli buffer and separated by electrophoresis on a SDS-PAGE gel for immunoblotting. IB was performed as described above.

Mobility shift detection of phosphorylated proteins (Phos-tag): Mn$^{2+}$-Phos-tag SDS-PAGE was prepared by mixing 1.5M Tris-HCl (0.4w/v SDS; pH 8.8), 30% Acrylamide/0.8% Bisacrylamide, 25µM Phos-tag, 50µM MnCl2, 10%APS and TEMED. Protein extracts were loaded to the Phos-tag gel, separated by electrophoresis, transferred to polyvinylidine difluoride (PVDF) membranes and probed with the specific antibodies that detect both total and phosphorylated forms of the proteins. The signal was detected by the ECL system.

Treatment with PMA and OAG: Sub-confluent (~70%) HL-1 and HL-1$^{PKP2\text{-}shRNA}$ myocytes were treated with 100 nM of Phorbol-12-Myristate-13-Acetate (PMA) and 50 µM of 1-Oleoyl-2-Acetyl-sn-Glycerol (OAG). PMA and OAG were purchased from Calbiochem (EMD Millipore). Control groups were treated with Vehicle (DMSO). Cells were treated with the PKC activators for 15 and 30 minutes and expression level and localization of pPKC-α (Thr638) were
detected by IB and IF, respectively. Likewise, the corresponding changes in the pNF2 levels were detected by IB.

**Inhibition of CK1, GSK3-β and Ubiquitin proteasome system (UPS):** Sub-confluent HL-1 and HL-1PKP2:shRNA myocytes were treated with 5, 10 and 20 µM concentration of IC261 (Sigma, Cat#:10658) for an hour to block CK1. To block GSK3-β, cells were treated with a 10 µM concentration of BIO (EMD Chemicals-Calbiochem, Gibbstown, NJ 08027; cat # 361550) for 24 hour. Then, the cells were washed twice with PBS before collected for protein analysis. To block protein ubiquitination cells were treated with 10µM of MG132 (Sigma, C2211) for 4 hours.

**Recombinant lentiviruses:** Plasmids (pLKO.1 puro) containing shRNAs against Pkp2, Lats1, or Lats2 were purchased from Sigma (http://www.sigmaaldrich.com/life-science/functional-genomics-and-rnai/shrna.html). The list and sequence of shRNAs used in these experiments are shown in Online Table II. To generate lentiviruses, three lentiviral plasmids (pLKO.1 puro, pPAX2 and pMD2.G) were mixed in Opti-MEM™ media containing X-tremeGENE 9 DNA transfection Reagent (Roche) to allow DNA complexes to form. Then, the newly formed DNA complexes were added to the 293T cells cultured in the presence of growth media containing serum and were incubated overnight. After one media exchange, lentiviruses were harvested at 48 hours post transfection by collecting and filtering the media through a 0.45µm syringe filter (Sigma).

**Quantitative PCR (qPCR):** Total RNA was extracted from HL-1 and HL-1PKP2:shRNA myocytes using either miRNeasy (Qiagen, Catalog: 217004) or RNeasy kit (Qiagen, Catalog: 74104). RNA was treated on column with DNAse to eliminate contaminating genomic DNA. The cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Life tech, Catalog: 4368814). The mRNA levels of selected Hippo and Wnt targets were quantified by qPCR using
specific TaqMan Gene expression assay and were normalized to Gapdh mRNA level. The list of probes used for qPCR is shown in Online Table III.

**RNA-Sequencing (RNA-Seq):** The transcriptomes of HL-1 and HL-1PKP2:shRNA myocytes were analyzed by RNA-Seq using an Illumina platform at the Genomic and RNA profiling core of Baylor College of Medicine (https://www.bcm.edu/garp/index.cfm?pmid=25179). In brief, an aliquot of each sample was run on an Agilent Bioanalyser RNA chip to assess integrity of the RNA preparation. The concentration of each RNA sample was determined using a NanoDrop spectrophotometer. Samples where depleted of rRNA prior to RNA-Seq (EpiCentre Ribo-Zero Gold Magnetic Kit). The library was prepared on rRNA depleted samples using the Illumina TruSeq RNA library preparation kit and sequenced on the Illumina HiSeq instrument using the kitted sequencing reagents for a pair end 100 base pair run. After completion of the sequencing reaction, the CASAVA software was used to convert the fluorescent dye values into sequence files.

Raw RNA sequencing reads were mapped to the Mouse reference genome build 9 and analyzed by Tophat. Differentially expressed genes were obtained from Cuff diff (Cufflink package)⁴. Cuffdiff reports the FPKM for each gene and transcript using a mean-variance model for each condition that has multiple replicates, averages these models to generate a "pooled" average for that condition. To compare expression levels of the transcripts across runs, the counts were normalized and presented as fragments per kilobase of transcript per million mapped fragments (FPKM). The RNA seq data were also analyzed by de novo transcriptome assembly, using RNA-Seq by Expectation Maximization (RSEM) software package and the normalized data were represented as Transcript per million (TPM). Heatmaps for the Hippo pathway targets and the canonical Wnt Signaling pathway targets were plotted using R (http://www.rstudio.com/) and Volcano plot were graphed using MATLAB and Graph pad prism software.
**Ingenuity Pathway Analysis (IPA):** IPA (Ingenuity Systems) was applied to analyze differentially expressed genes that showed more than 1.2-fold change and a minimum FPKM or TPM of one in the control group. Total genes used for this analyze were mapped to the Ingenuity Pathways Knowledge Base. IPA was employed to assign biological functions to the differentially expressed genes. The IPA output was focused on the canonical pathway gene sets and ranked based on statistical significance. The significance of the association between the data set and the canonical pathway was determined based on two parameters: (1) a ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that map to the canonical pathway threshold value and (2) a \( p \) value calculated using Fischer's exact test determining the probability that the association between the genes in the data set and the canonical pathway is due to chance alone. The false discovery rate (q value) was set at 0.05.

Integrin signaling pathways, which encompass components of the Wnt signaling, RhoA/Rac/RAS signaling with \( \alpha \) and \( \beta \) integrins, as the upstream regulators, were the most significantly altered pathways (\( p \) value=3.58e-8 and threshold ratio; 0.386 for the entire pathway and \( p \)-value: 7.32e-3 and a threshold Ratio = 54/173 molecules = 0.3 for the canonical Wnt signaling pathway. Pathway designer tool from IPA was used to generate schematic network of the Wnt signaling pathway. To analyze the Hippo pathway, “upstream regulator” function of the IPA on the differentially expressed genes was used and the TEAD/TAZ downstream targets were obtained. In addition, because of the incompleteness of the IPA data set for the Hippo pathway, the known targets were also manually curated based on the published literature.

**Luciferase Assays:** The TEAD (8×GT-IIC-Luciferase; 34615) and Wnt signaling (7TFP; 24308) reporter plasmids were obtained from Addgene (www.addgene.org). The 8×GT-IIC-Luc constructed was generated by cloning eight copies of the TEAD binding sites spanning the GT-IIC motif of SV40 enhancer into pδ51-LucII. The 7TFP clone was constructed by cloning 7 copies of TCF sequences downstream of a Firefly luciferase cDNA in the pCF768 vector.
followed by the SV40-Puro selection cassette (6). In brief, cells were transfected with TEAD and 7TFP reporter plasmids to assess Hippo and Wnt signaling, respectively. To serve as internal control for transfection, cells were co-transfected with a Renilla luciferase reporter plasmid. Luciferase activity was measured 48 hours after transfection. Vector alone was included in the experiments, as a control. Experiments were performed in triplicate and repeated 3-5 times. TEAD and TCF-mediated gene transcription was determined by the ratio of firefly luciferase and Renilla luciferase activities using Duol-Glo® luciferase assay from Promega. The mean values of the normalized ratios were compared.

**Induction of Adipogenesis:** The adipogenesis induction procedure was as published (1). In brief, HL-1 and HL-1PKP2:shRNA myocytes were plated at a density of 60,000 cells per well on 0.1% gelatin-coated cover glass and treated with Adipogenesis Induction Medium (α-MEM supplemented with 10% FBS, 1% antibiotic–antimycotic, 10 µg/mL insulin, 0.5 mmol/L 3-isobutyl-1-methylxanthine, and 1 µmol/L dexamethasone) for 7 days. The media was then changed to Maintenance Medium containing 10 µg/mL insulin. Two days later, myocytes were stained with Oil Red O and PPARG. To quantify the number of adipocytes, the number of Oil Red O-stained cells in 25 microscopic fields (100X magnification) and the number of PPARG-stained cells in 20 fields (63× magnification) per group were counted in 3 independent experiments.

**Statistics:** Distribution pattern of the continuous variables were analyzed and those that followed a normal distribution pattern were expressed as mean ± SD and compared between the two groups by the t test and among multiple groups by ANOVA, followed by pairwise comparisons. Continuous variables that did not follow normality and the categorical variables were analyzed by the Kruskal-Wallis or by the Chi Square test. All statistical analyses were performed using GraphPad Prism version 6.00 for Windows, GraphPad Software (www.graphpad.com).
REFERENCES


### ONLINE TABLE I:

**List of Antibodies**

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## ONLINE TABLE II

**Sequence of the shRNAs**

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GCTTTTTG |
| *Pkp2* | TRCN0000123351 | 51    | CDS            | CCGGCCTGAGTATGTCTACAAGCTA  
CTCGAGTAGCTTGTAGACACTACTCA  
GGTTTTTG |
| *Lats1* | TRCN0000274539 | 39    | 3’ UTR         | CCGGTAGTCAATTCTTGTACTTTAA  
CTCGAGTATAAGACACAAGAATTGAC  
TATTTTTG |
| *Lats1* | TRCN0000274541 | 41    | CDS            | CCGGCCTATTCAACAGCCCGTGAAA  
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GGTTTTTG |
| *Lats1* | TRCN0000274542 | 42    | CDS            | CCGGTGACCCTGATGGCCAATTTAA  
CTCGAGTTAATATGGCCATCAGGT  
CATTTTTG |
| *Lats1* | TRCN0000274543 | 43    | CDS            | CCGGGCAACATTCAATTAACCGAAA  
CTCGAGTTTCTGGTTAATTGATGT  
GCTTTTTG |
| *Lats2* | TRCN0000022705 | 705   | CDS            | CCGGCACAGAATAGCAGAGATGA  
ACTCGAGTTCATCTCTGCTATTCTT |
ONLINE TABLE III
Taqman Gene Expression Assays
302810-R1

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**Ankrd1**
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**Axin2**
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**Bmp4**
Mm00432087_m1, Cat. # 4331182

**Bves**
Mm00517902_m1, Cat. # 4331182

**cJun**
Mm00495062_s1, Cat. # 4331182

**cMyc**
Mm00487804_m1, Cat. # 4331182

**Ctgf**
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**CyclinD1**
Mm00432359_m1, Cat. # 4331182

**Cyr61**
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**Gapdh**
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**Jup**
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302810-R1

*Mhy6*  Mm00440359_m1, Cat. # 4331182

*Pkp2*  Mm00503159_m1, Cat. # 4331182

*Runx1*  Mm01213404_m1, Cat. # 4331182

*Snai1*  Mm00441533_g1, Cat. # 4331182

*Sox2*  Mm03053810_s1, Cat. # 4331182
Online Table IV

List of YAP-TEAD and YAP-TAZ-TEAD Targets that are Differentially Expressed in PKP2 Knock down HL-1 Myocytes

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<th>Test-stat</th>
<th>p value</th>
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REFERENCES

Figure Legends

Figure I. Quantitative data for PKC-α in the human hearts (A), mouse hearts (B) and HL-1 cells in response to treatment with general PKC activators (C and D). A. Active PKC-α was reduced significantly in the human hearts with arrhythmogenic cardiomyopathy (AC, N=2 for normal hearts and 4 for AC hearts). B. Levels of pPKC-α were also reduced in 2 mouse models of AC. C. Treatment with PKC activators increased pPKC-α levels in the wild type HL-1 myocytes, whereas pPKC-α levels were either unchanged or marginally increased in HL-1PKp2:shRNA myocytes, maintaining differential reduction in the pPKC-α levels in the PKP2-deficient myocytes. Corresponding quantitative levels of pNF2 and total NF2 are also shown.

Figure II. shRNA mediated Knockdown (KD) of Lats1 and Lats2. A. qPCR analysis of Lats1 KD using four independent lentiviruses expressing shRNAs against Lats1 mRNA. Data show ~50-60% knockdown efficiency. B. IB analysis using the same set of shRNAs (as in panel A) showing LATS1 protein KD and the corresponding blot representing pYAP levels. C. qPCR analysis of Lats2 mRNA KD using four independent lentiviruses expressing shRNAs against Lats2 mRNA. Data show ~50-75% knockdown efficiency. D. IB analysis of the same experimental groups using anti LATS2 antibody, which showed multiple bands and was inconclusive. Levels of pYAP showed a trend towards decreased expression level after transfection with the shRNA construct against Lats2.

Figure III. A schematic model, showing the Hippo pathway and its components involved in the pathogenesis of AC: A. Components of the Hippo way are illustrated along with their effects on their targets. B. Components of the Hippo pathway involved in the pathogenesis of AC. Molecular remodeling of the AC, reduces junctional pPKC-α level and activates neurofibromin (NF2), an upstream molecule to the Hippo pathway. Activation of NF2
phosphorylates the downstream Hippo molecules including MST1/2, LATS1/2 and YAP, the latter is the effector of the pathway. YAP binds to β-catenin and JUP forming a complex and sequestering these components at the membrane and/or cytosol. Consequently, gene expression through TEAD and TCF7L2, transcription factors of the Hippo and the canonical Wnt pathways, respectively, is reduced. Suppression of gene expression through YAP-TEAD and β-catenin-TCF7L2 leads to adipogenesis in AC.

**Figure IV. Morphological changes in the HL-1^{PKP2:shRNA} myocytes:** Bright field (A) and Phalloidin staining of HL-1 myocytes at 3 sets of magnifications (B). The HL-1^{PKP2:shRNA} exhibited altered cellular morphology and cytoskeletal organizing, which might serve as additional stimuli to activate the Hippo pathway.
**A** Human pPKC-α

![Graph showing fold change for Human pPKC-α](image)

- Normal vs AC
- Fold change: p=0.007 (N=2-4)

**B** Mouse pPKC-α

![Graph showing fold change for Mouse pPKC-α](image)

- NTG vs Myh6:Jup
- Fold change: p=0.002 (N=2)

**C** PMA TREATMENT

![Graphs showing fold change for PMA TREATMENT](image)

- Cells, DMSO, 15 min, 30 min

**D** OAG TREATMENT

![Graphs showing fold change for OAG TREATMENT](image)

- Cells, DMSO, 15 min, 30 min

Legend:
- HL-1<sup>WT</sup>
- HL-1<sup>Pkp2:shRNA</sup>