Mitochondrial death protein Nix is induced in cardiac hypertrophy and triggers apoptotic cardiomyopathy

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Loss of cardiomyocytes through programmed cell death is a key event in the development of heart failure, but the inciting molecular mechanisms are largely unknown. We used microarray analysis to identify a genetic program for myocardial apoptosis in Gq-mediated and pressure-overload cardiac hypertrophy. A critical component of this apoptotic program was Nix/Bnip3L. Nix localized to mitochondria and caused release of cytochrome *c*, activation of caspase-3 and apoptotic cell death, when expressed in HEK293 fibroblasts. A previously undescribed truncated Nix isoform, termed sNix, was not targeted to mitochondria but heterodimerized with Nix and protected against Nix-mediated apoptosis. Forced *in vivo* myocardial expression of Nix resulted in apoptotic cardiomyopathy and rapid death. Conversely, sNix protected against apoptotic peripartum cardiomyopathy in Gαq-overexpressors. Thus, Nix/Bnip3L is upregulated in myocardial hypertrophy, and is both necessary and sufficient for Gq-mediated apoptosis of cardiomyocytes and resulting hypertrophy decompensation.

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Programmed cell death, or apoptosis, is a normal event in most organs where it maintains tissue homeostasis by balancing cellular regeneration with programmed elimination. Dysregulation of normal apoptotic signaling can lead to unrestrained cellular proliferation or inappropriate tissue degradation[1]. Although there is no known physiological role for apoptosis in non-proliferative cells, compelling evidence exists that terminally differentiated cardiomyocytes can undergo apoptosis. Furthermore, cardiomyocyte apoptosis is greatly increased in ischemic and dilated cardiomyopathy, acute myocardial infarction and arrhythmogenic right-ventricular dysplasia[2–4]. In experimental mouse models, extrinsic induction of cardiomyocyte apoptosis causes progression of 'compensated' myocardial hypertrophy to heart failure in the context of either augmented cardiomyocyte apoptosis signaling or a defective cardiomyocyte survival pathway[5,6], thus demonstrating that apoptosis represents a critical stress-activated pathological response in this transition. This link between hypertrophy and apoptotic heart failure may help explain why hypertrophy is a major risk factor for sudden death[7].

The mechanisms for apoptosis induction in hypertrophied myocardium are unknown, and identifying genetic modifiers of apoptosis is a major investigative goal in cardiovascular biology[1,8,9]. In the heart, as in all tissues, caspases are essential downstream effectors of apoptotic signals[1,10], but multiple upstream pathways activate caspases, including death-ligand receptors and the primary mitochondrial pathway[1]. One approach for delineating molecular determinants of apoptosis in cardiac hypertrophy might be transcriptome analysis of a relevant experimental model of apoptotic hypertrophy decompensation[11,12], such as Gq-dependent cardiac hypertrophy progressing to apoptosis in the cardiac-specific Gαq overexpressing mouse[5,13]. Gq is the heterotrimeric G protein that couples membrane receptors for angiotensin II, endothelin-1 and epinephrine to the cardiac hypertrophy response. *In vivo* inhibition or gene ablation has demonstrated that Gq signaling is necessary for normal embryonic cardiac development and for pressure overload hypertrophy in the adult heart[14–16]. Indeed, enhanced receptor-mediated or autonomous cardiac Gαq signaling is sufficient to recapitulate cellular, molecular and functional characteristics of pressure-overload hypertrophy[5,13,17], and can progress to apoptotic cardiac failure[5,18].

Here we report delineation of an induced apoptosis gene program in Gq-mediated cardiac hypertrophy, and identification of one component of this gene program as Nix/Bnip3L. Nix, which is not significantly expressed in normal myocardial issue, is strikingly induced in Gq-dependent and pressure overload hypertrophy. Expressed *in vitro*, Nix localizes to mitochondria and causes rapid cell death with cytochrome *c* release, caspase-3 activation and apoptotic nuclear changes. A novel truncated Nix isoform, designated sNix, is a non-localizing soluble protein with no deleterious effects, and which protects against Nix-induced apoptosis. Expressed in the *in vivo* mouse heart, Nix provoked a dilated cardiomyopathy that was invariably lethal due to massive cardiomyocyte apoptosis within days of detectable protein expression. In contrast, sNix-expressing mice were normal and sNix protected against Gq-mediated apoptotic cardiac death. These data establish a molecular mechanism for induction of cardiomyocyte apoptosis in hypertrophied myocardium, and for its progression to heart failure.

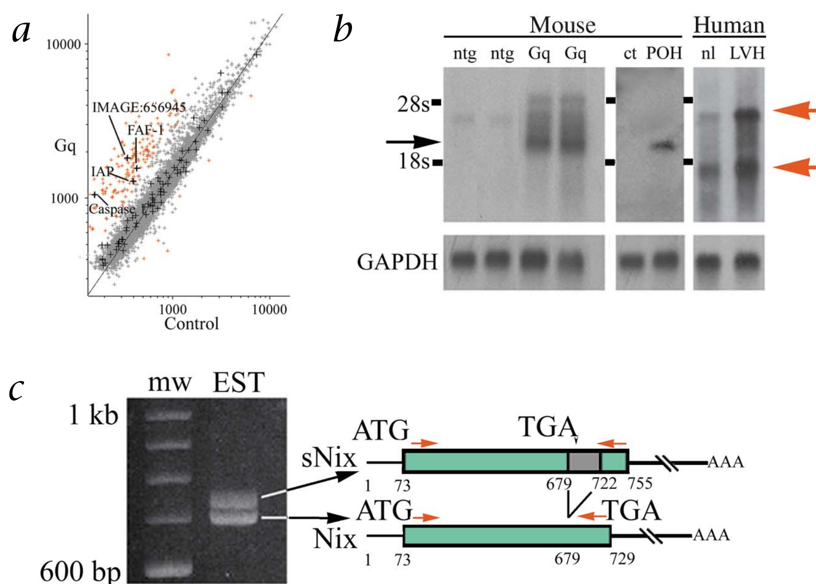

Fig. 1 Identification and cloning of mouse Nix and sNix. ***a***, DNA microarray analysis showing 153 regulated genes in Gq transgenic mice (red +), of which 4 of 88 were apoptosis genes (black +). IAP, baculoviral IAP repeat; FAF-1; Fas-associated factor-1. ***b***, Northern-blot analysis of IMAGE:656946/Nix showing increased expression in Gq transgenic (left) and transverse aortic coarcted (TAC; middle) mice, as well as human hypertensive left ventricular hypertrophy (LVH; right; representative of 3 individual experiments). Transcripts seen in mouse (black arrow) and human (red arrows) hearts are consistent with their respective known mRNA species[35,36]. ***c***, RT-PCR of Nix ORF from mouse hearts generated 2 products, identified by sequencing as: Nix and splice variant, sNix, which contains a 42-bp insert encoding a premature termination codon that truncates the protein by ten amino acids.

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Nix is upregulated in hypertrophied myocardium

Based on preliminary DNA microarray analysis of Gq-transgenic hearts suggesting that regulated expression of apoptosis modifiers might cause an apoptotic proclivity in Gq-mediated hypertrophy[19], we prospectively assayed expression of apoptosis-related genes in Gq hearts using duplicate Incyte microarrays. 153 of 8,799 analyzed sequences were regulated in Gq hearts. Of 88 apoptosis genes represented, only four were regulated in Gq hearts (Fig. 1*a*), caspase-1, FAF-1, baculoviral IAP repeat-containing 4 and IMAGE EST 656945, with homology to Bcl-2. Northern-blot analysis confirmed IMAGE:656945 upregulation in Gq-transgenic mouse hearts, and also demonstrated it in acute pressure overload from transverse aortic constriction[17] and human hypertensive cardiac hypertrophy (Fig. 1*b*).

To identify the regulated transcript encoded by IMAGE:656945, we probed a custom Gq-transgenic mouse-heart cDNA library. Seven independent cDNA clones were isolated, the largest of which contained an incomplete open reading frame (ORF) identifying the gene as a recently described Bcl-2 family member, Nix/Bnip3L (ref. 20). The full-length mouse cardiac Nix ORF, encoding a 218-amino-acid protein, was obtained by reverse transcriptase (RT)-PCR (Fig. 1*c* and *d*). Also identified was a novel Nix splice variant, sNix, with a 42-base insertion that encodes a premature termination codon, truncating the C terminus by 10 amino acids (Fig. 1*d*). Because Nix is a pro-apoptotic mitochondria-targeted protein[20] and mitochondrial degeneration is characteristic of Gαq-induced cardiomyocyte apoptosis[21], we postulated that increased Nix expression could contribute to apoptotic degeneration.

Nix induces apoptosis through mitochondria

The cellular effects of increased Nix expression were assessed by *in vitro* expression of FLAG epitope-tagged chimeric protein in HEK293 cells. FLAG–sNix-expressing cells were viable (data not shown), but FLAG–Nix expressing cells underwent apoptosis within 72 hours of transfection (Fig. 2). Nix-mediated apoptosis signaling was delineated by confocal microscopy. 24–48 hours after transfection, Nix occurred in a punctate perinuclear distribution colocalizing with mitochondrial heat-shock protein 60 (HSP60) (Fig. 2*a*). (In contrast, sNix was distributed diffusely throughout the cytoplasm (see below)). Redistribution of HSP60 and cytochrome *c* from mitochondria to cytosol, indicating loss of mitochondrial integrity and represented by increased fluorescence and a more diffuse staining pattern, was observed only in Nix-expressing cells (Fig. 2*b*), and never in sNix-expressing cells (data not shown). As cytosolic cytochrome *c* can induce apoptosis by complexing with Apaf-1 and activating the caspase cascade[22], caspase-3 activity was assayed by confocal microscopy of its processed form. Indeed, caspase-3 activation was widespread and, as with cytochrome *c* and HSP60 redistribution, was limited to Nix-expressing cells (Fig. 2*c*). Nix-expressing cells labeled strongly with terminal deoxynucleotide transferase (TdT) (Fig. 2*d*), whereas sNix cells did not (data not shown). Together, the concordance between HSP60 and cytochrome *c* redistribution, TdT staining, and caspase-3 activation in Nix-expressing cells demonstrates that Nix, but not sNix, mediates apoptotic cell death through the mitochondrial pathway.

Increased Nix expression causes apoptotic cardiomyopathy

The above results and recent published data[20] indicate that Nix can be a potent cell-suicide factor *in vitro*, but its *in vivo* function in myocardium or any other tissue is not known. Therefore, to determine if increased myocardial Nix expression is sufficient to cause cardiomyopathy, FLAG–Nix and –sNix were transgenically expressed in mouse hearts using the α-myosin heavy-chain promoter (α-MHC), chosen because its activity is minimal in the fetal ventricle, but induced shortly after birth[23]. sNix-expressing mice from two independent lines were viable for nine months, and were normal in histological appearance, ventricular function, and cardiac gene expression (data not shown). Four Nix founders (F0) were identified (from 35 possible) (Fig. 3*a*), but only two mosaic founders, with 60 and 13 copies of the transgene, produced transgenic progeny. Myocardial Nix expression of first generation Nix mice correlated with transgene copy number (Fig. 3*b*). Immunoblot analysis demonstrated detectable myocardial transgene expression on postnatal day three, with expression that was near maximal on day five (Fig. 3*b*), and assays of cardiac gene expression revealed significantly increased atrial natriuretic pep-

tide (ANF) (267 ± 29% of nontransgenic siblings (NTG); $P < 0.01$), and decreased sarcoplasmic reticulum calcium (SERCA) (30 ± 2% of NTG; $P < 0.01$) and phospholamban (25 ± 1% of NTG; $P < 0.01$) gene expression, suggesting a molecular heart-failure phenotype[24] (Fig. 3*c*). At birth, Nix transgenic pups were indistinguishable from nontransgenic, but by six days old were strikingly smaller than their non-transgenic littermates (Nix body weight: 2.21 ± 0.45 g, $n = 13$; nontransgenic littermates: 3.55 ± 0.49 g, $n = 25$; $P < 0.001$) (Fig. 3*d*). All high-expressing Nix mouse pups died on day 6 or 7, whereas low-expressing Nix mice died between days 11 and 14. Although hearts of Nix mice appeared grossly normal, they occupied a substantially greater proportion of the thoracic cavity than those of their nontransgenic littermates (Fig. 3*d*) and echocardiography showed relative ventricular dilation, bradycardia, and depressed contractile function (Fig. 3*d*, inset).

Immuno-confocal microscopy demonstrated transgenic FLAG–Nix protein expression specifically in myocardial tissue (Fig. 4*a*). Positive signals from the terminal transferase–mediated dUTP-biotin nick end labeling (TUNEL) assay were widespread in Nix expressors, with apoptotic indices (positive nuclei/total nuclei) of 20 ± 3% in Nix expressors versus 2 ± 1% in controls ($n = 5$ each; $P < 0.01$). Apoptosis was specific for cardiomyocytes, as shown by costaining with α-sarcomeric actin (Fig. 4*b*, left). Nuclear morphology was characteristic of apoptotic cells, with fragmentation and margination of DNA at the nuclear envelope (Fig. 4*b*, right). sNix-expressing mice showed none of the abnormalities seen in Nix mice (data not shown).

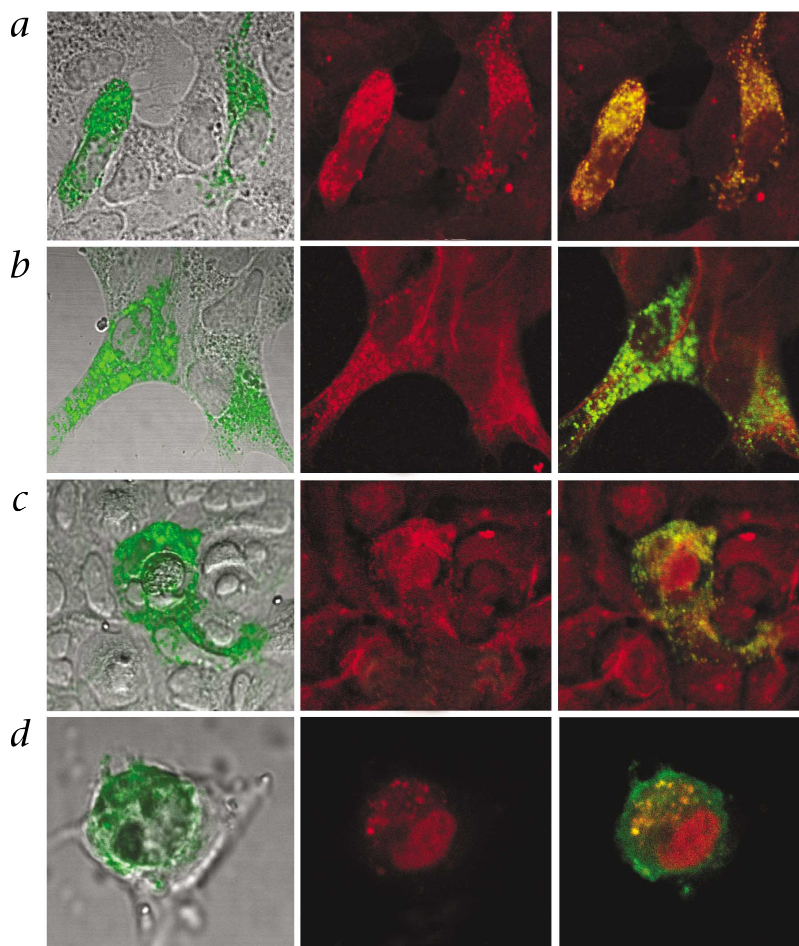

Fig. 2 Confocal analysis of Nix-expressing cells. ***a*–*d***, Left column is FLAG–Nix (green) with light micrographs showing transfected and non-transfected cells; middle column is mitochondrial HSP-60 (*a*), cytochrome *c* (*b*), activated caspase-3 (*c*), and TUNEL (*d*). Right column is overlay.

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sNix prevents Gq-mediated peripartum cardiomyopathy

Immunoblot analysis showed that, whereas FLAG–Nix was a membrane-bound protein of 44 kD, FLAG–sNix was a cytosolic protein of 42 kD (Fig. 5*a*). Furthermore, Nix colocalized with mitochondrial HSP60, but sNix did not (Fig. 5*b*). Migration of Nix and sNix at twice their predicted masses suggested homodimerization, which is common in this family of proteins[25,26]. We therefore considered that sNix might inhibit Nix-mediated apoptosis through heterodimerization. Indeed, Nix and sNix homodimerization and Nix–sNix heterodimerization was demonstrated by immunoprecipitation (Fig. 5*c*). The functional consequence of this intermolecular interaction was attenuated Nix-mediated apoptosis, as demonstrated by delayed apoptotic cell death in sNix–Nix-cotransfected HEK293 cells (Fig. 5*d*).

The apparent dominant inhibitory activity of sNix for Nix was used to determine if Nix was essential for apoptosis in Gq-mediated peripartum cardiomyopathy[5]. Double transgenic sNix/Gαq females were mated and followed for development of peripartum cardiomyopathy, including cardiac dilation, deterioration in contractile function, and death. At baseline, sNix did not affect Gαq expression or the characteristic features of cardiac hypertrophy and contractile depression in this transgenic model (Fig. 6). However, co-expression of sNix with Gαq delayed or prevented the early death seen in the peripartum period (Fig. 6*a*), strikingly diminished cardiomyocyte apoptosis (Fig. 6*b*), and largely prevented the characteristic ventricular postpartum dilation (Fig. 6*c*). However, cardiac mass (Gq = 220 ± 10 mg; sNix/Gq = 200 ± 20 mg) of Gq mice was not significantly changed by co-expression of Nix.

Discussion

Myocardial hypertrophy, defined as increased cardiac mass and cardiomyocyte volume associated with characteristic changes in gene and protein expression, is the universal response to hemodynamic stress. Enhanced myocardial force-generating capacity and favorable geometry in the hypertrophied heart compensate for increased load, and thus permit increased cardiac work[27]. However, in the face of unremitting hemodynamic overload, compensated hypertrophy inexorably and inevitably fails, leading to dilated cardiomyopathy through a poorly understood process termed decompensation[28]. Loss of cardiomyocytes contributes to hypertrophy decompensation, and studies over the past decade have increasingly suggested an important pathophysiological role for cardiomyocyte apoptosis in progression to heart failure[1–6,29]. To modify or abort decompensation, intrinsic determinants of myocardial apoptosis must be identified.

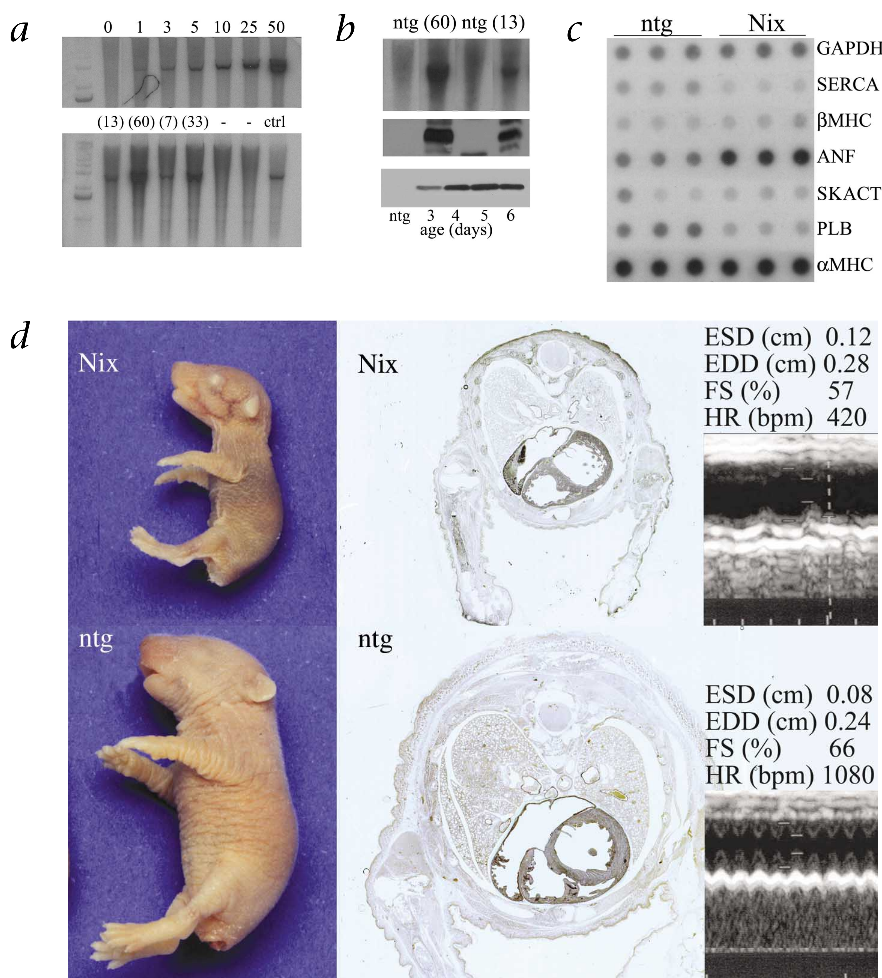

Fig. 3 Phenotypic analysis of Nix-expressing mice. ***a***, Southern-blot analysis of 4 Nix founders, showing numbers of incorporated transgenes; top is quantitative standards. ***b***, Southern (top) and western (middle) blots of Nix expression in neonatal F1 Nix-high (60) and Nix-low (13) mice. Bottom shows transgene protein expression as a function of mouse age. ***c***, RNA dot blot analysis of cardiac gene expression in 6-day-old Nix mice and NTG. GAPDH, control; SERCA, sarcoplasmic reticulum calcium ATPase; bMHC, b myosin heavy chain; ANF, atrial natriuretic peptide; SKACT, skeletal actin; PLB, phospholamban; aMHC, a myosin heavy chain. ***d***, Pathological analysis revealed stunted growth of 6-day-old mouse pups (left) and, on cross-section of mid thorax (right), relative cardiac enlargement (cardiac myosin light chains stained brown for visualization). Insets are m-mode echocardiograms; quantitative data are on top. ESD, end-systolic dimension; EDD, end-diastolic dimension; FS, fractional shortening; HR, heart rate.

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Toward this end, an increase in pro-apoptotic Bcl-2 family members has been reported after *in vitro* cardiomyocyte oxidative stress[30] or mechanical stretch[31], and pro-apoptotic Bax protein is increased after acute myocardial infarction in humans[32]. Particular importance as potential targets for therapeutic intervention would be assigned to apoptosis effectors that are induced in non-failing hypertrophy, thus preceding (and therefore possibly promoting) cardiomyocyte apoptosis and the heart-failure phenotype. The present studies identify Nix/Bnip3L as a hypertrophy-induced molecular trigger for cardiomyocyte suicide in Gαq overexpression, experimental murine pressure overload and non-failing human cardiac hypertrophy.

Both pro- and anti-apoptotic Bcl-2 proteins are highly expressed in embryonic and neonatal hearts, in which apoptosis is required for normal development. Pro-apoptotic family members are, however, barely detectable in terminally differentiated adult myocardium[30], thus assuring a balance of Bcl-2-related proteins in the normal adult heart that is heavily shifted toward cell survival. We detected increased Nix expression as part of an upregulated four-member apoptosis gene program in cardiac hypertrophy secondary to Gq-overexpression, and murine and human pressure overload, all of which represent Gq-dependent processes[5,13–16]. *In vitro* expression delineated the primary mitochondrial effects of Nix and revealed that its naturally occurring C-terminal truncated isoform, sNix, had no apoptotic activity, and was indeed a dominant inhibitor of Nix-mediated apoptosis. *In vivo* expression showed that forced Nix expression was sufficient to cause cardiomyocyte apoptosis and lethal dilated cardiomyopathy. Protection by sNix against apoptosis in the Gαq-peripartum model further demonstrated that Nix function/mitochondrial targeting is necessary for this form of apoptotic heart failure.

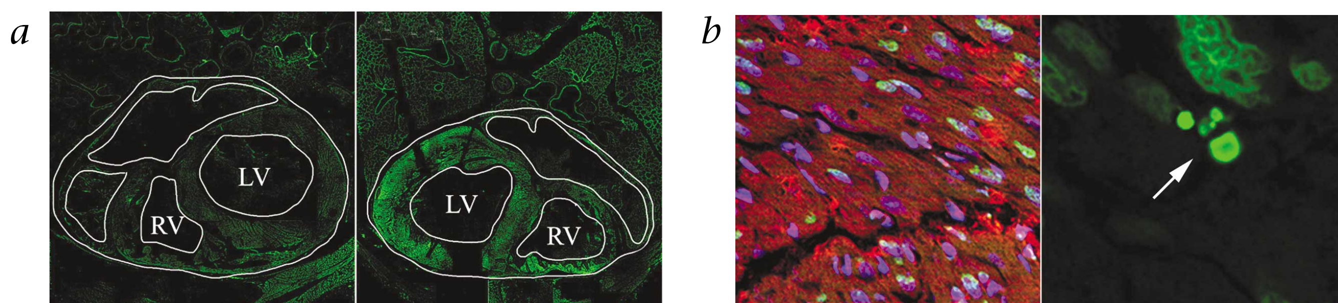
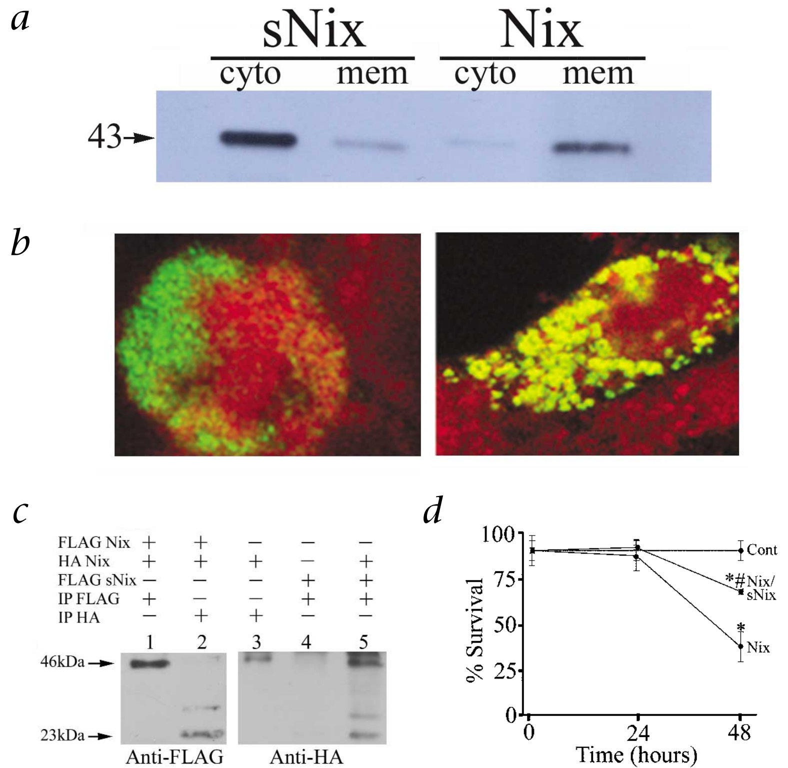


Fig. 4 Cellular effects of myocardial Nix expression. ***a***, Anti-FLAG fluorescence microscopy of Nix expression in ntg (left) and Nix (right) mice. LV, left ventricle; RV, right ventricle. Hearts are outlined and gain was increased to show non-staining tissues for clarity. ***b***, Left panel, Confocal analysis of Nix ventricular myocardium. TUNEL-positive myocyte nuclei are green; normal nuclei are blue and cardiomyocytes are labeled red with α-sarcomeric actin. Magnification, ×60. Right panel, cardiomyocyte apoptotic bodies (arrow) visualized by TUNEL labeling. Magnification, ×200.

Fig. 5 Expression of recombinant Nix and sNix in HEK 293 cells. ***a***, Immunoblot analysis demonstrated faster migration of FLAG–sNix, localized primarily in cytosol, compared to membrane-associated FLAG–Nix. ***b***, Confocal microscopy of Nix/sNix (green) and mitochondrial HSP-60 (red) demonstrates localization of Nix, but not sNix in mitochondria (yellow). ***c***, Immunoblot analyses of Nix homodimeric (column 2) and Nix/sNix heterodimeric (column 5) complexes. +, transfection or (IP) immunoprecipitation reagent. Columns 1 and 3, positive control; 4, negative control. ***d***, Enhanced viability of HEK293 cells co-expressing Nix/sNix, relative to Nix alone. *, $P < 0.001$ compared with control; #, $P < 0.001$ compared with Nix.



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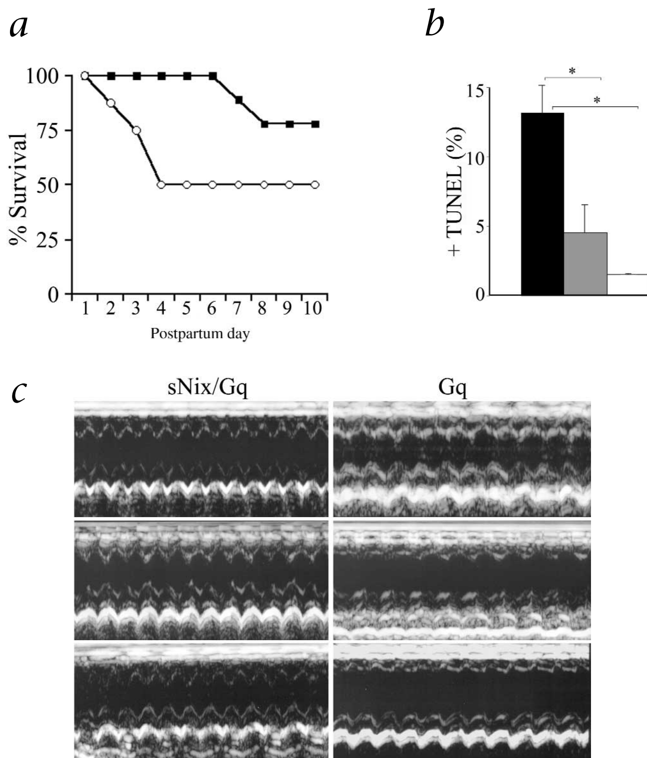
The existence of a genetic program for apoptosis in cardiomyocytes may at first seem surprising, but is less so in the context of a hypertrophy-associated 'fetal gene program', that is, re-expression of genes that are abundant in the embryonic heart (including ANF and noncardiac isoforms of myosin and actin)[24]. Because apoptosis is necessary for normal embryonic cardiac development, re-induction of apoptosis genes may represent another facet of this fetal program. However, only 4 of 88 assayed apoptosis genes were regulated in Gq-dependent myocardial hypertrophy; a generalized increase in apoptotic mediators did not occur. Generalized anti-apoptotic approaches for prevention of hypertrophy decompensation are therefore not likely to be as effective as specific targeting of a single critical regulated component, such as Nix. It must also be considered that apoptosis is not the only relevant mechanism for cardiomyocyte death in decompensating hypertrophy. A phenotype of neonatal heart failure from myocyte necrosis has been reported in the α-MHC Arg403Gly mutation mouse model of hypertrophic cardiomyopathy[33,34] suggesting that an effective approach to prevent cardiomyocyte death in decompensating hypertrophy must target both processes.

The current studies established that increased myocardial Nix expression occurs in genetic and naturally occurring Gq-dependent cardiac hypertrophy. Nix overexpression showed the sufficiency of Nix to provoke apoptotic heart failure and identified downstream events mediating this response, and should therefore prove useful as a model for testing potential therapeutic interventions directed at mitochondrial stabilization or caspase activation. Finally, prevention of Gq-mediated apoptotic cardiac failure with the Nix dominant inhibitor, sNix, has established the potential therapeutic utility of targeting regulated apoptosis effectors in hypertrophy decompensation.



Methods

Transgenic models. The cardiac-specific Gαq transgenic mouse model of myocardial hypertrophy, and its proclivity for apoptotic decompensation under defined conditions, have been described in detail[5,13,17,18]. Gαq ventricular poly(A)$^+$ RNA for DNA microarray and northern-blot analysis was prepared from 8-wk-old male mice. Transgenic mice expressing murine Nix, or its truncated variant sNix, were created using the full-length mouse α-MHC promoter[23] and were identified by genomic Southern-blot analysis of tail clip DNA using the Nix or sNix cDNAs as radioactive probes. Transverse aortic banding to induce pressure overload, was performed as described[17]. Transaortic gradients averaged 93 ± 3 mmHg in 3-day banded mice. Studies were performed in accordance with protocols approved by the University of Cincinnati Institutional Animal Care and Use Committee.

Fig. 6 sNix effects on Gq-peripartum cardiomyopathy. ***a***, Kaplan Meyer curve of peripartum survival. ○, Gq (n = 8); ■, sNix/Gq (n = 9). ***b***, Apoptosis, assayed as apoptotic index. ■, Gq; ■, sNix/Gq; □, NTG (*, P < 0.001). ***c***, Serial echocardiography demonstrating progressive postpartum ventricular dilation and failure in Gq (right column), but not sNix/Gq (left column) mice. Top, prepartum; middle, 3 d postpartum; bottom, 7 d postpartum.

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mRNA expression analysis. Mouse and human ventricular mRNA was prepared using Triazol (Gibco-BRL, Grand Island, New York) and the Oligotex mRNA isolation kit (Qiagen, Valencia, California) according to manufacturer's instructions. Total RNA was passed twice through oligo d(T) columns and mRNA was stored as ethanolic precipitates at –70 °C. Experiments were performed with RNA pooled from 2 (northern blotting, RT-PCR) or 8 (microarray analyses) individual mouse ventricles. DNA-microarray hybridizations were performed in duplicate on different samples of Gαq mRNA using Incyte mouse gene-expression microarrays (GEM), version 1.12 (Genome Systems, St. Louis, Missouri). Poly(A)$^+$ mRNA from Gαq transgenic hearts and their corresponding non-transgenic controls were used to prepare Cy3 and Cy5 derivatized cDNAs, which were competitively hybridized to the DNA chips. Data were examined and analyzed as described[19]. Northern-blot analysis used standard methodology and formaldehyde/agarose gels in which 2 μg (mouse) or 7 μg (human) of poly(A)$^+$ mRNA was loaded per lane. Mouse blots were probed with ^{32}P-labeled (random priming) IMAGE EST:656945 obtained from Incyte, and human northern blots were probed with a 2.6-kb fragment of human Nix 3′ UTR (Genbank Accession #AL132665).

Cloning and analysis of mouse Nix and sNix. A custom Gq mouse-heart cDNA library was screened using IMAGE:656945 as radioactive template. The longest cDNA clone was 2.7 kb with an incomplete ORF of 642 base pairs (bp), which permitted identification of the gene product as mouse Nix[20] (Genbank Accession #AF067395). The full mouse Nix coding region was obtained by RT-PCR of Gαq ventricular mRNA using, as forward primer (5′-CTCGAGAGCCGGATACTGTC-3′) and as reverse primer (5′-CGACTGAGCACACCTTCT-3′). On multiple occasions, from both Gq and non-transgenic mouse hearts, two products were obtained, subcloned into PCR2 vector, and verified by DNA sequencing. Amino terminal epitope tags were added using PCR mutagenesis and the modified cDNAs were subcloned into pcDNA3 for transient expression in HEK293 fibroblasts. Expression was quantified by immunoblotting or fluorescence immunohistochemistry. Cellular viability was assessed with Trypan blue 0.4% (Sigma).

Immunohistochemistry. Paraffin-embedded heart sections fixed in 10% neutral buffered formalin underwent antigen unmasking by heating to 95 °C in 10 mM sodium citrate buffer, pH = 6.0, for 20 minutes. Confocal imaging was performed on a dual laser Nikon PCM2000 system and a Nikon Eclipse E800 microscope with emission wavelengths of 515 ± 30 nm (fluorescein), 605 ± 32 nm (Texas red), and 650 nm long-pass (Cy5). Light micrographs used differential interference contrast optics. Antibodies to HSP60 (Santa Cruz Biotech, #SC-1052), cytochrome-c (Santa Cruz Biotech, #SC-7159), active caspase-3 (R&D Systems, #AF835) and α-sarcomeric actin (Zymed #18-0177) were identified with Texas Red, fluorescein, or Cy5-linked secondary antibodies (Vector Laboratories, Burlingame, California, and Amersham Pharmacia, Piscataway, New Jersey). Epitope tags were labeled using anti-FLAG M2 monoclonal antibody (Sigma #F3165) or anti-HA (12CA5) (Roche #1583816). Apoptosis was determined using the TUNEL assay and Promega fluorescein or horseradish peroxidase apoptosis detection systems. Immunoprecipitations were performed in 0.2% CHAPS and 150 mM NaCl buffer.

Statistical analysis. Data are expressed as mean ± SEM. Comparisons used Student's t test or ANOVA as appropriate. Survival of peripartum Gq and sNix/Gq mice was compared by Wilcoxon test. P value of < 0.05 was assigned statistical significance.

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Competing interests statement

The authors declare that they have no competing financial interests.

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