Calcium/Calmodulin-Dependent Protein Kinase II Couples Wnt Signaling With Histone Deacetylase 4 and Mediates Dishevelled-Induced Cardiomyopathy

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Abstract—Activation of Wnt signaling results in maladaptive cardiac remodeling and cardiomyopathy. Recently, calcium/calmodulin-dependent protein kinase II (CaMKII) was reported to be a pivotal participant in myocardial remodeling. Because CaMKII was suggested as a downstream target of noncanonical Wnt signaling, we aimed to elucidate the role of CaMKII in dishevelled-1–induced cardiomyopathy and the mechanisms underlying its function. Dishevelled-1–induced cardiomyopathy was reversed by deletion of neither CaMKIIδ nor CaMKIIγ. Therefore, dishevelled-1–transgenic mice were crossed with CaMKIIδ/γ double-knockout mice. These mice displayed a normal cardiac phenotype without cardiac hypertrophy, fibrosis, apoptosis, or left ventricular dysfunction. Further mechanistic analyses unveiled that CaMKIIδ/γ couples noncanonical Wnt signaling to histone deacetylase 4 and myosin enhancer factor 2. Therefore, our findings indicate that the axis, consisting of dishevelled-1, CaMKII, histone deacetylase 4, and myosin enhancer factor 2, is an attractive therapeutic target for prevention of cardiac remodeling and its progression to left ventricular dysfunction. (Hypertension. 2015;65:335-344. DOI: 10.1161/HYPERTENSIONAHA.114.04467.) • Online Data Supplement

Key Words: calcium-calmodulin-dependent protein kinase type 2 ▪ cardiomyopathies ▪ dishevelled proteins ▪ histone deacetylase 4 ▪ Wnt signaling pathway

Congestive heart failure is the leading cause of death in developed countries.1 There are ≥23 million patients experiencing worldwide.1–4 Incidence of heart failure, and its associated morbidity and mortality, is still on the rise.5 At present, therapeutic strategies to attenuate adverse cardiac remodeling are limited to β-adrenoceptor antagonists and inhibitors of the renin–angiotensin–aldosterone system. Unfortunately, these therapies are insufficient in a substantial number of patients. Given these clinical observations, new therapeutic approaches are needed and may potentially be directed at signaling pathways that transduce neuroendocrine or stretch-mediated signals. Pathological injuries reactivate several signaling pathways in the adult heart that traditionally are thought to be operative only in the developing heart. The Wnt signaling pathway, an evolutionarily conserved intracellular signaling pathway, is the most extensively investigated one of these pathways.6–8 Dishevelled, the hub of Wnt signaling, regulates and couples noncanonical Wnt signaling to histone deacetylase 4 and myosin enhancer factor 2. Therefore, our findings indicate that the axis, consisting of dishevelled-1, CaMKII, histone deacetylase 4, and myosin enhancer factor 2, is an attractive therapeutic target for prevention of cardiac remodeling and its progression to left ventricular dysfunction. (Hypertension. 2015;65:335-344. DOI: 10.1161/HYPERTENSIONAHA.114.04467.) • Online Data Supplement

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Calcium/calmodulin-dependent protein kinase II (CaMKII), a multifunctional serine/threonine protein kinase with a broad spectrum of substrates, plays an important role in the Wnt/Ca2+ pathway.12 Several isoforms of CaMKII are derived from channels ≥3 branches of Wnt signal transduction: the Wnt/β-catenin pathway, the Wnt/Ca2+ pathway, and the Wnt/c-Jun N-terminal kinase (JNK) pathway.13 Recent reports from our and other groups have shown that dishevelled expression is upregulated after aortic banding (rat) and in tachycardia-induced heart failure (porcine).13 Moreover, knockout of dishevelled-1 attenuates pressure-overload–induced left ventricular (LV) remodeling.15 In contrast, transgenic dishevelled-1 overexpression leads to progressive dilated cardiomyopathy, which is accompanied by the activation of all branches of Wnt signaling.10 Therefore, dishevelled-1 is both necessary and sufficient for cardiac remodeling and dysfunction. However, the mechanisms bridging chronic dishevelled-1 activation or upregulation to expressionional changes and successive cardiac dysfunction after pathological injuries remain to be elucidated.

Calcium/calmodulin-dependent protein kinase II (CaMKII), a multifunctional serine/threonine protein kinase with a broad spectrum of substrates, plays an important role in the Wnt/Ca2+ pathway.12 Several isoforms of CaMKII are derived from
4 genes (α, β, δ, and γ) that display distinct but overlapping expression patterns. The α and β isoforms are almost exclusively expressed in the brain, whereas the other isoforms are found more ubiquitously. In the heart, CaMKII δ and γ are the predominant CaMKII isoforms, with CaMKIIβ displaying the highest expression level. Upregulation of CaMKII expression and activity is a general characteristic of heart failure in humans and in animal models.14,15

Although CaMKII is a critical downstream target of dishevelled-1,16 a functional relationship between these proteins in cardiac remodeling has not been identified to date. In this study, deletion of neither CaMKII δ nor γ reverses dishevelled-1–induced cardiomyopathy. Interestingly, here we found that mice lacking both CaMKII δ and γ isoforms are resistant to dishevelled-1–induced cardiac hypertrophy, fibrosis, and LV dysfunction, which is mediated by the histone deacetylas 4/myosin enhancer factor 2 (HDAC4/MEF2) complex. Therefore, this investigation suggests that both the δ and γ isoforms of CaMKII serve as fundamental links between dishevelled and consecutive detrimental effects. Our findings reveal that the dishevelled–CaMKII–HDAC4–MEF2 axis holds a pivotal role in maladaptive cardiac remodeling and is a potential novel therapeutic target for the prevention of LV dysfunction.

**Methods**

Materials and Methods are available in the online-only Data Supplement.

**Results**

**CaMKIIδ-Knockout, CaMKIIγ-Knockout, CaMKIIδγ-Knockout, and Dishevelled-1-Transgenic Mice Lacking CaMKII Are Generated**

We previously reported that dishevelled-1–transgenic (DVL) mice exhibit a robust increase in both phosphorylated and total CaMKII levels,10 which are known to play a key role in cardiac dysfunction. In the same study, we have also found that Dvl-1 knockdown prevents cardiomyocyte hypertrophy in response to β-adrenergic stimulation. Using Western blot, here we further report that isoproterenol (Iso) induced hypertrophy accompanied with increased dishevelled-1, phosphorylated CaMKII, phosphorylated HDAC4 and MEF2, which could be reversed by dishevelled-1 knockdown (Figure S1 in the online-only Data Supplement). To assess the potential role of CaMKII in the detrimental effects after dishevelled-1 overexpression, we crossed DVL mice, which display cardiac hypertrophy and LV dysfunction, with CaMKIIδ-knockout mice. However, the phenotype of the DVL mice was not rescued by the deletion of CaMKIIδ (Figure S2).

Because we hypothesized that CaMKIIγ may also play a crucial role in dishevelled-1–induced pathological injuries, we crossed cardiac-directed CaMKIIγ-knockout and CaMKIIδγ-knockout animal lines with DVL mice (Figure 1A). All the mice were viable and developed normally until early adulthood. In the CaMKIIδ-knockout and CaMKIIδγ-knockout mice, as well as in the CaMKIIδγ-knockout animals, no overt baseline cardiac phenotype was observed.17,18 The phenotype of the CaMKIIδγ-knockout is described in detail in a separate article.19 Interestingly, CaMKIIδγ-knockout reversed the phenotype caused by dishevelled-1 overexpression, whereas CaMKIIδγ-knockout did not (Figure S3), indicating that both the δ and γ isoforms of CaMKII are crucial in dishevelled-1–induced cardiac remodeling. To gain further insight into the mechanisms underlying these outcomes, we selected 4 mouse lines that were generated by crossing DVL mice with CaMKIIδγ-knockout mice: wild-type mice as control (CTRL), CaMKIIδ-deficient, and dishevelled-1 wild-type mice as CKO, dishevelled-1 transgenic mice with normal level of CaMKIIδγ as DVL, and dishevelled-1–transgenic mice lacking CaMKIIδγ as DWC (Figure 1B; Table S1).

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Generation of dishevelled (Dvl)-1-transgenic (Tg; DVL) mice harboring a calcium/calmodulin-dependent protein kinase IIδγ-knockout (CaMKIIδγ-KO [CKO]). A, Genetic strategy for depleting CaMKII in DVL mice. B, Western blots of Dvl-1 overexpression in DVL mice and DVL mice lacking CaMKIIδγ (DWC), as well as a substantial reduction in CaMKII protein levels in heart homogenates from CKO and DWC mice at 12 weeks, indicating successful deletion of CaMKII in DVL mice. C, mRNA levels of CaMKII isoforms α in control (CTRL), CKO, DVL, and DWC mouse hearts; CaMKIIα was not detectable. All RNA levels were normalized to internal hypoxanthine guanine phosphoribosyl transferase (Hprt) expression and presented as fold-change relative to CTRL samples. Values are presented as mean±SEM. n=10 per group. MHC indicates myosin heavy chain; and ND, not detectable.
CaMKIIα expression was not detected in any of these groups (Figure 1C).

**Deletion of CaMKIIδγ Attenuates Dishevelled-1–Induced Severe Cardiac Hypertrophy, Fibrosis, and Apoptosis**

In accordance with our previous findings, the size and structure of the hearts of CKO mice displayed no baseline differences from the CTRL, whereas the DVL mice exhibited remarkable cardiac hypertrophy. Interestingly, deletion of CaMKIIδγ nearly normalized dishevelled-1–induced enhancement of heart size and cross-sectional myocyte area, using hematoxylin and eosin staining, which is as typical as the wheat germ agglutinin lectin staining, in DWC mice versus DVL mice (Figure 2A and 2B). DVL mice showed a significant augmentation in heart weight/tibia length ratio and LV weight/tibia length ratio, whereas in the DWC group, depletion of CaMKIIδγ inhibited the dishevelled-1–evoked increase in heart weight/tibia length and LV weight/tibia length ratio (Figure 2D). This effect is also highlighted by reduced expression of the hypertrophic markers atrial natriuretic factor and β-myosin heavy chain in DWC mice compared with DVL mice (Figure 2E). In vivo noninvasive transthoracic echocardiography revealed reduction of the LV posterior wall thickness in systole and end-diastolic diameter, further reflecting the attenuated hypertrophic response to dishevelled-1 overexpression by CaMKIIδγ silencing (Figure 3A, 3C, and 3D). Taken together, these data indicate that deletion of CaMKIIδγ reversed dishevelled-1–induced cardiac hypertrophy.

Apart from cardiac myocyte hypertrophy, it has been recognized that cardiac fibrosis and apoptosis are pivotal to the development of LV dysfunction. In this study, no significant difference in fibrosis and apoptosis was observed between CTRL mice and CKO mice, whereas DVL mice had sharply augmented interstitial and perivascular fibrosis and apoptosis that were significantly reduced by ablation of CaMKIIδγ (Figure 2A and 2C; Figure S4).

**Deletion of CaMKIIδγ Rescues Dishevelled-1–Induced LV Dysfunction**

Because cardiac hypertrophy, fibrosis, and apoptosis were ameliorated in DWC mice, we used transthoracic echocardiography and hemodynamic measurements to test whether cardiac function was improved in these animals. Compared with CTRL

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**Figure 2.** Diminished cardiac hypertrophy and fibrosis of dishevelled-1–transgenic (DVL) mice after deletion of calcium/calmodulin-dependent protein kinase IIδγ (CaMKIIδγ). A, Heart size was assessed to detect reduction of cardiac hypertrophy. Histological sections were stained with hematoxylin and eosin to measure myocyte size and with picrosirius red to detect fibrosis. B and C, Quantification of myocyte size (B) and fibrosis area (C) presented as fold-changes in cross-sectional area of myocyte size and fibrosis in each group compared with control (CTRL) mice. D, Heart weight/tibia length (HW/TL) ratio and left ventricular weight/tibia length (LVW/TL) ratio reflect blunted cardiac hypertrophy in DWC mice after ablation of CaMKIIδγ. E, Fold-changes in mRNA levels of the hypertrophic markers atrial natriuretic factor (ANF) and β-myosin heavy chain (MHC). Values are presented as mean±SEM. n>10 per group. *P<0.05 vs CTRL; #P<0.05 vs DVL.
mice, DVL mice displayed marked enhancement of end-diastolic volume, end-systolic volume, and end-diastolic pressure, as well as an apparent reduction in the maximal velocities of contraction (dp/dt max) and relaxation (dp/dt min and τ), thus reflecting severely impaired systolic and diastolic cardiac dysfunction. However, all of these hemodynamic parameters were completely normalized in DWC mice after depletion of CaMKIIδγ, suggesting a rescue of LV function (Table; Figure 3B).

**CaMKII Contributes to Noncanonical Wnt Signaling–Induced Cardiac Hypertrophy by Mediating MEF2 via Phosphorylation/Export of HDAC4**

Previously, dishevelled-1 overexpression led to robust activation of CaMKII, which induced an increase in the phosphorylation of HDAC4, a repressor of hypertrophy-related transcription factors, such as MEF2, serum response factor, and GATA.\(^\text{10,20,21}\) Furthermore, we found a strong increase in CaMKIIδγ-related HDAC4 phosphorylation in DVL mice when compared with CTRL littermates via in vitro HDAC4 kinase assay. Importantly, CaMKIIδγ deletion in DWC mice significantly blocked phosphorylation of HDAC4 (Figure 4A and 4B), implying that HDAC4 is a downstream target of CaMKII in response to dishevelled-1 overexpression. To gain further insight into the links among dishevelled-1, CaMKII, and HDAC4, adenoviral dishevelled-1 overexpression was used to stimulate cultured neonatal rat ventricular myocytes. KN93, a CaMKII inhibitor, effectively prevented dishevelled-1–induced phosphorylation of CaMKII and HDAC4, whereas its analogue KN92 did not (Figure 4C). In addition, pharmacological inhibition of CaMKII also inhibited the dishevelled-1–induced increase in myocyte size and atrial natriuretic factor expression (Figure 5A and 5B), indicating that CaMKII inhibition blocks cardiomyocyte hypertrophy resulting from dishevelled-1 overexpression.

To test whether HDAC4 inhibits dishevelled-1–related cardiac hypertrophy further, neonatal rat ventricular myocytes were treated with adenovirus encoding a phosphorylation-resistant HDAC4 mutant, HDAC4-S246,467,632A.
Interestingly, dishevelled-1 overexpression resulted in a remarkable increase in MEF2 activity, which was blocked by CaMKII inhibition and by HDAC4-3S/A overexpression (Figure 5C). Meanwhile, we observed Wnt5a-induced cardiomyocyte hypertrophy, complying with MEF2 activation. Both of them could be inhibited by CaMKII inhibition (Figure S5). Taken together, these observations confirm that CaMKII and HDAC4 are critical to dishevelled-1–evoked cardiomyocyte hypertrophy.

Dishevelled-1–Induced Activation of β-Catenin, Protein Kinase C, and JNK Is Not Affected by CaMKII Deletion

CaMKII was previously shown to interact with the Wnt/β-catenin signaling pathway, 22 raising the question of whether the reduction in dishevelled-1–induced cardiomyopathy after genetic deletion or pharmacological inhibition of CaMKII occurs via suppression of Wnt/β-catenin signaling. These 2 approaches of cardiomyopathy reduction resulted in neither the inhibition of β-catenin accumulation nor the downregulation of the β-catenin–dependent Wnt target gene Axin2 (Figure 6A–6C). Thus, attenuation of dishevelled-1–induced cardiomyopathy by CaMKII suppression is not mediated through interaction with the canonical Wnt/β-catenin signaling pathway. Furthermore, CaMKII-knockout in the DWC mice did not prevent the activation of protein kinase C and JNK, as assessed by Western blot (data not shown). Figure 7 illustrates the proposed mechanism by which dishevelled-1

(HDAC4-3S/A), which localizes predominantly to the nucleus. Interestingly, HDAC4-3S/A completely reversed dishevelled-1 overexpression-induced enhancement of expression of the hypertrophic marker atrial natriuretic factor and cardiomyocyte size (Figure 4D; Figure 5A and 5B), suggesting that HDAC4 is central to dishevelled-1–evoked cardiomyocyte hypertrophy.

Figure 4. Implication of the histone deacetylase 4/myosin enhancer factor 2 (HDAC4/MEF2) complex in dishevelled (Dvl)-1–induced cardiac hypertrophy. A, Western blot of increased levels of phosphorylated calcium/calmodulin-dependent protein kinase II (CaMKII) after Dvl-1 overexpression, phosphorylated HDAC4 at the CaMKII phosphorylation site Ser-632, and total HDAC4 levels. B, HDAC4 kinase activity assayed with GST-HDAC4 as substrate. HDAC4 phosphorylation was significantly increased in ventricular extracts from Dvl-1-transgenic (DVL) mice. C, The use of an adenovirus harboring wild-type Dvl-1 (Dvl-1-wt) stimulated cardiomyocytes in vitro and phosphorylated the Ser-632 site of HDAC4. These processes were inhibited by addition of KN93, a CaMKII inhibitor, but not by the inactive analogue KN92, as revealed by Western blot. D, Western blot of the change in concentration of adenoral Flag-HDAC4-3S/A. Values are reported as mean±SEM. n>10 per group. *P<0.05 vs CTRL; P<0.05 vs DWC. n≥14 per group.

### Table. Hemodynamic Parameters Demonstrate the Rescue of Cardiac Dysfunction in Dishevelled-1–Transgenic Mice by Calcium/Calmodulin-Dependent Protein Kinase IIδγ-Knockout

<table>
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<th>Parameters</th>
<th>CTRL</th>
<th>CKO</th>
<th>DVL</th>
<th>DWC</th>
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<tr>
<td>Heart rate, bpm</td>
<td>493±12</td>
<td>487±19</td>
<td>451±14</td>
<td>499±15</td>
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<td>End-systolic volume, µL</td>
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<td>6.9±0.5</td>
<td>12.7±1.5*</td>
<td>7.3±0.8</td>
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<td>End-diastolic volume, µL</td>
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<td>17.8±2.9</td>
<td>31.4±4.7*</td>
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<td>End-systolic pressure, mmHg</td>
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<td>82.9±2.9</td>
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<tr>
<td>End-diastolic pressure, mmHg</td>
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<td>5.0±0.5</td>
<td>9.8±1.2*</td>
<td>4.7±0.8</td>
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<td>Stroke volume, µL</td>
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<td>12.3±1.6</td>
<td>11.0±1.8</td>
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</tr>
<tr>
<td>dP/dt max, mmHg/s</td>
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<td>6283±404</td>
<td>4194±296*</td>
<td>6242±359</td>
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<tr>
<td>dP/dt min, mmHg/s</td>
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<td>-5672±354</td>
<td>-3853±289*</td>
<td>-5457±312</td>
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<tr>
<td>τ, ms</td>
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<td>12.8±0.9</td>
<td>15.5±1.3*</td>
<td>13.0±0.6</td>
</tr>
</tbody>
</table>

Values are demonstrated as mean±SEM. CKO indicates calcium/calmodulin-dependent protein kinase IIδγ-knockout; CTRL, control; DVL, dishevelled-1–transgenic; DWC, dishevelled-1–transgenic with calcium/calmodulin-dependent protein kinase IIδγ-knockout; and τ, tau (a parameter reflecting diastolic cardiac function). *P<0.05 vs CTRL; P<0.05 vs DWC. n≥14 per group.
activates CaMKII, phosphorylates HDAC4, and mediates MEF2-related gene transcription, which consequently leads to cardiac hypertrophy.

Discussion

Wnt signaling is centrally involved in myocardial remodeling after pathological injuries. Here, we show that combined deletion of CaMKII δ and γ rescues dishevelled-1–induced cardiac hypertrophy, fibrosis, and apoptosis, and also completely reverses dishevelled-1–induced LV dysfunction. Further analyses of signaling events indicated that cardioprotection in DWC mice via CaMKII δγ deletion was mediated by downregulation of MEF2 transcription through phosphorylation/export of HDAC4 from the nucleus. This is the first report to demonstrate a crucial role for the CaMKII axis in association with cardiomyopathy induced by activation of Wnt signaling components. Wnt5a-induced CaMKII activities are mediated via HDAC4 and MEF2 further downstream functionally linking Wnt signaling to HDAC4 for the first time.

Previous studies revealed that dishevelled-1 is necessary and sufficient to induce cardiac remodeling and dysfunction, but the main effecter acting after pathological activation of dishevelled-1 has not yet been determined. Although some studies support the notion that dishevelled activation is specific to the noncanonical Wnt pathway, other data indicate that dishevelled activation is critical for all 3 Wnt branches. During the process of cardiac remodeling, we observed that dishevelled-1 activation involves all 3 Wnt branches, with predominant signaling to the noncanonical pathway. In addition, we show that the dishevelled-CaMKII axis is more important in this context than protein kinase C and JNK, a conclusion supported by multiple lines of evidence. In the DWC group, deletion of CaMKIIδγ blocked dishevelled-1–induced increases in heart size, myocyte area, heart weight/tibia length, LV weight/tibia length ratio, hypertrophic marker gene expression, posterior wall thickness, and end-diastolic diameter in response to dishevelled-1 overexpression. Cardiac fibrosis and apoptosis were ameliorated by...
the loss of CaMKIIδγ, and more importantly, cardiac dysfunction induced by dishevelled-1 was completely rescued by CaMKIIδγ deletion. Dishevelled-1, CaMKII, and HDAC4 were activated simultaneously after aortic banding at 72 hours and 7 days. CaMKIIδγ-knockout attenuated aortic banding-induced cardiac dysfunction and interstitial fibrosis but not hypertrophy.19 Whereas after aortic banding and Iso stimulation, cardiac expression of Rcan1-4 as a measure for calcineurin activity was highly upregulated in CaMKIIδγ-knockout mice19; this was not the case in DVL mice (data not shown). As cardiac hypertrophy in CaMKIIδγ-knockout mice after aortic banding and Iso stimulation was clearly dependent on the calcineurin overactivation,19 the lack of that in DVL mice (data not shown) indicates that the canonical Wnt signaling is still activated in the dishevelled-1-transgenic with CaMKIIδγ-knockout mice. Notably, CaMKIIδγ deletion also did not result in significant changes in the levels of protein kinase C or JNK because both of these 2 proteins increased similarly in DVL mice in the presence or absence of CaMKIIδγ expression. Therefore, we conclude that dishevelled-1 upregulation activates all 3 branches of the Wnt signaling pathway, with CaMKII acting as the most pivotal component in the process of myocardial remodeling.

Apart from the implication of CaMKII in excitation–contraction coupling and gene transcription in the heart, CaMKII has also been associated with the development of cardiac remodeling and dysfunction.18,25–27

Interestingly, in this study, we observed that deletion of CaMKIIδ or CaMKIIγ alone is not sufficient to rescue the phenotype of DVL mice, but simultaneous knockout of CaMKIIδ and CaMKIIγ does rescue the phenotype, indicating that CaMKIIγ, which is not the most highly expressed CaMKII isoform in the heart, may also function after pathological injuries. Therefore, when developing new pharmacological

Figure 6. Genetic deletion and pharmacological inhibition of calcium/calmodulin-dependent protein kinase II (CaMKII) do not affect β-catenin accumulation. A and B, Western blots and densitometry of active and total β-catenin levels revealed β-catenin accumulation after dishevelled (Dvl-1) overexpression, even after genetic deletion and pharmacological inhibition of CaMKII. C, No significant changes in the levels of the Wnt/β-catenin target gene Axin2 were detected relative to Dvl-1 overexpression both in vivo and in vitro, indicating that the Wnt/β-catenin signaling pathway was not altered by CaMKII suppression. Values are reported as mean±SEM. n>10 per group. *P<0.05 vs control (CTRL; mice) or AdC KN92 (cardiomyocytes); #P<0.05 vs DWC (mice), or AdDvl KN93 (cardiomyocytes). All experiments with cardiomyocytes were performed ≥3×. AdC indicates control adenovirus; AdDvl, adenovirus harboring the Dvl-1-wt gene; CKO, CaMKII-knockout; DVL, Dvl-1-transgenic; DWC, DVL mice lacking CaMKIIδγ; and HDAC4-3S/A, HDAC4-S246,467,632A.
compounds to treat cardiac remodeling, both isoforms of CaMKII δ and γ should be taken into account.

Although the loss of CaMKII δγ considerably ameliorated cardiac hypertrophy and fibrosis in response to dishevelled-1 overexpression, 2 interesting questions arise from our observations of dishevelled-1 transgenic mice deficient in CaMKII δγ. First, why is atrial natriuretic factor expression in DWC mice when compared with CTRL animals, but much less than DVL mice (Figure 2E)? Second, why is cardiac fibrosis not completely normalized (Figure 2C)? We here suggest the involvement of the β-catenin–dependent Wnt signaling rather than the Wnt/JNK pathway. This hypothesis is in line with our recent findings that Dapper-1–induced activation of canonical Wnt signaling results in mild myocardial remodeling. And, it is also supported by previous findings that cardiomyocyte-specific β-catenin loss-of-function mutations lead to attenuated LV remodeling and improved cardiac function after pressure overload, chronic angiotensin II stimulation, or experimental infarction. Conversely, augmented mortality and dilated cardiomyopathy occur in conditional β-catenin gain-of-function mutations. Therefore, although CaMKII signaling predominates, canonical Wnt/β-catenin signaling may also partially contribute to dishevelled-1–induced cardiac remodeling. Further investigations are necessary to gain additional insight into this issue.

To examine the downstream signaling components of Wnt/ CaMKII further, we examined the class II HDACs, which can be phosphorylated by CaMK and protein kinase D. HDAC4, a class II HDAC, has recently been reported to have a specific CaMKII docking site that is not present in other HDACs. Zhang et al reported that HDAC4 (but not HDAC5) kinase activity is apparently increased in CaMKII transgenic mouse hearts when compared with wild-type controls. In comparison, ablation of CaMKII δ inhibits cardiac hypertrophy. Mechanistically, depletion of CaMKII δ suppresses phosphorylation/export of HDAC4 from the nucleus, leading to downregulation of MEF2 transcription, which is both necessary and sufficient for pathological cardiac remodeling.

We observed that total and phosphorylated HDAC4 levels are comparably augmented in the DVL mouse heart versus the CTRL mouse heart, and furthermore, ablation of CaMKII δ blocked HDAC4 phosphorylation in DWC mice. We note that the inclusion of HDAC4-3S/A, a signal-resistant HDAC4 mutant that localizes predominantly to the nucleus, reduced hypertrophic marker gene expression evoked by dishevelled-1, indicating that HDAC4 functions as a downstream target of CaMKII after dishevelled-1 overexpression. Interestingly, CaMKII δγ is among the splicing variants that are upregulated in cardiac hypertrophy. We found previously that CaMKII selectively signals to HDAC4 via binding to a unique docking site and phosphorylation of Ser-467 and Ser-632.

The transcription factor MEF2 has been suggested to serve as a common end point for hypertrophic signaling pathways in cardiomyocytes. HDAC4 is known to be associated with MEF2 and represses its activation; signal-dependent dissociation of HDAC4 from MEF2 results in activation of cardiac hypertrophy. Dishevelled (Dvl)-1 mediator, CaMKII, and MEF2 selectively both in vitro and in vivo by regulating phosphorylation and dissociation of HDAC4 from MEF2. In the present study, pharmacological inhibition and genetic elimination of CaMKII blocked dishevelled-1 upregulation-induced cardiac hypertrophy and was accompanied by decreased HDAC4 phosphorylation. Interestingly, knockdown of dishevelled-1 abrogated Iso-induced activation of CaMKII, HDAC4, and MEF2. Accordingly, we hypothesized that MEF2 serves as the end point of the dishevelled-1–induced hypertrophic signaling pathway, a conjecture strongly supported by the observation that dishevelled-1 overexpression induced a remarkable increase in MEF2 activity. This increase was completely inhibited by CaMKII antagonists and HDAC4-3S/A overexpression, reflecting that the dishevelled–CaMKII–HDAC4 axis converges at MEF2. Furthermore, we found that Wnt5a leads to cardiomyocyte hypertrophy and the activation of MEF2, and these could be reversed by KN93, a CaMKII inhibitor. This confirms that the noncanonical Wnt signal can
activate MEF2 via CaMKII. Certainly, KN93 is a nonselective CaMKII inhibitor; however, it should be emphasized here that the α and β isoforms of CaMKII are almost exclusively expressed in the brain. In the heart, CaMKII δ and γ are the predominant CaMKII isoforms. In Figure 1, we also confirmed this. Therefore, even though the KN compounds are nonselective, it will not affect the outcome in this specific context.

Findings of Kühl et al.9,10 support the link between intracellular Ca2+ release and the activation of CaMKII. Taken together previous observations10 with our findings, we think that there is no interaction between disevelled and CaMKII. In the heart, the binding of the noncanonical Wnt ligand to cognate Frizzled receptor activates disevelled. Receptor ligand interaction and activation of disevelled further activate phospholipase C, which leads to a short-lived increase in the concentration of inositol 1,4,5-triphosphate and 1,2 diacylglycerol. Inositol 1,4,5-triphosphate diffuses through the cytosol and interacts with the calcium channels present on the membrane of endoplasmic reticulum resulting in release of calcium ions. Calcium ions along with ubiquitously expressed eukaryotic protein calmodulin activate CaMKII.

Perspectives

Our present work demonstrates that disevelled-1 predominantly signals to CaMKII, which then induces phosphorylation/dissociation of HDAC4 from MEF2. Without repression of HDAC4, MEF2-related gene transcription contributes to cardiac hypertrophy and LV dysfunction. Therefore, our study yields insight into the mechanism of pathogenesis of cardiac dysfunction and has substantial implications for the development of novel interventions for preventing cardiac remodeling and its progression to heart dysfunction by targeting the disevelled–CaMKII–HDAC4–MEF2 axis.

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Disclosures

None.

References


**Novelty and Significance**

**What Is New?**

- Here, we crossed dishevelled-1–transgenic mice with calcium/calmodulin-dependent protein kinase IIiγ double-knockout mice. Interestingly, the dishevelled-1–transgenic mice lacking calcium/calmodulin-dependent protein kinase IIiγ displayed a normal cardiac phenotype without cardiac hypertrophy, fibrosis, or left ventricular dysfunction. Therefore, we report that dishevelled-1 upregulation activates all 3 branches of the Wnt signaling pathway, with calcium/calmodulin-dependent protein kinase IIiγ acting as the most pivotal component in the process of myocardial remodeling.

- Furthermore, mechanistic analyses revealed that calcium/calmodulin-dependent protein kinase IIiγ couples noncanonical Wnt signaling with histone deacetylase 4/myosin enhancer factor 2.

3 branches of Wnt signaling pathways. Some doubt that the overexpression of dishevelled-1 is too strong to exclude the nonspecific effect in this model.

**Summary**

All in all, our study demonstrates for the first time that dishevelled–calcium/calmodulin-dependent protein kinase II–histone deacetylase 4–myosin enhancer factor 2 axis might hold a critical role in cardiac remodeling and its progression to left ventricular dysfunction.

**What Is Relevant?**

- In our previous study, we generated dishevelled-1–transgenic mice with strong expression of dishevelled-1 and confirmable activation of all the
Calcium/Calmodulin-Dependent Protein Kinase II Couples Wnt Signaling With Histone Deacetylase 4 and Mediates Dishevelled-Induced Cardiomyopathy

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CAMKII COUPLES WNT SIGNALING TO HDAC4 AND MEDIATES DISHEVELLED-INDUCED CARDIOMYOPATHY

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Supplemental Information

Methods

Chemical reagents and adenovirus production.

KN92 and KN93 (1µmol/L) were obtained from Calbiochem. Adenovirus harbouring myosin enhancer factor 2 (MEF2) luciferase and HDAC4-S246,467,632A (HDAC4-3S/A) were produced as described previously[1, 2]. Recombinant adenovirus was generated with the AdMax™ Vector Creation Kit (Microbix) containing full-length cDNA-encodingDvl-1-wt (AdDvl). Empty adenovirus was used as control (AdC). After generation the adenoviruses were amplified, purified with the Adeno-X Purification Kit (BD) and its infectious units per µl were determined with the Adeno-X Rapid Titer Kit (BD). An infectious unit of 30 per µl was maintained throughout all experiments as well as the infection period of 48 hours, unless otherwise noted. Luciferase reporter assay kit was obtained from Promega.

Animals.

This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the local animal ethics review board (approval number: 40631) and the Government Bureau of Karlsruhe (project number: 35-9185.81/G-131/06). The generation of heart-directed DVL mice was described previously[2]. CaMKIIδ-KO, CaMKIIγ knockout (CaMKIIγ-KO) and CaMKIIδγ double knockout (CaMKIIδγ-KO) animal lines were produced with the protocol in Supplemental Material. To generate the homozygous CaMKIIδγ-KO mice, heterozygous CaMKIIδγ-KO offspring carrying the Dvl-1 transgene were inbred with a line lacking the transgene. The C57BL/6 background DVL mice were crossed with 129SvEv/C57BL6/CD1 background CaMKIIδγ-KO mice, leading to four genotypes: Dvl-1 wild-type mice with normal levels of CaMKIIδγ (CTRL), CaMKIIδγ-deficient with non-Dvl-1-Tg mice (CKO), Dvl-1-Tg mice with normal CaMKIIδγ levels (DVL), and Dvl-1-Tg mice deficient for CaMKIIδγ (DWC). DVL mice were also crossed with CaMKIIδ-KO mice and CaMKIIγ-KO mice using the same mating plan. There is no genetic variability between the groups. All mice used in the present investigation were 12 weeks old.

Culture and adenoviral infection of NRVMs.

NRVMs were isolated from 1- to 3-day-old BR-Wistar rats (Charles River Laboratories) and purified using a discontinuous Percoll gradient. We consistently obtained cell populations containing more than 95% cardiomyocytes using this protocol. For adenoviral infection or siRNA treatment, NRVMs were infected 24 hours after changing to serum-free medium, grown for an additional 24 hours, and if necessary, then stimulated with Wnt5a conditional medium for another 24 hours. Before Wnt5a treatment, CaMKII inhibitor was applied. Cells were then harvested for western blot, quantitative real-time PCR, and/or fixed for immunocytochemical staining. For cardiomyocyte hypertrophy, the myocyte cell surface area was measured as previously[3]. For the MEF2 reporter assay, adenovirus harboring MEF2 luciferase was applied to cultured cardiomyocytes 8 hours prior to all the above stimulation. After treatment, cellular extracts were prepared to determine luciferase expression using a
luciferase reporter assay kit (Promega).

**Dvl-1 knockdown and isoproterenol stimulation.**

siRNAs were synthesized by MWG (Ebersberg, Germany). For siRNA transfection, cells were cultured in serum free medium for 40 hours and followed at least 5 hours in both serum and antibiotic free myomedium or OptiMEM (Invitrogen). Then, they were incubated for 24 hours at 37°C with siRNA treatment. After this, the medium was changed to serum-free myomedium for another 24 hours with isoproterenol (50nM) stimulation. siRNAs used were Dvl-1 5'-AGA UCA CCA UUG CCA AUG C-3' and non-specific control siRNA 5'-AGG UAG UGU AAU CGC CUU G-3'. In all siRNA mediated knockdown experiments myocytes transfected with non-specific siRNA were used as controls.

**Echocardiography.**

Mice were lightly anesthetized by isoflurane inhalation (~2% isofluorane) and kept warm on a heating platform in a supine position. *In vivo* non-invasive transthoracic echocardiography was performed in a modified setting as previously described[2]. In brief, to evaluate cardiac function, a two-dimensional parasternal short axis view and M-mode tracings of the LV were obtained with an ATL 5000 echocardiogram. LV dimensions were determined on the M-mode tracings and averaged from at least three consecutive cardiac cycles. LV fractional shortening was calculated as \((\text{LVIDd} - \text{LVIDs})/\text{LVIDd}\) and expressed as a percentage. The observer was unaware of the genotype of the mice.

**Hemodynamic measurement.**

Before sacrificing the mice, cardiac performance was detected as described previously[2]. Briefly, a 1.4F micromanometer-tipped catheter (Millar Instruments) was inserted retrogradely into the LV through the right carotid artery. Data were recorded using the software package Chart 5 (AD Instruments). The following indices were measured with the PVAN 3.5 software (Millar Instruments Inc.) and averaged from 10 sequential beats: heart rate, LV end-systolic volume, LV end-diastolic volume, stroke volume, peak rates of LV pressure development (dP/dt max) and relaxation (dP/dt min), LV end-systolic pressure, LV end-diastolic pressure, and the time needed for relaxation of 50% maximal LV pressure to baseline value (τ), which has been reported to be a useful indicator of LV diastolic dysfunction.

**HDAC4 kinase activity assay.**

HDAC4 kinase activity assay was performed in ventricular homogenates as previously[1, 4]. Briefly, GST-HDAC4 fusion proteins (amino acids 419–670 of HDAC4) were used as substrates. The GST-HDAC proteins were conjugated to glutathione–agarose beads. GST-HDAC-bound beads were incubated with ventricular lysates (100 µg) in lysis buffer for 4 hours at 4 °C. Beads were washed one time with the same buffer. Then beads resuspended in kinase reaction buffer (containing 12.5 µM ATP and 5 µCi of [32P]ATP and reactions) were allowed to proceed for 30 min at room temperature. Samples were boiled. Phosphoproteins were resolved by SDS/PAGE, visualized by autoradiography, and quantified by using a PhosphorImager.

**Heart tissue preparation and histopathology.**

Mice were euthanized and heart was excised in diastole by injection of saturated potassium chloride solution. After dissecting LV, part of myocardial samples were snap-
frozen with liquid nitrogen for protein as well as mRNA analysis and the other part was fixed for 24 hours in 4% formalin dissolved in 0.1 M PBS (pH 7.4), subsequently embedded in paraffin, and transversely cut into 5 μm sections for further histological analysis.

Sections were stained with haematoxylin and eosin (H&E) to determine the myocyte size[5]. To quantify cardiac fibrosis and apoptosis, we stained sections with picrosirius red (PSR)[6] and tunnel staining, respectively. Images were captured with a digital camera and analyzed using Image J program (myocyte size and fibrosis) and Image 2 (apoptosis) in a blind manner.

**Western blotting.**

Total ventricular extracts were prepared from the LV myocardium and cultured cardiomyocytes on ice as described previously[2]. Protein concentrations were measured by the BCA assay. Equal amounts of protein extracts were separated with SDS-PAGE and transferred to a nitrocellulose membrane (Millipore). Primary antibodies used were anti Dvl-1 (Santa Cruz), anti β-catenin (Santa Cruz), anti active β-catenin (Millipore), anti CaMKII (BD Bioscience), anti active CaMKII (Thr286) (Promega), anti HDAC4 (Cell Signaling), anti p-HDAC4 (Ser632) (Santa Cruz), anti PKCα (Cell Signaling), anti p-PKCα/βII (Thr638/841) (Cell Signaling), anti PKCd (Cell Signaling), anti p-PKCd (Tyr311) (Cell Signaling), anti JNK1 (Santa Cruz), anti p-JNK (Santa Cruz), anti p-CREM (Ser133) (Cell Signaling) and anti α-actin (Sigma-Aldrich). Anti-rabbit IgG and anti-mouse IgG horseradish peroxidase-conjugated antibodies (Santa Cruz) were used as secondary antibodies. Bands were detected by ECL and quantified by densitometry using the Image J program.

**Quantitative real-time PCR.**

Total RNA was extracted from the LVs of the animals and cultured cardiomyocytes using TRIzol (Invitrogen, Germany). First-strand cDNA was synthesized with the RevertAid First Strand cDNA Synthesis Kit (Fermentas). Quantitative real-time PCR was performed as described previously[2]. For rat, HPRT 5´-gtcaaggggccataaaag-3´ and 5´-tgccatgctttacagtga-3´, probe #22; ANF 5´-ccgcagccaatg-3´, and 5´-caacgctttctgaaggggtg-3´, probe #25; Axin-2 5´-gaggagtgcggcagc-3´ and 5´-cgctgctgtctggcct-3´, probe #116. For mouse, HPRT 5´-gtcagggggcataaaag-3´ and 5´-tgacagtctttacagtga-3´, probe #22; ANF 5´-ccgacccagaggtg-3´, and 5´-caacgctttctgaaggggtg-3´, probe #25; βMHC 5´-caaggctaatctgacagg-3´ and 5´-ccatgcgcaacctttcttc-3´, probe #78; Axin2 5´-ctgctggtccagcgag-3´ and 5´-tgagctttctttcct-3´, probe #50; CaMKIIα 5´-gtgccaaggtataca-3´, and 5´-cgctcagctttc-3´, probe #106; CaMKIIβ 5´-gccatcctcactatgtc-3´ and 5´-ctccatcctgtttgtagt-3´, probe #66; CaMKIIδ 5´-agttcagggcactga-3´ and 5´-cgctttgactgctgctt-3´, probe #68; and CaMKIIγ 5´-gttgccatcctcacaaccat-3´ and 5´-catgtgacttcttgatagc-3´, probe #80.

**Statistics.**

Data are summarized as mean ± standard error of the mean. Statistical analysis was performed with the Graph-Pad Prism Software Package Version 5.0 (GraphPad, Inc.). Differences between groups were compared by one-way ANOVA followed by Student-Newman-Keuls post hoc testing for the analysis of multiple groups. A value of P<0.05 in a two-tailed distribution was considered statistically significant unless otherwise stated.
**Discussion**

Recently, several studies have highlighted the role of CaMKIIδ in heart disease. Cardiac-specific overexpression of CaMKIIδ induces hypertrophy and dilated cardiomyopathy with ventricular dysfunction, loss of intracellular Ca^{2+} homeostasis, and premature death, as compared to wild-type littermates[7-9]. In contrast, CaMKII inhibition by pharmacological or genetic approaches reverses heart failure-associated changes such as arrhythmias, hypertrophy, and dysfunction in animal models of structural heart disease[10-12]. Interestingly, the use of mice lacking CaMKIIδ, which is the dominant isoform of CaMKII in the heart significantly attenuates the development of pressure overload-induced heart failure and improves survival, concomitant with decreases in cardiac dysfunction, apoptosis, and fibrosis[11, 12].

In an attempt to further explore the nuclear mediator of Wnt/CaMKII in cardiac remodeling and dysfunction, we first monitored activating transcription factor-1 (ATF1) and cAMP response element-binding protein (CREM), since they are transcription factors mediated by Wnt/CaMKII signaling[13], and gene regulation by CREM is an important mechanism of Wnt-directed mammalian myogenic gene expression[14] and β1-adrenoceptor-induced cardiac damage in mice[15]. Although these observations raise the possibility that ATF1 or CREM may be a potential downstream target in Dvl-1 overexpression-induced cardiac remodeling, we did not detect any significant changes in the activation of ATF1 and CREM in DVL mice (data not shown).
References


Supplemental table
Supplemental table S1: Detailed genotype of each group.

<table>
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CTRL equals control (Dvl-1 wildtype mice with normal levels of CaMKIIδγ), CKO CaMKII-KO (Dvl-1 wildtype mice without CaMKIIδγ expression), DVL Dvl-1-Tg (Dvl-1-Tg mice with normal levels of CaMKIIδγ), and DWC (Dvl-1-Tg with CaMKIIδγ-KO). The third to sixth generation of offspring was used in this study. There is no genetic variability between the littermates.
Figure S1: Dvl-1 knockdown inhibits ISO-induced activation of CaMKII, HDAC4 and MEF2. (A, B) Western blots and densitometry of increased levels of Dvl-1, phosphorylated CaMKII, phosphorylated HDAC4 and MEF2 following ISO stimulation, and all of them were inhibited by Dvl-1 knockdown. ISO, isoproterenol; Dvl-KD, Dvl-1 knockdown; Values are reported as mean ± standard error of the mean. *P < 0.05 vs. CTRL; #P < 0.05 vs. ISO + KD. All experiments with cardiomyocytes were carried out at least three times.
Figure S2: Dvl-1-induced cardiac hypertrophy and LV dysfunction was not rescued Deletion of CaMKIIδ alone. (A–D) Demonstrating quantitative analysis of left ventricular PWTs, EDD, EF and FS. EDD means end-diastolic diameter, PWTs posterior wall thickness in systole, EF ejection fraction, and FS fraction shortening. CTRL equals control, CKOδ CaMKIIδ knockout, DVL Dvl-1 transgenic, and DWCδ Dvl-1 transgenic with CaMKIIδ knockout. Values are demonstrated as mean ± SEM. * denotes $P < 0.05$ vs. CTRL, and # denotes $P < 0.05$ vs. DWCδ. n > 4 per group.
Figure S3: Dvl-1-induced cardiac hypertrophy and LV dysfunction was also not rescued Deletion of CaMKIIγ. (A–D) Demonstrating quantitative analysis of left ventricular PWTs, EDD, EF and FS. EDD means end-diastolic diameter, PWTs posterior wall thickness in systole, EF ejection fraction, and FS fraction shortening. CTRL equals control, CKOγ CaMKIIγ knockout, DVL Dvl-1 transgenic, and DWCγ Dvl-1 transgenic with CaMKIIγ knockout. Values are demonstrated as mean ± SEM. * denotes $P < 0.05$ vs. CTRL, and # denotes $P < 0.05$ vs. DWCγ. n > 4 per group.
Figure S4: Reduced cardiac apoptosis of DWC mice by deletion of CaMKIIδγ. (A) As determined by TUNEL assay, frequency of apoptosis in the LV was robustly enhanced in DVL mice, whereas deletion of CaMKIIδγ significantly reduced the increase of apoptosis in the DWC mice. (B) Bar graph summarized changes of the apoptosis frequency in all groups. Values are presented as mean ± standard error of the mean. *P < 0.05 vs. CTRL; #P < 0.05 vs. DWC. n = 12 per group.
Figure S5: The role of Wnt5a on cardiac hypertrophy. (A) Conditional Wnt5a stimulation resulted in cardiomyocytes hypertrophy. This could be rescued by CaMKII inhibition. (B) MEF2-dependent luciferase activity was markedly upregulated by conditional Wnt5a stimulation, and KN93 inhibited the upregulation of luciferase activity. Values are reported as mean ± standard error of the mean. *P < 0.05 vs. KN92; #P < 0.05 vs Wnt5a with KN93. All experiments with cardiomyocytes were carried out at least three times.