The interaction of nucleoside diphosphate kinase B with G$\beta\gamma$ dimers controls heterotrimeric G protein function

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Heterotrimeric G proteins in physiological and pathological processes have been extensively studied so far. However, little is known about mechanisms regulating the cellular content and compartmentalization of G proteins. Here, we show that the association of nucleoside diphosphate kinase B (NDPK B) with the G protein $\beta\gamma$ dimer (G$\beta\gamma$) is required for G protein function in vivo. In zebrafish embryos, morpholino-mediated knockdown of zebrafish NDPK B, but not NDPK A, results in a severe decrease in cardiac contractility. The depletion of NDPK B is associated with a drastic reduction in G$\beta_1\gamma_2$ dimer expression. Moreover, the protein levels of the adenylyl cyclase (AC)-regulating Gα_s and Gα_i subunits as well as the caveolae scaffold proteins caveolin-1 and -3 are strongly reduced. In addition, the knockdown of the zebrafish Gβ_1 orthologs, Gβ_1 and Gβ_{1like}, causes a cardiac phenotype very similar to that of NDPK B morphants. The loss of Gβ_1/Gβ_{1like} is associated with a down-regulation in caveolins, AC-regulating Gα-subunits, and most important, NDPK B. A comparison of embryonic fibroblasts from wild-type and NDPK A/B knockout mice demonstrate a similar reduction of G protein, caveolin-1 and basal cAMP content in mammalian cells that can be rescued by re-expression of human NDPK B. Thus, our results suggest a role for the interaction of NDPK B with G$\beta\gamma$ dimers and caveolins in regulating membranous G protein content and maintaining normal G protein function in vivo.

cAMP | cardiac contractility | G proteins | NDPK | zebrafish

Signaling through the activation of G proteins represents the most widely used signaling pathway in mammalian biology (1). A variety of G protein-coupled receptors (GPCRs) mediate extracellular signals via heterotrimeric G proteins, which are composed of a guanine nucleotide binding α-subunit (Gα), as well as a β-subunit (Gβ) and a γ-subunit (Gγ). Upon GPCR activation, the bound GDP in Gα is exchanged for GTP and both the GTP-liganded Gα and the stable dimer G$\beta\gamma$ regulate downstream effectors (2).

Nucleoside diphosphate kinases (NDPKs), which catalyze the transfer of γ-phosphate between NTPs and NDPs, represent a family of multifunctional proteins encoded by nine human nm23 genes. The two major isoforms, NDPK A and B (17–21 kDa), play crucial roles in a wide array of cellular processes [for review see (3)]. Despite their high sequence homology and the well known formation of heterohexamers to perform their house keeping enzyme activity (4), NDPK A and B have distinct cellular functions, which are based on the possibility of both isoforms contributing to multimeric protein complexes like the SET complex (5) and a complex formed with Ca^{2+}-activated potassium channel KCa3.1 (6). In such complexes, NDPK not only supplies NTPs but also acts as protein kinase (6). We have previously shown that NDPK B, but not NDPK A, forms a complex with G$\beta\gamma$ (7, 8) and acts as a histidine kinase for Gβ. The high energetic phosphate on Gβ can be specifically transferred to GDP and the GTP that is formed, induces G protein activation (3). Recent in vitro findings suggest that this phosphorelay can regulate G_s protein activity independently of GPCRs in cardiomyocytes, thereby stimulating cAMP synthesis and contractility (9). However, data supporting a role of the NDPK B/G$\beta\gamma$ complex in G protein signaling in vivo are still missing.

Therefore, we used the zebrafish model to selectively decrease the expression of NDPK A, B and the zebrafish Gβ_1 isoforms by injection of morpholino-modified antisense oligonucleotides. In addition, we analyzed embryonic fibroblasts (MEFs) from NDPK A and B double knockout mice to explore the function of the NDPK B/G$\beta\gamma$ complex in mammals. We report that heterotrimeric G proteins and NDPK B depend on each other in the maintenance of their membranous protein content and additionally regulate caveolin expression. Apparently, NDPK B is an essential partner of heterotrimeric G proteins, indispensable for intact G protein content and function in vivo.

Results

Morpholino-Mediated Knockdown of the Zebrafish NDPK A and B. To investigate the role of the NDPK B/G$\beta\gamma$ complex in vivo, we used antisense-morpholino oligonucleotides (MO) to inhibit the gene expression of the two major NDPK isoforms in zebrafish embryos. Zebrafish NDPK B (NDPK Z1) and NDPK A (NDPK Z2) exhibit 85% and 77% amino acid sequence identity to their human orthologs (Fig. S1*A*), and are thus evolutionarily conserved. Specific MOs, targeted against the translational start site of zebrafish NDPK B or NDPK A, were injected into the embryo at one-cell-stage. The resulting morphants were analyzed at 48–72 h post-fertilization (hpf). Wild-type (WT) embryos and embryos injected with a standard control morpholino (MO-Ctrl) served as controls. As shown in Fig. 1*A*, injection of both, MO-NDPK A and MO-NDPK B strongly reduced total NDPK content. As an antibody specifically recognizing zebrafish NDPK A is not available, we analyzed total NDPK expression by an antibody recognizing both isoforms. Using an NDPK B specific antibody, the decreased NDPK B expression by MO-NDPK B injection could be demonstrated, while MO-NDPK A did not

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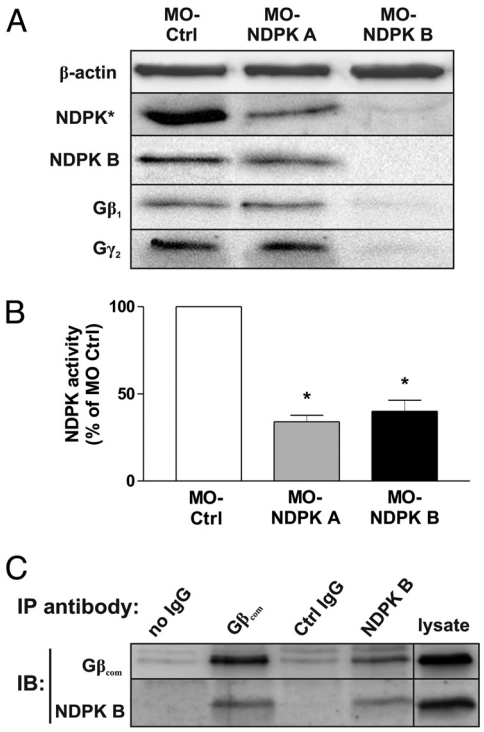

Fig. 1. NDPK A and B depletion in zebrafish embryos. Zebrafish embryos were injected with MO-Ctrl (4 ng), MO-NDPK A (8 ng) and MO-NDPK B (4 ng). (*A*) Immunoblot analysis of lysates prepared 72 hpf using specific antibodies against total fish NDPK (NDPK*), NDPK B, Gβ_1 and Gγ_2. β-actin served as loading and specificity control. (*B*) Relative NDPK activity (normalized to MO-Ctrl) was quantified as formation of ^{3}H-GTP from ^{3}H-GDP and ATP in lysates of embryos injected as indicated. Data are means ± SEM, $n = 3$, *, $P <$ 0.05 vs. MO-Ctrl. (*C*) Co-immunoprecipitation of NDPK B and Gβ in zebrabrafish lysates. Endogenous Gβ or NDPK B was immunoprecipitated (IP) from zebrafish lysates. Association of NDPK B or Gβ was detected by immunoblotting (IB). Representative immunoblots are shown.

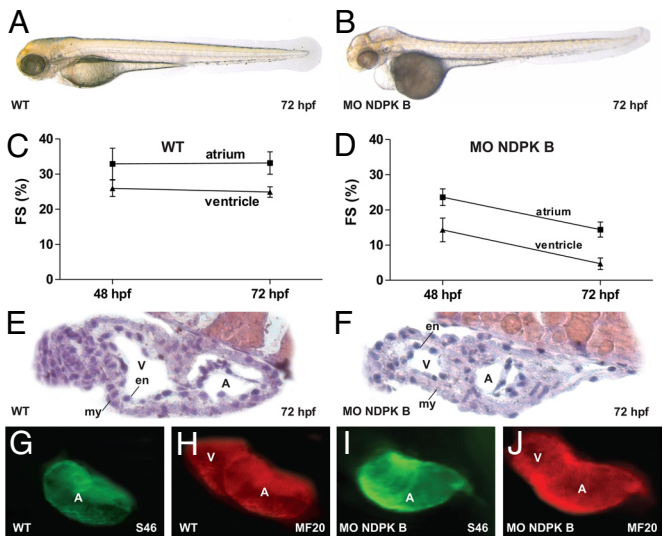

Fig. 2. Cardiac dysfunction in NDPK B-depleted zebrafish embryos. Cardiac phenotype of wild-type (WT) (*A*) and NDPK B knockdown (*B*) embryos at 72 hpf. Fractional shortening (FS) of the atrial and ventricular chamber of WT (*C*) and NDPK B morphants (*D*) at 48 and 72 hpf. Data are means ± SEM, $n = 11$–21. Histology sections of a WT (*E*) and NDPK B knockdown (*F*) heart region stained with hematoxylin/eosin. A, atrium, V, ventricle, en, endocardial, my, myocardial layer. (*G*–*J*) Whole-mount immunofluorescence following staining with specific antibodies against atrial myosin heavy chain [S46, (*G*) and (*I*)] and entire heart tube myosin heavy chain [MF20, (*H* and *J*)] showing unaltered expression of structure proteins.

diminish the NDPK B content. In agreement with the reduced abundance of the protein, the MO-injection against NDPK A or B largely reduced the total NDPK phosphate transfer activity to 34 ± 4% and 40 ± 6% of WT activity, respectively (Fig. 1*B*). Most important, the protein levels of Gβ_1 and Gγ_2 were markedly reduced upon depletion of NDPK B, but not upon depletion of NDPK A. In contrast, the expression of β-actin (Fig. 1*A*) was not altered. To verify the previously detected complex formation of NDPK B with G$\beta\gamma$ (7, 8) in zebrafish, we performed co-immunoprecipitation experiments. As shown in Fig. 1*C*, an anti-Gβ antibody precipitated both endogenous Gβ and NDPK B from zebrafish lysates. Reciprocally, NDPK B and Gβ were co-immunoprecipitated with an anti-NDPK B antibody. These data confirm the association of NDPK B with G$\beta\gamma$ in the zebrafish.

Cardiac Dysfunction in NDPK B-Depleted Zebrafish Embryos. Despite the reduction of protein expression and total NDPK activity, zebrafish depleted of NDPK A did not reveal any obvious phenotypic alterations (Fig. S2) and were indistinguishable from wild-type (WT, Fig. 2*A* and Movie S1) or MO-Ctrl (see Fig. 4*A*) injected animals. In contrast, the NDPK B morphants showed severe alterations, such as smaller eyes and an impairment of the heart function (Fig. 2*B*). A decrease in cardiac contractility was associated with pericardial edema, insufficient blood flow and decelerated heart rate (Fig. 2*B* and Movie S2). Quantitative analysis of cardiac contractility revealed a progressive diminution in atrial and ventricular fractional shortening (FS): in the atrial chamber we found a reduction from 33 ± 4.4% in WT embryos to 23.6 ± 6.7% and 14.4 ± 7.1% in NDPK B morphants at 48 and 72 hpf, respectively. In the ventricle, fractional shortening was reduced from 25%±3% in control animals to 14.3 ± 3.4% (48 hpf) and 4.7 ± 1.6% (72 hpf) in embryos with NDPK B depletion (Fig. 2 *C* and *D*). Aside from the reduced contractility, the hearts of MO-NDPK B injected embryos appeared morphologically normal with two distinct heart chambers. At 72 hpf, appropriate growth of an epicardial, myocardial as well as an endocardial layer was observed by HE-staining similar to WT and MO-Ctrl embryos (Fig. 2 *E* and *F*). As shown by immunofluorescence, the expression of the heart-specific thick myofilament components, such as atrial and ventricular myosin heavy chain (MHC) was also not affected by NDPK B knockdown (Fig. 2 *G*–*J*). Altogether, these data indicate a structurally intact development of the heart in NDPK B morphants.

To confirm that the cardiac phenotype is specific to NDPK B knockdown, we performed RNA rescue experiments (Fig. S3) by co-injection of a zebrafish NDPK B mRNA, that is morpholino-resistent due to silent mutations in the MO target site. Up to 53% of the embryos co-injected with MO-NDPK B and the mutated mRNA displayed a wild-type-like phenotype (Fig. S3 *B*–*E*) and restored NDPK B expression as well as cardiac contractility (Fig. S3*F*).

Morpholino-Mediated Knockdown of Zebrafish Gβ_1 Isoforms. In zebrafish, two Gβ_1 orthologs have been identified, Gβ_1 and Gβ_{1like}, which differ in only three amino acids outside known functional domains. Both are >98% identical to the human Gβ_1 protein (Fig. S1*C*). Whole-mount in situ hybridization using specific probes against the 5′-untranslated regions of Gβ_1 or Gβ_{1like} revealed an identical mRNA-pattern (Fig. S4), indicating that both genes might be functionally relevant. G protein β_1-subunit forms a complex with NDPK B in mammalian cells (3, 7, 8) and the co-immunoprecipitation experiments shown in Fig. 1*C* indicate an identical association in zebrafish. We therefore sought

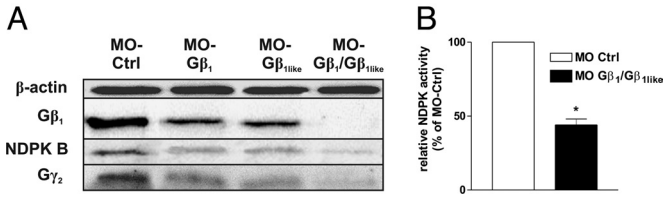

Fig. 3. Gβ$_1$ and Gβ$_{1like}$ depletion in zebrafish embryos. Zebrafish embryos were injected with MO-Ctrl (4 ng), MO-Gβ$_1$ (3 ng) and MO-Gβ$_{1like}$ (3 ng) singly or in combination (3 + 3 ng). (*A*) Immunoblot analysis of lysates prepared 72 hpf using specific antibodies against NDPK B, Gβ$_1$ and Gγ$_2$. β-actin served as loading and specificity control. (*B*) Relative NDPK activity (normalized to MO-Ctrl) was quantified as formation of ^{3}H-GTP from ^{3}H-GDP and ATP in lysates of embryos injected as indicated. Data are means ± SEM, $n = 3$, *, $P <$ 0.05 vs. MO-Ctrl.

to suppress the expression of the Gβ$_1$ isoforms by two specific MOs, MO-Gβ$_1$ and MO-Gβ$_{1like}$, targeted against the translational start site. The MOs were injected singly or in combination into zebrafish embryos. Immunoblot analyses of the lysates revealed that injection of a single MO (MO-Gβ$_1$ or MO-Gβ$_{1like}$) reduced the total Gβ$_1$ ortholog expression by about 50%, whereas the double-knockdown by co-injection of both MOs (MO-Gβ$_1$/Gβ$_{1like}$) led to a nearly complete loss of zebrafish Gβ$_1$ (Fig. 3*A*). In accordance with mRNA data from the in situ hybridization, these data show that total zebrafish Gβ$_1$ is equally composed of both Gβ$_1$ and Gβ$_{1like}$. As Gγ subunits form heterodimers with Gβ$_1$ and only Gβγ dimers are stable protein entities (2), corresponding reduction in Gγ$_2$ protein expression was detected (Fig. 3*A*). To further substantiate the specificity of the knockdown effects, we performed Gβ$_1$ knockdown using a splice MO [MO(s)-Gβ$_{1like}$] instead of the translation start MO. Injection of MO(s)-Gβ$_{1like}$ caused a similar phenotype as MO-Gβ$_{1like}$. Immunoblot analysis confirmed the depletion of Gβ$_1$ and RT-PCR verified altered splicing (10) in MO(s)-Gβ$_{1like}$ injected embryos (Fig. S5).

Most importantly, the knockdown of the Gβ$_1$ isoforms by either approach proportionally decreased the amount of the NDPK B protein (Fig. 3*A*). In accordance with the data obtained by NDPK B knockdown, the total NDPK activity in the zebrafish lysates following Gβ$_1$/Gβ$_{1like}$ knockdown was reduced to 44 ± 4% of control (Fig. 3*B*).

Loss of Gβ$_1$ in Zebrafish Embryos Phenocopies NDPK B Morphants. Injection of MOs targeted against the translation start of Gβ$_1$ or Gβ$_{1like}$ as well as of the splice MO against Gβ$_{1like}$ causes identical cardiac phenotypes, which were very similar to those obtained in NDPK B morphants. Consistent with the findings on the protein level, the double-knockdown of both Gβ$_1$ isoforms caused the most severe phenotype (Fig. 4*B*) characterized by significantly impaired cardiac contractility. The fractional shortening of the atrial and ventricular chamber is severely decreased from 34.4 ± 3.9% and 24.9 ± 3.7% in MO-Ctrl to 12.6 ± 3.7% and 2.6 ± 1.1%, respectively, in MO-Gβ$_1$/Gβ$_{1like}$ double-injected embryos at 72 hpf (Fig. 4 *C* and *D*). Similar to NDPK B knockdown embryos, the heart of the Gβ$_1$/Gβ$_{1like}$ morphants appeared normally developed and structurally intact (Fig. 4 *E*–*J*).

Together with the strong reduction of Gβ$_1$γ$_2$ expression by NDPK B depletion and the co-immunoprecipitation experiments (Fig. 1), these data argue for a close association of NDPK B and Gβγ in the zebrafish. To further substantiate this hypothesis, we injected subeffective doses of either MO-NDPK B (2 ng) or MO-Gβ$_1$/Gβ$_{1like}$ (1 ng each) and observed no significant effect on cardiac contractility (Fig. S6). However, co-injection of both MOs at these subeffective doses resulted in a severe reduction of atrial and ventricular fractional shortening at 72 hpf

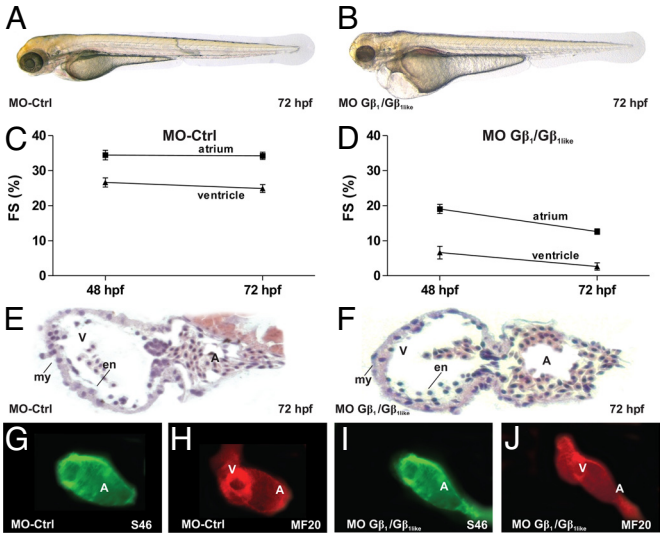

Fig. 4. Loss of zebrafish Gβ$_1$ phenocopies NDPK B morphants. Cardiac phenotype of control morpholino-injected (MO-Ctrl) (*A*) and MO-Gβ$_1$/Gβ$_{1like}$ double-injected (*B*) embryos at 72 hpf. Fractional shortening (FS) of the atrial and ventricular chamber of MO-Ctrl fishes (*C*) and Gβ$_1$/Gβ$_{1like}$ morphants (*D*) at 48 and 72 hpf. Data are means ± SEM, n = 10–12. Histology sections of a MO-Ctrl (*E*) and Gβ$_1$/Gβ$_{1like}$ knockdown (*F*) heart region stained with hematoxylin/eosin. A, atrium, V, ventricle, en, endocardial, my, myocardial layer. (*G*–*J*) Whole-mount immunofluorescence following staining with specific antibodies against atrial myosin heavy chain [S46, (*G* and *I*)] and entire heart tube myosin heavy chain [MF20, (*H* and *J*)] showing unaltered expression of structure proteins.

(Fig. S6) pointing to a synergistic role of NDPK B and Gβ for cardiac contractility in the zebrafish.

Impairment of G Protein Signaling by NDPK B/Gβγ Depletion in Zebrafish Embryos. Increasing evidence (11, 12) indicates that Gβγ is required for the membrane insertion and stability of the associated Gα subunits. As heterotrimeric G proteins play a key role in the regulation of cardiac contractility by stimulating or inhibiting adenylyl cyclase activity, we studied the effects of either NDPK B or Gβ$_1$ depletion on the expression of the Gα subunits in zebrafish embryos by immunoblot analysis. The protein content of the adenylyl cyclase-regulating Gα subfamilies, Gα$_s$, and Gα$_i$, was diminished in whole zebrafish lysates, following NDPK B as well as Gβ$_1$/Gβ$_{1like}$ depletion (Fig. 5*A*). In NDPK B as well as Gβ$_1$/Gβ$_{1like}$ morphants, the Gα protein levels were reduced by at least 50% compared to WT or MO-Ctrl embryos. Most importantly, the expression of several marker proteins from different cellular compartments, cadherin, α-tubulin, β-actin and GAPDH, was not affected by the depletion of either NDPK B or Gβ$_1$/Gβ$_{1like}$ (Fig. 5*A*). Because of the marked cardiac phenotype of the morphant embryos, we additionally determined G protein levels in heart-specific tissue prepared from the transgenic zebrafish line Tg(*cmlc2*:gfp) (25) with heart-specific GFP expression. As shown in Fig. 5*B*, knockdown of NDPK B resulted in similar alterations of G protein α- and β-subunit content in tissue from zebrafish hearts. Interestingly, while the expression of the plasma membrane marker cadherin was not altered, the knockdown of NDPK B or Gβ$_1$ resulted in a decrease in the caveolae forming membrane proteins caveolin-1 and -3 (Fig. 5*A*), which are known as important scaffold proteins for a large number of signaling molecules (13), including GPCRs, G proteins and effectors (14). The amount of muscle specific caveolin-3 was also strongly reduced in isolated fish hearts (Fig. 5*B*).

As this coordinated loss of caveolins with NDPK B and Gβ$_1$

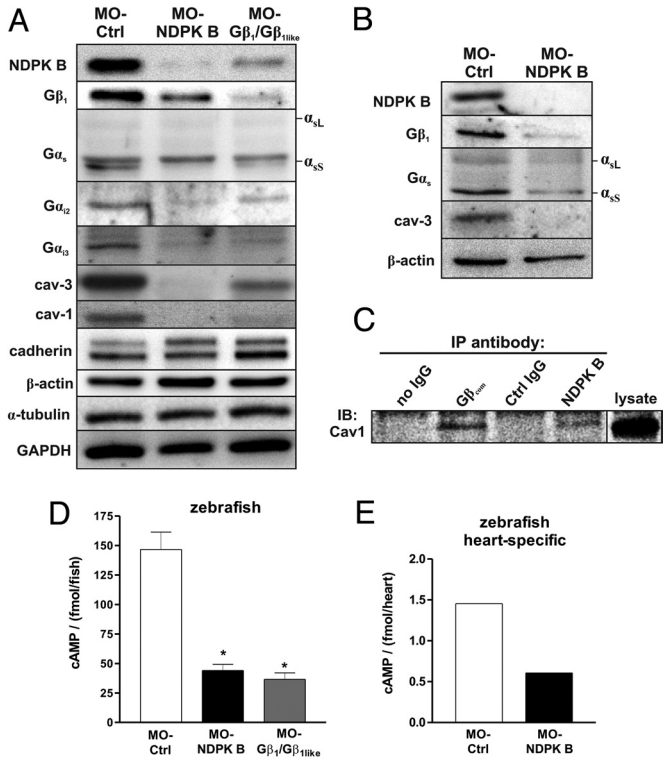

Fig. 5. Alterations of G protein content and cAMP formation in NDPK B and Gβ knockdown zebrafish embryos. (*A* and *B*) Representative immunoblots of zebrafish embryos at 72 hpf following knockdown of either NDPK B or Gβ_1/Gβ_{1like} in total fish lysates (*A*) and heart-specific extracts (*B*) using specific antibodies against the indicated G protein subunit and caveolin isoform. Positions of the long (α_{sL}) and short (α_{sS}) Gα_s splice variants are indicated. Note that Gα_{sS} is migrating below a nonspecific immunoreactive band. Marker proteins (cadherin, β-actin, α-tubulin, GAPDH) representing different cell compartments are shown as loading and specificity control. (*C*) Co-immunoprecipitation of caveolin-1 with NDPK B and Gβ. Endogenous Gβ or NDPK B was immunoprecipitated (IP) from zebrafish lysates (refer to Fig. 1*C*). Association of caveolin-1 was detected by immunoblotting (IB). A representative immunoblot is shown. (*D*) cAMP was measured in lysates from whole embryos at 72 hpf injected with the indicated MOs. Data are means ± SEM, n = 3, *, $P < 0.05$ vs. MO-Ctrl. (*E*) cAMP content of zebrafish hearts isolated from MO-Ctrl vs. MO-NDPK B-injected fishes with heart-specific GFP expression [Tg(*cmlc2*:gfp)] at 72 hpf. n = 365 hearts were pooled and lysed for each condition, respectively. The average of two independent experiments is shown.

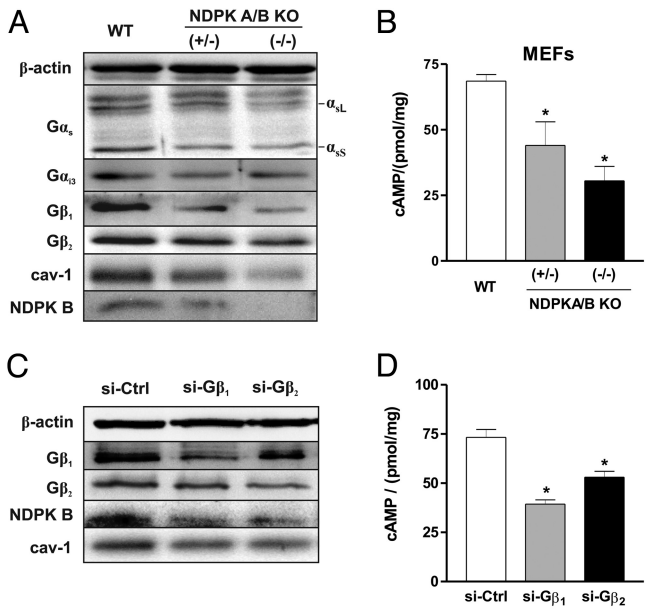

Fig. 6. Alterations of G protein content and cAMP formation in NDPK A/B double-knockout and Gβ subunit knockdown MEFs. (*A*) Membrane fraction of mouse embryonic fibroblasts (MEFs) from wild-type (WT), heterozygous (+/−), and homozygous (−/−) NDPK A/B mice were subjected to immunoblot analysis with the indicated antibodies, showing the loss of G protein α and β subunits as well as caveolin-1 in the membrane fraction of NDPK A/B KO MEFs. Positions of Gα_s splice variants (long, α_{sL}, short, α_{sS}) are indicated. (*B*) Basal cAMP production in WT, (+/−) and (−/−) NDPK A/B MEFs in the presence of 1 mM IBMX and 1 μM propranolol. Data are means ± SEM, n = 3, *, $P < 0.05$ vs. WT. (*C*) Immunoblot analysis of WT MEFs transfected with scrambled siRNA (si-Ctrl) and specific siRNA (si-) against Gβ_1 and Gβ_2 as indicated, demonstrating reduced expression of NDPK B and caveolin-1 in MEFs following Gβ_1 or Gβ_2 depletion. Expression levels of β-actin served as loading control. (*D*) Basal cAMP contents were measured 72 h after transfection with the indicated siRNA in the presence of 1 mM IBMX and 1 μM propranolol. Data are means ± SEM, n = 3, *, $P < 0.05$ vs. si-Ctrl.

pointed to a close association of these proteins, we analyzed the co-immunoprecipitations obtained from zebrafish lysates with the primary antibodies against Gβ or NDPK B (see Fig. 1*C*). Caveolin-1 was detected in both precipitates (Fig. 5*C*), indicating that caveolins are likely part of protein complexes including amongst others, Gβ and NDPK B.

NDPK/G$\beta\gamma$ complexes are involved in the basal cAMP production of cardiomyocytes (9). Thus, the reduced pump function of the hearts of the NDPK B and Gβ_1 knockdown animals (see Figs. 2 and 4) suggested the analysis of basal cAMP levels in whole zebrafish and isolated hearts. Consistent with our results in rat cardiac myocytes (9), depletion of NDPK B or Gβ_1 in zebrafish caused a significant decrease in basal cAMP production to 44 ± 5.3 fmol/fish and 36.5 ± 5.5 fmol/fish, respectively, compared to MO-Ctrl-injected fish with 146.7 ± 14.7 fmol/fish (Fig. 5*D*). Particularly, basal cAMP levels in isolated hearts from NDPK B morphants (0.6 fmol/heart) are markedly diminished compared to MO-Ctrl hearts (1.38 fmol/heart, Fig. 5*E*).

Our results indicate that NDPK B, caveolins, and heterotrimeric G proteins might depend on each other for maintenance of their protein expression levels. We therefore asked, whether this phenomenon occurs on the mRNA or on the protein level. We detected the relative content of mRNA encoding NDPK B and Gβ_1 following MO-injection by quantitative real-time-PCR. The content of both mRNAs, encoding either NDPK B or Gβ_1, showed no significant difference between morphants and control embryos (Fig. S7), arguing for post-translational regulation.

Alterations of G Protein Signaling in NDPK A/B Double-Knockout Mouse Embryonic Fibroblasts. The regulation of G protein abundance and function by NDPK B in zebrafish embryos raised the question, whether this is a general phenomenon that also occurs in mammals. To address this question, we used mouse embryonic fibroblasts (MEFs) that have been derived from homozygous (−/−) and heterozygous (+/−) NDPK A and B double-knockout (KO) mice, respectively (15). As signal transduction by heterotrimeric G proteins as well as caveolae formation is located at the plasma membrane, we compared the protein amounts of caveolin-1 and different G protein subunits of WT MEFs, (+/−) and (−/−) NDPK A/B MEFs (Fig. 6*A*) in the membrane fraction. Similar to zebrafish morphants, loss of NDPK in MEFs was paralleled by diminished caveolin-1 content. Interestingly, the reduction of caveolin-1 was gene dose-dependent. The decrease in caveolin-1 paralleled the decreasing NDPK protein expression in heterozygous and homozygous NDPK KO MEFs (Fig. 6*A*). The reduction in NDPK B and caveolin-1 also resulted in decreased protein levels of the G protein subunits Gβ_1, Gβ_2, Gα_s and Gα_{i3} in NDPK KO MEFs.

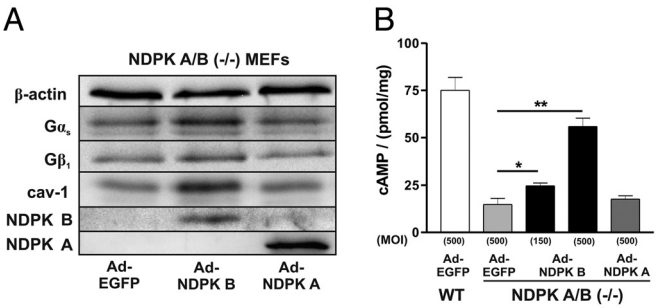

Fig. 7. Rescue of the phenotype of NDPK A/B double-knockout MEFs by adenoviral expression of human NDPK B. NDPK A/B (−/−) MEFs were infected with the adenoviruses Ad-EGFP, Ad-NDPK A and Ad-NDPK B at the multiplicity of infection (MOI) of 500. (*A*) Representative immunoblots of the membrane fraction of (−/−) MEFs infected as indicated confirmed the re-expression of NDPK A and B and showed elevated levels of caveolin-1, $G\beta_1$ and $G\alpha_s$ compared with EGFP control infected cells. (*B*) cAMP production in (−/−) MEFs following infection with the adenoviruses at the indicated MOIs. Data are means ± SEM, $n = 3$, *, $P < 0.05$, **, $P < 0.001$ vs. Ad-EGFP infected (−/−) MEFs.

Moreover, in line with our results in isolated rat cardiomyocytes (9) and zebrafish embryos, we found a significant reduction of the basal cAMP levels by 56% (44 ± 9 pmol/mg) in heterozygous and by 69% (30.5 ± 5.5 pmol/mg) in homozygous NDPK KO MEFs compared to WT MEFs (68.5 ± 2.5 pmol/mg) (Fig. 6*B*).

To confirm the mutual dependence of NDPK B and $G\beta\gamma$ content in MEFs, we performed siRNA-mediated knockdown of $G\beta_1$ and $G\beta_2$ subunits in WT MEFs (16). Compared to control transfected cells, depletion of either $G\beta_1$ or $G\beta_2$ in MEFs resulted in a reduced NDPK B protein expression that is paralleled by a decrease in caveolin-1 (Fig. 6*C*) and a diminished basal cAMP production (69.2 ± 4 pmol/mg in si-Ctrl vs. 39.4 ± 2.2 pmol/mg in si-$G\beta_1$ and 53.3 ± 2.2 pmol/mg in si-$G\beta_2$) (Fig. 6*D*).

To test whether the alterations observed in the NDPK A/B KO MEFs can be attributed to the loss in NDPK B, we adenovirally re-expressed human NDPK B or NDPK A up to five-fold the WT level. Infection with an EGFP encoding adenovirus was used as a control. NDPK B expression (Ad-NDPK B) induced a significant increase in membranous $G\alpha_s$, $G\beta_1$ as well as caveolin-1 levels at 72 h post infection. In contrast, expression of NDPK A (Ad-NDPK A) was without effect (Fig. 7*A*). The basal cAMP content in the KO MEFs increased up to the WT levels by the expression of NDPK B, but not by NDPK A, in a dose-dependent manner [Fig. 7*B*, Ad-EGFP 14.7 ± 3.3, pmol/mg, Ad-NDPK A 17.6 ± 1.8 pmol/mg (MOI 500), Ad-NDPK B 24.6 ± 1.5 pmol/mg (MOI 150), and 55.9 ± 4.4 pmol/mg (MOI 500)]. Thus, re-expression of human NDPK B could at least partially rescue the reduction in G protein and cAMP content of NDPK KO MEFs.

Discussion

As central regulators in signal transduction heterotrimeric G proteins are interacting with numerous proteins (1). We have previously complemented the classical concept of G protein activation by showing that a complex of NDPK B and $G\beta\gamma$ dimers can activate G proteins independently from GPCRs (7–9). To study the role of NDPK B and $G\beta\gamma$ in vivo, we used the zebrafish as model organism. After MO-mediated knockdown of NDPK B, as well as $G\beta_1$, we detected the appearance of a phenotype with dramatically reduced cardiac contractility. Similar to injection of control morpholinos, the knockdown of NDPK A did not cause an obvious phenotype, although the total NDPK activity was reduced to a similar extent as by NDPK B depletion. Most likely, the remaining NDPK phosphate transfer activity is sufficient to maintain the housekeeping function of NDPK in nucleotide metabolism. The specificity in the requirement of the NDPK B isoform was further substantiated by the rescue experiments in which MO-resistant zebrafish NDPK B RNA was co-injected. Thus, the NDPK B morphant zebrafish is a living animal model with the loss of NDPK B gene function.

Our results further show that zebrafish $G\beta_1$ is composed of two isoforms which apparently do not differ in their biological function and are obviously derived to a similar extent from two active genes encoding $G\beta_1$ and $G\beta_{1like}$. Hence, the double-knockdown of both $G\beta_1$ and $G\beta_{1like}$ resulted in lesser $G\beta_1$ protein expression which correlated to the severity of the cardiac phenotype. As for NDPK B, the $G\beta_1/G\beta_{1like}$ morphants generated in this study represent a living vertebrate model that allows for the analysis of cardiovascular physiology with a specific depletion of a single $G\beta$ subtype, while former genetic approaches were restricted to cell culture models, or lower organisms such as *Dictyostelium discoideum* and *Caenorhabditis elegans* (17–19).

We obtained several lines of evidence that the observed phenotypic alterations can be related to a lack of the interaction of NDPK B with $G\beta\gamma$. First, with regard to the impaired cardiac contractility, the NDPK B and $G\beta_1/G\beta_{1like}$ morphants developed a very similar phenotype and the MOs directed against NDPK B and $G\beta_1/G\beta_{1like}$ act apparently synergistic (See Fig. S6). Second, the knockdown of NDPK B, but not of NDPK A, caused a loss in $G\beta_1\gamma_2$ paralleling the depletion of NDPK B protein expression. Reciprocally, the knockdown of $G\beta_1$ and $G\beta_{1like}$ singly or in combination, caused a loss in NDPK B expression paralleling the reduction in $G\beta_1\gamma_2$ dimer content. Third, the NDPK B as well as the $G\beta_1/G\beta_{1like}$ knockdown led to decreased abundance of the α-subunits of the adenylyl cyclase-regulating G_s and G_i protein subfamilies, whereas the expression of several marker proteins (cadherin, α-tubulin, β-actin, GAPDH, MHC) was not affected. Forth, co-immunoprecipitation experiments confirmed a close association of NDPK B with $G\beta\gamma$ in the zebrafish and thus argue for the existence of the previously purified NDPK B/$G\beta_1\gamma$ complex (7) also in the zebrafish. Our data therefore indicate that NDPK B and $G\beta_1\gamma_2$ are mutually dependent for the maintenance of their protein levels and both are essential for cardiac contractility in zebrafish embryos (7–9). In line with this interpretation, the NDPK A knockdown embryos reported herein as well as the published NDPK A KO mice (20) did not develop obvious defects.

Taking into account the pivotal role of heterotrimeric G proteins in the signaling of many cellular processes, we asked whether the mutual dependence of $G\beta\gamma$ and NDPK B expression levels is specific for the zebrafish or is a more general phenomenon. Using MEFs from non-viable NDPK A/B knockout mice, heterozygous and wild-type littermates, we could indeed confirm the findings in a mammalian genetic model. The gradual depletion and complete absence of NDPK A/B in membranes of MEFs from heterozygous and homozygous animals, respectively, was paralleled by a gradual loss of $G\beta_1$ and $G\beta_2$ as well as a reduction in basal cAMP levels. In addition, also in the MEF model, the reciprocal experiment, that is, the siRNA-mediated knockdown of $G\beta_1$ or $G\beta_2$ expression, resulted in a reduction of the NDPK B level and basal cAMP content. Similar to the zebrafish, re-expression of human NDPK B, but not NDPK A, in KO MEFs rescued the loss in G protein content and restored basal cAMP levels. Therefore, the data in mammalian cells are in complete agreement with the data obtained in the zebrafish and argue for an essential, evolutionarily conserved interaction of NDPK B with $G\beta\gamma$ which is required for G protein stabilization and function.

Interestingly, the depletion of NDPK B and $G\beta\gamma$ had a major impact on the abundance of caveolins and α-subunits of heterotrimeric G proteins in the zebrafish as well as in MEFs. It is well known that the individual members of the G protein heterotrimers confer to the stability of their binding partners (16, 21). In particular, by using RNAi-technology, recent studies showed

(16, 17) that Gβ subunits are necessary for the stable accumulation of Gα proteins. Similarly, interfering with the membrane attachment of Gβγ dimers induced a loss of $G\alpha_s$ protein content in cardiac myocytes, and thus resulted in impaired contractility (12). On the other hand, caveolins are essential for the formation of caveolae, small plasma membrane invaginations, that are involved in compartmentalization of various signaling molecules, including components of GPCR-induced signal transduction cascades [for review see (14)]. Recent evidence suggests that in the heart, β-adrenoceptors, G_s, G_i and adenylyl cyclase type 5/6 are localized in caveolae which thereby locally confines cAMP generation (22). Our functional data together with the co-immunoprecipitation suggest that caveolins are together with NDPK B and Gβγ part of a larger protein complex. This complex is apparently required for the posttranslational stabilization and function of the participating proteins, particularly of heterotrimeric G proteins. Accordingly, the loss of NDPK B or Gβγ induces a depletion of caveolins, NDPK B and heterotrimeric G proteins at the plasma membrane as well as a reduction in basal cAMP production. In the heart, this is associated with a loss of contractile function as observed in NDPK B and Gβ morphants. In agreement with our hypothesis, the morpholino-induced knockdown of caveolin-3 in the zebrafish causes a cardiomyopathy-like phenotype (23). Mice with a genetic depletion of the muscle-specific caveolin-3 in the heart develop a severe cardiomyopathy (24).

In summary, our findings identify NDPK B as an essential complex partner of heterotrimeric G proteins regulating adenylyl cyclase activity. This complex is apparently associated with caveolin-enriched plasma membrane domains and is required to maintain stable levels of heterotrimeric G_s and G_i proteins at the plasma membrane. Thus, NDPK B contributes to G protein signaling not only by direct G protein activation via the previously described phosphorelay (7–9), but also by modulating the cellular amount and compartmentalization of heterotrimeric G proteins.

Materials and Methods

Zebrafish Maintenance and Manipulation. Zebrafish *Danio rerio* (TE4/6, TüAB, Tg(*cmlc2*:gfp) strains) were maintained under standard conditions (25). Details on MOs, injections, histology and immunostaining are provided in the *SI Text*.

Measurement of Fractional Shortening. Fractional shortening was assayed by measuring the systolic contractile function normalized to the diameters of the heart of immobilized embryos as described (26).

Immunoblot Analysis and Co-Immunoprecipition. Embryos were treated with deyolking buffer (55 mM NaCl; 1.8 mM KCl; 1.25 mM $NaHCO_3$) and washed twice with washing buffer (110 mM NaCl; 3.5 mM KCl; 2.7 mM $CaCl_2$; 10 mM Tris-HCl, pH 8.5). Single zebrafish hearts were prepared from the zebrafish line Tg(*cmlc2*:gfp) with heart-specific GFP expression. Lysates were prepared in buffer H (10 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM PMSF, and protease inhibitors) and homogenized with two 10-s bursts applied by a Polytron (Kinematica) at a setting of 20,000 rpm. Co-immunoprecipitation in these lysates was performed as described (9). Immunoblotting was performed according to standard procedures. Details on the used antibodies are provided in the *SI Text*.

NDPK Activity Assay. NDPK phosphate transfer activity was measured as formation of ^{3}H-GTP from ^{3}H-GDP and ATP exactly as described previously (8) in whole zebrafish lysates prepared without detergents at 72 hpf.

Mouse Embryonic Fibroblasts. Generation of NDPK A/B double-knockout mice in the genetic background of C57BL/6, preparation and culture of WT, (+/−) and (−/−) NDPK A/B KO MEFs have been described (15). Cell lysates were homogenizied as described above in buffer H without Triton X-100 and centrifuged at 100,000 × *g* to obtain membrane fractions (8). Details on recombinant adenoviruses and siRNAs are provided in the *SI Text*.

Measurement of cAMP Levels. Generation of cAMP was assayed as previously described (8) using a cAMP immunoassay (Assay Designs).

Statistical Analysis. All results were expressed as mean ± SEM. Statistical analysis was performed with Student's *t* test or ANOVA with Bonferroni post-hoc test as appropriate. $P < 0.05$ was considered significant.

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