Valvular Heart Disease

Protein Kinase D2 Controls Cardiac Valve Formation in Zebrafish by Regulating Histone Deacetylase 5 Activity

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Background—The molecular mechanisms that guide heart valve formation are not well understood. However, elucidation of the genetic basis of congenital heart disease is one of the prerequisites for the development of tissue-engineered heart valves.

Methods and Results—We isolated here a mutation in zebrafish, bungee (bng)<sup>Y849N</sup>, which selectively perturbs valve formation in the embryonic heart by abrogating endocardial Notch signaling in cardiac cushions. We found by positional cloning that the bng phenotype is caused by a missense mutation (Y849N) in zebrafish protein kinase D2 (pkd2). The bng mutation selectively impairs PKD2 kinase activity and hence Histone deacetylase 5 phosphorylation, nuclear export, and inactivation. As a result, the expression of Histone deacetylase 5 target genes Krüppel-like factor 2a and 4a, transcription factors known to be pivotal for heart valve formation and to act upstream of Notch signaling, is severely downregulated in bungee (bng) mutant embryos. Accordingly, the expression of Notch target genes, such as Hey1, Hey2, and HeyL, is severely decreased in bng mutant embryos. Remarkably, downregulation of Histone deacetylase 5 activity in homozygous bng mutant embryos can rescue the mutant phenotype and reconstitutes notchlb expression in atrioventricular endocardial cells.

Conclusions—We demonstrate for the first time that proper heart valve formation critically depends on Protein kinase D2-Histone deacetylase 5-Krüppel-like factor signaling. (Circulation. 2011;124:324-334.)

Key Words: histone deacetylase 5, human heart valves protein kinase D2 Zebrafish

In humans, cardiac valve defects account for 20% to 30% of all congenital cardiovascular malformations, with an incidence of ~5% of live births. Each year, >100 000 patients receive cardiac valve replacement therapies with mechanical or xenogenous biological prosthesis in the United States. The recent discovery that somatic cells can be reprogrammed back to pluripotent stem cells has provided a potential avenue for autologous cardiac valve engineering, because induced pluripotent stem cells are capable of forming any cell type of the body. However, the molecular signals that orchestrate cardiac valve formation are only poorly deciphered.

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One essential milestone in heart valve morphogenesis is the formation of endocardial cushions, which arise from a subset of endothelial cells at the atrioventricular (AV) canal (AVC) and the outflow tract (OFT). Endocardial cushion formation is a multistep process starting with the specification of endocardial cells of the AVC, their subsequent delamination and migration into the cardiac jelly, and their epithelial-to-mesenchymal transformation (EMT). Endocardial cushions then fuse to form the AV complex, which is remodeled into fibrous tissue to build up mature valve leaflets. On a molecular level, orchestration of several myocardial and endocardial growth factor–mediated signaling cascades is essential during endocardial cushion formation. For instance, both myocardial Bone morphogenetic protein 2 and 4 (BMP2/4) signaling and endocardial Notch1 signaling were recently found to be crucial for the transformation of AVC and OFT endocardial cells to mesenchymal cells. In addition, several protein kinases such as the Mitogen-activated protein 3 kinase and Rho-associated kinase are involved in the development of endocardial cushions and the induction of EMT. In contrast, although members of the Protein Kinase D (PKD) family are also strongly expressed in the vertebrate heart, their exact role in heart development has not yet been deciphered.

In mammals, the PKD family consists of 3 highly homologous proteins, PKD1 (PKCμ), PKD2, and PKD3 (PKCr), that share an amino-terminal autoregulatory domain and a...
carboxy-terminal catalytic serine/threonine kinase domain.\textsuperscript{10} In a catalytic inactive state, PKDs reside within the cytosol. Activation of cytosolic PKD by different mechanisms such as binding of diacylglycerol or site-specific phosphorylation by PKC leads to its translocation to distinct subcellular compartments such as the plasma membrane, the Golgi network, and the nucleus.\textsuperscript{10} In the nucleus, PKDs can phosphorylate and thus inactivate class II Histone deacetylases (HDACs) such as HDAC4 and HDAC5.\textsuperscript{11} Recently, PKD-HDAC signaling has been found to be involved in cell migration,\textsuperscript{12} proliferation,\textsuperscript{13} and differentiation.\textsuperscript{14} In mice, all 3 PKD family members are strongly expressed in the embryonic heart, suggesting a crucial role of PKDs in heart development.\textsuperscript{9} Whereas PKD1-HDAC signaling was recently found to be essential for pathological remodeling of the heart,\textsuperscript{15} the specific roles of PKD2 and PKD3 in the vertebrate heart are not known yet.

In searching for key regulators of cardiac valve formation, we isolated in a large-scale ethylnitrosourea (ENU)-mutagenesis screen the recessive, embryonic lethal zebrafish mutation \textit{bungee} (\textit{bng}\textsuperscript{bnt77}). \textit{Bng} mutant embryos fail to develop AV valves owing to impaired endocardial Notch1 signaling. Using positional cloning, gene knockdown, and rescue experiments, we demonstrate that the \textit{bng} phenotype is caused by a missense mutation (Y849N) in zebrafish \textit{protein kinase D2} (\textit{pkd2}). The \textit{bng} mutation selectively impairs PKD2 kinase activity and hence HDAC5 phosphorylation, nuclear export, and inactivation. As a result, we find targets of HDAC5-dependent transcription known to be involved in cardiac valve formation such as Krüppel-like factor (KLF) 2a and KLF4a,\textsuperscript{16,17} significantly downregulated in \textit{bungee} mutant zebrafish embryos, suggesting for the first time a pivotal role of PKD2-HDAC5-KLF signaling during cardiac valve formation.

\textbf{Methods}

\textit{Histology, Antisense RNA In Situ Hybridization, and Immunostaining}

For histology, embryos were fixed in 4% paraformaldehyde and embedded in JB-4 (Polysciences, Inc). Then, 5-\textmu{}m sections were cut, dried, and stained with hematoxylin and eosin.\textsuperscript{18} Whole-mount antisense RNA in situ hybridization was carried out essentially as described.\textsuperscript{18} Immunostaining of Dent's fixed zebrafish embryos was carried out with antibodies against distinct muscle myosin heavy chains (MF20 and S46). HEK293A cells were fixed with 4%

\textit{Injection Procedures}

Morpholino-modified oligonucleotides were directed against the splice donor site of exon 16 of zebrafish \textit{pkd2}. A standard control oligonucleotide (MO-control) (GENETOOLS, LLC) was injected at the same concentration. Sense-capped RNA was synthesized with the \textit{mMESSAGE mMASCHINE} system (Ambion).

\textbf{Expression and Purification of Recombinant Protein Kinase D2}

For expression of zebrafish GST fusion proteins, zebrafish \textit{pkd2} (\textit{zpkd2}) variants were subcloned into pDEST24 (Invitrogen), expressed in \textit{Escherichia coli}, and harvested by centrifugation. Bacterial cells were disrupted by sonication, and cell debris was removed by centrifugation. The GST fusion proteins were purified with glutathione-sepharose matrix (Amersham) and eluted with 0.03 mol/L reduced glutathione.

\textbf{In Vitro Kinase Assay}

To test zPKD2 kinase activity, zPKD2 was incubated with HDAC5-GST fusion protein. Incubations were carried out with 10 \mu{}Ci of \textit{[^32P]}-yATP at 30°C for 10 minutes in kinase buffer. Phosphorylated proteins were separated by SDS-PAGE, and \textit{[^32P]} autoradiography was performed.

\textbf{Cell Culture and Transfection Assays}

HEK293A cells were maintained in Dulbecco modified Eagle medium (10% FCS, 2 mmol/L L-glutamine and penicillin-streptomycin) and transfected with pCDNA3.2-V5-\textit{zpkd2}\textit{S749E} [\textit{zpkd2}\textit{S749/753E} and -\textit{zpkd2}\textit{S749/753E,Y849N} and pCDNA3hHDAC5-Flag. Transfections were performed with lipofectamine (Invitrogen) according to manufacturer's instructions.

\textbf{Coimmunoprecipitation and Immunoblotting}

HEK293A cells were harvested 2 days after transfection in lysis buffer and disrupted, and cell debris was removed by centrifugation. FLAG-tagged proteins were immunoprecipitated with an M2-agarose conjugate (Sigma) and washed with lysis buffer. Bound proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and immunoblotted with anti-V5 (Invitrogen) or anti-Flag (Sigma) antibodies.

\textbf{Quantitative Real-Time Polymerase Chain Reaction}

Quantitative real-time polymerase chain reaction was carried out according to standard protocols with the SYBR-Green method (Thermo Scientific) using an ABI 7000. cDNA was generated from RNA of 72-hours-after-fertilization (hpf) \textit{bng} mutant and wild-type embryonic hearts using oligo(dT) primer and SuperScript II reverse transcriptase (Invitrogen).

More detailed information regarding the methods used is provided in the expanded Methods section in the online-only Data Supplement.

\textbf{Results}

\textit{Disturbed Endocardial Signaling Leads to Impaired Heart Valve Development in Bungee Mutants}

In searching for novel regulators of cardiac valve formation, we isolated here in a large-scale ENU-mutagenesis screen the recessive, embryonic lethal zebrafish mutant \textit{bungee} (\textit{bnt77}). Between 24 and 48 hpf, the \textit{bng} heart jogs leftwards and loops rightwards, myocardial and endocardial cell layers are clearly defined, atrial and ventricular cardiomyocyte populations express chamber-specific myosin heavy chains, indicating regular chamber differentiation and specification, and a peristaltic contraction wave passes through it, propelling the first blood cells through the vascular bed (Movies I and II and Figures IA through IH and IIA through IIP in the online-only Data Supplement). However, starting at 48 hpf, a developmental time point when in wild-type zebrafish heart atrium and ventricle usually get demarcated by the AVC, in \textit{bng} mutant hearts, the AV junction is dilated, resulting in regurgitation of blood from the ventricle to the atrium at each ventricular systole, blood congestion in the atrium
and sinus venosus area, and reduced forward blood flow through the aorta (Movies III and IV in the online-only Data Supplement and Figure 1A through 1D). In contrast to wild-type embryos, in which endocardial cells accumulate at the AVC and give rise to endocardial cushions, the precursors of the cardiac AV valves, in bng mutants, endocardial cells fail to cluster at the AVC by 48 hpf. By 96 hpf, a time point when wild-type embryos usually have developed multilayered endocardial cushions at the AVC, homozygous bng embryos show no evidence of endocardial cushion formation (Figure 1C and 1D and Figure IIIA and IIIB in the online-only Data Supplement). As shown in Figure IIIC through IIIG in the online-only Data Supplement, we find that cell proliferation does not differ between bng mutant and wild-type hearts at 48 and 72 hpf. In particular, we find no differences in BrdU-positive AV endocardial cells between wild-type and bng mutant zebrafish (Figure IIIC through IIIG in the online-only Data Supplement). Moreover, the development of other endothelial cell populations, such as the chamber endocardium, dorsal aorta, or posterior cardinal vein seems to be unaffected in homozygous bng mutants (Figures IIIA and IIIB and IVG through IVL in the online-only Data Supplement).19 In addition, development of the eyes, brain, pectoral fins, and inner ear also appears unaltered in bng mutants compared with wild-type littermates (Figure IVA through IVF in the online-only Data Supplement).

Cardiac cushion development is known to be initiated by molecular signals such as BMP4, transmitted from the AV myocardial cell population to the AV endocardial cell population.2 On these stimuli, endocardial cells usually accumulate at the AVC, increasingly express Notch1b, and then migrate into the AV jelly to undergo EMT.20 To evaluate whether defective transformation of AV epithelial cells into mesenchymal cells is responsible for impaired cardiac valve formation in bng mutant embryos, we first investigated the expression of bmp4 and found that, similar to the situation in wild-types, in bng mutant hearts, bmp4 mRNA regularly accumulates at the AVC, the ventricular-aortic border (cardiac OFT), and the sinus-atrial junction at 72 hpf (Figure 1E through 1H). In contrast, we found endocardial notch1b RNA levels, which are usually enhanced at the AV border in wild-type hearts by 72 hpf, not to be increased at the AVC and the ventricular-aortic border in bng mutants (Figure II through 1L). To further evaluate whether EMT is specifically disrupted in bng mutant hearts, we next evaluated the expression of secreted phosphoprotein 1 (spp1; osteopontin), a factor known to be upregulated in activated AV endocardial cells that migrate into the cardiac jelly and subsequently undergo EMT.21-22 As shown in Figure 1M through 1P, in contrast to wild-type embryos, in which we found high levels of spp1 mRNA at the AV boundary, spp1 is completely absent from the AVC in bng mutant hearts. Interestingly, expression of general endothelial markers, such as fli1a, fli-1, or tec2 is not affected by the bng mutation (Figures IIIA and IIIB and IVG, IVH, IVJ, IVK, and IVM in the online-only Data Supplement).

The bng<sup>h177</sup> Locus Encodes zpkd2

To identify the ENU-induced mutation causing the recessive bng mutant phenotype, we performed a positional walk. Using bulk segregant analysis, we mapped bng to zebrafish linkage group 15. Recombination analysis of 1050 bng mutant embryos restricted bng to a genomic interval flanked by the microsatellite markers z23908 and z34586. Further fine-mapping restricted the bng mutation interval to 2 open reading frames encoding proteins highly homologous to human PKD2 and a protein of unknown function (zgc:152692) (Figure 2A). To identify the site of the ENU-induced mutation in bng, we sequenced the entire zebrafish coding sequence of the 2 open reading frames from wild-type and bng mutant cDNA and genomic DNA. We identified the bng mutation to be a thymine-to-adenine nucleotide transversion (TAT→AAT) in codon 849 of the zpkd2 gene, predicted to change the amino acid tyrosine to an asparagine (Y849N; Figure 2B). As in humans, zpkd2 is encoded by 18 exons. The protein consists of 923 amino acids and shows 74% amino acid identity to the human ortholog (Figure V in the online-only Data Supplement). zPKD2 contains a conserved serine/threonine-kinase domain in the C-terminal region and 2 cysteine-rich domains (C1a and C1b) in the amino-terminus of the protein and a pleckstrin homology domain, both known to be important for the regulation of the kinase activity of PKD2.10 The bng mutation (Y849N) resides within the kinase domain of zPKD2 (Figure V in the online-only Data Supplement).

In mammals, pkd2 is expressed ubiquitously both during embryonic development and in the adult.9 In the zebrafish embryo, at 48 hpf, we find pronounced expression of zpkd2 mRNA in the brain, gastrointestinal tract, and heart (Figure 2C and 2D). Within the heart, we find enhanced zpkd2 mRNA levels in the endocardium of the AVC and OFT, whereas AV myocardial cells seem to be devoid of PKD2 mRNA (Figure 2E). zpkd2 mRNA expression levels and tissue distribution do not differ between wild-type and bng mutant embryos, indicating that bng mutant zpkd2 mRNA is not degraded by nonsense-mediated RNA decay (data not shown).

To substantiate our finding that impaired cardiac valve formation in bng mutant zebrafish is indeed due to altered PKD2 function, we injected wild-type or bng mutant pkd2 mRNA into embryos derived from intercrossing heterozygous bng zebrafish. After injection of 1.6 ng wild-type zpkd2 mRNA (zpkd2<sup>wt</sup>), 58.7±14.5% (4 independent experiments, n=90; P<0.001) of injected bng mutant embryos showed properly developed endocardial cushions at 72 hpf (Figure 3A and 3B). On a molecular level, we find in these genotypically homozygous mutant embryos reconstitution of notch1b and spp1 expression in the AV endocardium (Figure 3D through 3G). Similar rescue rates (47±6.4%; 3 independent experiments, n=98; P<0.001) were achieved by injection of 1.6 ng mouse pkd2 mRNA (mpkd2<sup>wt</sup>; Figure 3A), implicating an evolutionarily conserved role of PKD2 in AV valve development. In contrast, injection of 1.6 ng bng mutant zpkd2 mRNA (zpkd2<sup>y849n</sup>) into homozygous mutant bng embryos (4 independent experiments, n=75) does not restore cardiac valve formation (Figure 3A and 3C), indicating a loss-of-function effect of the bng mutation on PKD function. Accordingly, during injection of 1.2 ng Morpholino-modified antisense oligonucleotides directed against the splice donor site of exon 16 of zpkd2 into 1-cell-stage wild-type zebrafish embryos, 95% of the injected embryos (n=285; P<0.001) displayed defective endocardial cushion development similar to bng mutants (Figure 3H through 3J) that was accompanied
Figure 1. Effects of the bng mutation on embryonic heart morphology. 
A and B, Lateral view of wild-type (wt; A) and bng mutant (B) embryos at 72 hours after fertilization (hpf). The bng mutant embryos show intracardial blood congestion and pericardial edema. C and D, Hematoxylin and eosin staining of sagittal histological sections of wt (C) and bng (D) hearts at 96 hpf. Similar to wild-types, in bng atria and ventricles, myocardial (my) and endocardial (en) cell layers are clearly defined, heart chambers are separated by an atrioventricular ring, but no endocardial cushions develop at the atrioventricular canal (AVC). E through P, Defective endocardial signaling in bng mutant hearts. Schematic descriptions of the spatial expression of bmp4, notch1b, and spp1 are shown on the right of the whole-mount antisense RNA in situ hybridization images of wt and bng embryos at 72 hpf. E through H, Similar to wild types, bmp4 staining is observed at the inflow tract (IFT), AVC, and outflow tract (OFT) myocardium of bng mutant embryos. I through L, Endocardial notch1b expression is enhanced at the AVC and OFT of wt embryos (I and J) but is diffusely expanded throughout the endocardium in bng embryos (K and L). M through P, spp1 is expressed in the AVC and OFT endocardium of wt embryos (M and N), whereas spp1 expression is completely absent within the atrioventricular canal of bng mutant hearts (O, P). Black arrow shows the AVC; white arrow, OFT; and black arrowhead, IFT. A indicates atrium; V, ventricle.
by the absence of notch1b and spp1 expression at the AV boundary (Figure 3K through 3N) and ventriculo-atrial blood regurgitation (Movie V in the online-only Data Supplement).

In summary, these findings confirm that loss of zPKD2 function indeed accounts for the bng mutant phenotype.

The bng PKD2Y849N Mutation Significantly Affects Protein Kinase D Kinase Activity

The bng zPKD2Y849N mutation resides within the highly conserved serine/threonine kinase domain of PKD2 (Figure V in the online-only Data Supplement). Substitution of aspartate 738 by an alanine (D738A) was recently found to result in a kinase-deficient PKD2 protein, whereas substitution of serine 749 and 753 by glutamic acid (S749/753E) leads to constitutive activation of PKD2 (CA-PKD2) kinase function.23 One of the potential targets of PKD2 kinase activity in the mammalian heart is HDAC5.11

To evaluate whether, similar to the human ortholog, zebrafish PKD2 acts as a kinase, thereby phosphorylating targets such as HDAC5, we first assayed zPKD2 kinase activity in vitro. Because PKD2 has to be activated via site-specific phosphorylation by PKC before "acting as a kinase,"10 we used CA-PKD2 for the assay. As shown in Figure 4A, both human (CA-hPKD2wt) and zebrafish (CA-zPKD2wt) PKD2 can strongly phosphorylate HDAC5. Next, because the bng mutation (zPKD2Y849N) resides within the PKD2 kinase domain, we examined whether it affects zPKD2 kinase activity. As shown in Figure 4A, in contrast to CA-hPKD2wt, bng mutant CA-zPKD2bng cannot phosphorylate HDAC5, implicating that tyrosine 849 residue is essential for zPKD2 kinase activity. To rule out that loss of HDAC5 phosphorylation by bng mutant PKD2 is due to impaired binding, we performed coimmunoprecipitation experiments with FLAG-tagged HDAC5 and V5-tagged wild-type and bng mutant PKD2. However, as shown in Figure 4B, both wild-type (CA-PKD2wt, lane 1) and bng mutant PKD2 (CA-PKD2bng, lane 2) strongly bind HDAC5, indicating that loss of HDAC5 phosphorylation is not due to impaired binding of bng mutant PKD2 to HDAC5.

Finally, to evaluate whether diminished zPKD2 kinase activity accounts for the bng mutant heart phenotype in vivo, we evaluated the ability of kinase-deficient PKD2 and CA-PKD2 to reconstitute AV valve formation in bng mutant embryos. Strikingly, in contrast to CA-PKD2, injection of 3 ng kinase-deficient pkd2 mRNA does not rescue the cardiac valve defects in bng mutant embryos (Figure 4C), suggesting that PKD2 kinase activity is essential for cardiac AV valve formation.

Protein Kinase D2 Controls Atrioventricular Valve Formation via Histone Deacetylase 5 Signaling

Activated PKD2 is known to phosphorylate class II HDACs such as HDAC5. Phosphorylated HDAC5 is then bound by 14-3-3 chaperones, exported to the cytosol, and thereby inactivated.11 To evaluate whether impaired phosphorylation of HDAC5 by kinase-deficient bng mutant PKD2 (PKD2Y849N) leads to diminished nuclear export of HDAC5, we evaluated cytosolic Flag-tagged human HDAC5 expression in HEK293A cells cotransfected with V5-tagged wild-type and bng mutant PKD2 (CA-PKD2bng, lane 2) strongly bind HDAC5, indicating that loss of HDAC5 phosphorylation is not due to impaired binding of bng mutant PKD2 to HDAC5.

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cytosol, whereas its nuclear fraction is significantly reduced (Figure 4D and 4E). In contrast, similar to wild-type bng, mutant PKD2 (CA-PKD2\textsuperscript{bng}) localizes to both the cytoplasm and the nucleus in HEK293A cells, HDAC5-FLAG remains mainly in the nucleus in the presence of CA-PKD2\textsuperscript{bng} (Figure 4D and 4E), implying that CA-PKD2\textsuperscript{bng} is unable to inactivate HDAC5 by nuclear export.

Hence, to assess whether enhanced nuclear activity of HDAC5 also accounts for the bng mutant phenotype in vivo, we evaluated the effect of enhancing nuclear HDAC activity in the sensitized heterozygous bng mutant background. To do so, we injected CA-hHDAC5\textsuperscript{2+} into heterozygous bng (\textit{bng}+/\textit{H}11001/\textit{H}11002) or wild-type embryos and analyzed the resulting cardiac phenotype. After injection of 0.8 ng CA-hHDAC5...
mRNA, 21.2% (n=4; P<0.05) of injected heterozygous bng zebrafish embryos display the homozygous bng mutant phenotype, including loss of notch1 expression at the ventricular-aortic border and the absence of AV valves accompanied by regurgitation of blood from the ventricle to the atrium, whereas injection of the same amount of CA-hHDAC5 mRNA in homozygous wild-type embryos does not affect cardiac valve formation (Figure 5A through 5C and Movie VI).
in the online-only Data Supplement). Additionally, down-regulation of HDAC5 activity in homozygous mutant bng embryos by the microinjection of HDAC5-specific Morpholinos can rescue the mutant phenotype in 16.1% (n=4; P<0.001) of injected embryos and reconstitutes the expression of notch1b (Figure 5A through 5C). These findings imply that the bng mutant phenotype is mediated mainly by PKD2-HDAC5 signaling.

Next, to evaluate the molecular signals by which increased nuclear activity of HDAC5 impairs cardiac valve formation, we assayed the expression of several known HDAC5 transcriptional targets such as the KLF2a and KLF4a (klf2a and klf4a) in bng mutant hearts by whole-mount antisense RNA in situ hybridization and quantitative real-time polymerase chain reaction. Interestingly, both KLF2a and KLF4a were recently found to be specifically expressed in endocardial cells and to be essential for AV valve formation in zebrafish.16,17 As shown in Figure 5G through 5K, compared with the wild-type, RNA levels of klf2a and klf4a are severely reduced at the AV boundary and the cardiac OFT in bng mutant hearts. Interestingly, KLF2a is known to regulate notch expression in endothelial cells,16 implying that loss of notch1b expression in bng AV endocardium is a direct consequence of impaired PKD2-HDAC5-KLF signaling. In mammals, members of the helix-loop-helix hairy/enhancer of split (Hes)-related repressor protein (Hey) transcription fac-

Figure 5. Ectopic expression of Histone deacetylase 5 (HDAC5) leads to cardiac valve defects in heterozygous bng embryos, whereas HDAC5 knockdown can rescue the bng AVC defects. A, Injection of constitutive active human HDAC5 (CA-hHDAC5) RNA into heterozygous bng embryos, usually devoid of a cardiac phenotype, leads to the bng mutant heart phenotype. B and C, Endocardial notch1b expression is enhanced at the atrioventricular canal (AVC) and outflow tract (OFT) of heterozygous bng embryos (bng+/−) control injected with KCl (C) but is diffusely expanded throughout the endocardium in heterozygous bng embryos (bng+/−) injected with CA-hHDAC5 (B). D, Knockdown of zebrafish HDAC5 can rescue the heart phenotype in 16.1% of bng mutant embryos, whereas injection of a control Morpholino has no effect. E and F, Whole-mount antisense RNA in situ hybridizations demonstrate that RNA distribution of notch1b in AVC and OFT endocardium is rescued in bng mutant embryos injected with HDAC5 Morpholinos (E), whereas MO-control injection has no effect (F). G, Quantitative real-time polymerase chain reaction of the HDAC5 target genes klf2a and klf4a and the control targets nr4a1 and nr4a2 (nuclear receptor subfamily 4 group A member 1 and 2) demonstrates that their transcripts are strongly decreased in bng mutant hearts (relative expression of klf2a/H11005 0.18 ± 0.14, P<0.01, n=6; klf4a/H11005 0.28 ± 0.24, P=NS, n=5; nr4a1/H11005 0.22 ± 0.18, P<0.05, n=6; nr4a2/H11005 0.11 ± 0.05, P<0.05, n=9). H through K, In contrast to wild-type (wt) hearts in which klf2a and klf4a is specifically expressed in the AVC (H and J), bng mutant hearts are completely devoid of klf2a and klf4a RNA at 72 hours after fertilization as revealed by whole-mount antisense RNA in situ hybridization (I and K).
Heart valve defects are one of the most common congenital and acquired cardiac diseases. However, the molecular programs that guide vertebrate heart valve formation are only poorly deciphered but are of essential importance for future autologous cardiac valve engineering. Assessment of cardiac valve formation is facilitated in zebrafish embryos because they are transparent and not dependent on intact cardiovascular function during the first week of development. In searching for molecular programs that guide vertebrate heart valve formation, we dissected the molecular cause of the zebrafish heart valve mutant bng and found that endocardial PKD2 plays an essential role during AV valve development by regulating HDAC5-KLF signaling.

Valvulogenesis in zebrafish proceeds in multiple steps, including the differentiation of AV endocardial cells, their migration into the extracellular matrix, the development of endocardial cushions by EMT of the activated AV endocardial cells, and finally the remodeling of the endocardial cushions into mature valve leaflets. With the use of forward and reverse genetics strategies in zebrafish and mice, various signaling pathways such as Wnt/β-catenin, Notch,\(^6\) Nuclear factor of activated T cells,\(^31\) T-box,\(^32\) and BMP signaling\(^33\) were unraveled to guide heart valve formation. Interestingly, expression of bnp4 in the AV myocardium is unaltered in bng mutant zebrafish. In mice, myocardial BMP4 signaling mediates AV septation and valvulogenesis.\(^34\) Defective BMP signaling by ablation of BMP receptor 1a in the endocardium leads to loss of endocardial cushion formation at the AV border.\(^35\) Additionally, mice lacking the BMP inhibitor Smad6 develop hyperplastic endocardial cushions.\(^35\) Aberrant BMP signals lead to the downregulation of several pathways important for EMT in AV endocardial cushions such as Transforming growth factor-β and Notch1.\(^36\) Notch1 signals within AV endocardial cells induce the expression of Snail, a transcription factor that directly suppresses the expression of VE-cadherin and promotes EMT.\(^6\) Remarkably, endocardial notch1b is misexpressed in bng mutants, suggesting an AV endocardium-specific defect. Furthermore, spp1, a marker of activated and migrating endocardial cells,\(^22\) is completely absent in bng mutant hearts, implying that the onset of EMT is already blocked in bng mutant hearts.

We find zPKD2 to specifically localize to endocardial cells of the AVC, the cells that undergo EMT and finally form AV valves. In vertebrates, the PKD family of serine/threonine kinases comprises 3 members (PKD1, PKD2, and PKD3) that share similar domains, a C-terminal catalytic/kinase domain, 2 cysteine-rich domains, and 1 pleckstrin homology domain, whereas the 2 latter are known to be important for the regulation of PKD kinase activity and for its subcellular localization.\(^10\) We show here that the bng mutation (Y849N) resides within the kinase domain of zebrafish PKD2. As a result, PKD2\(^{Y849N}\) kinase activity is severely diminished, whereas its subcellular localization is completely unaffected by the bng mutation.

The PKDs are usually activated either directly via phorbol esters or indirectly by distinct mechanisms, including G protein–coupled receptors,\(^36\) resulting in the translocation of PKD into the cell nucleus.\(^38\) It has recently been shown that nuclear PKD phosphorylates class II HDACs (HDAC4, HDAC5, HDAC7, and HDAC9)\(^{40,41}\) which are known repressors of transcriptional processes such as Myocyte enhancer factor 2 (MEF2)–mediated transcription.\(^{40,41}\) We demonstrate here that bng mutant PKD2\(^{Y849N}\) is unable to phosphorylate HDAC5. Usually, phosphorylation of HDACs promotes 14–3-3 binding, their subsequent nuclear export, and thus their inactivation.\(^42\) Indeed, we find that transfection of HDAC5, together with bng mutant PKD2, into HEK293A cells leads to the inhibition of HDAC5 nuclear export and thus its func-

![Figure 6](image-url)

**Figure 6.** The Notch target genes hey1, hey2, and heyL are severely downregulated in bng. A and B, Whole-mount antisense RNA in situ hybridizations demonstrate that hey2 is highly expressed in the atrioventricular canal (AVC) in wild-type (wt) zebrafish embryos (A), whereas hey2 expression is completely absent in the AVC of bng mutant hearts (B). C, Quantitative real-time polymerase chain reaction of the Notch target genes hey1, hey2, and heyL demonstrates that their transcripts are strongly decreased in bng mutant hearts (relative expression of hey1=0.09±0.13, P<0.05; hey2=0.09±0.09, P<0.05; heyL=0.15±0.16, P<0.05; n=4).
tional activation in the nucleus. Additionally, injection of CA-HDAC5 into heterozygous bng embryos results in severe heart valve anomalies, whereas knockdown of HDAC5 in homozygous bng zebrafish can rescue the bng mutant phenotype. These findings substantiate our hypothesis that PKD2-HDAC5 signaling controls valvulogenesis in the zebrafish embryo.

Recent studies revealed an essential role of PKD-HDAC-mediated signaling in response to pathological cardiac stress stimuli.15,43 Mice with cardiomyocyte-specific deletion of PKD1 show decreased hypertrophy and cardiac fibrosis after transverse aortic constriction.15 Nullizygous HDAC5 mice develop cardiac hypertrophy with age, and the heart becomes severely enlarged in response to constitutive Calcineurin activation or pressure overload.44 However, until now, the exact role of PKD2 in the vertebrate heart has not been elucidated. Our studies demonstrate for the first time an essential role of PKD2-HDAC5 signaling within AV endocardial cells.

In cardiac and skeletal muscle cells, MEF2 transcription factor activity is negatively regulated by HDAC5.45 In addition, HDAC5-dependent regulation of MEF2 transcriptional activity plays an important role in endothelial cells where regular KLF expression crucially depends on HDAC5-MEF2 signaling.46,47 Consistent with an increased nuclear HDAC5 activity in bng mutants, expression of klf2a and klf4a, 2 transcription factors usually expressed in AV endocardial cells,16,17,48 is found to be abrogated in bng mutant hearts. Interestingly, klf2a is known to regulate Notch expression in endothelial cells and to be essential for heart valve formation in zebrafish,16 implying that loss of Notch expression in bng AV endocardium and impaired heart valve formation might be a direct consequence of impaired PKD2-HDAC5-KLF signaling.

In mammals, the best-known Notch effector molecules are members of the basic Hes family and the related Hey transcription factor family. We found that expression of Hey1, Hey2, and HeyL is reduced in bng mutant embryos. Interestingly, it was recently shown that reduced Hey gene expression in endocardial cells leads to impaired EMT of endocardial cells and thus defective cardiac valve formation.25–27

The recent discovery that somatic cells can be first reprogrammed back to pluripotent stem cells and then driven by defined transcriptional cocktails to various cell fates5 provides a potential avenue for autologous cardiac tissue engineering. We demonstrate here for the first time that PKD2-HDAC5-KLF signaling plays a crucial role in vertebrate AV valve formation. It will be interesting to evaluate in future studies whether activation of bng/PKD2-HDAC5-KLF signaling by itself is sufficient to initiate EMT and hence heart valve formation in reprogrammed human stem cells.

Acknowledgments
We thank J. Rudloff for excellent technical assistance.

Sources of Funding
This work was supported by the Deutsche Forschungsgemeinschaft Ro2173/2-1, Ro2173/2-2, and Ro2173/3-1; by Bundesministerium für Bildung und Forschung 01GS0108, 01GS0420, 01GS0836, 01GS1104 (NGFNplus) and Insight-DCM; and the European Union (EU-Inheritance).

Disclosures
None.

References

**CLINICAL PERSPECTIVE**

Defective development of the heart valves occurs in 20% to 30% of congenital malformations. However, in most cases, the underlying causes have not been identified. There is increasing evidence that the regulatory mechanisms governing normal valve development also contribute to human valve pathology. In searching for novel molecular signaling pathways that orchestrate vertebrate heart valve development, we isolated the molecular cause of the ethylisourea (ENU)-induced recessive embryonic-lethal zebrafish mutant *bungee* (*bng*), which shows defective endocardial cushion development and subsequently impaired heart valve formation. We found that the *bng* mutation selectively impairs Protein kinase D2 kinase activity, which leads to reduced Histone deacetylase 5 phosphorylation, nuclear export, and inactivation. As a result of enhanced Histone deacetylase 5 repressor activity, Notch signaling is severely impaired in *bungee* mutant embryos. Accordingly, expression of the well-known Notch target genes Hey1, Hey2, and HeyL is decreased in *bng* mutant embryos. Hence, it will be interesting to evaluate in future studies whether mutations in components of this novel signaling pathway such as Protein kinase D2, Histone deacetylase 5, Krüppel-like factor, Notch1, and members of the Hey family are also involved in human congenital heart disease, especially those that arise from defective endocardial cushion development such as septal and valvular defects. Furthermore, our findings might affect future strategies aiming to engineer human heart valve tissue for improved therapeutics or replacement strategies.
Protein Kinase D2 Controls Cardiac Valve Formation in Zebrafish by Regulating Histone Deacetylase 5 Activity


_Circulation_. published online July 5, 2011;
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Zebralsh strains

Care and breeding of zebrafish *Danio rerio* was as described ¹. The present study was performed after securing appropriate institutional approvals. It conforms to the Guide for the Care and Use of Laboratory Animals published by the “US National Institute of Health” (NIH Publication No. 85-23, revised 1996). Pictures and movies were recorded 72 hours post fertilization. To inhibit pigmentation, 0.003 % 1-phenyl-2-thiourea was added to the embryo medium ².

Injection procedures

Morpholino-modified oligonucleotides were directed against the splice donor site of exon 16 of zebrafish *pkd2* (MO-*zpkd2* = 5’- TGGTGCTAAGTGGTTGTACCGTCAG-3’) and the splice donor site of exon 5 of zebrafish *hdac5* (MO-*hdac5* = 5’-CACACTTGAAGTCTACTCACCGGT-3’). A standard control oligonucleotide (MO-*control*) (GENETOOLS, LLC) was injected at the same concentration. Sense-capped RNA was synthesized using the mMESSAGE mMASCHINE system (Ambion) from pCS2zpkd2wt, pCS2zpkd2Y849N, pCS2zpkd2D738A, pCS2zpkd2S749/753E, pCS2mpkd2wt and pCS2hHDAC5. RNA was microinjected into one-cell stage embryos. Siblings from the same pool were injected using 0.2 M KCl as control.

Antisense RNA in situ hybridization

Whole-mount antisense RNA in situ hybridization was carried out essentially as described ¹ using digoxigenin labelled antisense probes for *pkd2*, *cmlc1*, *cmlc2*, *vmhc*, *amhc*, *nexilin*, *anf*, *tpm4*, *tnnt*, *bmp4*, *notch1b*, *spp1*, *klf2a*, *klf4a*, and *hey2*. 
Expression and purification of recombinant PKD2

Constitutive active \( zpkd2^{S749/753E} \) and kinase deficient \( zpkd2^{D738A} \) was generated by PCR mutagenesis of \( zpkd2 \). For expression of zebrafish GST fusion proteins, constitutive active wild-type \( zpkd2^{S749/753E} \), constitutive active bng mutant \( zpkd2^{S749/753E,Y849N} \) and kinase-deficient \( zpkd2^{D738A} \) were subcloned into pDEST24 (Invitrogen). GST-zPKD2 fusion proteins were expressed in \( E. coli \) BL21-AI by inducing bacteria with 0.1 % Arabinose over night at 30°C. Bacteria were harvested by centrifugation and resuspended in lysis buffer (150 mM NaCl, 50 mM Tris/HCl at pH 7.4, 5 mM EDTA at pH 7.4, 1% Triton X-100, and protease inhibitors (Roche)). Bacterial cells were disrupted by sonification, and cell debris were removed by centrifugation (12,000 rpm, 30 min, 4°C). GST fusion proteins were purified with Glutathione-Sepharose matrix (Amersham) and eluted with 0.03 M reduced Glutathione.

**In vitro kinase assay**

To test zPKD2 kinase activity, zPKD2 was incubated with HDAC5-GST fusion protein. Incubations were carried out with 10 µCi of \([^{32}P]-\gamma\text{ATP}\) at 30°C for 10 min in kinase buffer containing 20 mM HEPES, pH 7.4, 2.5 mM MgAc, 0.03% Triton X-100, and 0.1 mM ATP. Kinase reaction was terminated by SDS-sample buffer. Phosphorylated proteins were separated by SDS-PAGE and and \(^{32}P\) autoradiography was performed.

**Co-immunoprecipitation and immunoblotting**

HEK293A cells were harvested two days after transfection in lysis buffer (50 mM Tris at pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100, protease inhibitors (Roche), and 1 mM PMSF). The cells were disrupted by passage through a 25-gauge needle, and cell debris were removed by centrifugation. FLAG-tagged proteins were immunoprecipitated with an M2-agarose conjugate (Sigma) and thoroughly washed with lysis buffer. Bound proteins were
separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and immunoblotted with either an anti-V5 (1:5000, Invitrogen) or anti-Flag (1:1000, Sigma) antibody.

**Phospho-Histone H3 staining and BrdU labeling**

Whole-mount anti-Phospho-Histone H3 antibody staining was performed as described[^3]. Embryos were mounted in a tissue culture dish with glass bottom with 3 % methylcellulose and confocal analysis was performed on a Nikon A1R.

Embryos were incubated in 10 mM BrdU/E3 for 20 min on ice. Embryos were washed 3 times with E3 and incubated in E3 for 2 h at 28.5 °C. They were fixed in Dent’s fix (80 % methanol : 20 % dimethyl sulfoxide) over night at room temperature (RT), bleached in Dent’s bleach (70 % methanol : 20 % dimethyl sulfoxide : 10 % H₂O₂) over night at RT and stored for 1 h in 100 % methanol at – 20 °C. Embryos were rehydrated to PBT and permeabilized with 10 μg/ml Proteinase K (Roche). After a few washes in PBT embryos were refixed in 4 % PFA (20 min, RT) and additionally washed in H₂O. Embryos were incubated in 2 N HCl 1 h and washed with PBT. Embryos were blocked in 1.5 % FCS/PBDT for 30 min and 10 min in incubation buffer (Roche BrdU Labeling and Detection Kit). They were incubated with anti-BrdU antibody 1:10 (Roche BrdU Labeling and Detection Kit) for 2 h at RT and washed 4 times (15 min each) in 1,5 % FCS/PBDT before the addition of anti-mouse HRP secondary antibody 1:100 (Abcam) at 4 °C over night. After a few washes in 1,5 % FCS/PBDT the BrdU positive cells were DAB stained (Sigma). Embryos were sectioned and slightly HE stained.

**Statistical analyses**

Unpaired two tailed students’ t-test was performed to assess statistical significance of biological replicates of the quantitative data. For the qRT-PCR data t-test was performed to
the delta Ct values. For each replicate, pooled samples (heart specific analyses: pools of 100 embryonic hearts, whole embryo analyses: pools of 50 zebrafish embryos) were analyzed. Shapiro test was applied and significance values were corrected by Benjamini-Hochberg in order to assess approximate normal distribution for the real-time PCR data. For multi comparison scenarios (Fig. 3 A and 4 D), 1-way ANOVA using Tukey-Kramer post hoc test was applied. In general, an alpha level of 0.05 was set to accept statistical significance.
BrdU positive cells

<table>
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<td><strong>wt</strong></td>
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<td><strong>bng</strong></td>
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**G**

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(A) eye, pectoral fin (wt)
(B) somites (wt)
(C) otoliths (wt)
(D) eye, pectoral fin (bng)
(E) somites (bng)
(F) otoliths (bng)

(G) wt, tg(fli1a:GFP)
(H) Se, DA, PCV (wt)
(I) noto, DA, PCV (wt)

(J) bng, tg(fli1a:GFP)
(K) Se, DA, PCV (bng)

(M) relative expression

\[ \begin{align*}
\text{fli1a} & : 0.8 \\
\text{tie2} & : 0.8
\end{align*} \]

ns
**Figure S1:** Early cardiac development proceeds normally in *bng* mutant embryos. (A, E) Hematoxylin/Eosin staining of sagittal histological sections of wt (A) and *bng* mutant (E) hearts at 36 hpf. Similar to the wild-type situation (A), *bng* mutant hearts (E) consist of normally developed endo- and myocardial cell layers. Similar to wild-types (F, G), *bng* mutant embryos (B, C) show a normal distribution of *mlc1* RNA at 24 and 48 hpf as revealed by whole-mount antisense RNA *in situ* hybridization. The heart regularly jogs to the left and loops to the right in wild-type (B, C) and *bng* mutant (F, G) zebrafish embryos. A, atrium; V, ventricle; my, myocardium; en, endocardium; R, right; L, left.

**Figure S2:** Differentiation and specification of cardiomyocytes proceeds normally in *bng*. (A-L, O, P) Whole-mount antisense RNA *in situ* hybridisation analyses show that the expression patterns of *cmlc2* (A, B), *anf* (C, D), *vmhc* (E, F), *tpm4* (G, H), *amhc* (I, J), *nexn* (K, L) and *tnnt* (O, P) in *bng* mutants are indistinguishable from the wild-type at 72 hpf. (M, N) Double immunofluorescent staining with antibodies directed against atrial and ventricular meromyosin (MF20, red) and against the atrial-specific isoform of myosin heavy chain (S46, green). Similar to wild-types, cardiac myosin heavy chain proteins are expressed in the normal chamber-specific manner in *bng* embryos at 72 hpf.

**Figure S3:** Proliferation in *bng* mutant hearts is not affected by the *bng* mutations. (A, B) Confocal images of transgenic tg(*mlc2:rasGFP/ flk1:rasCherry*) wt (A) and *bng* mutant (B) embryonic hearts. Similar to the wild-type situation (A), myo- and endocardial cell layers are normally developed in *bng* mutant hearts (B). By contrast, endocardial cushions are completely absent in *bng* mutant hearts. Phospho-Histone H3 staining (green) (C, E) and BrdU labelling (D, F) reveal unaltered proliferation in *bng* (E, F) compared to wt (C, D) hearts at 72 hpf. Cell nuclei are counterstained with propidium iodide (red) (C, E). (D, F) In addition, BrdU positive AV endocardial cells are present in wt (D) and *bng* mutant (F) hearts.
(G) The number of BrdU positive cells is unaffected in bng mutant compared to wt hearts at 48 hpf and 72 hpf (n = 10, P>0.05, ns).

Figure S4: Development of the eyes, pectoral fins, somites and the vasculature appears unaffected in bng mutant embryos. (A, D) Dorsal view of wt (A) and bng (D) mutant embryos at 72 hpf. Eyes and pectoral fins are regularly formed in bng mutants (D) compared to wild-type littermates (A). (B, C, E, F) Unaltered structure of somites (B, E) and otoliths (C, F) in bng mutant embryos (E, F) in comparison to wild-types (B, C) at 72 hpf. (G, H, J, K) Vasculogenesis and angiogenesis proceeds normally in wt (G, H) and bng mutant (J, K) embryos at 24 hpf (G, J) and 72 hpf (H, K) as revealed by the analysis of the transgenic fli1a:GFP zebrafish line (tg(fli1a:GFP)). (I, L) Transversal sections of wt (I) and bng mutant (L) embryos at the mid-trunk region reveals regular development of the dorsal aorta (DA) and the posterior cardinal vein (PCV) in bng mutant embryos at 72 hpf. (M) Quantitative real-time PCR of fli1a and tie2 demonstrates that their transcripts are not affected in bng mutant hearts (relative expression of fli1a=0.91 ± 0.12, P>0.05, ns and tie2=0.87 ± 0.39, P>0.05, ns; n=3, respectively). DA, dorsal aorta; PCV, posterior cardinal vein; Se, intersegmental vessels; noto, notochord.

Figure S5: Amino acid sequence alignment of zebrafish (zPKD2) and human (hPKD2) PKD2 demonstrates high amino acid identity cross-species. Black boxes indicate similar amino acids and grey boxes indicate amino acids with similar chemical properties. Functional domains, such as two cysteine-rich domains (C1a and C1b, red), a Pleckstrin homology domain (PH, blue) and a C-terminal serine/threonine kinase domain (green) are indicated with bars above the alignment. The bng mutation PKD2Y849N resides within the kinase domain of PKD2 (red arrow).
References


**Movies S1 and S2:** Contracting heart of a wild-type (Movie S1) and a *bng* mutant (Movie S2) zebrafish embryo at 36 hpf. In wild-type and *bng* mutant zebrafish embryos both cardiac chambers vigorously contract with a regular rhythm. Lateral view, head to the left, ventral side downwards.

**Movies S3 and S4:** Contracting heart of a wild-type (Movie S3) and a *bng* mutant (Movie S4) zebrafish embryo at 72 hpf. In wild-type and *bng* mutant zebrafish embryos both cardiac chambers vigorously contract with a regular rhythm and sequentially, first the atrium (A) and then the ventricle (V). In contrast to wild-types (Movie S3) where the endocardial cushions prevent retrograde blood flow, blood regurgitates between the atrium and the ventricle of *bng* mutant hearts (Movie S4) due to abolished endocardial cushion formation. Lateral view, head to the left, ventral side downwards.

**Movie S5:** Inhibition of PKD2 gene function by injection of target-specific Morpholino-modified antisense oligonucleotides (MO-**pkd2**). Similar to *bng* mutant zebrafish embryos, *pkd2* morphant embryos show severe blood regurgitation between the atrium and the ventricle. Lateral view, head to the left, ventral side downwards.

**Movie S6:** CA-hHDAC5 was ectopically expressed in heterozygous *bng* zebrafish embryos by injection of CA-hHDAC5 mRNA. After 72 hpf, injected heterozygous embryos exhibit the *bng* mutant phenotype.