Nucleoside Diphosphate Kinase-C Suppresses cAMP Formation in Human Heart Failure

BACKGROUND: Chronic heart failure (HF) is associated with altered signal transduction via β-adrenoceptors and G proteins and with reduced cAMP formation. Nucleoside diphosphate kinases (NDPKs) are enriched at the plasma membrane of patients with end-stage HF, but the functional consequences of this are largely unknown, particularly for NDPK-C. Here, we investigated the potential role of NDPK-C in cardiac cAMP formation and contractility.

METHODS: Real-time polymerase chain reaction, (far) Western blot, immunoprecipitation, and immunocytochemistry were used to study the expression, interaction with G proteins, and localization of NDPKs. cAMP levels were determined with immunoassays or fluorescent resonance energy transfer, and contractility was determined in cardiomyocytes (cell shortening) and in vivo (fractional shortening).

RESULTS: NDPK-C was essential for the formation of an NDPK-B/G protein complex. Protein and mRNA levels of NDPK-C were upregulated in end-stage human HF, in rats after long-term isoprenaline stimulation through osmotic minipumps, and after incubation of rat neonatal cardiomyocytes with isoprenaline. Isoprenaline also promoted translocation of NDPK-C to the plasma membrane. Overexpression of NDPK-C in cardiomyocytes increased cAMP levels and sensitized cardiomyocytes to isoprenaline-induced augmentation of contractility, whereas NDPK-C knockdown decreased cAMP levels. In vivo, depletion of NDPK-C in zebrafish embryos caused cardiac edema and ventricular dysfunction. NDPK-B knockout mice had unaltered NDPK-C expression but showed contractile dysfunction and exacerbated cardiac remodeling during long-term isoprenaline stimulation. In human end-stage HF, the complex formation between NDPK-C and $G\alpha_{i2}$ was increased whereas the NDPK-C/$G\alpha_s$ interaction was decreased, producing a switch that may contribute to an NDPK-C–dependent cAMP reduction in HF.

CONCLUSIONS: Our findings identify NDPK-C as an essential requirement for both the interaction between NDPK isoforms and between NDPK isoforms and G proteins. NDPK-C is a novel critical regulator of β-adrenoceptor/cAMP signaling and cardiac contractility. By switching from $G\alpha_s$ to $G\alpha_{i2}$ activation, NDPK-C may contribute to lower cAMP levels and the related contractile dysfunction in HF.

Issam H. Abu-Taha, PhD*
Jordi Heijman, PhD*
Hans-Jörg Hippe, MD*
Nadine M. Wolf, PhD
Ali El-Armouche, MD
Viacheslav O. Nikolaev, PhD
Marina Schäfer, MSc
Christina M. Würtz, PhD
Stefan Neef, MD
Niels Voigt, MD
István Baczkó, MD, PhD
András Varró, MD, PhD, DSc
Marion Müller, MSc
Benjamin Meder, MD
Hugo A. Katus, MD
Katharina Spiger, PhD
Christiane Vettel, PhD
Lorenz H. Lehmann, MD
Johannes Backs, MD
Edward Y. Skolnik, MD
Susanne Lutz, PhD
Dobromir Dobrev, MD†
Thomas Wieland, PhD†

*Drs Abu-Taha, Heijman, and Hippe contributed equally.

†Drs Dobrev and Wieland share senior authorship.

Correspondence to: Thomas Wieland, PhD, Maybachstrasse14, 68169 Mannheim, Germany or Dobromir Dobrev, MD, Hufelandstrasse 55, 45122 Essen, Germany. E-mail thomas.wieland@medma.uni-heidelberg.de or dobromir.dobrev@uk-essen.de

Sources of Funding, see page 895

Key Words: heart failure
- myocardial contraction
- receptors, adrenergic, beta
- signal transduction

Clinical Perspective

What Is New?

- We show for the first time that the nucleoside diphosphate kinase (NDPK)-C is required and indispensable for the interaction of NDPKs with both stimulatory G_s and inhibitory G_i proteins.
- NDPK-C–mediated targeting of NDPKs to the plasma membrane is increased in response to β-adrenoceptor stimulation and enhances intracellular cAMP levels, cardiomyocyte contractility, and in vivo cardiac function.
- We provide novel mechanistic insights into remodeling of β-adrenoceptor signaling in heart failure (HF), showing that the HF-related increase in NDPK-C expression may cause constitutive G_i-mediated inhibition of adenylyl cyclases, providing a plausible explanation for the lower cAMP levels in HF.

What Are the Clinical Implications?

- The increased NDPK-C membrane content in human HF could potentially counteract a fading β-adrenoceptor response in the early stages of HF by increasing the amount of $G\alpha_s$ proteins in the plasma membrane. However, by switching to $G\alpha_{i2}$ activation, NDPK-C may play a role in HF progression by reducing cAMP levels, typical for end-stage human HF.
- A better understanding of the molecular processes underlying altered G-protein signaling in HF may help to develop new HF therapies. We identify NDPK-C as a novel therapeutic target involved in the regulation of aberrant G-protein signaling and cardiac contractility in HF.

Heart failure (HF) is a common cause of death and disability.[1] Altered signal transduction via β-adrenoceptors (βARs) and G proteins is a hallmark of chronic HF and contributes to impaired cardiac contractility.[2,3] The exact molecular pathophysiological mechanisms contributing to contractile dysfunction in patients with HF are incompletely understood, and a better understanding of these processes is expected to foster the development of improved treatment options for patients with HF.[3]

The plasma-membrane content of nucleoside diphosphate kinases (NDPKs) is increased in patients with end-stage HF,[4] pointing to a potential role for NDPKs in HF. NDPKs represent a family of multifunctional proteins encoded by 10 human *nm23* genes, of which the class I subfamily (consisting of NDPK-A, -B, -C, and -D) exerts enzymatic activity.[5] NDPKs form heterohexamers and catalyze the transfer of γ-phosphate between nucleotide triphosphates and nucleotide diphosphates.[6,7] NDPK-A and NDPK-B play a role in numerous cellular processes, often as part of larger signaling complexes. For example, NDPK-B, but not NDPK-A, forms complexes with Gβγ dimers and acts as a protein histidine kinase that can activate cardiac G proteins in a receptor-independent manner.[8–10] Nevertheless, our previous studies indicated that the complex between NDPK-B and G proteins cannot be reconstituted in vitro, suggesting that NDPK-B alone is insufficient to regulate G-protein signaling and that an as-yet unidentified key cofactor is required for the complex formation of NDPK-B and G proteins at the plasma membrane.[10]

NDPK-C exerts enzymatic activity and is able to form heterohexamers with NDPK-A and NDPK-B.[11] NDPK-C shares 72% homology with NDPK-A and NDPK-B but has an additional hydrophobic N-terminal domain (Figure I in the online-only Data Supplement), which could serve as a membrane anchor.[12,13] NDPK-C is generally less abundantly expressed than the major isoforms NDPK-A and NDPK-B[11] but is highly enriched at the cardiac plasma membrane of patients with HF.[14] Thus, NDPK-C might be the limiting factor targeting NDPK hexamers to membranous G proteins and could be the most relevant NDPK isoform for cAMP regulation in the heart and for the progression of HF. However, the function of NDPK-C in the heart is unknown.

To investigate the role of NDPK-C in HF, we performed biochemical studies of NDPK-C and G-protein signaling in human and rat tissue samples, assessed the functional impact of NDPK-C on cAMP levels and cardiac contractility in isolated rat cardiomyocytes, and determined the in vivo effects of NDPKs on contractility in zebrafish and mice. We identify NDPK-C as the critical isoform for the regulation of G-protein function and cAMP levels in the heart, with important consequences for cardiac contractility. Our results show that NDPK-C can interact promiscuously with both $G\alpha_s$ and $G\alpha_i$ proteins. The switch to $G\alpha_{i2}$-dominant regulation of G proteins by NDPK-C in human HF may contribute to the lower cAMP levels and impaired contractility characteristic of this clinical condition.

METHODS

A detailed overview of all methods is provided in the online-only Data Supplement. Key aspects are summarized below.

Tissue Procurement

Nonfailing human myocardium from the free wall of the left ventricle was obtained from organ donors with no apparent heart disease and normal left ventricular function (determined by echocardiography) for whom no suitable heart transplant recipients had been identified. Left ventricular myocardial samples of failing human hearts were obtained from patients with HF (New York Heart Association class III–IV) who underwent cardiac transplantation. The experimental protocol was approved by the ethics review board of the University of Szeged Medical Center. Informed written consent was obtained for the use of nondiseased human hearts. All procedures conformed to the Helsinki Declaration of the World Medical Association.

Circulation. 2017;135:881–897. DOI: 10.1161/CIRCULATIONAHA.116.022852

Rat and Mouse Models of Isoprenaline Stimulation and Isolation of Rat Cardiomyocytes

Male Wistar rats received 4 days of either isoprenaline or vehicle administered via osmotic minipumps, as previously described.[15] Male C57Bl/6 wild-type and NDPK-B knockout mice received the same treatment but for 7 days. All animals were anesthetized with 2% isoflurane inhalation and received carprofen (5 μg/kg SC) as analgesic. All procedures concerning the care and use of animals were in accordance with institutional guidelines (Az. G-12\10 Regierungspräsidium Karlsruhe, Germany, and Az. G10\65 LAVES Niedersachsen, Germany).

Generation of Recombinant Adenoviruses

Recombinant adenoviruses were generated as previously described.[8,16–18]

mRNA Analysis, Transfection and Transduction, Membrane Fractionation, Western Blots, Immunoprecipitation, Far Western Blotting, and ATP/GTP Hydrolysis Assays

Details are provided in the online-only Data Supplement. The primers that were used are listed in Table I in the online-only Data Supplement.

Visualization of the Subcellular Localization of NDPK-C

Neonatal rat cardiomyocytes (NRCMs) and adult rat cardiomyocytes (ARCMs) were cultured with serum-free medium on coverslips and infected with Ad-Flag–NDPK-C. Twenty-four hours later, cells were stimulated with solvent or isoprenaline for up to 6 hours. The subcellular localization of NDPK-C was visualized by confocal fluorescence microscopy.

Measurement of Intracellular cAMP

cAMP levels in NRCM or zebrafish lysates were assayed with a cAMP immunoassay.[8,9,19] For cAMP assays in living cells, ARCMs were isolated and transduced with Epac2-camps adenovirus, as previously described,[18] together with a control (LacZ) or NDPK-C–encoding adenovirus. Cells were stimulated with isoprenaline, forskolin, and 3-isobutyl-1-methylxanthine.

Fractional Shortening in ARCMs

ARCMs were infected with an adenovirus encoding enhanced green fluorescent protein (Ad-EGFP) or Ad-Flag–NDPK-C. Sarcomere shortening was assessed during field stimulation with a video-based sarcomere-length detection system at 1.25 mmol/L Ca^{2+} in the bath solution.[20]

Zebrafish Maintenance, Morpholino Injection, Measurement of Fractional Shortening, and Histology

Zebrafish *danio rerio* were maintained under standard conditions.[21] Embryos at a 1-cell stage were injected with the indicated amounts of the NDPK-B or NDPK-C targeting morpholino or a standard control oligonucleotide at the same concentration. Injections and analyses of fractional shortening, ventricular diameters, immunohistochemistry, and electron microscopy were performed as previously described.[19,21]

Statistics

Results are presented as mean±SEM. Normality was assessed with the D'Agostino and Pearson omnibus test. The Mann-Whitney test (for nonnormally distributed data or data with too few points to assess normality), an unpaired 2-tailed Student *t* test, or 1- or 2-way ANOVA followed by a post hoc Bonferroni or the Tukey test was used to compare means between groups. Values of $P<0.05$ were considered statistically significant.

RESULTS

Interactions Among NDPK-C, NDPK-B, and G Proteins

NDPK-C forms hetero-oligomers with NDPK-A and NDPK-B.[11] Moreover, NDPK-B/Gβγ complexes regulate G-protein activity and membrane content in cardiomyocytes.[8,19,22] Therefore, we assessed whether NDPK-C is involved in the complex formation of NDPKs with G proteins using far Western blotting.[10] When the purified heterotrimeric G protein transducin ($G_t\alpha\beta\gamma$)[10] was spotted on cellulose membranes and incubated with lysates of control NRCMs or NRCMs with adenovirus-mediated overexpression of NDPK-B, no direct interaction occurred (Figure 1A). In contrast, purified $G_t\alpha\beta\gamma$ showed a strong direct interaction with exogenous Flag–NDPK-C, detected with an anti-Flag antibody, in Flag–NDPK-C overexpressing NRCMs. The putative interaction between NDPK-B and endogenous NDPK-C was confirmed with recombinant NDPK-B spotted on cellulose membranes and incubated with NRCM lysates. Adenovirus-mediated overexpression of NDPK-C further increased the interaction between NDPK-B and NDPK-C (Figure 1A). Furthermore, raising the relative NDPK-C content in NRCM lysates with a constant overexpression of NDPK-B (multiplicity of infection, 100) by increasing the multiplicity of infection of the Ad-Flag–NDPK-C virus from 0 to 100 produced a concentration-dependent increase in the binding of NDPK-B to $G_t\alpha\beta\gamma$ (Figure 1B). These data suggest that NDPK-C is essential for the previously detected complex formation between NDPK-B and Gβγ.

ATP/GTP hydrolysis assays were used to analyze the effects of NDPK-B and NDPK-C on the activation of $G_t\alpha\beta\gamma$, as monitored by the GTPase activity of its Gα subunit, which does not hydrolyze ATP. Thus, only in the presence of NDPKs can the radiolabeled phosphate group from [^{32}P]ATP be transferred to GDP, forming [^{32}P]GTP, the appropriate $G_t\alpha$-GTPase substrate. The $G_t\alpha\beta\gamma$ preparation exhibited some ATP hydrolysis capacity, as indicated by the inhibitory effect of the NDPK activity–suppressing uridine 5'-diphosphate (UDP; Figure 1C).[23] This basal activity can be attributed to the NDPKs copurified in bovine $G_t\alpha\beta\gamma$.[10] Addition of purified recombinant NDPK-C,

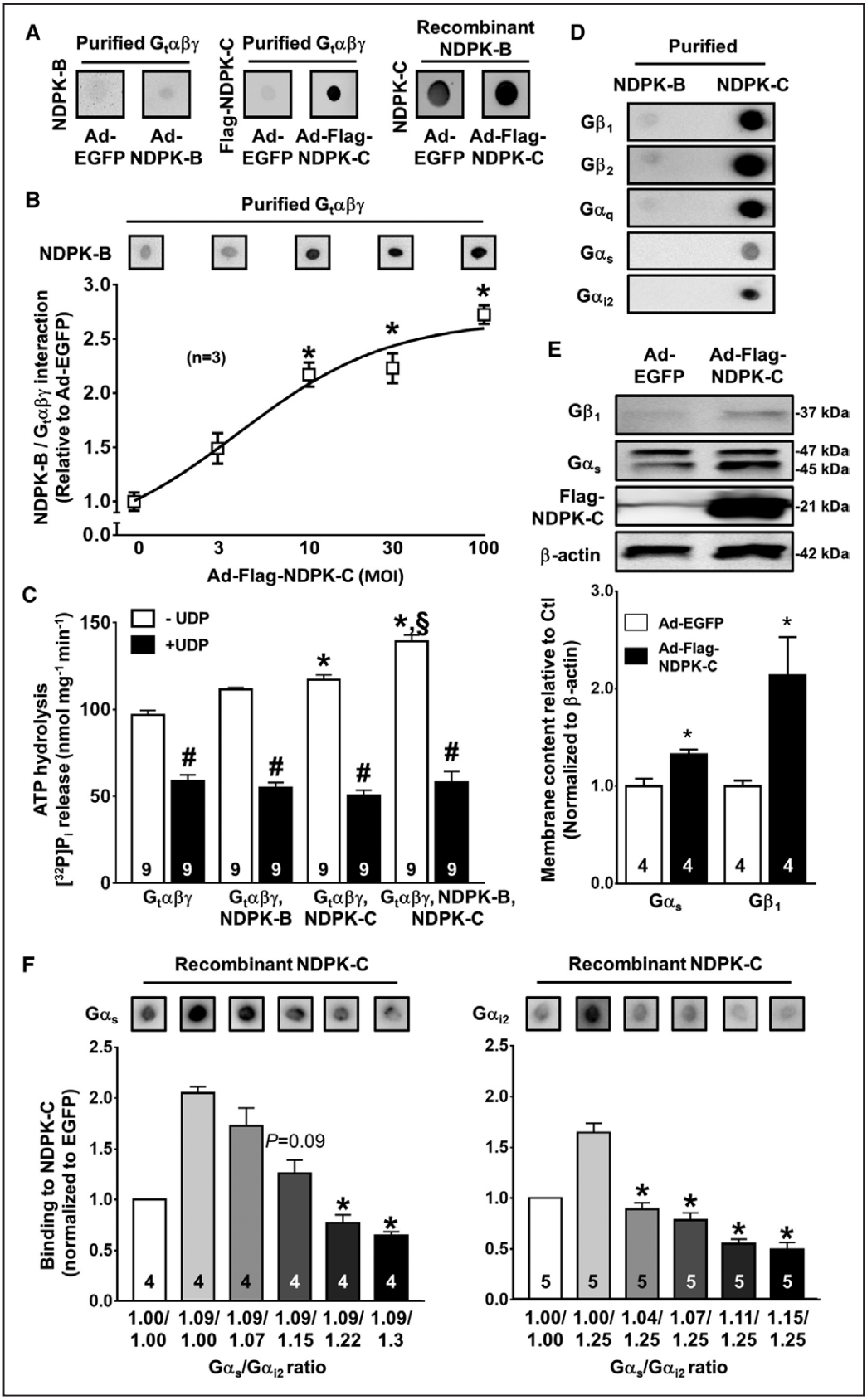

Figure 1. Nucleoside diphosphate kinase (NDPK)-C interacts directly with NDPK-B and G proteins.
A, Representative far Western blots: The purified heterotrimeric G protein transducin ($G_t\alpha\beta\gamma$) was incubated with lysates of control neonatal rat cardiomyocytes (NRCMs) or NRCMs overexpressing NDPK-B (**left**) or flag-tagged NDPK-C (*Continued*)

but not NDPK-B, significantly increased ATP hydrolysis. Combining both NDPKs produced a significant additional increase. All increments in ATP hydrolysis were sensitive to UDP, confirming the involvement of the enzymatic activity of NDPKs.[23] Similar results were obtained when GTP hydrolysis was measured (Figure II in the online-only Data Supplement), supporting an NDPK-C–dependent activation of $G_t\alpha\beta\gamma$.

To investigate the interaction between individual G-protein family members and NDPK-B or NDPK-C, purified NDPK-B and NDPK-C were spotted on cellulose membranes and incubated with lysates of mouse embryonic fibroblasts. Binding of G proteins to these NDPKs was subsequently detected with specific antibodies against $G\beta_1$, $G\beta_2$, $G\alpha_q$, $G\alpha_s$, and $G\alpha_{i2}$. Purified NDPK-B showed no direct interaction with any tested G-protein subunit, whereas NDPK-C strongly interacted with all G-protein subunit compositions tested (Figure 1D). To test whether NDPK-C alone can regulate G proteins, independently of NDPK-B, mouse embryonic fibroblasts of NDPK-A/NDPK-B double-knockout mice were used. Cardiac myocytes of these animals were not available because they die shortly after birth.[24] Adenovirus-mediated overexpression of Flag–NDPK-C in NDPK-A/NDPK-B double-knockout mouse embryonic fibroblasts significantly increased the membrane content of $G\alpha_s$ and $G\beta_1$ (Figure 1E), showing that NDPK-C alone is sufficient to regulate G proteins. We then determined whether $G\alpha_s$ and $G\alpha_{i2}$ compete for binding to NDPK-C. Different amounts of NRCM lysates overexpressing $G\beta_1\gamma_2$, $G\alpha_s$, or $G\alpha_{i2}$ (Figure III in the online-only Data Supplement) were combined to obtain specific ratios of $G\alpha_{i2}$ to $G\alpha_s$, and the interaction between recombinant NDPK-C and $G\alpha_s$ or $G\alpha_{i2}$ proteins was determined with Far Western blots. As expected,[8,9] recombinantly expressed $G\alpha_s$ or $G\alpha_{i2}$ in NRCM lysates bound to NDPK-C in the presence of sufficient amounts of recombinantly expressed $G\beta_1\gamma_2$ (Figure 1F). However, increasing the levels of $G\alpha_{i2}$ with a fixed amount of $G\alpha_s$ produced a ratio-dependent decrease in the amount of $G\alpha_s$ binding to NDPK-C (Figure 1F, left). Likewise, increasing $G\alpha_s$ levels for a fixed $G\alpha_{i2}$ amount produced a ratio-dependent decrease in the amount of $G\alpha_{i2}$ binding to NDPK-C (Figure 1F, right). Together, these data strongly suggest that NDPK-C is essential for both complex formation of NDPKs with G proteins and the resulting G-protein activation. Moreover, $G\alpha_s$ and $G\alpha_{i2}$ proteins directly compete for binding to NDPK-C, and even modest changes in the $G\alpha_{i2}$ to $G\alpha_s$ ratio apparently modulate the interaction between NDPK-C and G proteins.

NDPK-C Levels Increase in Response to βAR Stimulation

Using human tissue samples from a previous study,[14] we found a significantly increased membrane protein content of NDPK-A, NDPK-B, and NDPK-C isoforms in failing versus nonfailing hearts (Figure IV in the online-only Data Supplement), pointing to a role of NDPKs in HF pathophysiology. mRNA levels of NDPK-C, but not NDPK-A and NDPK-B, were significantly increased in HF hearts (Figure IV in the online-only Data Supplement), suggesting a selective transcriptional upregulation of NDPK-C in human HF. Patients with HF show a hyperactive sympathetic nervous system with elevated plasma catecholamine levels and subsequent chronic activation of βARs. Therefore, we asked whether chronic βAR stimulation contributes to the NDPK-C upregulation in HF. Compared with saline-infused controls, chronic in vivo βAR stimulation with isoprenaline in rats induced a significant 5- and 14-fold increase in NDPK-B and NDPK-C mRNA content, respectively (Figure 2A) but enhanced the protein levels of NDPK-C only (Figure 2B). Thus, the increased expression of NDPK-C in patients with HF could directly result from the chronic activation of βARs.

As shown in Figure 2C, NDPK-C and $G\alpha_s$ could be detected in Gβ precipitates from rat ventricular tissue lysates, indicating that NDPK-C interacts with the heterotrimeric G protein. Furthermore, the amount of NDPK-C and $G\alpha_s$ coimmunoprecipitating with Gβ apparently increased after long-term isoprenaline treatment, sug-

Figure 1 Continued. (**middle**). Recombinant NDPK-B was incubated with lysates of control NRCMs (**left**) or NRCMs overexpressing flag-tagged NDPK-C. NDPK-C was detected with an antibody against NDPK-C (**right**). **B**, Representative far Western blot and quantification of the NDPK-B/$G_t\alpha\beta\gamma$ interaction in NRCM lysates infected with Ad-NDPK-B (multiplicity of infection [MOI], 100) and varying combinations of an adenovirus encoding enhanced green fluorescent protein (Ad-EGFP) and Ad-Flag–NDPK-C (total combined MOI, 100). **P*<0.05 vs 100-MOI Ad-EGFP (0-MOI Ad-Flag–NDPK-C). **C**, ATP hydrolysis by $G_t\alpha\beta\gamma$ (1 μmol/L) and $G_t\alpha\beta\gamma$ plus NDPK-B (1 μmol/L), plus NDPK-C (0.25 μmol/L), or plus NDPK-B and NDPK-C determined by the amount of [^{32}P] P_i released in the absence or presence of 500 μmol/L uridine 5′-diphosphate (UDP). **P*<0.05 vs $G_t\alpha\beta\gamma$. #*P*<0.05 vs without UDP. §*P*<0.05 vs $G_t\alpha\beta\gamma$+NDPK-C. **D**, Far Western blot analysis of purified NDPK-B and NDPK-C incubated with cell lysates from mouse embryonic fibroblasts (MEFs). G-protein subunits were subsequently detected with antibodies against $G\beta_1$, $G\beta_2$, $G\alpha_q$, $G\alpha_s$, and $G\alpha_{i2}$. **E**, Representative Western blots and quantification of $G\beta_1$, $G\alpha_s$, Flag–NDPK-C, and β-actin in membrane fractions of NDPK-A/NDPK-B double-knockout MEFs infected with Ad-EGFP or Ad-Flag–NDPK-C. **P*<0.05 vs Ad-EGFP. **F.** Far Western blot analysis of NDPK-C incubated with mixtures of lysates of NRCMs overexpressing $G\beta_1\gamma_2$, $G\alpha_s$, or $G\alpha_{i2}$ to obtain specific ratios of both G proteins (indicated below the corresponding bars). The interaction between NDPK-C and $G\alpha_s$ or $G\alpha_{i2}$ was subsequently quantified (bars). **P*<0.05 vs incubation with lysates from NRCMs overexpressing only $G\alpha_s$ plus $G\beta_1\gamma_2$ (**left**) or $G\alpha_{i2}$ plus $G\beta_1\gamma_2$ (**right,** second bar in each chart).

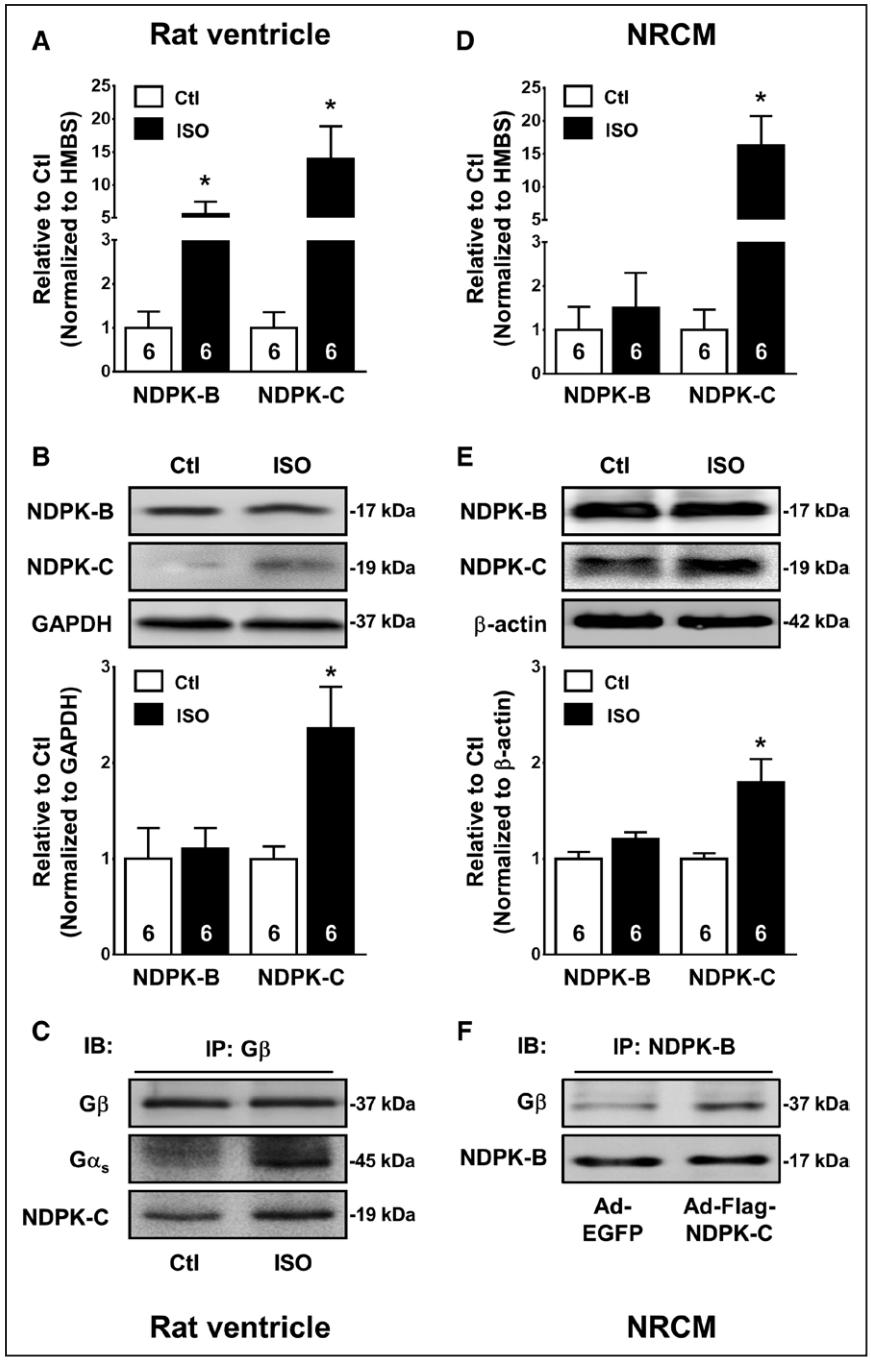

Figure 2. Nucleoside diphosphate kinase (NDPK)-C expression is increased after long-term isoprenaline (ISO) administration. **A** and **B**, NDPK-B and NDPK-C in control rats or rats after 4 days of ISO administration (2.4 mg·kg^{-1}·d^{-1}). **A**, mRNA content determined by quantitative polymerase chain reaction in ventricular samples. Hydroxymethylbilane synthase (HMBS) was used as housekeeping gene for normalization. **B**, Representative Western blots (**top**) and quantification of protein expression (**bottom**) in ventricular lysates. GAPDH was used as loading control. **C**, Immunoprecipitation (IP) of Gβ in ventricular lysates of control rats or rats after 4 days of ISO administration. Coimmunoprecipitated proteins were detected with antibodies against $G\alpha_s$ and NDPK-C. IB indicates immunoblot **D** and **E**, Similar to **A** and **B** for control neonatal rat cardiomyocytes (NRCMs) or NRCMs after 7 days of incubation with ISO (1.0 μmol/L). Data are shown relative to controls (Ctl). Numbers in bars indicate number of samples. *P<0.05 vs corresponding control. **F**, Immunoprecipitation of NDPK-B in lysates from NRCMs infected with an adenovirus encoding enhanced green fluorescent protein (Ad-EGFP) or Ad-Flag–NDPK-C, for 48 hours. Coimmunoprecipitated proteins were detected with antibodies against NDPK-B and Gβ.

gesting that isoprenaline not only increases NDPK-C expression but also may enhance the interaction between NDPK-C and G proteins. To verify that these alterations occur in cardiomyocytes, NRCMs were incubated with isoprenaline or solvent for 7 days in vitro. Consistent with the data obtained in human ventricular tissue samples, both mRNA content and protein expression of NDPK-C were increased by in vitro βAR stimulation, whereas the expression of NDPK-B remained unchanged (Figure 2D and 2E). The interaction between NDPK-B and Gβγ was confirmed in NRCMs and was enhanced by overexpression of NDPK-C (Figure 2F), suggesting that NDPK-C is critical for the interaction between NDPK-B and Gβγ in cardiomyocytes.

Subcellular Localization of NDPK-C After βAR Stimulation

NDPK-C was detected predominantly in the cytosol of unstimulated NRCMs. In vitro stimulation of NRCMs with isoprenaline for 6 hours significantly increased the protein levels of NDPK-C at the plasma membrane, with a membrane/cytosol fluorescence ratio of 2.26±0.22 (n=27) versus 0.83±0.10 (n=28) under control condi-

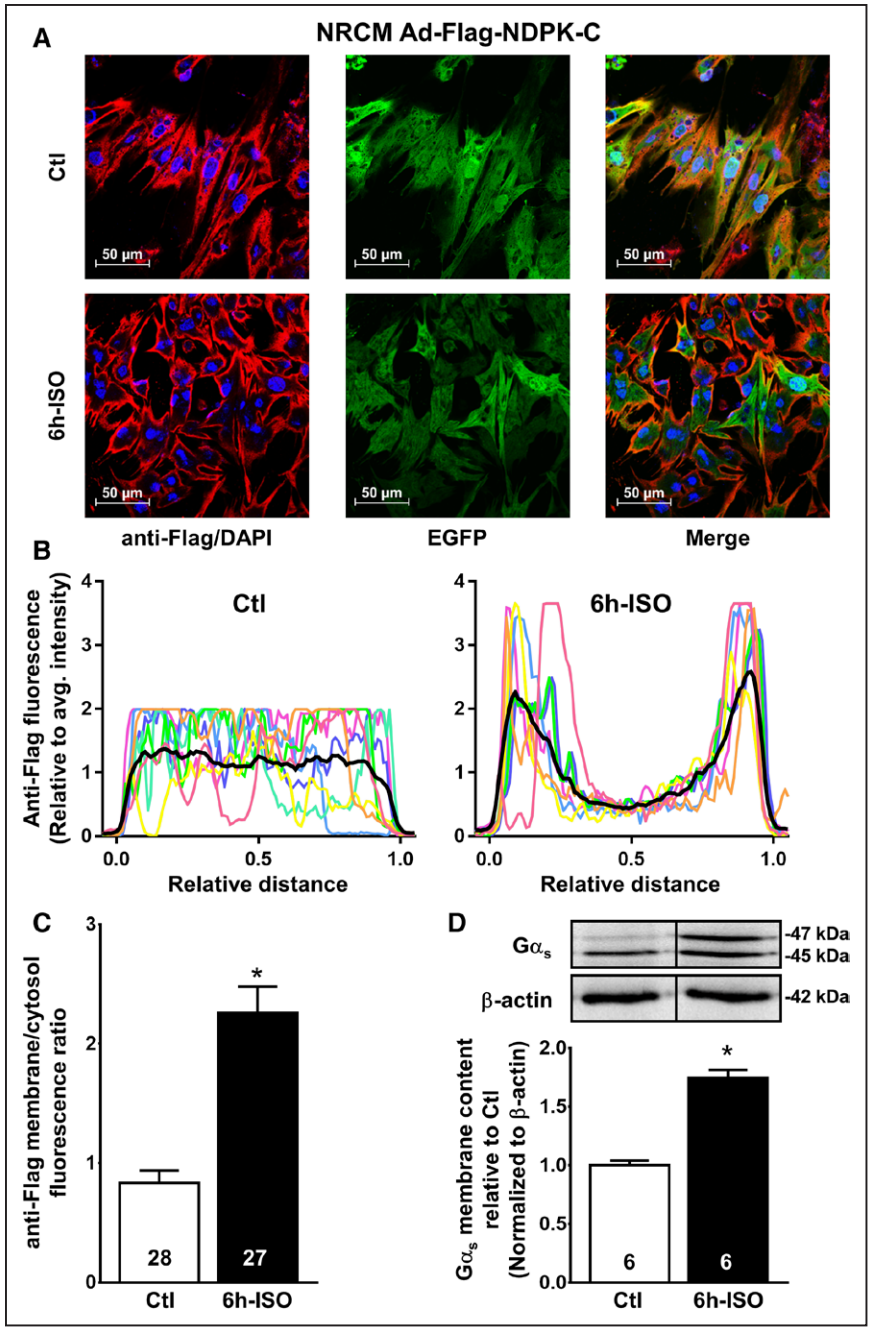

Figure 3. $G\alpha_s$ and nucleoside diphosphate kinase (NDPK)-C membrane content are enhanced after short-term isoprenaline (ISO) administration.
A, Confocal microscopy showing Flag–NDPK-C (red), DAPI (blue), and enhanced green fluorescent protein (EGFP; green) in neonatal rat cardiomyocytes (NRCMs) overexpressing Flag–NDPK-C (multiplicity of infection, 50) under control conditions (Ctl; **top row**) or after 6 hours of stimulation with 1.0 μmol/L ISO (6h-ISO; **bottom row**). **B**, Line profiles (colored lines) of Flag–NDPK-C in 8 representative NRCMs in Ctl (**left**) or 6h-ISO groups (**right**) and average line profiles across all NRCMs (black lines). **C**, Fluorescence intensity in membrane region (first and last 15% of line profile) vs cytosol in the Ctl and 6h-ISO groups. **D**, Representative Western blots of Gβ and $G\alpha_s$ (**top**) and quantification of $G\alpha_s$-protein expression (**bottom**) in membrane fractions from Ctl or 6h-ISO NRCMs. β-Actin was used as loading control. Numbers in bars indicate number of samples. **P*<0.05 vs Ctl.

tions (*P*<0.001; Figure 3A–3C), pointing to translocation of NDPK-C to the plasma membrane by isoprenaline in addition to the transcriptional upregulation of NDPK-C observed after 7 days of isoprenaline treatment. The interaction between NDPK-C and Gβγ determined by coimmunoprecipitation (Figure V in the online-only Data Supplement), as well as the membrane content of $G\alpha_s$ (Figure 3D), were also increased. Taken together, these data support the formation of NDPK-C, NDPK-B, and G-protein complexes, resulting in their enrichment at the plasma membrane after isoprenaline stimulation, and point to a potential role of NDPK-C in the regulation of cellular cAMP synthesis.

NDPK-C Modulates cAMP and Cardiomyocyte Contractility

To study the functional consequences of NDPK-C regulation on cellular cAMP levels, NDPK-C protein levels were reduced by siRNA-mediated knockdown (si–NDPK-C; Figure 4A) or increased by adenovirus-mediated overexpression (Ad-Flag–NDPK-C; Figure 4B). NDPK-C expression was reduced by 34.8±6.4% with si–NDPK-C compared with si-control (Figure 4C), but the use of Ad-Flag–NDPK-C and corresponding anti-Flag antibody precluded quantification of NDPK-C overexpression levels. Modification of NDPK-C protein levels produced parallel changes in

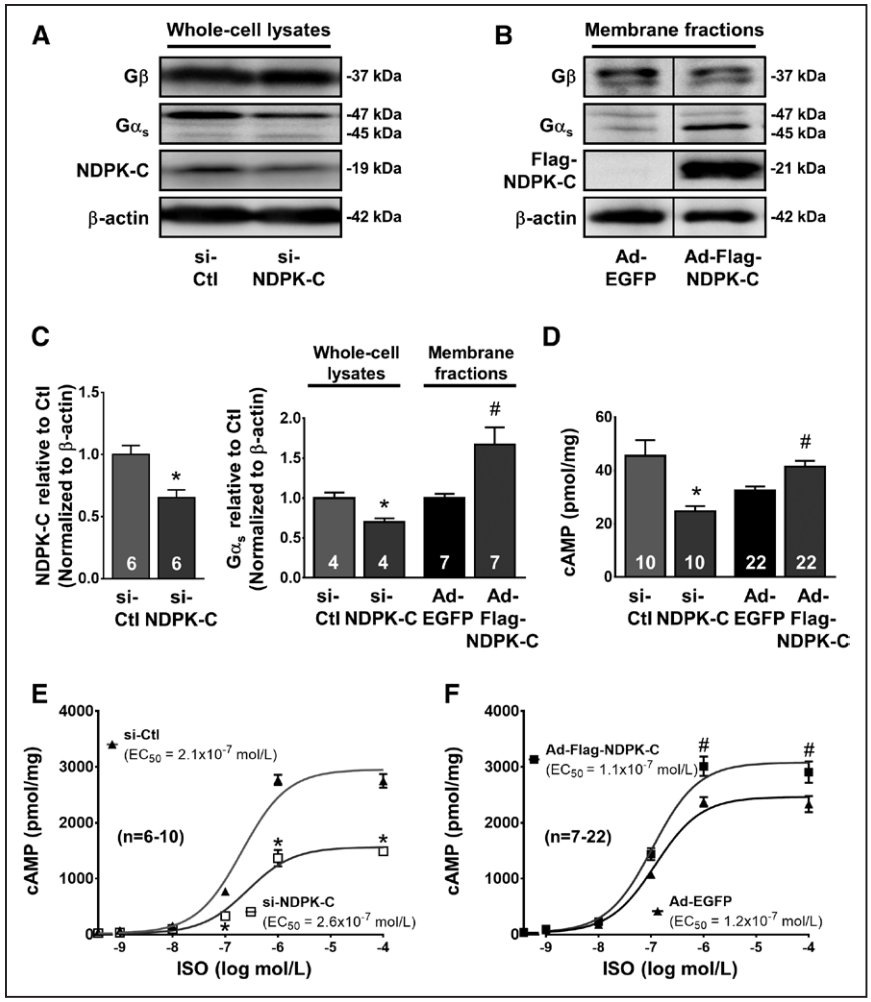

Figure 4. Nucleoside diphosphate kinase (NDPK-C) modulates cAMP levels.

A and **B**, Representative Western blots of Gβ, $G\alpha_s$, and NDPK-C or Flag–NDPK-C in lysates from neonatal rat cardiomyocytes (NRCMs) transfected with siRNA (si)-Ctl or si–NDPK-C (96 hours) and in membrane fractions from NRCMs infected with an adenovirus encoding enhanced green fluorescent protein (Ad-EGFP) or Ad-Flag–NDPK-C (multiplicity of infection, 500 for 48 hours). β-Actin levels served as loading control. **C**, Quantification of NDPK-C protein levels normalized to β-actin in si-Ctl vs si–NDPK-C (**left**) or $G\alpha_s$-protein levels from **A** and **B** normalized to β-actin relative to corresponding controls (**right**). **D**, cAMP levels in NRCMs transfected with si-Ctl or si–NDPK-C and NRCMs infected with Ad-EGFP or Ad-Flag–NDPK-C NRCMs in the presence of 1.0 mmol/L 3-isobutyl-1-methylxanthine (IBMX) and 1.0 μmol/L propranolol. **E** and **F**, Isoprenaline (ISO)-induced cAMP levels in NRCMs with si-RNA–mediated reduction in NDPK-C (**left**), or in NRCMs overexpressing NDPK-C (**right**) in the presence of 1.0 mmol/L IBMX and the indicated ISO concentration compared with corresponding controls. Numbers in bars indicate number of samples. **P*<0.05 vs si-Ctl. #*P*<0.05 vs Ad-EGFP.

$G\alpha_s$, with significantly reduced $G\alpha_s$ levels in lysates of si–NDPK-C NRCMs, and increased $G\alpha_s$ membrane content in Ad-Flag–NDPK-C NRCMs (Figure 4C). Knockdown of NDPK-C expression also significantly reduced cAMP levels, whereas NDPK-C overexpression enhanced cAMP content (Figure 4D). Isoprenaline induced a dose-dependent increase in cAMP content with a >50-fold increase in cAMP levels compared with control conditions after maximal stimulation (Figure 4E). The increase in isoprenaline-induced cAMP content was significantly reduced in si–NDPK-C NRCMs (Figure 4E) but further enhanced by overexpression of NDPK-C (Figure 4F). These findings establish a causal relationship between NDPK-C content at the plasma membrane and cellular cAMP levels in NRCMs.

We next determined whether NDPK-C also regulates G proteins and cAMP in ARCMs. NDPK-C levels at the plasma membrane were significantly increased after 6 hours of isoprenaline stimulation in ARCMs (Figure 5A and 5B). To assess the effects of NDPK-C in living ARCMs, we compared cAMP formation and fractional shortening[8,20] in ARCMs overexpressing NDPK-C with control ARCMs overexpressing EGFP or LacZ. Isoprenaline-induced cAMP levels were quantified in isolated ARCMs via fluorescent resonance energy transfer with an Epac-derived cAMP sensor.[25,26] Stimulation of control ARCMs with isoprenaline produced 55±3% of the maximal cAMP response achieved by direct stimulation of adenylyl cyclases with forskolin in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (Figure 5C). In NDPK-C–overexpressing ARCMs, the isoprenaline-induced cAMP response was significantly increased to 70±3% of the maximal cAMP response (Figure 5C).The isoprenaline concentrations producing half-maximal cell shortening were significantly higher in the control (4.5±0.9 nmol/L) than in NDPK-C–overexpressing ARCMs (0.8±0.1 nmol/L; Figure 5D), suggesting that the NDPK-C–induced increase in cAMP affects cardiomyocyte contraction by sensitizing single-cell shortening to βAR stimulation.

Knockdown of NDPK-C Modulates Contractility in Zebrafish

To analyze whether NDPK-C modulates contractility in vivo in a vertebrate organism, we performed morpholino-mediated knockdown of NDPK-C in zebrafish embryos. The injection of 300 μmol/L morpholino–NDPK-C caused

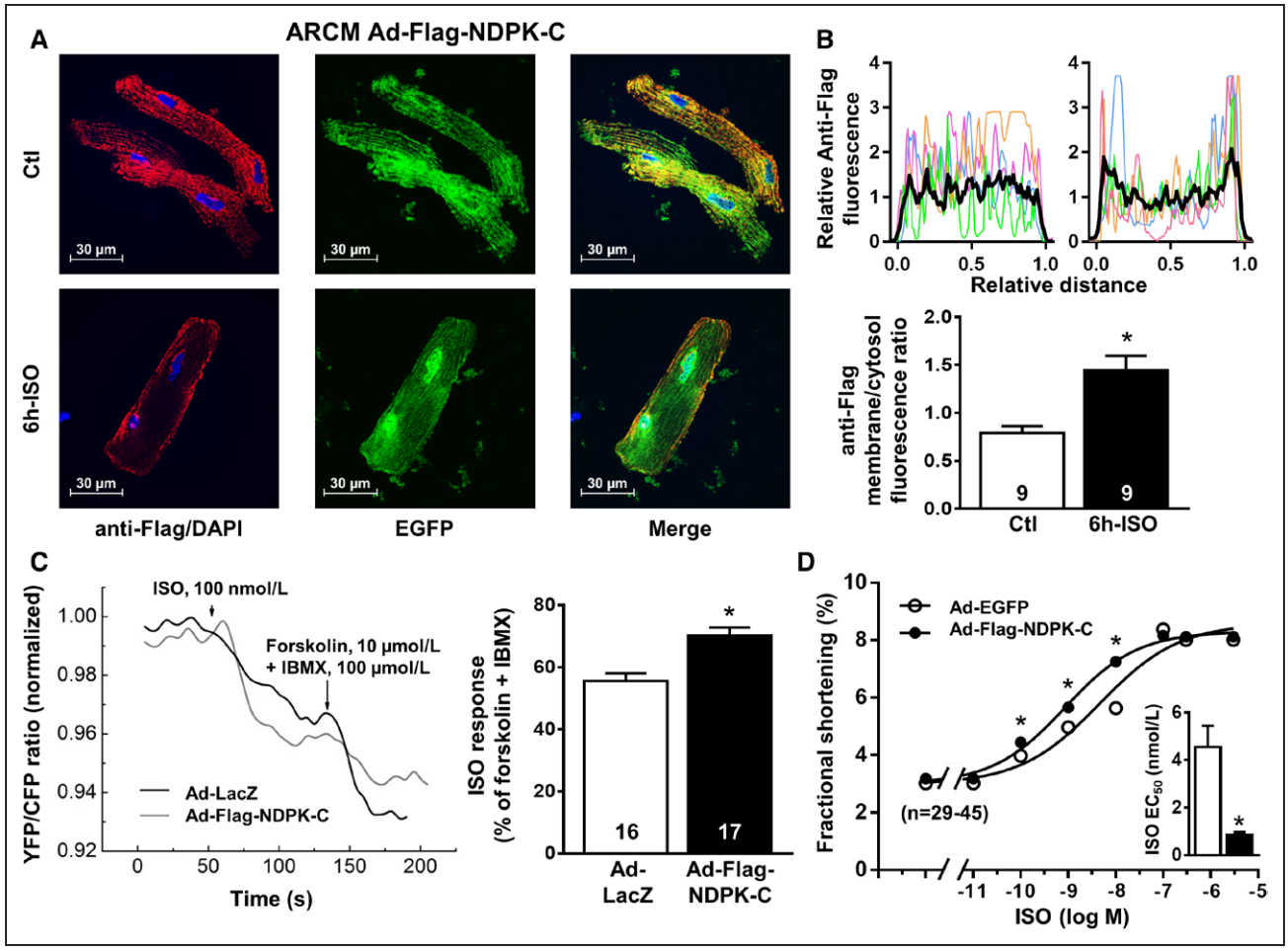

Figure 5. Consequences of nucleoside diphosphate kinase (NDPK)-C modulation in adult rat cardiomyocytes (ARCMs).

A, Confocal microscopy showing Flag–NDPK-C (red), DAPI (blue), and enhanced green fluorescent protein (EGFP; green) in ARCMs overexpressing Flag–NDPK-C (multiplicity of infection [MOI], 50) under control conditions (Ctl; **top row**) or after 6 hours of stimulation with ISO (6h-ISO; 1.0 µmol/L; **bottom row**). **B**, Line profiles of Flag–NDPK-C in 4 representative ARCMs (colored lines) in Ctl (**top left**) or 6h-ISO groups (**top right**) and average line profiles across all ARCMs (black lines). Bar chart shows fluorescent intensity in membrane region (first and last 15% of line profile) vs cytosol in the Ctl and 6h-ISO groups. **C**, Representative fluorescent resonance energy transfer (FRET; cyan fluorescent protein/yellow fluorescent protein [CFP/YFP]) ratio traces recorded from control and Ad-Flag–NDPK-C–overexpressing ARCMs (MOI, 300) expressing the cAMP sensor Epac2-camps after stimulation with ISO (100 nmol/L) and subsequent maximal stimulation of cAMP levels by forskolin (10 µmol/L) and 3-isobutyl-1-methylxanthine (IBMX; 100 µmol/L). Decreases in the FRET ratio represent increases in intracellular cAMP. The magnitude of the cAMP response to ISO as a percent of maximal response induced by forskolin plus IBMX in control and NDPK-C–overexpressing ARCMs is shown on the **right**. n=16 to 17 cells obtained from 2 independent infections. **D**, Concentration-dependent increase in relative fractional shortening of isolated ARCMs infected with Ad-EGFP or Ad-Flag–NDPK-C (MOI, 300) after short-term stimulation with the indicated concentrations of ISO. n=29 to 42 cells per condition from 3 independent infections. **Inset** shows the EC_{50} of ISO-induced augmentation of cellular fractional shortening. Numbers in bars indicate number of samples. **P*<0.05 vs corresponding control.

a loss of NDPK-C protein levels, resulting in phenotypic alterations, including reduced cardiac pump function and pericardial edema, compared with zebrafish injected with a control morpholino at 72 hours after fertilization (Figure 6A and Supplementary Videos I and II in the online-only Data Supplement). NDPK-C morphant hearts had a normal morphology of endocardial and myocardial cell layers and a regular expression of atrial and ventricular myosin heavy chains (Figure VIA–VID in the online-only Data Supplement). In addition, electron microscopy revealed no differences in the ultrastructure of sarcomeres, z line, and thick and thin filaments (Figure VIE and VIF in the online-only Data Supplement). Therefore, the structural development of the NDPK-C morphant hearts appears to be unhampered. Functionally, depletion of NDPK-C resulted in decreased basal cAMP levels (Figure 6B), similar to that reported previously for zebrafish with knockdown of NDPK-B.[19] Ventricular fractional

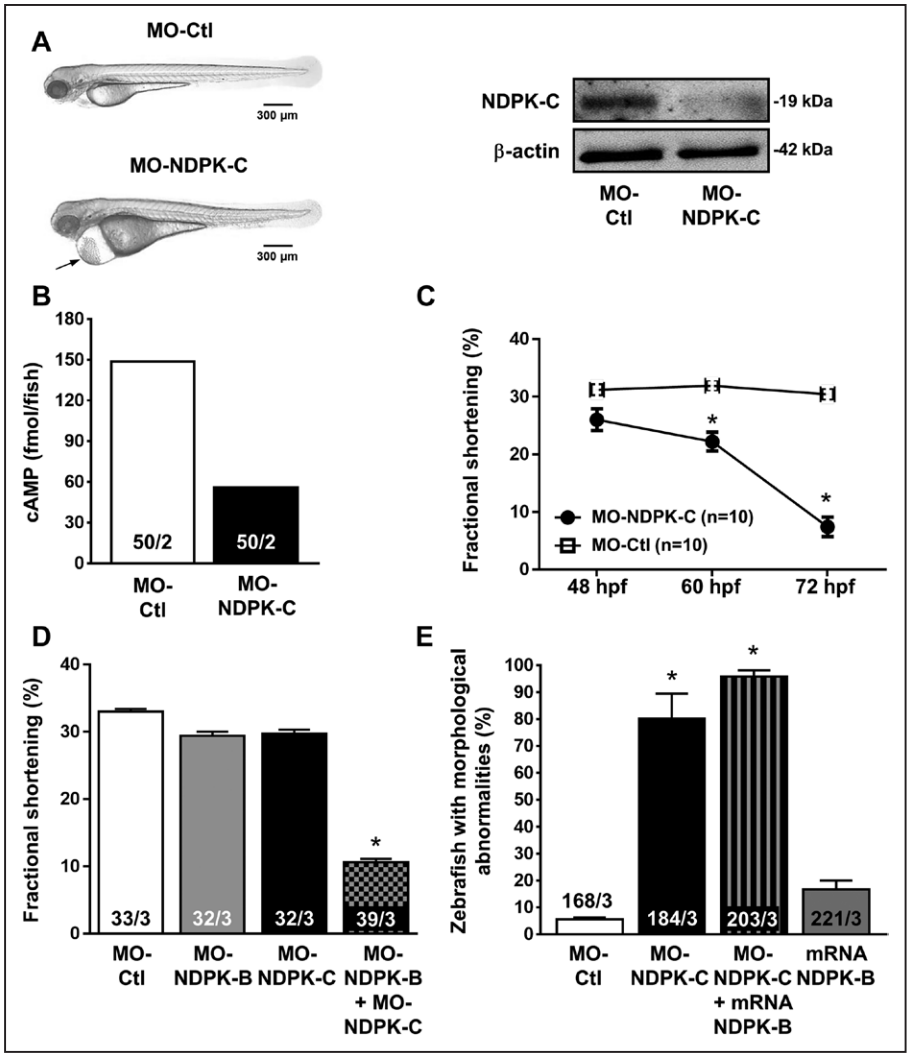

Figure 6. Nucleoside diphosphate kinase (NDPK)-C knockdown in zebrafish reduces ventricular contractility. **A**, Lateral view of representative zebrafish larvae injected with 300 µmol/L control or NDPK-C morpholinos (MO-Ctl and MO–NDPK-C, respectively) 72 hours post fertilization (hpf; **left**). NDPK-C–depleted embryos develop a pericardial edema (**arrow**) as a result of cardiac dysfunction. **Right**, Representative Western blot of NDPK-C and β-actin as loading control. **B**, cAMP levels in zebrafish injected with control or NDPK-C morpholinos 72 hpf in the presence of 3-isobutyl-1-methylxanthine (IBMX; 1.0 mmol/L). Shown is the average of 2 independent cAMP determinations in fish lysates. Numbers in bars indicate the total number of fish used for the determination of this value. **C**, In vivo fractional shortening of zebrafish ventricle in zebrafish injected with control or NDPK-C morpholinos at 48, 60, and 72 hpf. **P*<0.05 vs control morpholinos (repeated-measures 2-way ANOVA). **D**, In vivo fractional shortening of zebrafish ventricle in zebrafish injected with control, NDPK-B (125 µmol/L), NDPK-C (150 µmol/L), or both NDPK-B (125 µmol/L) and NDPK-C (150 µmol/L) morpholinos at 72 hpf. **P*<0.05 vs control morpholinos. **E**, Percentage of zebrafish developing morphological abnormalities under control conditions and after knockdown of NDPK-C, knockdown of NDPK-C with NDPK-B overexpression, or NDPK-B overexpression alone. **P*<0.05 vs control.

shortening was progressively reduced from 26.0±1.9% at 48 hours after fertilization to 7.4±1.7% at 72 hours after fertilization in NDPK-C morphants, whereas fractional shortening remained stable in morpholino-control zebrafish (Figure 6C). To evaluate synergism between NDPK-C and NDPK-B in vivo, a partial knockdown of NDPK-B and/or NDPK-C was performed with the use of low morpholino concentrations, and ventricular fractional shortening was assessed. Morpholino–NDPK-B (125 µmol/L) or morpholino–NDPK-C (150 µmol/L) alone did not significantly impair ventricular function, whereas their combination severely reduced fractional shortening (Figure 6D). To analyze whether an increased NDPK-B expression can substitute for the loss of NDPK-C, we overexpressed NDPK-B, an approach that previously rescued the phenotype caused by morpholino-mediated knockdown of NDPK-B.[19] Compared with controls, NDPK-C morphants frequently showed phenotypic abnormali-

ties (eg, pericardial edema or impaired cardiac function; Figure VII in the online-only Data Supplement). NDPK-B overexpression in NDPK-C knockdown embryos did not reduce the incidence of these phenotypic abnormalities (Figure 6E). Thus, in accordance with the in vitro data, NDPK-C is apparently indispensable for the complex formation between NDPKs and G proteins, which is involved in cAMP formation and the subsequent alterations in ventricular contractility in vivo.

NDPK-B–Deficient Mice Are Prone to Cardiac Dysfunction

To verify the importance of the complex formation of NDPK-C, NDPK-B, and G proteins described above for cardiac function in mammals, we took advantage of NDPK-B knockout mice.[27] These mice are viable, without obvious phenotype, and have a normal life span. The loss of NDPK-B does not alter the expression of NDPK-C in the heart of these mice (Figure VIII in the online-only Data Supplement). Nevertheless, at the age of 5 months, they start to develop cardiac dysfunction, as revealed by decreased fractional shortening (Figure 7A). To test whether the complex formation of NDPK-B with NDPK-C and G proteins is functionally relevant in the response to chronic βAR stimulation in the mammalian heart, we subjected these mice to the long-term isoprenaline stimulation protocol we used in rats (Figure 2A) and studied cardiac contractility and remodeling. As shown in Figure 7B through 7D, long-term isoprenaline treatment decreased fractional shortening more strongly in NDPK-B knockout mice than in wild-type controls. In addition, NDPK-B knockout mice were more susceptible to isoprenaline-induced cardiac hypertrophy and fibrosis. Taken together, these data indicate that the complex formation of NDPKs with $G\alpha_s$ proteins, which apparently requires the pres-

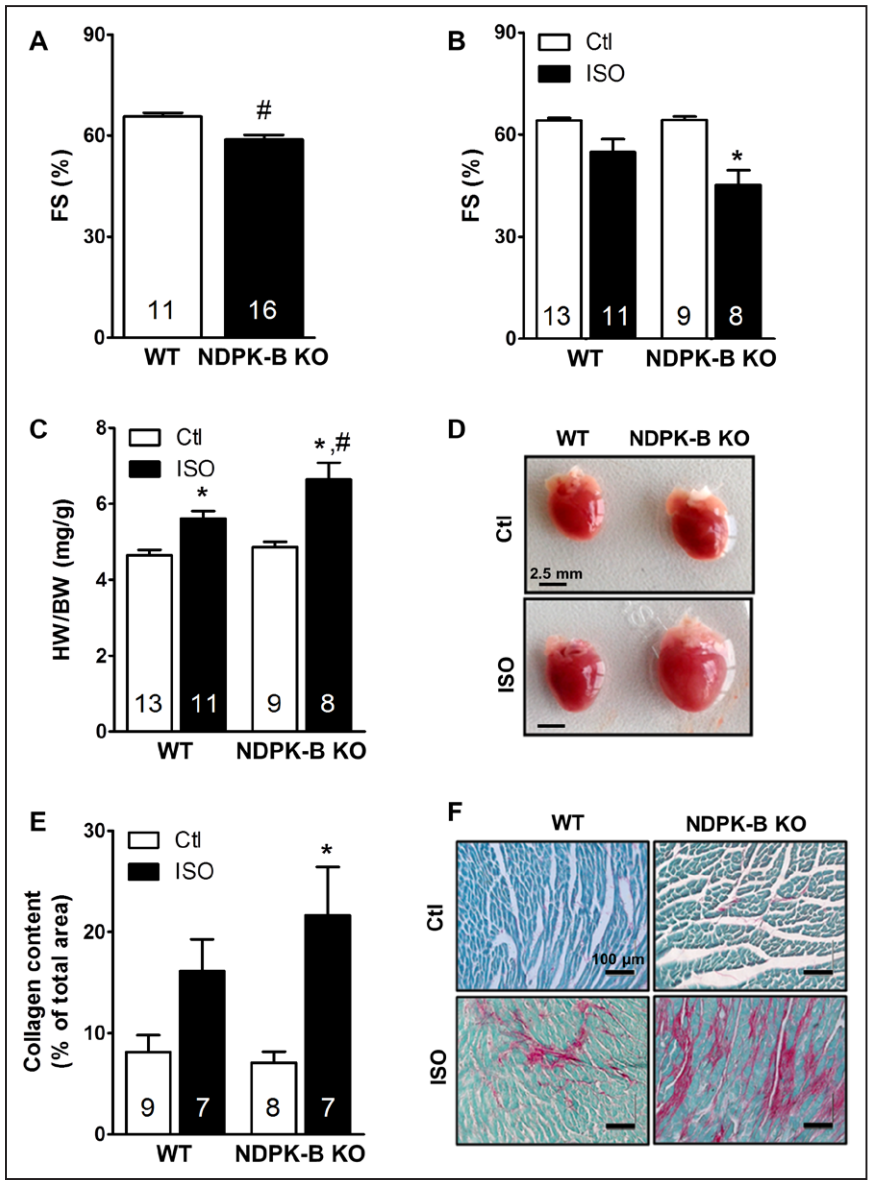

Figure 7. Ablation of nucleoside diphosphate kinase (NDPK)-B reduces cardiac function in aging mice and aggravates catecholamine-induced cardiac remodeling in young mice. **A**, Fractional shortening (FS) of 5-month-old male wild-type (WT) and NDPK-B knockout (NDPK-B KO) mice calculated from echocardiographic analysis in conscious animals. **B** and **C**, Two-month-old male WT and NDPK-B KO mice were either subjected to control conditions or treated with isoprenaline (ISO; 30 $mg \cdot kg^{-1} \cdot d^{-1}$) for 7 days via osmotic minipumps. **B**, FS in conscious animals at day 7 of the treatment. **C**, Quantification of hypertrophic growth by ratios of heart weight (HW) to body weight (BW). **D**, Representative images of explanted hearts. **E**, Quantification of fibrotic remodeling by Sirius Red/Fast Green Collagen staining of paraffin-embedded heart sections normalized to total tissue area. **F**, Representative image of collagen-stained (magenta) heart sections. *P<0.05 vs respective control. #P<0.05 versus WT, 2-way ANOVA with Bonferroni correction.

ence of both NDPK-B and NDPK-C, is beneficial during catecholamine-induced cardiac stress and protects the heart from an early onset of remodeling.

NDPK-C Causes a Switch From $G\alpha_s$- to $G\alpha_i$-Predominant Signaling in Human HF

The data presented so far identify NDPK-C, by mediating complex formation and membrane targeting of NDPK-B and $G\alpha_s$, as a regulator with stimulatory effects on cAMP levels and contractility. However, the increased expression of NDPK-C in human HF appears at odds with the reduced contractility observed in patients with HF. On the other hand, our far Western blots indicate that NDPK-C, but not NDPK-B, interacts with both $G\alpha_s$ and $G\alpha_i$, and the ratio of these proteins determines which G protein is preferentially bound (Figure 1D and 1F). Therefore, we hypothesized that NDPK-C may switch from $G\alpha_s$- to $G\alpha_i$-predominant signaling in human HF, thereby contributing to the lower cAMP levels and reduced cardiomyocyte contractility. To address this hypothesis, we analyzed NDPK-B and NDPK-C expression in ventricular tissue samples from a separate patient cohort. In accordance with the data obtained in the previous collective,[14] NDPK-B and NDPK-C protein levels were significantly increased in tissue lysates of patients with HF compared with healthy donor hearts (Figure 8A). The $G\alpha_{i2}$- and $G\alpha_s$-protein expression in membrane fractions of patients with HF appeared to be slightly increased (20%) and decreased (−15%), respectively, without reaching statistical significance (Figure 8A and Figure IXA in the online-only Data Supplement).

Next, we assessed the interactions among NDPK-C, $G\alpha_{i2}$, and $G\alpha_s$ in ventricular tissue lysates of control samples and those from patients with HF using coimmunoprecipitation. The NDPK-C content of the $G\alpha_{i2}$ precipitates was increased 2.8±0.8-fold in patients with HF (P=0.11), whereas the NDPK-C content in the $G\alpha_s$ precipitates of HF samples was decreased to 0.54±0.14-fold of nonfailing controls (P=0.07; Figure IXB in the online-only Data Supplement). Conversely, the amount of $G\alpha_{i2}$ that coprecipitated with NDPK-C was increased 2.39±0.35-fold (P<0.05), whereas the amount of $G\alpha_s$ that coprecipitated with NDPK-C was decreased to 0.55±0.08 of nonfailing controls (P<0.05) in human HF, pointing to a switch in the interaction of NDPK-C from $G\alpha_s$ to $G\alpha_{i2}$ (Figure 8B). cAMP levels were significantly reduced by 42% in patients with HF (Figure 8C), consistent with previous results.[28] To determine whether the switch in NDPK/G-protein signaling affects cAMP levels, NRCMs overexpressing EGFP, NDPK-C, $G\alpha_{i2}$, or NDPK-C plus $G\alpha_{i2}$ (Figure 8D) were incubated with increasing concentrations of isoprenaline. Overexpression of NDPK-C increased the basal and isoprenaline-induced cAMP levels in NRCMs. In contrast, overexpression of $G\alpha_{i2}$ reduced the basal cAMP content and the isoprenaline-induced increase in cAMP levels compared with EGFP-expressing controls (Figure 8E and 8F). It is notable that overexpression of both NDPK-C and $G\alpha_{i2}$ further reduced basal cAMP levels and isoprenaline-induced cAMP production compared with $G\alpha_{i2}$ overexpression alone (Figure 8E and 8F). These data indicate that the HF-related increase in NDPK-C and the stronger interaction between $G\alpha_{i2}$ and NDPK-C (Figure 8A and 8B) amplify the inhibitory effects of $G\alpha_{i2}$ proteins on cardiomyocyte cAMP levels and positions NDPK-C as a novel, potentially critical regulator of $G\alpha_i$-protein signaling and cellular cAMP in human HF.

DISCUSSION

In the present study, we identified NDPK-C as an essential and indispensable component of the interaction between NDPKs and G proteins. NDPK-C anchors these complexes at the plasma membrane, thereby being an important regulator of cAMP levels and cardiomyocyte contractility. We also discovered that a switch from predominantly $G\alpha_s$ stimulation to $G\alpha_i$ signaling by NDPK-C in human HF might cause the lower cAMP levels in patients with HF. Together, our findings identify NDPK-C as a novel critical regulator of βAR/cAMP signaling that may contribute to contractile dysfunction in HF.

G-Protein Signaling in HF

HF induces complex remodeling, with changes in G-protein signaling as a hallmark of this remodeling process. Long-term sympathetic stimulation results in desensitization of βARs, including reduced expression of β_1ARs and upregulation of inhibitory G-protein–coupled receptor kinases.[29,30] In addition, the expression and activity of inhibitory $G\alpha_i$ proteins are increased ≈30% in end-stage HF,[31,32] and a shift from a prevalence of $G\alpha_s$-mediated adenylyl cyclase stimulation to $G\alpha_i$-mediated inhibition of adenylyl cyclases through β_2-ARs in HF has been reported.[33] The increase in $G\alpha_i$-protein activity may result partly from transcriptional upregulation of $G\alpha_{i2}$ and likely contributes to the impaired βAR responsiveness associated with HF.[34] A modest upregulation of $G\alpha_{i2}$ was also identified in the rat model with long-term isoprenaline administration in our study,[35] which suggests that these effects are an adaptive response to chronic sympathetic stimulation. However, in a chronic setting, these changes result in reduced ventricular cardiomyocyte cAMP levels that decrease the activation of protein kinase A in HF. Together with the augmentation of global protein phosphatase activity in HF,[36] the restricted cAMP signaling results in reduced phosphorylation of key cardiac Ca^{2+}-handling proteins with subsequent decreases in Ca^{2+}-transient amplitude and cellular shortening,[37] which are well-established contributors to the reduced ventricular contractility in HF.[38]

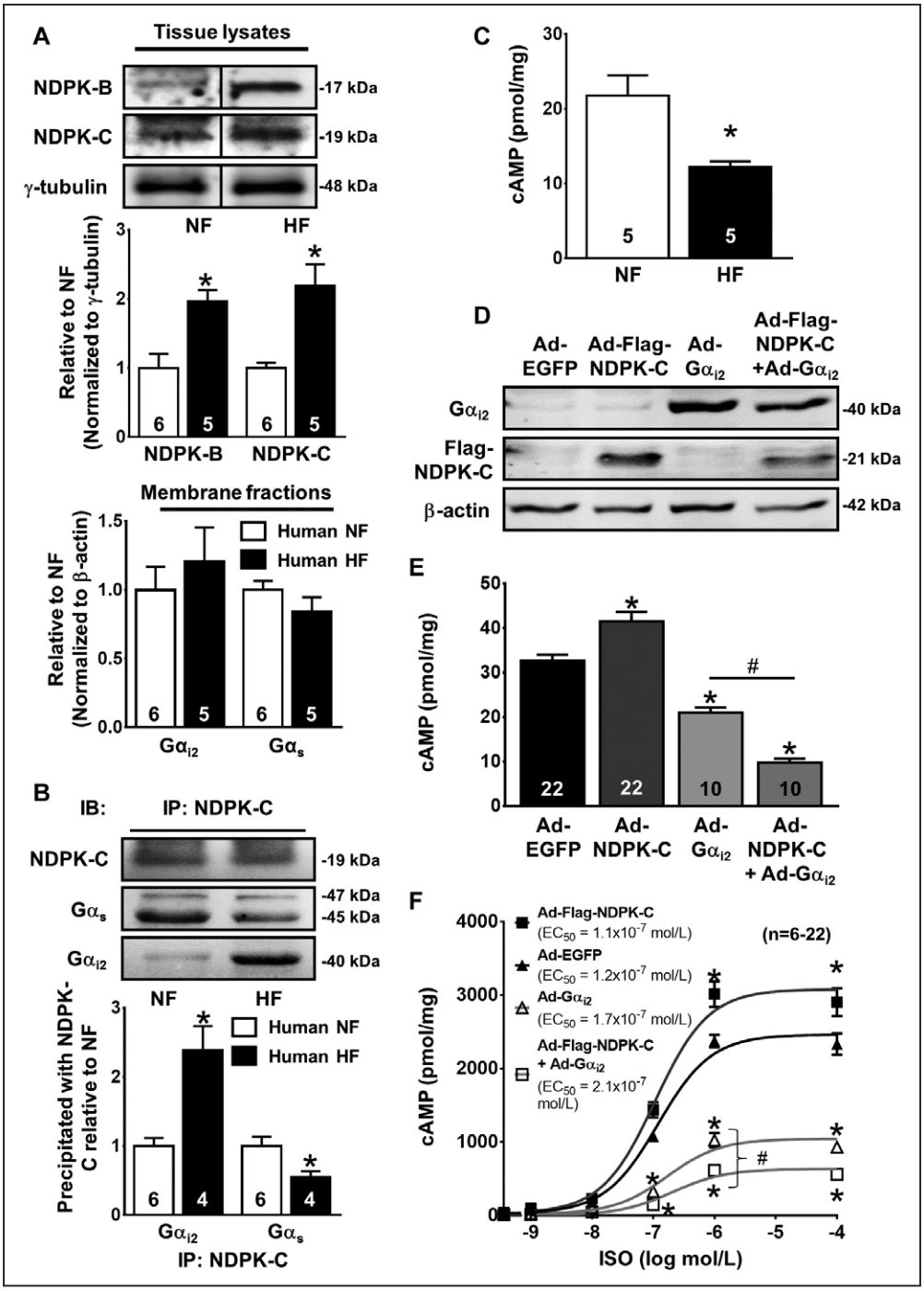

Figure 8. Nucleoside diphosphate kinase (NDPK)-C interaction with G proteins shifts toward $G\alpha_{i2}$ in heart failure (HF). **A**, Representative Western blots (**top**) and quantification of protein expression (**middle**) of NDPK-B and NDPK-C in ventricular whole-tissue lysates obtained from explanted hearts from patients with end-stage HF or nonfailing (NF) control donor hearts. γ-Tubulin served as loading control. **Bottom**, Quantification of $G\alpha_{i2}$ and $G\alpha_s$ in ventricular membrane fractions obtained from HF or NF control donor hearts. β-Actin served as loading control. Original blots shown in Figure IX in the online-only Data Supplement. **B**, Immunoprecipitation (IP) of NDPK-C in ventricular lysates obtained from patients with HF or NF donor hearts and quantification of the amount of $G\alpha_s$ and $G\alpha_{i2}$ immunoprecipitating with NDPK-C relative to NF controls. IB indicates immunoblot. **C**, cAMP levels in ventricular samples of patients with end-stage HF or NF control donor hearts in the presence of 3-isobutyl-1-methylxanthine (IBMX; 1.0 mmol/L). Numbers in bars indicate number of hearts. From 1 HF sample, not enough lysate could be obtained for immunoprecipitation. **P*<0.05 vs NF. **D** through **F**, Representative Western blots of $G\alpha_{i2}$, Flag–NDPK-C, and β-actin as loading control (**D**), basal cAMP levels (**E**), and ISO-induced cAMP levels with EC_{50} values (**F**) in neonatal rat cardiomyocytes (NRCMs) infected with an adenovirus encoding enhanced green fluorescent protein (Ad-EGFP, multiplicity of infection [MOI], 500), Ad-Flag–NDPK-C (MOI, 500), Ad-$G\alpha_{i2}$ (MOI, 50), or Ad-Flag–NDPK-C (MOI, 500) plus Ad-$G\alpha_{i2}$ (MOI, 50). **P*<0.05 vs Ad-EGFP controls. #*P*<0.05 for the effect of Ad-Flag–NDPK-C plus Ad-$G\alpha_{i2}$.

Previous work has shown that the plasma membrane content of NDPK-A, NDPK-B, and NDPK-C is increased in patients with end-stage HF[4] and that long-term activation of βARs increases anchoring of NDPKs to the plasma membrane.[39] Here, we discovered that this increase is due at least in part to a transcriptional upregulation and that NDPK-C is required and indispensable for membrane localization of NDPKs. The human *nm23-H3* gene encoding NDPK-C contains active AP-2 sites and a putative CREBP-1 binding site in its promoter region,[40] which are involved in cAMP-dependent regulation of gene transcription[41,42] and could contribute to the enhancement of NDPK-C protein levels after isoprenaline administration or in end-stage human HF with chronic sympathetic stimulation. Further work is needed to directly test these hypotheses.

Our data on the interaction of NDPK-C with NDPK-B and heterotrimeric G proteins also strongly suggest that NDPK-C is an essential prerequisite for the complex formation of NDPK-C/NDPK-B hetero-oligomers with G proteins. In addition, the lipophilic nature of NDPK-C may allow an easier association with the plasma membrane[43,44] and is therefore likely the limiting factor for the plasma membrane localization of NDPK isoforms.

Novelty and Potential Clinical Implications

We have identified a novel mechanism by which the plasma membrane content of heterotrimeric G proteins is regulated in cardiomyocytes through NDPK-C. In particular, we show for the first time that NDPK-C is required and indispensable for the interaction between NDPKs and both stimulatory $G\alpha_s$ and inhibitory $G\alpha_i$ proteins, find that NDPK-C–mediated targeting of NDPKs to the plasma membrane is increased in response to βAR stimulation and enhances intracellular cAMP levels, demonstrate that cardiomyocyte contractility in vitro and in vivo is strictly modulated by NDPK-C levels, and provide novel mechanistic insights into cAMP signaling in HF, identifying previously unrecognized molecular targets for the development of new and potentially more effective HF therapy options. Our data put previous studies about regulation of cAMP signaling through NDPKs in a novel and conclusive perspective: NDPK-B can exert its effects on cAMP signaling and contractility only in the presence of NDPK-C, which is responsible for both the membrane targeting and interaction of NDPKs with G proteins. Furthermore, our findings provide a potential mechanistic explanation for why even a small increase in $G\alpha_{i2}$-protein levels and increased membrane-associated NDPKs in HF may inhibit cAMP synthesis[14] and could cause a profound negative inotropic effect. It is interesting to note that modest changes in the $G\alpha_s/G\alpha_i$ ratio (Figure 1F), which are in the same range as those reported in HF,[31,32] may determine which of the G-protein subtypes is bound to NDPK-C. Thus, in the absence of $G\alpha_i$ upregulation, the increased membrane content of NDPK-C in human HF is a mechanism that could potentially counteract a fading βAR response in the early stages of HF by increasing the amount of $G\alpha_s$ proteins at the plasma membrane. However, by switching to $G\alpha_{i2}$ activation, NDPK-C could play a role in the progression of the disease and the reduced cAMP levels observed in end-stage human HF. Together, these data provide new insights into the complex alterations in G-protein signaling that are a hallmark of the vicious cycle of chronic sympathetic stimulation in HF and, for the first time, establish increased NDPK-C function as a novel possible molecular facilitator of detrimental $G\alpha_i$-protein signaling in patients with HF.

Treatment with β-blockers has been the standard therapy for patients with HF. Despite their positive effects on morbidity and mortality, β-blockers are not without limitations. Accordingly, other ways to regulate G-protein signaling and cardiac proteins involved in cardiac contractility are currently being investigated in the treatment of HF.[45,46] A better understanding of the molecular processes involved in dysregulation of G-protein signaling in HF is expected to foster the development of improved HF therapies. On the basis of our studies, we identify NDPK-C as a novel potential therapeutic target involved in the regulation of aberrant G-protein signaling and cardiac contractility in HF. Even short-term stimulation of cardiomyocytes with isoprenaline increases the plasma membrane content of NDPK-C and strengthens the interaction between NDPK-C and G proteins, thereby increasing cAMP synthesis. The increased expression or plasma membrane content of NDPK-C in human HF might initially allow the heart to compensate a beginning loss in βAR-induced contractile response in the onset of HF. However, because of the promiscuous nature of NDPK-C, which is able to interact with both $G\alpha_s$ and $G\alpha_i$, this compensatory mechanism might become detrimental for the heart when the interaction with G_{i2} increases over time. Along these lines, we show that if $G\alpha_{i2}$ levels are enhanced, an increase in NDPK-C abundance reduces cAMP levels (Figure 8E and 8F), which may contribute to the detrimental cardiomyocyte phenotype of human HF. Thus, inhibition of NDPK-C might represent a novel adjuvant therapy for patients with end-stage HF.

Limitations

The causes of HF are diverse. We used human ventricular tissue samples from a selected group of patients with end-stage HF. Our finding of increased NDPK-C expression may not hold true for all types and stages of HF. In addition, experimental conditions in vitro may not fully reflect the dynamic regulation of cAMP and contractility in vivo. For example, cAMP levels may change rapidly after tissue excision, and in situ cAMP levels were found to be similar between nonfailing patients and patients with HF, possibly as a result of increased plasma norepinephrine

levels counteracting intrinsic downregulation of cAMP in cardiomyocytes.[47]

Isoprenaline increased the plasma membrane content of the primarily cytosolic NDPK-C within several hours after stimulation. The mechanisms involved could include translocation, increased protein stability at the plasma membrane, or reduced degradation and should be addressed in future studies.

Here, we characterized the role of NDPK-C in modulating cAMP levels. However, as histidine kinases, NDPKs could also directly phosphorylate various targets within the cardiomyocyte, thereby participating in the control of various cardiac functions.[48] For example, NDPK-B activates transient-receptor potential vallinoid type-5 channels through phosphorylation of histidine 711, thereby controlling Ca^{2+} reabsorption in the kidney.[49] Similarly, NDPK-B activates small-conductance Ca^{2+}-activated K^+ channels in vascular smooth muscle cells, controlling neointima formation in carotid arteries.[50] Subsequent work should test these possibilities in the heart.

Conclusions

NDPK-C is indispensable for the interaction between NDPKs and G proteins and the anchoring of these complexes at the plasma membrane, thereby dynamically regulating cAMP levels and cardiomyocyte contractility. The switch from predominantly $G\alpha_s$ stimulation to $G\alpha_i$ activation by NDPK-C in human HF might cause lower cAMP levels in patients with HF, potentially contributing to the progression of HF. Together, our findings position NDPK-C as a novel critical determinant of βAR/cAMP signaling that could contribute to impaired cardiac function and remodeling in human HF.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the expert technical assistance from Doris Baltus, Kristina Stephan-Schnatz, Heike Rauscher, Felicia Radtke, Geronimo Heilmann, Annette Kötting-Dorsch, Katrin Kupser, Barbara Langer, Claudia Liebetrau, Alexandra Müller, Ramona Nagel, and Lisa Walter. The authors also thank Ioan Lascu, University Bordeaux Segalen, for kindly providing purified NDPKs and the custom-made NDPK-C antibody.

SOURCES OF FUNDING

The authors' work was supported by grants from the Deutsche Forschungsgemeinschaft (Wi1373/9-3 to Dr Wieland and EL 270/7-1 to Dr El-Armouche), the Fondation Leducq (European North-American Atrial Fibrillation Research Alliance to Dr Dobrev), the European Network for Translational Research in Atrial Fibrillation (No. 261057 to Dr Dobrev), and the German Federal Ministry of Education and Research through the DZHK (German Center for Cardiovascular Research to Drs Dobrev, Katus, Backs, and Wieland).

DISCLOSURES

None.

AFFILIATIONS

From Institute of Experimental and Clinical Pharmacology and Toxicology, Mannheim Medical Faculty (I.H.A.-T., N.M.W., K.S., C.V., S.L., T.W.), and Department of Internal Medicine III (H.-J.H., N.M.W., M.M., B.M., H.-A.K., L.H.L., J.B.), Heidelberg University, Heidelberg-Mannheim, Germany; Institute of Pharmacology, West German Heart and Vascular Center, University Duisburg-Essen, Essen, Germany (I.H.A.-T., J.H., M.S., N.V., D.D.); Institute of Pharmacology and Toxicology, University Medical Center Göttingen, Germany (A.E.-A., C.M.W., S.L.); Department of Pharmacology and Toxicology, Medical Faculty Carl Gustav Carus, Dresden University of Technology, Germany (A.E.-A.); Institute of Experimental Cardiovascular Research, University Medical Center Hamburg-Eppendorf, Germany (V.O.N.); Department of Internal Medicine II, University of Regensburg, Germany (S.N.); Department of Pharmacology and Pharmacotherapy, Faculty of Medicine, University of Szeged, Hungary (I.B., A.V.); Division of Nephrology, New York University Langone Medical Center, New York (E.Y.S.); and DZHK (German Center for Cardiovascular Research), Partner Site HD/MA, Heidelberg-Mannheim, Germany (B.M., H.A.K., C.V., J.B., T.W.). The current affiliation for H.-J.H. is the Department of Cardiology and Angiology, University Hospital Schleswig-Holstein, Kiel, Germany.

FOOTNOTES

Received April 7, 2016; accepted November 23, 2016.

The online-only Data Supplement is available with this article at http://circ.ahajournals.org/lookup/suppl/doi:10.1161/CIRCULATIONAHA.116.022852/-/DC1.

Circulation is available at http://circ.ahajournals.org.

REFERENCES

1. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Blaha MJ, Dai S, Ford ES, Fox CS, Franco S, Fullerton HJ, Gillespie C, Hailpern SM, Heit JA, Howard VJ, Huffman MD, Judd SE, Kissela BM, Kittner SJ, Lackland DT, Lichtman JH, Lisabeth LD, Mackey RH, Magid DJ, Marcus GM, Marelli A, Matchar DB, McGuire DK, Mohler ER 3rd, Moy CS, Mussolino ME, Neumar RW, Nichol G, Pandey DK, Paynter NP, Reeves MJ, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Wong ND, Woo D, Turner MB; American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Heart disease and stroke statistics–2014 update: a report from the American Heart Association. *Circulation.* 2014;129:e28–e292. doi: 10.1161/01.cir.0000441139.02102.80.
2. Movsesian MA. Cyclic AMP-mediated signal transduction in heart failure: molecular pathophysiology and therapeutic implications. *J Investig Med.* 1997;45:432–440.

3. El-Armouche A, Eschenhagen T. Beta-adrenergic stimulation and myocardial function in the failing heart. *Heart Fail Rev*. 2009;14:225–241. doi: 10.1007/s10741-008-9132-8.
4. Lutz S, Hippe HJ, Niroomand F, Wieland T. Nucleoside diphosphate kinase-mediated activation of heterotrimeric G proteins. *Methods Enzymol*. 2004;390:403–418. doi: 10.1016/S0076-6879(04)90025-0.
5. Boissan M, Dabernat S, Peuchant E, Schlattner U, Lascu I, Lacombe ML. The mammalian Nm23/NDPK family: from metastasis control to cilia movement. *Mol Cell Biochem*. 2009;329:51–62. doi: 10.1007/s11010-009-0120-7.
6. Gilles AM, Presecan E, Vonica A, Lascu I. Nucleoside diphosphate kinase from human erythrocytes: structural characterization of the two polypeptide chains responsible for heterogeneity of the hexameric enzyme. *J Biol Chem*. 1991;266:8784–8789.
7. Janin J, Dumas C, Moréra S, Xu Y, Meyer P, Chiadmi M, Cherfils J. Three-dimensional structure of nucleoside diphosphate kinase. *J Bioenerg Biomembr*. 2000;32:215–225.
8. Hippe HJ, Luedde M, Lutz S, Koehler H, Eschenhagen T, Frey N, Katus HA, Wieland T, Niroomand F. Regulation of cardiac cAMP synthesis and contractility by nucleoside diphosphate kinase B/G protein beta gamma dimer complexes. *Circ Res*. 2007;100:1191–1199. doi: 10.1161/01.RES.0000264058.
9. Hippe HJ, Lutz S, Cuello F, Knorr K, Vogt A, Jakobs KH, Wieland T, Niroomand F. Activation of heterotrimeric G proteins by a high energy phosphate transfer via nucleoside diphosphate kinase (NDPK) B and Gβ subunits: specific activation of Gαs by an NDPK B/Gβγ complex in H10 cells. *J Biol Chem*. 2003;278:7227–7233. doi: 10.1074/jbc.M210305200.
10. Cuello F, Schulze RA, Heemeyer F, Meyer HE, Lutz S, Jakobs KH, Niroomand F, Wieland T. Activation of heterotrimeric G proteins by a high energy phosphate transfer via nucleoside diphosphate kinase (NDPK) B and Gβ subunits: complex formation of NDPK B with Gbeta gamma dimers and phosphorylation of His-266 in Gβ. *J Biol Chem*. 2003;278:7220–7226. doi: 10.1074/jbc.M210304200.
11. Erent M, Gonin P, Cherfils J, Tissier P, Raschellà G, Giartosio A, Agou F, Sarger C, Lacombe ML, Konrad M, Lascu I. Structural and catalytic properties and homology modelling of the human nucleoside diphosphate kinase C, product of the DRnm23 gene. *Eur J Biochem*. 2001;268:1972–1981.
12. Moréra S, Lacombe ML, Xu Y, LeBras G, Janin J. X-ray structure of human nucleoside diphosphate kinase B complexed with GDP at 2 Å resolution. *Structure*. 1995;3:1307–1314.
13. Webb PA, Perisic O, Mendola CE, Backer JM, Williams RL. The crystal structure of a human nucleoside diphosphate kinase, NM23-H2. *J Mol Biol*. 1995;251:574–587.
14. Lutz S, Mura R, Baltus D, Movsesian M, Kübler W, Niroomand F. Increased activity of membrane-associated nucleoside diphosphate kinase and inhibition of cAMP synthesis in failing human myocardium. *Cardiovasc Res*. 2001;49:48–55.
15. El-Armouche A, Gocht F, Jaeckel E, Wittköpper K, Peeck M, Eschenhagen T. Long-term beta-adrenergic stimulation leads to downregulation of protein phosphatase inhibitor-1 in the heart. *Eur J Heart Fail*. 2007;9:1077–1080. doi: 10.1016/j.ejheart.2007.09.006.
16. He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B. A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci USA*. 1998;95:2509–2514.
17. Luo J, Deng ZL, Luo X, Tang N, Song WX, Chen J, Sharff KA, Luu HH, Haydon RC, Kinzler KW, Vogelstein B, He TC. A protocol for rapid generation of recombinant adenoviruses using the AdEasy system. *Nat Protoc*. 2007;2:1236–1247. doi: 10.1038/nprot.2007.135.
18. Nikolaev VO, Moshkov A, Lyon AR, Miragoli M, Novak P, Paur H, Lohse MJ, Korchev YE, Harding SE, Gorelik J. β2-adrenergic receptor redistribution in heart failure changes cAMP compartmentation. *Science*. 2010;327:1653–1657. doi: 10.1126/science.1185988.
19. Hippe HJ, Wolf NM, Abu-Taha I, Mehringer R, Just S, Lutz S, Niroomand F, Postel EH, Katus HA, Rottbauer W, Wieland T. The interaction of nucleoside diphosphate kinase B with Gβγ dimers controls heterotrimeric G protein function. *Proc Natl Acad Sci USA*. 2009;106:16269–16274. doi: 10.1073/pnas.0901679106.
20. Grote-Wessels S, Baba HA, Boknik P, El-Armouche A, Fabritz L, Gillmann HJ, Kucerova D, Matus M, Müller FU, Neumann J, Schmitz M, Stümpel F, Theilmeier G, Wohlschlaeger J, Schmitz W, Kirchhefer U. Inhibition of protein phosphatase 1 by inhibitor-2 exacerbates progression of cardiac failure in a model with pressure overload. *Cardiovasc Res*. 2008;79:464–471. doi: 10.1093/cvr/cvn113.
21. Rottbauer W, Wessels G, Dahme T, Just S, Trano N, Hassel D, Burns CG, Katus HA, Fishman MC. Cardiac myosin light chain-2: a novel essential component of thick-myofilament assembly and contractility of the heart. *Circ Res*. 2006;99:323–331. doi: 10.1161/01.RES.0000234807.16034.fe.
22. Hippe HJ, Abu-Taha I, Wolf NM, Katus HA, Wieland T. Through scaffolding and catalytic actions nucleoside diphosphate kinase B differentially regulates basal and β-adrenoceptor-stimulated cAMP synthesis. *Cell Signal*. 2011;23:579–585. doi: 10.1016/j.cellsig.2010.11.010.
23. Mourad N, Parks RE Jr. Erythrocytic nucleoside diphosphokinase, II: isolation and kinetics. *J Biol Chem*. 1966;241:271–278.
24. Postel EH, Wohlman I, Zou X, Juan T, Sun N, D'Agostin D, Cuellar M, Choi T, Notterman DA, La Perle KM. Targeted deletion of Nm23/nucleoside diphosphate kinase A and B reveals their requirement for definitive erythropoiesis in the mouse embryo. *Dev Dyn*. 2009;238:775–787. doi: 10.1002/dvdy.21887.
25. Börner S, Schwede F, Schlipp A, Berisha F, Calebiro D, Lohse MJ, Nikolaev VO. FRET measurements of intracellular cAMP concentrations and cAMP analog permeability in intact cells. *Nat Protoc*. 2011;6:427–438. doi: 10.1038/nprot.2010.198.
26. Iancu RV, Ramamurthy G, Warrier S, Nikolaev VO, Lohse MJ, Jones SW, Harvey RD. Cytoplasmic cAMP concentrations in intact cardiac myocytes. *Am J Physiol Cell Physiol*. 2008;295:C414–C422. doi: 10.1152/ajpcell.00038.2008.
27. Di L, Srivastava S, Zhdanova O, Sun Y, Li Z, Skolnik EY. Nucleoside diphosphate kinase B knock-out mice have impaired activation of the K^+ channel KCa3.1, resulting in defective T cell activation. *J Biol Chem*. 2010;285:38765–38771. doi: 10.1074/jbc.M110.168070.
28. Böhm M, Reiger B, Schwinger RH, Erdmann E. cAMP concentrations, cAMP dependent protein kinase activity, and phospholamban in non-failing and failing myocardium. *Cardiovasc Res*. 1994;28:1713–1719.
29. Lohse MJ, Engelhardt S, Danner S, Böhm M. Mechanisms of beta-adrenergic receptor desensitization: from molecular biology to heart failure. *Basic Res Cardiol*. 1996;91 Suppl 2:29–34.
30. Lohse MJ, Engelhardt S, Eschenhagen T. What is the role of beta-adrenergic signaling in heart failure? *Circ Res*. 2003;93:896–906. doi: 10.1161/01.RES.0000102042.83024.CA.
31. Feldman AM, Cates AE, Veazey WB, Hershberger RE, Bristow MR, Baughman KL, Baumgartner WA, Van Dop C. Increase of the 40,000-mol wt pertussis toxin substrate (G protein) in the failing human heart. *J Clin Invest*. 1988;82:189–197. doi: 10.1172/JCI113569.
32. Neumann J, Schmitz W, Scholz H, von Meyerinck L, Döring V, Kalmar P. Increase in myocardial Gi-proteins in heart failure. *Lancet*. 1988;2:936–937.
33. He JQ, Balijepalli RC, Haworth RA, Kamp TJ. Crosstalk of beta-adrenergic receptor subtypes through Gi blunts beta-adrenergic stimulation of L-type Ca^{2+} channels in canine heart failure. *Circ Res*. 2005;97:566–573. doi: 10.1161/01.RES.0000181160.31851.05.
34. Eschenhagen T, Mende U, Nose M, Schmitz W, Scholz H, Haverich A, Hirt S, Döring V, Kalmár P, Höppner W. Increased messenger

RNA level of the inhibitory G protein alpha subunit Giα2 in human end-stage heart failure. *Circ Res*. 1992;70:688–696.
35. Mende U, Eschenhagen T, Geertz B, Schmitz W, Scholz H, Schulte am Esch J, Sempell R, Steinfath M. Isoprenaline-induced increase in the 40/41 kDa pertussis toxin substrates and functional consequences on contractile response in rat heart. *Naunyn Schmiedebergs Arch Pharmacol*. 1992;345:44–50.
36. Heijman J, Dewenter M, El-Armouche A, Dobrev D. Function and regulation of serine/threonine phosphatases in the healthy and diseased heart. *J Mol Cell Cardiol*. 2013;64:90–98. doi: 10.1016/j.yjmcc.2013.09.006.
37. Ravens U, Dobrev D. Regulation of sarcoplasmic reticulum Ca^{2+}-ATPase and phospholamban in the failing and nonfailing heart. *Cardiovasc Res*. 2000;45:245–252.
38. Schmidt U, Hajjar RJ, Kim CS, Lebeche D, Doye AA, Gwathmey JK. Human heart failure: cAMP stimulation of SR Ca^{2+}-ATPase activity and phosphorylation level of phospholamban. *Am J Physiol*. 1999;277(pt 2):H474–H480.
39. Lutz S, Mura RA, Hippe HJ, Tiefenbacher C, Niroomand F. Plasma membrane-associated nucleoside diphosphate kinase (nm23) in the heart is regulated by beta-adrenergic signaling. *Br J Pharmacol*. 2003;140:1019–1026. doi: 10.1038/sj.bjp.0705527.
40. Martinez R, Venturelli D, Perrotti D, Veronese ML, Kastury K, Druck T, Huebner K, Calabretta B. Gene structure, promoter activity, and chromosomal location of the DR-nm23 gene, a related member of the nm23 gene family. *Cancer Res*. 1997;57:1180–1187.
41. Müller FU, Loser K, Kleideiter U, Neumann J, von Wallbrunn C, Dobner T, Scheld HH, Bantel H, Engels IH, Schulze-Osthoff K, Schmitz W. Transcription factor AP-2α triggers apoptosis in cardiac myocytes. *Cell Death Differ*. 2004;11:485–493. doi: 10.1038/sj.cdd.4401383.
42. Müller FU, Lewin G, Baba HA, Bokník P, Fabritz L, Kirchhefer U, Kirchhof P, Loser K, Matus M, Neumann J, Riemann B, Schmitz W. Heart-directed expression of a human cardiac isoform of cAMP-response element modulator in transgenic mice. *J Biol Chem*. 2005;280:6906–6914. doi: 10.1074/jbc.M407864200.
43. Barraud P, Amrein L, Dobremez E, Dabernat S, Masse K, Larou M, Daniel JY, Landry M. Differential expression of nm23 genes in adult mouse dorsal root ganglia. *J Comp Neurol*. 2002;444:306–323.
44. Mitchell KA, Szabo G, de S Otero A. Direct binding of cytosolic NDP kinases to membrane lipids is regulated by nucleotides. *Biochim Biophys Acta*. 2009;1793:469–476. doi: 10.1016/j.bbamcr.2008.12.009.
45. Pleger ST, Brinks H, Ritterhoff J, Raake P, Koch WJ, Katus HA, Most P. Heart failure gene therapy: the path to clinical practice. *Circ Res*. 2013;113:792–809. doi: 10.1161/CIRCRESAHA.113.300269.
46. Vlahos CJ, McDowell SA, Clerk A. Kinases as therapeutic targets for heart failure. *Nat Rev Drug Discov*. 2003;2:99–113. doi: 10.1038/nrd1009.
47. Regitz-Zagrosek V, Hertrampf R, Steffen C, Hildebrandt A, Fleck E. Myocardial cyclic AMP and norepinephrine content in human heart failure. *Eur Heart J*. 1994;15(suppl D):7–13.
48. Wieland T, Attwood PV. Alterations in reversible protein histidine phosphorylation as intracellular signals in cardiovascular disease. *Front Pharmacol*. 2015;6:173. doi: 10.3389/fphar.2015.00173.
49. Cai X, Srivastava S, Surindran S, Li Z, Skolnik EY. Regulation of the epithelial Ca^{2+} channel TRPV5 by reversible histidine phosphorylation mediated by NDPK-B and PHPT1. *Mol Biol Cell*. 2014;25:1244–1250. doi: 10.1091/mbc.E13-04-0180.
50. Zhou XB, Feng YX, Sun Q, Lukowski R, Qiu Y, Spiger K, Li Z, Ruth P, Korth M, Skolnik EY, Borggrefe M, Dobrev D, Wieland T. Nucleoside diphosphate kinase B-activated intermediate conductance potassium channels are critical for neointima formation in mouse carotid arteries. *Arterioscler Thromb Vasc Biol*. 2015;35:1852–1861. doi: 10.1161/ATVBAHA.115.305881.

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Circulation. 2017;135:881-897; originally published online December 7, 2016;
doi: 10.1161/CIRCULATIONAHA.116.022852
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0009-7322. Online ISSN: 1524-4539

Supplemental Material

Nucleoside Diphosphate Kinase-C Suppresses cAMP Formation in Human Heart Failure

Issam H. Abu-Taha, PhD[1,2]*, Jordi Heijman, PhD[2]*, Hans-Jörg Hippe, MD[3]*†, Nadine M. Wolf, PhD[1,3], Ali El-Armouche, MD[4,5], Viacheslav O. Nikolaev, PhD[6], Marina Schäfer[2], Christina M. Würtz, PhD[4], Stefan Neef, MD[7], Niels Voigt, MD[2], István Baczkó, MD, PhD[8], András Varró, MD, PhD, DSc[8], Marion Müller[3], Benjamin Meder, MD[3,10], Hugo A. Katus, MD[3,10], Katharina Spiger, PhD[1], Christiane Vettel, PhD[1,10], Lorenz H. Lehmann, MD[3],Johannes Backs, MD[3,10], Edward Y. Skolnik, MD[9], Susanne Lutz, PhD[1,4], Dobromir Dobrev, MD[2‡], and Thomas Wieland, PhD[1,10‡]

[1]Institute of Experimental and Clinical Pharmacology and Toxicology, Mannheim Medical Faculty, Heidelberg University, Mannheim, Germany; [2]Institute of Pharmacology, Faculty of Medicine, University Duisburg-Essen, Essen, Germany; [3]Department of Internal Medicine III, Heidelberg University, Heidelberg, Germany; [4]Institute of Pharmacology and Toxicology, University Medical Center Göttingen, Göttingen, Germany; [5]Department of Pharmacology and Toxicology, Medical Faculty Carl Gustav Carus, Dresden University of Technology, 01307 Dresden, Germany; [6]Institute of Experimental Cardiovascular Research, University Medical Center Hamburg-Eppendorf, D-20246 Hamburg, Germany; [7]Department of Internal Medicine II, University of Regensburg, D-93053 Regensburg, Germany; [8]Department of Pharmacology and Pharmacotherapy, Faculty of Medicine, University of Szeged, Szeged, Hungary; [9]Division of Nephrology, New York University Langone Medical Center, New York, USA [10], DZHK (German Center for Cardiovascular Research), Partner Site, Heidelberg-Mannheim, Germany

*These authors contributed equally

†Present address: Department of Cardiology and Angiology, University Hospital Schleswig-Holstein, Campus Kiel, D-24105 Kiel, Germany

‡These authors share senior authorship

Extended Methods

Tissue procurement

Non-failing human myocardium from the free wall of the left ventricle was obtained from organ donors with no apparent heart disease and normal left ventricular function (determined by echocardiography) for whom no suitable heart transplant recipients had been identified. Left ventricular myocardium of failing human hearts was obtained from patients that underwent cardiac transplantation due to end-stage heart failure (HF, NYHA III–IV). Patient characteristics are given in[1] for samples used in mRNA analyses. Samples for the additional cohort used for Western blot analyses were obtained from 5 patients (all male, aged 52.4±1.2 years) with end-stage HF (EF<25%) and from 6 organ donors (all male, aged 54.5±2.7 years). The experimental protocol was approved by the Ethical Review Board of the Medical Center of the University of Szeged. Informed written consent was obtained for the use of non-diseased human hearts in this research study. All procedures conformed to the Helsinki Declaration of the World Medical Association.

Rat model of isoprenaline-stimulation and isolation of rat cardiomyocytes

For the rat HF model, male Wistar rats (240-300 g) received 4 days of either isoprenaline (2.4 mg/kg/day, ISO). Control rats received vehicle (0.9% NaCl), administered via osmotic minipumps (Alzet, USA), as previously described.[2] NRCM and ARCM were isolated from normal Wistar rats using a collagenase digestion method as previously described. All procedures regarding care and use of animals were in accordance with institutional guidelines (Az. G10/65 LAVES Niedersachsen, Germany).

Isolation of RNA and quantification of mRNA content

Total RNA was isolated using a high purity RNA isolation kit (Roche) from human, mouse and rat ventricular tissue samples, or from NRCM. The mRNA in 1 µg of total RNA was transcribed into cDNA using the first strand cDNA synthesis kit (Fermentas) according to the manufacturer's protocol and mRNA content was quantified by real-time PCR from reverse-transcribed cDNA samples using the Light Cycler System (Roche). Specificity of the reactions was confirmed using a dissociation protocol after each cycle, and comparison of the results with the expected melting-point temperature of the amplicon, as well as by verification of the expected size of the product. PCR amplifications were carried out in duplicates, using the following conditions: 10 minutes at 95°C, followed by a total of 35-45 three temperature cycles (10 sec at 95°C, 10 seconds at 55°C and 25 sec at 72°C), and 5 min at 72°C. The primers that were used are listed in **Supplementary Table 1**. Target genes were normalized using oligonucleotide primers for either, human porphobilinogen deaminase (PBGD), ribosomal protein L10 (RPL10) or rat hydroxymethylbilanesynthetase (HMBS) as an internal standard.

Supplementary Table 1. Primers

Human		
Gene	**Forward**	**Reverse**
NDPK-A	CAACTGTGAGCGTACCTTC	GTCTTCACCACATTCAGCC
NDPK-B	TGAAGCAGCACTACATTGAC	GGCTGATTTCTTTTTCAGCAC
NDPK-C	AGGTTGGCAAGAACCTGATTCAC	CCTGTACGCCAGGGATTG
Rat		
Gene	**Forward**	**Reverse**
NDPK-B	TGAAAGACCGTCCTTTCTTCC	CTCTCCACTGAATCACTGCC
NDPK-C	CAGTGATTCGGTGGAAAGTG	AAATAGAGGGTGGGATGTGG

Generation of recombinant adenoviruses

EGFP and Human cDNA of NDPK-A, NDPK-B and Flag-NDPK-C were subcloned in the pAdTrack or pIRES shuttle vector, and recombinant adenoviruses were generated using the

AdEasy system, as described.[3, 4] The adenoviruses encoding EGFP, $G\beta_1\gamma_2$ ($G\beta\gamma$) and LacZ have previously been described.[5, 6]

Transfection and transduction

NRCM or MEFs were infected with recombinant adenoviruses at the indicated MOI under serum-free conditions 48 hours after plating, and were subsequently cultured supplemented with 4% FCS for 48 hours. Knockdown of NDPK-C was performed using siRNA against rat NDPK-C (GCTTTGAAAGGAAGGGCTT) for 96 hours. Control siRNA was a scrambled siRNA (Ambion).[5] ARCM were infected 1 hour after plating and further cultured for 24 hours in a HEPES-modified medium 199 (M199, Sigma S7528, supplemented with 5 mmol/L taurine, 5 mmol/L carnitine, 5 mmol/L creatine, 5 mmol/L N-mercaptoproprionyl glycine, 0.1 µmol/L insulin, 10,000 U/mL penicillin and 10 mg/mL streptomycin, pH 7.25).

Immunoblot analysis and co-immunoprecipitation

Preparation of whole cell lysates, membrane fractions and zebrafish lysates has been described previously.[7, 8] Co-immunoprecipitation and immunoblotting were performed according to standard procedures, as described,[7, 8] by using specific antibodies against the Flag epitope (F3165, Sigma), $G\alpha_{s,olf}$ (C-18, Santa Cruz), $G\alpha_{i2}$ (T-19, Santa Cruz), $G\alpha_q$ (C-19, Santa Cruz), $G\beta_1$ (sc-379, Santa Cruz), $G\beta$ (T-20, Santa Cruz), zebrafish NDPK B (L-16, Santa Cruz), mammalian NDPK-B (MC-412, Kamiya, or a custom antibody generously provided by Edith Postel, Robert Wood Johnson Medical School, New Brunswick, NJ),[9] NDPK-C (PAB2028, Abnova, or a custom antibody generously provided by Ioan Lascu, University Bordeaux Segalen, France),[10] β-actin (AC-74, Sigma), GAPDH (5G4, HyTest), pan-cadherin (C1821, Sigma) and γ-tubulin (4D11, Thermo Scientific). Detection was performed with suitable secondary antibodies and an enhanced chemiluminescence (ECL) reagent (Thermo Scientific). Protein bands were visualized with a FluorS-MultImager (Bio-

Rad) or a ChemoCam Imager (Intas) and quantified using ImageJ software. The signal intensity of the indicated loading control was used for normalization. For calibration of the amounts of $G\alpha_s$ or $G\alpha_{i2}$ in Western blots, fluorescent secondary antibodies were used and visualized using Odyssey CLx (Licor).

Preparation of membrane fractions

Preparation of highly-purified sarcolemma from ventricular tissue samples obtained from human HF and NF patients was performed as previously described.[8] NRCM or MEFs were homogenized twice for 15 seconds in a buffer containing 10 mmol/L Tris-HCl, pH 7.4 and 1 mmol/L EDTA with a homogenizer at 20,000 rpm interrupted by a 30 seconds cooling period on ice. To obtain membrane fractions, the homogenate was cleared from debris and nuclei by centrifugation at 600 g for 5 minutes. Subsequently, membranes were pelleted at 100,000 g for 30 minutes at 4°C.

Far Western blotting

Purified heterotrimeric G protein transducin $G_t\alpha\beta\gamma$, recombinant NDPK-B, or NDPK-C (Genway) were spotted on a nitrocellulose membrane and blocked in Roti-Block® for 1 hour at room temperature. The membrane was incubated with 200 µg of cell lysate obtained from MEFs or NRCM infected with either Ad-EGFP (MOI 100), Ad-NDPK-B (MOI 100), or Ad-NDPK-B (MOI 100) plus NDPK-C with increasing MOIs (3-100), for 2 hours. For interaction assay between $G\alpha_s$ or $G\alpha_{i2}$ and NDPK-C, the membrane was incubated with a total amount of 150 µg cell lysate obtained from different combinations of NRCM infected with Ad-EGFP (MOI 50), Ad-$G\alpha_s$ (MOI 50), Ad-$G\alpha_{i2}$ (MOI 50), or Ad-$G\beta_1\gamma_2$ (MOI 50). The membrane was washed 9 times for 10 minutes and followed by Western blot procedures. To estimate the amount of $G\alpha_s$ and $G\alpha_{i2}$ that interact with NDPK-C, 1000 ng, 500 ng, 80 ng or 50 ng of

recombinant $G\alpha_s$ and $G\alpha_{i2}$ (Calbiochem) were blotted and a standard curve was determined based on the integrated density of the bands.

ATP hydrolysis and GTP hydrolysis assays

The assays were performed as previously described.[11-13] In brief, 1 µmol/L purified heterotrimeric G protein transducin $G_t\alpha\beta\gamma$, 0.5 - 2 µmol/L recombinant NDPK-B or 0.25 - 0.5 µmol/L recombinant NDPK-C (Genway) were incubated in the reaction buffer containing 0.5 mmol/L $MgCl_2$, 0.1 mmol/L EGTA, 1 mmol/L dithiothreitol and 0.2% (w/v) bovine serum albumin in 50 mmol/L triethanolamine/HCL, pH 7.4. For ATP hydrolysis, 0.5 µmol/L [γ-^{32}P]ATP (0.1 µCi/tube), 50 µmol/L ATP, and 0.5 µmol/L GDP were added to the reaction buffer. For GTP hydrolysis 0.5 µmol/L [γ-^{32}P]GTP (0.1 µCi/tube) and 50 µmol/L GTP were added to the reaction buffer. To inhibit the effect of NDPKs on ATP hydrolysis, 500 µmol/L UDP was added to the reaction tubes, as described.[14] The reaction tubes were incubated for 40 minutes at 30°C. The reaction was stopped by adding ice cold sodium phosphate buffer (20 mmol/L, pH 2.0) with 5% (w/v) activated charcoal followed by centrifugation at 10,000 g for 10 min. The amount of [^{32}P]P_i release was determined in 500 µL of the supernatant in 3 mL water using the Tri-Carb liquid scintillation analyzer (Packard).

Visualization of the subcellular localization of NDPK C

NRCM and ARCM were cultured with serum free medium on coverslips for 12 hours and infected with Ad-Flag-NDPK-C at a MOI of 50. 24 hours later, the cells were stimulated with solvent or 1 µmol/L ISO for up to 6 hours. The medium was withdrawn and the cells were washed twice with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde for 10 minutes. After fixation, cells were permeabilized with 0.1% Triton X-100 in PBS for 10 minutes, treated with Roti-Block (Roth) for 1 hour and incubated with the anti-Flag antibody at a dilution of 1:1000 in PBS containing 0.1% Tween 20 (Tween-PBS) for 3 hours at RT.

The coverslips were washed 3 times with Tween-PBS and incubated with the Cy3 labeled secondary antibody (1:200 dilution, Dianova) for 1 hour in a humidified chamber protected from light. The coverslips were again washed twice with Tween-PBS and mounted with Vectashield containing DAPI. The subcellular localization of NDPK-C was subsequently visualized by confocal fluorescence microscopy at the Nikon Imaging Center, University of Heidelberg, Heidelberg, Germany.

Measurement of intracellular cAMP

Generation of cAMP in NRCM or Zebrafish lysate was assayed as previously described using a cAMP immunoassay (Assay Designs or Cayman Chemical).[5, 7, 15] cAMP was also assayed in living ARCM that were isolated as previously described, plated on laminin-coated glass coverslips and transduced with Epac2-camps adenovirus[6] at MOI 300 together with either a control (LacZ) or a Flag-NDPK-C-encoding adenovirus at the same MOI. 48 hours later, cells were washed once and maintained in a physiological buffer containing 144 mmol/L NaCl, 5.4 mmol/L KCl, 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, and 20 mmol/L HEPES, pH 7.4 at room temperature, placed on a Zeiss Axio Observer A1 microscope equipped with Plan-Apochromat 63x/1.4 oil immersion objective, Polychrome V light source, DV2 DualView beam splitter and CoolSNAP-HQ2 CCD-camera (Visitron Systems, Puchheim, Germany). Cells were stimulated with ISO, forskolin and 3-isobutyl-1-methylxanthine (IBMX; all from Sigma). The YFP/CFP emission ratio was monitored upon 436 nm excitation (filters YFP 535 ± 15 nm, CFP 480 ± 20 nm). After each measurement, emission values were corrected for bleed-through of CFP into YFP channel and for photo-bleaching as described.[16] The imaging data were acquired using the VisiView software (Visitron) and analyzed with Excel and Origin 8.5 (OriginLab) packages.

Fractional shortening in ARCM

ARCM were infected with Ad-EGFP or Ad-Flag-NDPK C at a MOI of 300 for 24 hours. Thereafter, sarcomere shortening was assessed on field stimulation (1 Hz with 4 ms duration, 20 V bipolar) using a video-based sarcomere length detection system (IonOptix) at 1 mmol/L Ca^{2+} in the bath solution. Myocytes were exposed to increasing concentrations of ISO (0.1 to 300 nmol/L). Sarcomere length measurements were performed at room temperature on intact, rod-shaped cells, which had no spontaneous contractions or microblebs, as previously described.[17]

Zebrafish maintenance, MO-injection, measurement of fractional shortening and histology

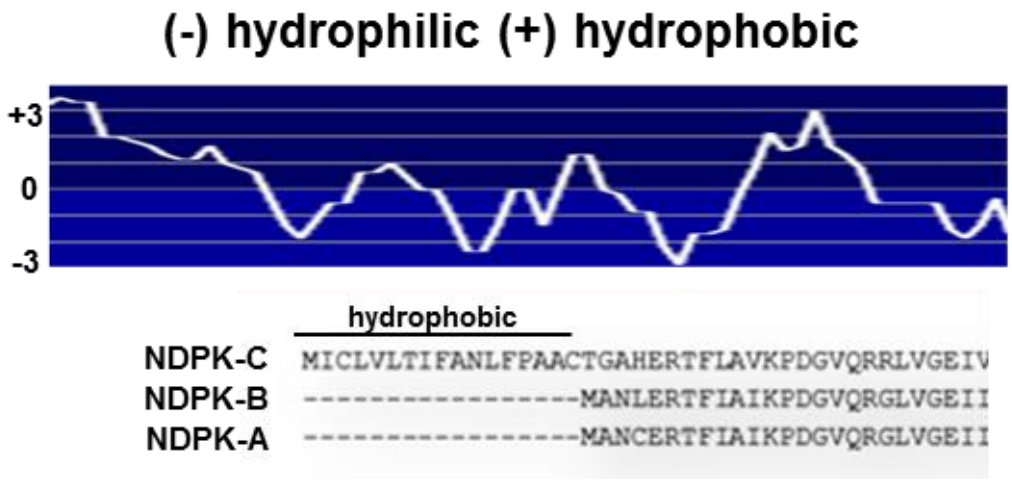
Zebrafish danio rerio (TE4/6 strain) were maintained under standard conditions.[17] Morpholino-modified oligonucleotides (Gene Tools, USA) were directed against the splice-donor site of exon2 (MO-NDPK-C: 5'-ATGCTGTATTAGGGTTCTCACCTGC) of zebrafish NDPK-C or against the translation start site of zebrafish NDPK-B (5'-GGTGCGCTCGGTCTTAGCAGACATG).[7] Embryos at one-cell stage were injected with the indicated amounts of morpholino or a standard control oligonucleotide (MO-Ctl, Genetools, LCC) at the same concentration. For rescue experiments, embryos were injected with NDPK-C morpholino and/or NDPK-B mRNA (130 ng/µL) and phenotypic abnormalities (pericardial edema, macroscopic morphological abnormalities, and/or macroscopic cardiac dysfunction; **Supplementary Figure 7**) were assessed. Injections and analyses were performed as previously described.[7] Still images and video films were recorded and digitized with a Zeiss microscope/MCU II. Fractional shortening (FS) and ventricular diameters were assayed as described. For sectioned hematoxylin/eosin- and whole mount immunostainings, embryos were fixed in 4% paraformaldehyde. Sections were stained with or incubated with the indicated antibody (S46; MF20), as described.[17] For electron microscopy embryos were pre-fixed with 3% glutaraldehyde and 0.1% picric acid in 0.1 mol/L sodium cacodylate, and

post-fixed in 1% OsO_4. Samples were embedded in Epon (Serva) according to standard procedure and electron micrographs of fish embryos were obtained as described.[17]

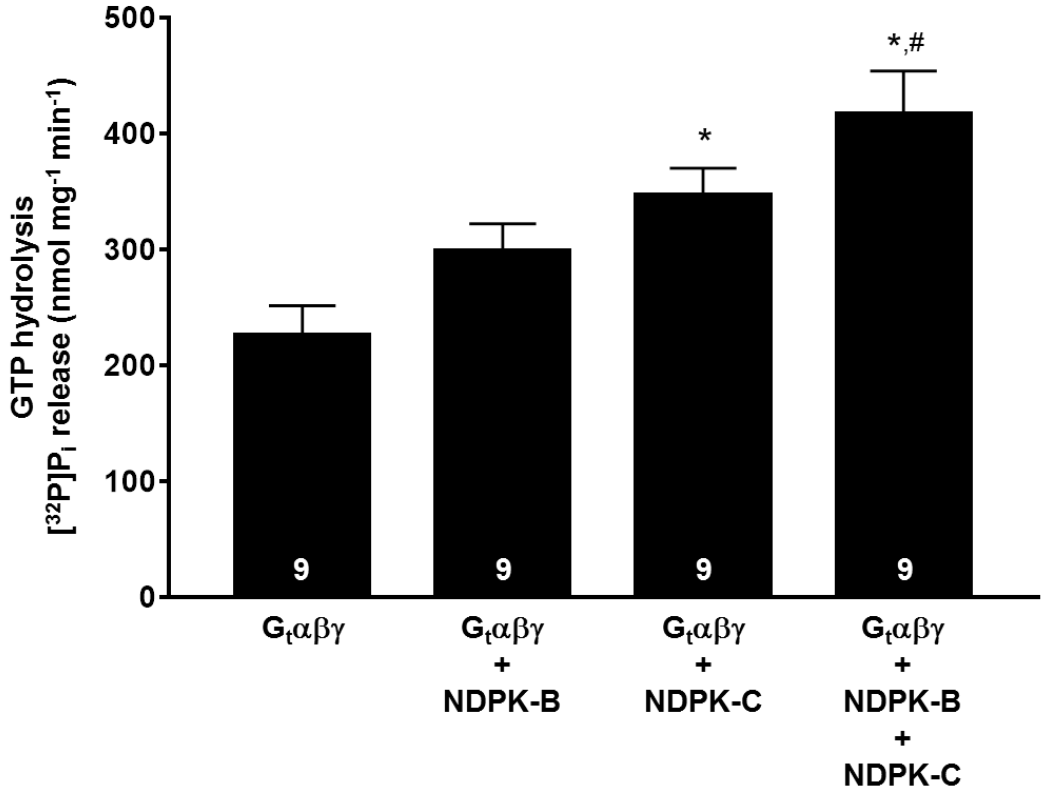
Chronic isoprenaline administration, echocardiography and collagen staining in control and NDPK-B knock-out mice

The study was performed with 2 or 5 months old male C57Bl/6 NDPK-$B^{-/-}$ and NDPK-$B^{+/+}$ littermates.[18] ISO (Sigma-Aldrich) was delivered to 2 months old mice through subcutaneously implanted osmotic minipumps (Alzet, model 1007D) that released ISO in 0.9% NaCl at a dose of 30 mg/kg/d.[19] For the procedure animals were anesthetized with 2% isoflurane inhalation and received carprofen (5 µg/kg s.c.) as analgesic. All procedures regarding care and use of animals were in accordance with institutional guidelines and authorized by the Regierungspräsidium Karlsruhe, Germany (AZ: G-12\10). After 7 days cardiac function was monitored by 2D echocardiography (Sonos 5500) on conscious mice, as described previously.[20] M-mode tracings were used to measure left ventricular internal end-diastolic (LVEDD) and end-systolic diameters (LVESD). The % fractional shortening (FS) was calculated using FS (%) = ((LVEDD-LVESD)/LVEDD) * 100. Animals were subsequently sacrificed and hearts were harvested for further analysis. Middle transverse heart sections (1 mm) were fixed in 4% formaldehyde (4°C, overnight) and embedded in paraffin. Deparaffination, rehydration and staining of 10 µm sections were performed according to the manufacturer's instructions (Sirius Red/Fast Green Collagen Staining Kit, Chondrex). Collagen content was determined by ImageJ and normalized to the total tissue area.

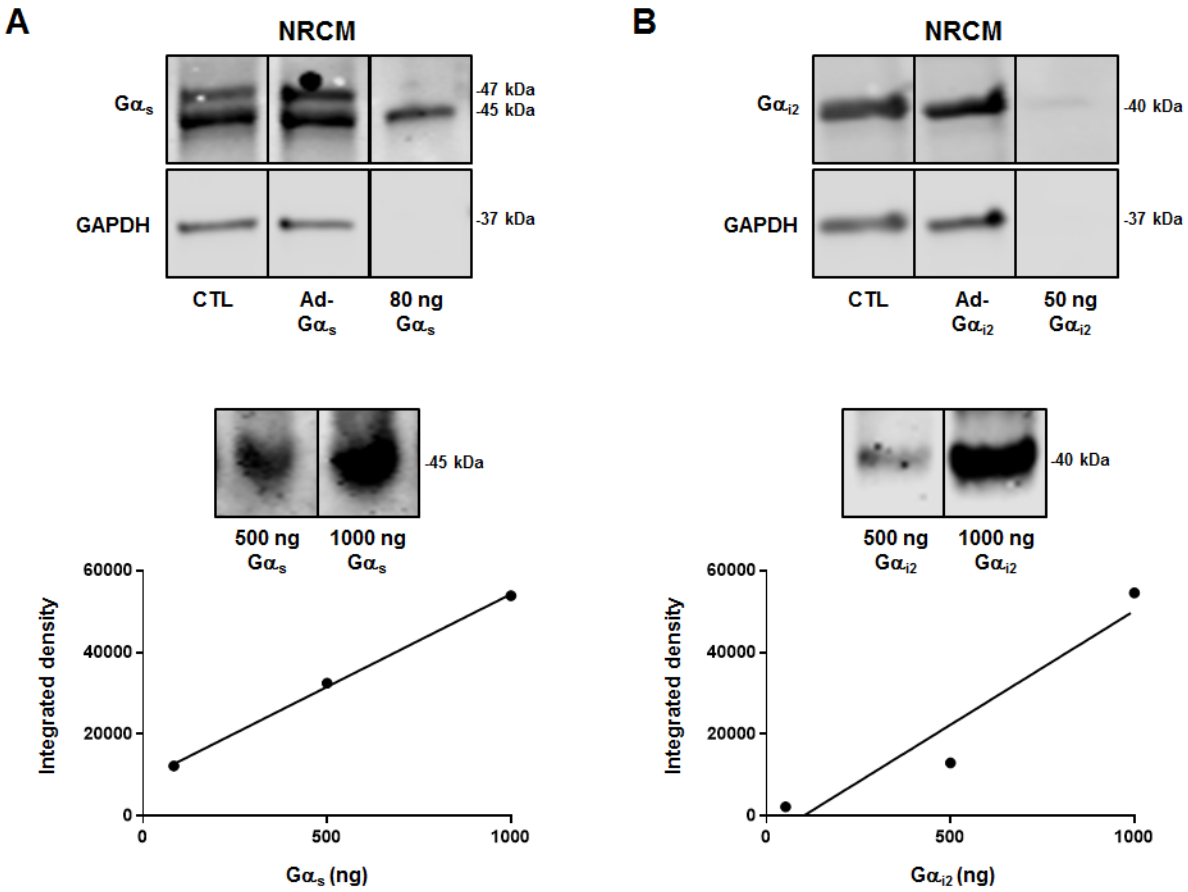
Supplementary Figures



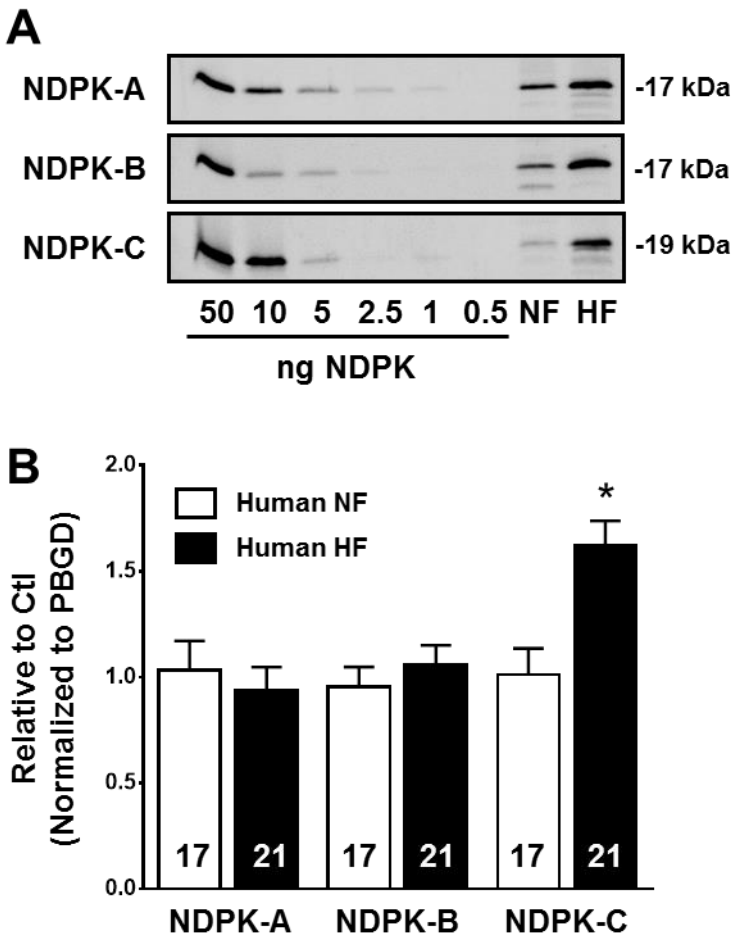
Supplementary Figure 1. Kyte-Doolitle plot of NDPK-C and sequence alignment of N-terminal domains of NDPK-A, NDPK-B and NDPK-C showing the hydrophobicity of the NDPK-C N-terminal domain.



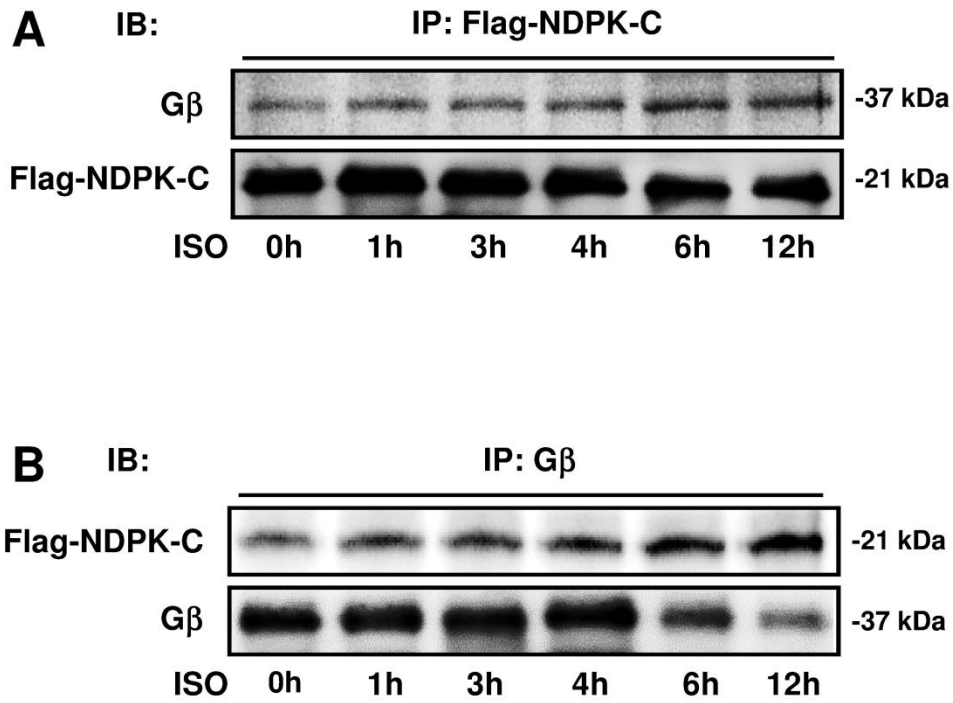
Supplementary Figure 2. NDPK-C regulates GTPase activity of the heterotrimeric G protein transducin ($G_t\alpha\beta\gamma$). GTP hydrolysis was determined by the amount of $[^{32}P]P_i$ release in the presence of 1 µmol/L $G_t\alpha\beta\gamma$, $G_t\alpha\beta\gamma + 2$ µmol/L NDPK-B, $G_t\alpha\beta\gamma + 0.5$ µmol/L NDPK-C, or $G_t\alpha\beta\gamma$ + NDPK-B + NDPK-C. *P<0.05 vs. $G_t\alpha\beta\gamma$; $^{\#}$P<0.05 vs. $G_t\alpha\beta\gamma$ + NDPK-B.



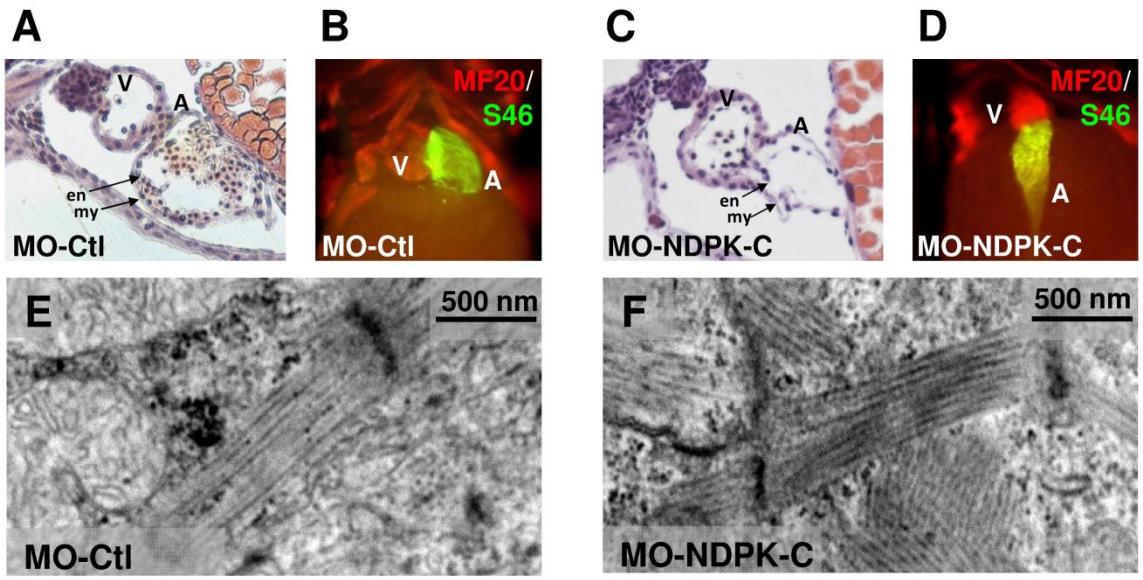
Supplementary Figure 3. Calibration of the amounts of $G\alpha_s$ or $G\alpha_{i2}$ in Western blots of NRCM lysates. A, Representative Western blots of $G\alpha_s$ and GAPDH in control (CTL) NRCMs, NRCMs overexpressing $G\alpha_s$ and 80 ng recombinant $G\alpha_s$ (top) and Western blot of 500 ng or 1000 ng recombinant $G\alpha_s$ (middle) that were employed to create a calibration curve for $G\alpha_s$ (bottom). **B,** Similar to panel A for $G\alpha_{i2}$.



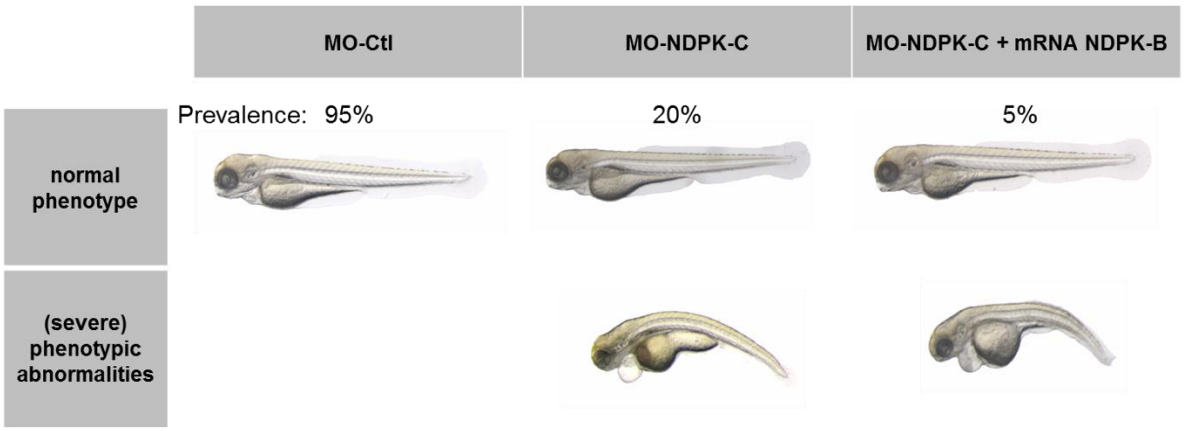
Supplementary Figure 4. NDPK-C expression is increased in heart failure (HF). **A,** Representative Western blots of increasing amounts of purified NDPK-A, -B, or -C and plasmalemmal fractions of ventricular samples obtained from hearts explanted from patients with end-stage HF or non-failing (NF) donor hearts. **B,** mRNA content of NDPK-A, -B , and -C in ventricular samples from patients with HF or NF controls. All heart samples were obtained from the patient cohort described in Lutz et al.[1]



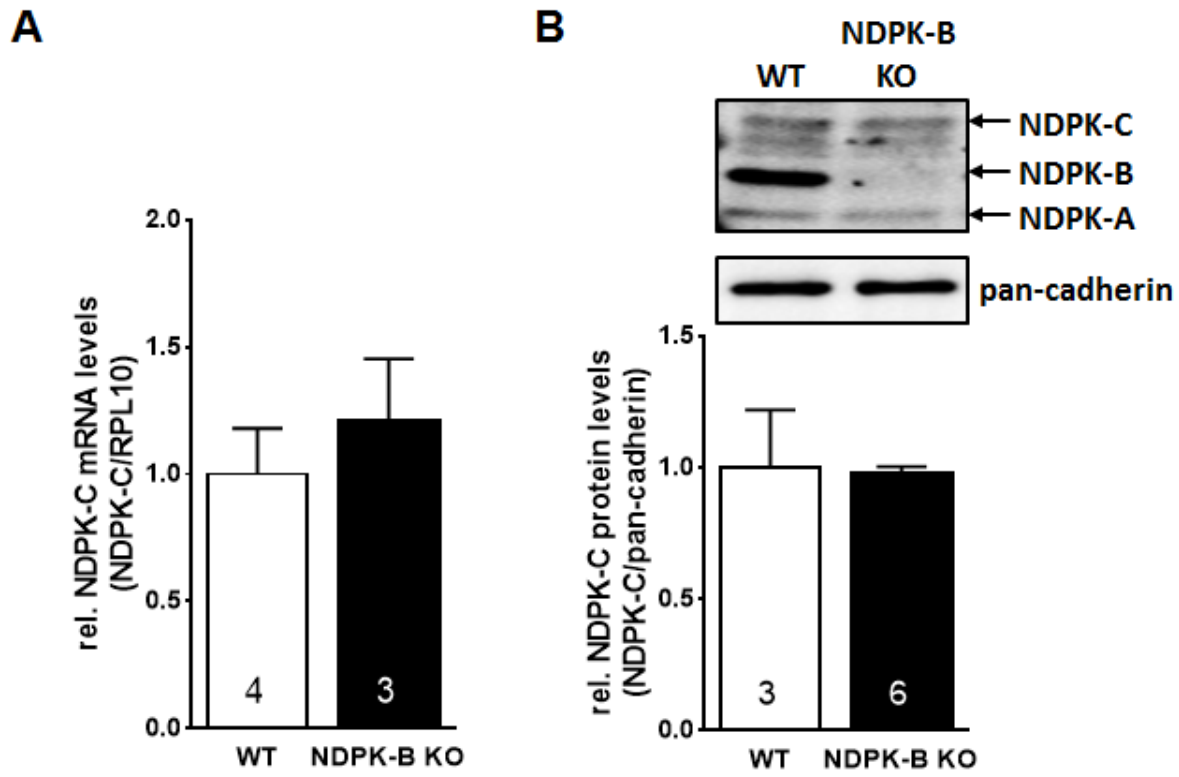
Supplementary Figure 5. NDPK-C/Gβ interaction is enhanced following ISO stimulation. **A,** Immunoprecipitation of Flag-NDPK-C in lysates obtained from NRCM infected with Ad-Flag-NDPK-C (MOI=500) after stimulation with 1.0 μmol/L ISO for the indicated time. **B,** Similar to panel A for Flag-NDPK-C co-immunoprecipitating with Gβ. Co-immunoprecipitation was detected using antibodies against Flag-NDPK-C and Gβ.



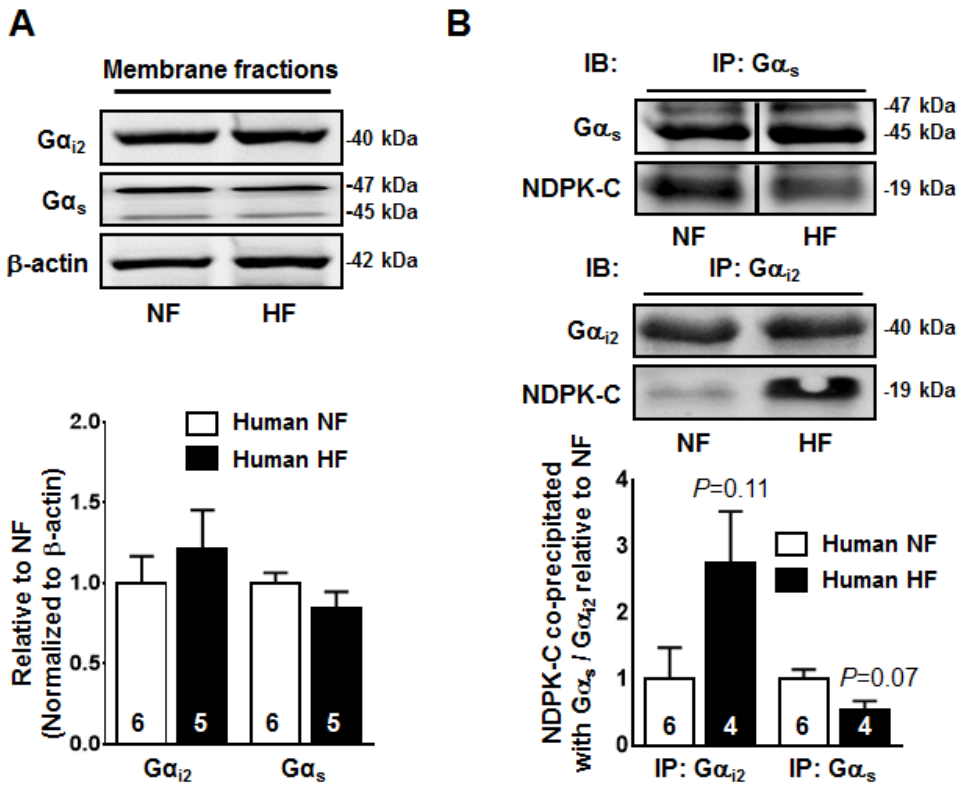
Supplementary Figure 6. Structural properties of zebrafish hearts. **A,C,** Histology sections of a representative control morpholino-injected zebrafish heart **(A)** and a zebrafish heart with morpholino-induced NDPK-C knockdown **(C)** stained with hematoxylin/eosin. Morphants display normal heart morphology with distinct endocardial and myocardial cell layers in atrium and ventricle. A, atrium, V, ventricle, en, endocardial layer, my, myocardial layer. **B,D,** Whole mount immunofluorescence following staining with specific antibodies against atrial myosin heavy chain (S46) and entire heart tube myosin heavy chain (MF20) showing unaltered expression of structural proteins in control (**B**) or NDPK-C knockdown (**D**) zebrafish hearts. **E,F,** Transmission electron microscopy of a control **(E)** and a NDPK-C knockdown **(F)** heart at 72 hpf. The sarcomeres of cardiomyocytes of NDPK-C morphant hearts show no ultrastructural differences.



Supplementary Figure 7. Lateral view of representative zebrafish larvae injected with control morpholino (MO-Ctl), with NDPK-C morpholino (MO-NDPK-C), or with MO-NDPK-C with concomitant increase in NDPK-B expression (MO-NDPK-C + mRNA NDPK-B) at 72 hours post fertilization exhibiting a normal phenotype (top row) or phenotypic abnormalities (bottom row).



Supplementary Figure 8. Ablation of NDPK-B has no impact on NDPK-C mRNA or protein levels. **A**, quantitative PCR of NDPK-C transcripts normalized to RPL10. Values are given relative to wildtype (WT) control. **B**, representative immunoblot of NDPK-A, B, and C and quantification of NDPK-C expression levels normalized to pan-cadherin. Lysates prepared from ventricular myocardium were analyzed by immunoblot and probed with an antibody detecting indicated isoforms of NDPKs. Values are given relative to WT control.



Supplementary Figure 9. Expression and interaction of NDPKs and G proteins in human end-stage heart failure (HF). **A,** Representative Western blots (top), and quantification of protein expression (bottom) of $G\alpha_{i2}$ and $G\alpha_s$ in ventricular membrane fractions obtained from explanted hearts from patients with end-stage HF or non-failing (NF) control donor hearts. β-actin served as loading control. The bar chart is also shown in **Figure 8A**. **B,** Immunoprecipitation of $G\alpha_s$ or $G\alpha_{i2}$ in ventricular lysates obtained from HF patients or NF donor hearts and quantification of immunoprecipitated NDPK-C, relative to NF controls. Numbers in bars indicate number of hearts. From one HF sample not enough lysate could be obtained for immunoprecipitation.

Supplementary Videos

Supplementary Video 1. Control morpholino-injected zebrafish embryo at 72 hpf. After 72 hours of development, cardiac chambers contract vigorously in wild-type (WT) zebrafish embryos.

Supplementary Video 2. NDPK-C knockdown zebrafish embryo at 72 hpf. NDPK-C expression was inhibited by injection of Morpholino-modified antisense oligonucleotides (MO) against NDPK-C. After 72 h of development, MO-NDPK-C injected embryos suffer from severely impaired cardiac contractility of the ventricle.

References

1. Lutz S, Mura R, Baltus D, Movsesian M, Kubler W, Niroomand F. Increased activity of membrane-associated nucleoside diphosphate kinase and inhibition of cAMP synthesis in failing human myocardium. *Cardiovasc Res*. 2001;49:48-55
2. El-Armouche A, Gocht F, Jaeckel E, Wittkopper K, Peeck M, Eschenhagen T. Long-term beta-adrenergic stimulation leads to downregulation of protein phosphatase inhibitor-1 in the heart. *Eur J Heart Fail*. 2007;9:1077-1080
3. He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B. A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci U S A*. 1998;95:2509-2514
4. Luo J, Deng ZL, Luo X, Tang N, Song WX, Chen J, Sharff KA, Luu HH, Haydon RC, Kinzler KW, Vogelstein B, He TC. A protocol for rapid generation of recombinant adenoviruses using the AdEasy system. *Nat Protoc*. 2007;2:1236-1247
5. Hippe HJ, Luedde M, Lutz S, Koehler H, Eschenhagen T, Frey N, Katus HA, Wieland T, Niroomand F. Regulation of cardiac cAMP synthesis and contractility by nucleoside diphosphate kinase B/G protein βγ dimer complexes. *Circ Res*. 2007;100:1191-1199
6. Nikolaev VO, Moshkov A, Lyon AR, Miragoli M, Novak P, Paur H, Lohse MJ, Korchev YE, Harding SE, Gorelik J. β2-adrenergic receptor redistribution in heart failure changes cAMP compartmentation. *Science*. 2010;327:1653-1657
7. Hippe HJ, Wolf NM, Abu-Taha I, Mehringer R, Just S, Lutz S, Niroomand F, Postel EH, Katus HA, Rottbauer W, Wieland T. The interaction of nucleoside diphosphate kinase B with Gβγ dimers controls heterotrimeric G protein function. *Proc Natl Acad Sci U S A*. 2009;106:16269-16274
8. Lutz S, Hippe HJ, Niroomand F, Wieland T. Nucleoside diphosphate kinase-mediated activation of heterotrimeric G proteins. *Methods Enzymol.* 2004;390:403-418
9. Feng Y, Gross S, Wolf NM, Butenschon VM, Qiu Y, Devraj K, Liebner S, Kroll J, Skolnik EY, Hammes HP, Wieland T. Nucleoside diphosphate kinase B regulates angiogenesis through modulation of vascular endothelial growth factor receptor type 2 and endothelial adherens junction proteins. *Arterioscler Thromb Vasc Biol*. 2014;34:2292-2300
10. Erent M, Gonin P, Cherfils J, Tissier P, Raschellà G, Giartosio A, Agou F, Sarger C, Lacombe ML, Konrad M, Lascu I. Structural and catalytic properties and homology modelling of the human nucleoside diphosphate kinase C, product of the DRnm23 gene. *Eur J Biochem.* 2001;268:1972-1981
11. Aktories K, Schultz G, Jakobs KH. Islet-activating protein impairs α_2-adrenoceptor-mediated inhibitory regulation of human platelet adenylate cyclase. *Naunyn Schmiedebergs Arch Pharmacol*. 1983;324:196-200
12. Gierschik P, Bouillon T, Jakobs KH. Receptor-stimulated hydrolysis of guanosine 5'-triphosphate in membrane preparations. *Methods Enzymol*. 1994;237:13-26
13. Klinker JF, Seifert R, Damm H, Rommelspacher H. Activation by β-carbolines of G-proteins in HL-60 membranes and the bovine retinal G-protein transducin in a receptor-independent manner. *Biochem Pharmacol*. 1997;53:1621-1626
14. Mourad N, Parks RE, Jr. Erythrocytic nucleoside diphosphokinase. II. Isolation and kinetics. *J Biol Chem*. 1966;241:271-278
15. Hippe HJ, Lutz S, Cuello F, Knorr K, Vogt A, Jakobs K, Wieland T, Niroomand F. Activation of heterotrimeric G proteins by a high energy phosphate transfer via nucleoside diphosphate kinase (NDPK) B and Gβ subunits. Specific activation of Gsα by an NDPK B.Gβγ complex in H10 cells. *J Biol Chem*. 2003;278:7227-7233
16. Borner S, Schwede F, Schlipp A, Berisha F, Calebiro D, Lohse MJ, Nikolaev VO. FRET measurements of intracellular cAMP concentrations and cAMP analog permeability in intact cells. *Nat Protoc*. 2011;6:427-438

17. Rottbauer W, Wessels G, Dahme T, Just S, Trano N, Hassel D, Burns CG, Katus HA, Fishman MC. Cardiac myosin light chain-2: a novel essential component of thick-myofilament assembly and contractility of the heart. *Circ Res*. 2006;99:323-331
18. Di L, Srivastava S, Zhdanova O, Sun Y, Li Z, Skolnik EY. Nucleoside diphosphate kinase B knock-out mice have impaired activation of the K^+ channel KCa3.1, resulting in defective T cell activation. *J Biol Chem*. 2010;285:38765-38771
19. El-Armouche A, Wittkopper K, Degenhardt F, Weinberger F, Didie M, Melnychenko I, Grimm M, Peeck M, Zimmermann WH, Unsold B, Hasenfuss G, Dobrev D, Eschenhagen T. Phosphatase inhibitor-1-deficient mice are protected from catecholamine-induced arrhythmias and myocardial hypertrophy. *Cardiovasc Res*. 2008;80:396-406
20. Backs J, Backs T, Neef S, Kreusser MM, Lehmann LH, Patrick DM, Grueter CE, Qi X, Richardson JA, Hill JA, Katus HA, Bassel-Duby R, Maier LS, Olson EN. The delta isoform of CaM kinase II is required for pathological cardiac hypertrophy and remodeling after pressure overload. *Proc Natl Acad Sci U S A*. 2009;106:2342-2347