Marques, F. Z., Prestes, P. R., Byars, S. G., Ritchie, S. C., Würtz, P., Patel, S. K., ... Charchar, F. J. (2017). Experimental and Human Evidence for Lipocalin-2 (Neutrophil Gelatinase-Associated Lipocalin [NGAL]) in the Development of Cardiac Hypertrophy and heart failure. Journal of the American Heart Association, 6(6), [e005971]. https://doi.org/10.1161/JAHA.117.005971

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Experimental and Human Evidence for Lipocalin-2 (Neutrophil Gelatinase-Associated Lipocalin [NGAL]) in the Development of Cardiac Hypertrophy and Heart Failure

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Background—Cardiac hypertrophy increases the risk of developing heart failure and cardiovascular death. The neutrophil inflammatory protein, lipocalin-2 (LCN2/NGAL), is elevated in certain forms of cardiac hypertrophy and acute heart failure. However, a specific role for LCN2 in predisposition and etiology of hypertrophy and the relevant genetic determinants are unclear. Here, we defined the role of LCN2 in concentric cardiac hypertrophy in terms of pathophysiology, inflammatory expression networks, and genomic determinants.

Methods and Results—We used 3 experimental models: a polygenic model of cardiac hypertrophy and heart failure, a model of intrauterine growth restriction and Lcn2-knockout mouse; cultured cardiomyocytes; and 2 human cohorts: 114 type 2 diabetes mellitus patients and 2064 healthy subjects of the YFS (Young Finns Study). In hypertrophic heart rats, cardiac and circulating Lcn2 was significantly overexpressed before, during, and after development of cardiac hypertrophy and heart failure. Lcn2 expression was increased in hypertrophic hearts in a model of intrauterine growth restriction, whereas Lcn2-knockout mice had smaller hearts. In cultured cardiomyocytes, Lcn2 activated molecular hypertrophic pathways and increased cell size, but reduced proliferation and cell numbers. Increased Lcn2 was associated with cardiac hypertrophy and diastolic dysfunction in diabetes mellitus. In the YFS, LCN2 expression was associated with body mass index and cardiac mass and with levels of inflammatory markers. The single-nucleotide polymorphism, rs13297295, located near LCN2 defined a significant cis-eQTL for LCN2 expression.

Conclusions—Direct effects of LCN2 on cardiomyocyte size and number and the consistent associations in experimental and human analyses reveal a central role for LCN2 in the ontogeny of cardiac hypertrophy and heart failure. (J Am Heart Assoc. 2017;6:e005971. DOI: 10.1161/JAHA.117.005971.)

Key Words: concentric hypertrophy • C-reactive protein • gene coexpression networks • GlycA • hypertrophy • lipocalin-2 • NGAL • systems biology

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Accompanying Data S1, Tables S1 through S12, and Figures S1 through S4 are available at http://jaha.ahajournals.org/content/6/6/e005971/DC1/embed/inline-supplementary-material-1.pdf

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Received March 7, 2017; accepted May 2, 2017.

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Clinical Perspective

What is New?
- Using several animal models, in vitro and human studies, we identified LCN2 as a central gene in the developmental origins of cardiac hypertrophy leading to heart failure.
- Increased LCN2 expression has defined effects of cardiomyocyte proliferation and hypertrophy that might explain cardiac hypertrophy and is likely to reflect chronic activation of inflammatory pathways.

What are the Clinical Implications?
- The experimental effects of LCN2 on cultured cardiomyocytes and in hearts of neonatal animals need to be corroborated in clinical studies of relationships between LCN2, heart size, and, if possible, cardiomyocyte numbers.
- LCN2 could be targeted as a therapeutic target and also developed as an early marker for cardiac hypertrophy and heart failure.

Cardiac hypertrophy is, after age, the single most important risk factor for cardiovascular death, often as a result of heart failure. Hypertrophic remodeling of the heart is usually in response to increased workload, and the response to such stress has been shown to involve inflammatory pathways. Indeed, chronic inflammatory processes have been implicated not only in response to stress, but also more generally as primary etiological factors in cardiovascular disease (CVD). Cardiovascular remodeling depends on refashioning the interstitium, and inflammation stimulates molecules, such as matrix metalloproteinase-9 (MMP9), that degrade the interstitial matrix. MMP9 levels have been associated with cardiovascular disease prognosis, and MMP9 is stimulated by the protein lipocalin-2 (LCN2), also known as neutrophil gelatinase-associated lipocalin (NGAL). LCN2 levels have been used to reflect tissue damage, particularly of the kidney, but more recently also for CVD manifestations, including hypertensive cardiac hypertrophy, coronary artery disease and acute heart failure. LCN2 has also been associated with long-term mortality following acute heart failure, independent of renal function. However, it is unclear whether LCN2 is simply a marker of an inflammatory process or capable of direct effects on the heart that might contribute to cardiac hypertrophy and failure.

In this study, we investigated the association of LCN2 with concentric cardiac hypertrophy in genetic and environmental experimental models and in relation to the normal variation of human heart size and cardiac hypertrophy in diabetes mellitus. We examined transcriptional associations with LCN2 and identified genetic polymorphisms influencing LCN2 expression. We determined the direct cellular effects of LCN2 in cultured cardiomyocytes. Our findings reveal increased LCN2 levels as a consistent association with cardiac hypertrophy in a variety of models and human cohorts, and our in vitro studies support a direct role for LCN2 in the origins of cardiomyocyte hypertrophy and reduced cardiomyocyte proliferation.

Methods

Detailed methods are available in the Data S1.

Genetic Model of Cardiac Hypertrophy and Heart Failure

The hypertrophic heart rat (HHR) is a normotensive inbred polygenic model of adult cardiac hypertrophy, heart failure, and premature death generated by us (Prof Stephen Harrap and Prof Lea Delbridge, University of Melbourne, Parkville, Australia). HHRs have a reduced endowment of cardiomyocytes from very early life, a situation predisposing to hypertrophy and failure in later life. Aged-matched male animals were sampled during the following periods: neonatal (postnatal day 2, n=11 HHR, n=10 Normal Heart Rat [NHR]), adolescent (4 weeks old, n=4 HHR and n=4 NHR for cardiomyocyte isolation), young adult (13 weeks old, n=7 HHR, n=7 NHR; 35 weeks old, n=8 NHR, n=11 HHR), and old adult (50 weeks old, n=11 HHR, n=10 NHR).

Animals were euthanized by decapitation (neonatal) or with an overdose of pentobarbitone (Lethobarb; adult animals). The heart was immediately removed, and ventricles were dissected from the atri. Cardiac weight index (mg/g) was calculated from the total heart weight (mg) relative to total body weight (g) of the animal. The studies involving animals were approved by the Animal Ethics Committee of Deakin University and the University of Melbourne and ratified at Federation University Australia. They were performed according to the “Code of Practice for the Care and Use of Animals for Scientific Purposes” from the National Health & Medical Research Council of Australia.

Rat Microarray Experiments

RNA was extracted from the left ventricle of 2-day-old HHRs and NHRs (n=8/group, no pooling), and Affymetrix GeneChip Rat Gene 1.0 ST Arrays (Affymetrix, Santa Clara, CA) was used to assess genes differentially expressed with the assistance of the Ramaciotti Centre for Gene Function Analysis. The data set obtained has been deposited in the National Center for Biotechnology Information Gene Expression Omnibus.
Lcn2 in Cardiac Hypertrophy  Marques et al

Differential expression analysis was performed using the Partek Genomics Suite (version 6.6; Partek Inc, Chesterfield, MO), with Bonferroni-adjusted P<0.05 and fold difference higher than 2.

Lcn2 mRNA and Protein Levels in Models of Cardiac Hypertrophy and Heart Failure

Primers and conditions used for all real-time quantitative PCR (qPCR) are shown in Table S1. Amplification reactions used the SensiFast SYBR Low-ROX Kit qPCR reagent system (Bioline Reagents Ltd, London, UK) or empty vector (pExpress) were transfected into rat embryonic ventricular myocardial cells (H9c2) using Lipofectamine 2000 (Life Technologies). We counted the number of cells by hemocytometry with the use of the Countess Automated Cell Counter (Life Technologies). Wheat germ agglutinin and Hoechst staining was used to measure cell size, and phospho-histone H3 staining was used to determine cell proliferation. Apoptosis was investigated by flow cytometry using an Annexin-V: FITC Apoptosis Detection Kit I. All in vitro experiments were independently repeated 3 times, each time in triplicates.

RNA-Sequencing and Molecular Pathways

RNA was extracted from Lcn2-KO mice and cells transfected with Lcn2 plasmid for 48 hours (and respective controls). RNA from 3 samples of each group was sent to RNA-sequencing at the Australian Genome Research Facility using the Illumina HiSeq platform (v3 chemistry 100 bp paired-end sequencing). Each sample was considered an individual sample and no pooling was performed. Analysis of differential expression was performed in the R statistical programming environment (version 3.1.0) using Rsusead (version 1.14.2) and edgeR (version 3.6.8) Bioconductor packages (Table S2). P values were adjusted for multiple testing using the Benjamini-Hochberg correction with a false discovery rate <0.05. Gene ontology enrichment analysis was performed on filtered lists of differentially expressed genes to ask which pathways were enriched in genes differentially expressed.

Intrauterine Growth Restriction Rat Model

An environmental model of cardiac hypertrophy was developed using Wistar Kyoto rats by intrauterine growth restriction, induced by uteroplacental insufficiency on day 18 of pregnancy (term being 22 days), was also investigated. Six-month-old operated female and male rats (n=9) were compared to Wistar Kyoto female and male sham rats (n=16).

Human Echocardiography Measurements

Briefly, 114 individuals with echocardiographic measures were selected from a prospective cohort of type 2 diabetic subjects whose basic characteristics are shown in Table S3. In addition, subjects with echocardiographic measures from the Young Finns Study (YFS) analyzed, shown in Table S4. The YFS is a longitudinal population-based study of 3596 individuals recruited during childhood in 1980. Genome-wide genotype data, transcriptome-wide microarray profiling, C-reactive protein (CRP), glycoprotein acetylation (GlycA), and echocardiographic measurements were available for different subsets of 2064 individuals aged 34 to 48 years, participating in the 2011 follow-up study.  The cohort studies complied with the Declaration of Helsinki and were approved by the human ethics committee at each institution. All subjects gave informed consent.

In both cohorts, echocardiographic examinations were performed using transthoracic echocardiography by an Acuson Sequoia 512 (Acuson, Mountain View, CA) with a 3.5-MHz scanning frequency phased-array transducer. From the ultrasound images, LV structure, systolic, and diastolic function were measured following the guidelines of the American Society of Echocardiography, as previously described.
Cardiac hypertrophy was defined as LV mass indexed to the body surface of >95 g/m² in women and >115 g/m² in men.\(^{30}\) E/E' ratio was calculated using the average values of lateral and septal e' velocity.\(^{29}\)

**Human Plasma LCN2 Measurement**

Human plasma was used to measure LCN2 levels in duplicates in 121 subjects with type 2 diabetes mellitus using the Quantikine ELISA Human Lipocalin-2 Immunoassay (R&D Systems, Minneapolis, MN), according to the supplier.

**LCN2 mRNA Levels in Human Heart**

We used data in the repository Gene Expression Omnibus series GSE1145 to investigate the levels of LCN2 in human idiopathic dilated hearts (n=11 control hearts and n=15 idiopathic dilated hearts). We performed a whole-genome analysis using the Gene Expression Omnibus tools, including false discovery rate <0.05, to determine whether LCN2 was overexpressed in human idiopathic dilated hearts.

**GlycA Measurement**

GlycA reflects the integrated concentrations and glycosylation states of several of the most abundant inflammatory acute-phase glycoproteins\(^{31,32}\) measured with a proton nuclear magnetic resonance metabolomics platform.\(^{33}\)

**CRP Measurement**

High-sensitivity CRP was quantified from serum samples using an automated analyzer with a latex turbidimetric immunoassay kit.

**Coexpression Networks and Quantitative Trait Loci**

Transcriptome-wide microarray profiling was performed on whole blood for 1650 individuals in the YFS as previously described.\(^{24}\) Briefly, stabilized total RNA was obtained from whole blood for individuals in the YFS. RNA was hybridized to Illumina HT-12 (version 4; Illumina, San Diego, CA) BeadChip arrays, and raw probe data were exported with the Illumina BeadStudio software. Both positive and negative control probes were used to quantile normalize using the *limma* R package.\(^{34}\) Probe intensities were reported on a log₂ scale.

Identification and characterization of the gene coexpression network analyzed in this study is described in Ritchie et al.\(^{32}\) Here, we defined the neutrophil module’s coexpression as the Spearman’s correlation coefficient between its 27 genes.\(^{32}\) The average expression was used for genes with multiple microarray probes. Edges in the coexpression network were defined as the magnitude of the correlation exponentiated to the power of 4. A vector summarizing module expression was calculated for association testing as the first eigenvector of a principal components analysis on module expression. This summary expression profile captured 57% of the total variation in module gene expression. Association analyses are described in the Statistical Analyses section below.

Module quantitative trait loci (QTLs) were identified for 1386 individuals with matched genotype and gene expression data in the YFS through a genome-wide scan for SNPs associated with the summary expression profile using PLINK 1.90 beta (version 3.32). Individual associations were tested using a linear model of minor allele dosage on neutrophil module summary expression. An SNP was considered a module QTL where \(P<5 \times 10^{-8}\) (genome-wide significance). Models were adjusted for age, sex, and the first 2 principal components of the genotype data. The module QTL, rs13297295, on chromosome 9 was further tested for an association with LCN2 expression levels using the same model. Rs13297295 was also tested for association with GlycA and CRP in the 1712 individuals with matched genotype and GlycA or CRP data.

**Statistical Analyses**

R software (version 3.13; R Foundation for Statistical Computing, Vienna, Austria) was used for the analyses of the YFS data. The *NetRep* package (version 0.54) was used for network analyses.\(^{35}\) Measurements of GlycA, routine lipids, CRP, body mass index (BMI), heart function (measured as early filling [E] to early diastolic mitral annular velocity \([E']—E/E’\) ratio, and E to late \([A]\) diastolic filling—\(E/A\) ratio) were normalized using a natural logarithm transformation, and all continuous measurements were standardized to SD units in both cohorts. Module associations with the inflammatory biomarkers were assessed by linear regression of: neutrophil module expression on GlycA and CRP; linear regression of LCN2 expression on GlycA and CRP. To assess whether LCN2 was a mediator of the relationship between these biomarkers and the neutrophil module, we used linear regression of: GlycA and CRP on LCN2 expression and neutrophil module expression; CRP on LCN2 expression and neutrophil module expression; and GlycA on LCN2 expression and neutrophil module expression. All terms in the models were additive, and all models were adjusted for age and sex. Matched gene expression, GlycA, and CRP data.
were available for 1650 individuals. Associations between LCN2 expression and echocardiographic measurements in the YFS (Table S5) were tested by linear regression of each echocardiographic measurement on LCN2 expression, adjusting for age and sex. Each association was considered significant where \( P<0.05 \). Matched gene expression and echocardiographic data were available for between 1482 and 1573 individuals depending on the LV phenotype.

Inter- and intraassay coefficients of variability were calculated for ELISAs, and only less than 15\% variability was accepted (hence 7 human samples from the type 2 diabetes mellitus cohort were excluded from further analyses). Human plasma LCN2 levels were not normally distributed; therefore, LCN2 was log transformed for the association analyses presented in Table S3. An independent \( t \) test was used to assess differences in continuous variables between those with and without cardiac hypertrophy or chi-square analyses for dichotomous variables. A general linear model analysis was performed to test for associations between presence of cardiac hypertrophy and plasma LCN2 levels after adjusting for variables from the univariable analysis with a \( P \) value of <1.0 (age, sex, BMI, estimated glomerular filtration rate, and systolic blood pressure). We used untransformed LCN2 levels to perform Spearman Rho correlations between human plasma LCN2 and LV left ventricle mass and function in the type 2 diabetes mellitus cohort (Table S6). Significance was set at \( P<0.05 \).

Results from the animal groups were tested for normal distribution using the Skewness and Kurtosis tests. Independent sample \( t \) tests (with Welch’s correction in the case of different variance) and ANOVA were used to compare the data between the animal groups. A 2-way ANOVA was used to compare between Lcn2 expression in the different cell types in HHRs and NHRs.

Results

Lcn2 Is Associated With Cardiac Size in Experimental Genetic and Environmental Models

HHR and NHR

A transcriptome analysis of neonatal P2 LV tissue identified 21 genes with significant differential expression between HHRs and NHRs (Table S7 and Figure S1) involving pathways for cardiovascular system development and function, and cell growth and proliferation (Tables S8 and S9), with Lcn2 showing the greatest differential expression (\( q=7\times10^{-11} \); Table S7). Elevated cardiac Lcn2 expression was validated by qPCR at postnatal day 2 (Figure 1A) and was found to persist with established hypertrophy at 13 weeks of age and with the emergence of heart failure at 35 or 50 weeks of age (Figure 1B) and further confirmed by 25-kDa Lcn2 monomer protein analyses (Figure 1C and 1D). Compared with its control strain, the NHR, we found significantly higher circulating Lcn2 in adult HHRs with established hypertrophy at 35 weeks of age (Figure 1E), but also soon after birth (Figure 1F) before hypertrophy is evident but cardiomyocyte numbers are already reduced. RNA and immunohistochemical studies (Figure 1G and 1H) showed Lcn2 expression in cardiomyocytes and noncardiomyocyte (fibroendothelial) cells. Correlation between cardiac Lcn2 mRNA and plasma Lcn2 in the HHRs and NHRs was \( r=0.996 (P<0.001) \).

Sequencing the HHR and NHR Lcn2 genes for comparison with the published sequence for spontaneously hypertensive rats and Fisher 344 (original progenitors of HHR and NHR), we found 3 unique SNPs in the HHR, all inherited from the spontaneously hypertensive rats, with 1 being intronic and 2 being upstream of the coding sequence (Figure 2A through 2D). In the HHR heart, both Lcn2 mRNA and pre-mRNA levels were increased (Figure 2E), suggesting a transcriptional dysregulation of Lcn2 in the HHR. Transcription Factor Affinity Prediction (sTRAP) analysis suggested that one of these SNPs (rs196968512) created a binding site for the enhancer, RAR-related orphan receptor A (Figure 2B and 2F).

Lcn2 knockout mice

Hearts from adult mice with double knockout of the Lcn2 gene (Lcn2-KO)19 were significantly smaller than age-matched wild-type mice (cardiac weight index 5.4 versus 5.9 mg/g, \( P=0.03 \); Figure 3A). RNA-sequencing of murine heart tissue from Lcn2-KO versus wild-type identified 16 significantly differentially expressed genes (Table S10) that are relevant to pathways related to hypertrophic cardiomyopathies (Table S11; Figure 3B and 3C).

Intrauterine growth restricted rats

Last, in an environmental model of intrauterine growth restriction,18,19 we demonstrated that subsequent adult cardiac hypertrophy was associated with significantly higher levels of cardiac Lcn2 mRNA (\( P=0.0214 \); Figure 4).

Lcn2 Overexpression Induces Hypertrophy in Cardiomyocytes and reduces proliferation

We next sought to determine whether increased Lcn2 transcription in cardiomyocytes results in a hypertrophic phenotype. We transfected rat embryonic ventricular myocardial cells with a plasmid containing the Lcn2 mRNA sequence and performed imaging and RNA-sequencing analyses. Significant increase in the expression of Lcn2 in transfected cells (log fold change=4.44; \( q=0.0005 \); Figure S2) resulted in a significant increase in the size of transfected cells (Figure 5A and Figure S3A). In these hypertrophic cells, we found significantly increased expression of 2 genes previously linked...
with hypertrophic cardiomyopathy—thrombospondin 2 (log fold change=−0.36; q=0.0005) and dynamin 1 (log fold change=−0.48; q=0.044). In addition to hypertrophy, overexpression of Lcn2 resulted in a significant decrease in cell numbers (Figure 5B) with a concomitant reduction in cells positive for phosphorylated histone H3, reflecting reduced cell mitosis (Figure 5C and Figure S3B). No change in apoptosis was observed (Figure S4). Pathway enrichment analysis of the 529 genes with nominally significant evidence of differential expression (unadjusted P<0.05) between transfected and control myocytes suggested dysregulation of genes related to cell cycle (Kyoto Encyclopedia of Genes and Genomes mro04110; P=0.006). There was suggestive evidence for genes related to hypertrophic cardiomyopathy (Kyoto Encyclopedia of Genes and Genomes mro05410; P=0.07) and dilated cardiomyopathy (Kyoto Encyclopedia of Genes and Genomes mro05414; P=0.09; Figure 5D and 5E; Table S12).

**Human LCN2 Is Associated With LV Hypertrophy in Diabetes Mellitus**

Obesity and type 2 diabetes mellitus have been associated with elevated levels of plasma LCN2. Independently from those findings, cardiac hypertrophy has been associated with diastolic dysfunction and is recognized as a diabetic complication. However, whether cardiac hypertrophy and diastolic dysfunction in type 2 diabetic patients is associated with high

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**Figure 1.** Overexpression of lipocalin-2 (Lcn2) in a polygenic model of cardiac hypertrophy. A, Relative expression levels of Lcn2 mRNA measured by real-time PCR in the heart of 2-day-old hypertrophic heart rat (HHR; n=10) compared to normal heart rat (NHR; n=8; P<0.0001), (B) 13-week-old (P=0.016; n=9/strain), 35-week-old (P<0.001; n=8 NHR and n=11 HHR), and 50-week-old (P=0.0015; n=8 NHR and n=11 HHR) HHR compared to NHR. Heart Lcn2 (25-kDa monomer) protein is significantly higher in the HHR compared with NHR, measured by both (C) western blot (P=0.039; n=3/strain) and (D) ELISA (P=0.029; n=4/strain). E, Rat plasma Lcn2 in 35-week-old (P=0.0009; n=5/strain) and (F) 2-day-old (P<0.0001; n=5/strain) HHR compared to NHR. G, Lcn2 mRNA in cardiomyocytes (P=0.013; n=4/strain) and noncardiomyocytes (P=0.03) in the NHR and HHR. The interaction explained 5.135% of total variation (P=0.138), the cell type 6.91% of variation (P=0.0899), and the strain explained the majority of variation (63.58%; P=0.0001). H, Lcn2 staining in NHR and HHR hearts (×400 magnification; scale bar=200μm). *P<0.05; **P<0.01; ***P<0.001. Data shown as mean and error bars represent SEM.

DOI: 10.1161/JAHA.117.005971

Journal of the American Heart Association
LCN2 levels is not known. Echocardiographic assessment of 114 patients with type 2 diabetes mellitus and normal renal function revealed significantly higher levels of mean plasma LCN2 in the 30 diabetic subjects with LV hypertrophy than those 84 without 44.0 ng/mL [95% CI, 38.3–50.6] versus 36.0 ng/mL [33.1–39.2] P=0.017 that remained after adjustment for age, sex, BMI, estimated glomerular filtration rate, and systolic blood pressure (P=0.034; Figure 6A). There

Figure 2. Variants in the lipocalin-2 (Lcn2) gene, showing regions with variants in the HHR, according to the Rat Genome Database (RGD; version 5). A, Genotype analysis of the region of 10 000 bp around the Lcn2 gene, showing the origin of the variants observed in the HHR. Highlighted in gray are variants that differ from the reference genome, showing that the HHR carries 3 unique variants which were inherited from the SHR. B, Single-nucleotide polymorphism (SNP) on chr3: 16 767 791 (rs19696512 C/T) 1401 bp upstream of Lcn2 gene. C, SNP on chr3: 16 767 398 (G/A) in a highly conserved region 1001 bp upstream the Lcn2 gene. D, Nonfunctional intronic SNP originally from SHR on position chr3: 16 763 494 (rs198262931 C/T). E, Lcn2 pre-mRNA is also upregulated in the HHR compared to the NHR (n=5/strain), suggesting that it is dysregulated at the transcriptional level. F, The SNP, rs19696512, creates a new binding site for the transcription factor, Rora (region underlined in B), which acts as an enhancer for expression of Lcn2 and is exclusive of the HHR. The figure shows the binding site score and the P values for the binding of Rora to that region. chr3 indicates rat chromosome 3; F344, Fisher 344 rat; HHR, hypertrophic heart rat; N/A, nonannotated SNP; NHR, normal heart rat; RGD, reference sequence from the Rat Genome Database v5; SHR, spontaneously hypertensive rat.
was a positive correlation between LCN2 levels and LV mass (n=114; Spearman’s r=0.22; P=0.018; Figure 6B and Table S6). In diabetic subjects with cardiac hypertrophy, there was evidence of diastolic dysfunction (Table S3) with a significantly increased E/E₀ ratio (mean±SD 15.2±4.5 versus 11.4±3.5; P<0.0001), but there was no association between these measurements and LCN2 (Table S6).

**LCN2 mRNA Is the Human Heart**

From subjects with idiopathic dilated cardiomyopathy, cardiac RNA expression data in a public repository (GSE1145) revealed overexpression of cardiac LCN2 after adjustment for multiple comparisons (Figure 6C; false discovery rate, q=0.008).

**LCN2 Expression, Cardiac Size and Function, and BMI in the YFS**

In the 1590 YFS individuals (mean age, 42 years) with matched echocardiographic and whole-blood gene expression data, linear regression analysis adjusted for age and sex showed that LCN2 expression was associated with various structural and functional LV phenotypes (Table S5). Higher LCN2 expression was associated with increased heart rate (P=6×10⁻⁶), LV end-diastolic volume (P=0.02), and cardiac output (P=3×10⁻⁵). LV mass (P=5×10⁻⁵) and thickness of the interventricular septum (P=8×10⁻⁴) were also positively correlated with LCN2 expression. Although the negative correlation between LCN2 expression and E/A ratio (P=5×10⁻⁴) suggested diastolic impairment, this might have been confounded by the increased heart rate, given that other measures of diastolic function (E/E₀ ratio, mitral E-wave declaration time, and isovolumic relaxation time) did not show significant
correlations with LCN2 expression (Table S5). There was also no significant correlation between LCN2 expression and indices of systolic function, including ejection fraction and systolic wall velocities (Table S5). In addition, LCN2 expression correlated significantly with BMI ($r=0.33$; 95% CI, 0.28–0.37; $P=8\times10^{-42}$). In terms of LV phenotypes, BMI was associated with increased LV size, heart rate, end-diastolic volume, and cardiac output (data not shown). There was also more consistent evidence of reduced diastolic function with increasing BMI, although systolic function was normal. Regression models that included LCN2 expression and BMI revealed that the correlation between BMI and LCN2 expression could account for the associations observed for LCN2 expression alone (data not shown). This has been reported previously and interpreted as part of the low-grade inflammatory activation that accompanies obesity and predisposes to insulin resistance and type 2 diabetes mellitus.42

**LCN2 Is Central to a Neutrophil Gene Coexpression Network and Is Under Genetic Control**

Previous analysis of whole-blood gene expression data identified a reproducible tightly coexpressed gene module
associated with elevated levels of inflammatory markers in 2 independent healthy population studies. This module was significantly enriched for genes involved in the innate immune response, in particular neutrophil function. Among these, the expression of LCN2 showed high centrality to the neutrophil module (Figure 7), with a scaled connectivity (Data S1) of 0.65 and a correlation of 0.89 with the module’s summary expression profile. In a healthy population of 1650 individuals from the YFS cohort (Table S4), we found that the module summary expression was independently associated with both GlycA ($\beta$=0.16; 95% CI, 0.11–0.21; $P$=2×10$^{-9}$) and CRP ($\beta$=0.15; 95% CI, 0.093–0.20; $P$=5×10$^{-8}$) when both were included in the same model, suggesting the module is related to inflammatory processes reflected by both biomarkers. Although LCN2 expression itself was independently associated with both GlycA ($\beta$=0.18; 95% CI, 0.12–0.23; $P$=7×10$^{-11}$) and CRP ($\beta$=0.19; 95% CI, 0.14–0.24; $P$=3×10$^{-12}$), GlycA and CRP were no longer significant when LCN2 was included in the model. This suggests that LCN2 on its own is a better predictor of the module summary profile. To determine the potential genetic determinants of the neutrophil module function, we performed a QTL scan on the neutrophil module’s summary expression (Data S1) in 1650 healthy individuals from the YFS cohort. We found that rs13297295, the top module QTL, was located 750 kb downstream from LCN2 to which it was a cis-eQTL, with each “C” allele at rs13297295 increasing expression of LCN2 by 0.39 SD ($P$=2×10$^{-9}$; adjusted for age, sex, and 2 genetic principal components). There was no detectable association between rs13297295 and CRP or GlycA. Taken together, these results suggest that increased LCN2 expression is central to the inflammatory gene module.

Discussion

Our experimental and human studies reveal increased levels of LCN2 as a consistent correlate of cardiac hypertrophy (summarized in Figure 8). This relationship existed in the HHR polygenic model of spontaneous cardiac hypertrophy leading to heart failure, in Lcn2 gene knockout mice, but also in an environmental model of cardiac hypertrophy following intrauterine growth retardation. In human studies, LCN2 was associated with cardiac hypertrophy in healthy subjects of the YFS and in patients with type 2 diabetes mellitus. Importantly, our in vitro studies of Lcn2 overexpression showed that it can activate hypertrophic pathways and cause an increase in cardiomyocyte size but a decrease in their proliferation. Irrespective of the primary cause of increased LCN2, these direct cellular effects provide a common fundamental pathophysiology for the contribution of LCN2 to cardiac hypertrophy. This is the first time that a specific cardiomyocyte hypertrophic effect of LCN2 has been demonstrated. Previous studies have focused on the effects of LCN2 on the interstitial matrix through induction of the proteinase, MMP9. This is relevant to the degradation of intercellular matrix as part of the remodeling of the heart during the development of hypertrophy. However, we could find no significant variation in MMP9 expression in association with changes in Lcn2 in HHR and Lcn2 knockout mice (data not shown). It would also be beneficial to submit Lcn2 knockout mice to stressors such as transverse aortic constriction to further understand the role of Lcn2 in heart disease, but this was outside the scope of this study.

Interestingly, we observed that Lcn2 expression reduced in vitro cardiomyocyte proliferation and cell numbers. It might seem counterintuitive that a limitation of cardiomyocyte

Figure 6. Lipocalin-2 (LCN2) is associated with human cardiac hypertrophy. A, Plasma levels of LCN2 were higher in patients with echocardiographically determined left ventricular hypertrophy (n=30) compared with those without (n=84; $P$=0.017, showing the median and 95% CI). B, There was a positive correlation between LCN2 levels and left ventricular mass (n=114; Spearman’s $r$=0.22; $P$=0.018). C, LCN2 was overexpressed in human idiopathic dilated hearts compared with normal hearts ($P$=0.008 after adjustment for false discovery rate). Data shown as mean and error bars represent SEM. *$P$<0.05; **$P$<0.01.
numbers would contribute to cardiac hypertrophy. However, in very early life, when cardiomyocyte replication establishes the endowment of cardiac contractile cells, the actions of Lcn2 to reduce cell numbers could have long-lasting effects.46 Fewer cells means greater individual workload resulting in hypertrophy. We have shown previously that the HHR has a reduced complement of cardiomyocytes in early postnatal life, an age at which we discovered cardiac Lcn2 to be highly expressed. Although we have no measurements of cardiomyocyte numbers in the early postnatal period following intrauterine growth restriction, other studies have shown that birth weight is associated with reduced numbers of cardiomyocytes,47 and very early protein restriction has been associated with increased cardiomyocyte apoptosis.48 Therefore, increased Lcn2 very early in life (whether genetic or environmental in origin) could predispose to hypertrophy through effects on cell number. The propensity for hypertrophy would be magnified by any persistent increase in Lcn2 levels into adulthood, as we saw in the HHR and in adult animals that had experienced intrauterine growth retardation.

In our human analyses, we found that LCN2 expression correlated significantly with cardiac size in healthy subjects in the YFS. Cardiac size also correlated with BMI in these subjects. Increased BMI is known to augment LCN2, probably as part of the induction of a chronic mild inflammatory state.49 Given the direct effects of LCN2 on cardiomyocyte hypertrophy, it is not unreasonable to suggest that at least part of the influence of BMI on heart size might be mediated through increases in Lcn2. Diabetes mellitus is also characterized as a state of chronic inflammation and cardiac hypertrophy is a common finding patients with type 2 diabetes mellitus.50 We found that diabetic patients with cardiac hypertrophy had significantly higher plasma concentrations of LCN2, even after adjustment of BMI, renal function, and blood pressure. In the absence of other measures of inflammatory markers, we cannot be certain of the explanation of the elevated LCN2 in those diabetics with cardiac hypertrophy.

The factors that might increase LCN2 deserve consideration. LCN2 exhibits complex and tissue-specific regulation and pathophysiology relevant to a broad portfolio of biological functions and disease involvements, including bacterial

Figure 7. Neutrophil module: An inflammatory biomarker associated coexpression network is under genetic control of a cis-eQTL of lipocalin-2 (LCN2). A, coexpression heatmap (Spearman’s correlation) and scaled network connectivity (Methods) of genes composing the neutrophil module in the YFS (n=1650). B, Box plots of age- and sex-adjusted LCN2 expression in individuals with differing dosages of the rs13297295 minor allele (“C”). C, Locus zoom plot of the 1-MB region around LCN2 showing association on the y-axis (–log10 P value) between each single-nucleotide polymorphism (points) and LCN2 expression, recombination rate in the EUR population in that region (blue line underneath the points), and r² between each SNP and rs13297295 (point color).
infection, inflammation, cancer, renal damage, obesity, and, more recently, CVD.51

In the YFS, the associations between LCN2 and the biomarkers, CRP and GlycA, suggest that underlying activation of inflammatory pathways might be responsible. LCN2 is released from neutrophils in response to inflammatory cytokines, including tumor necrosis factor-α, interleukin-1β, and interleukin-6.51 We analyzed the transcriptional subnetworks associated with the biomarkers of low-grade inflammation in otherwise healthy individuals of the YFS. LCN2 expression appeared central to a coexpression module representing proteins secreted or expressed by neutrophils as part of the innate immune response.32 The expression of this neutrophil module was significantly associated with levels of both CRP and GlycA. Expression QTL analyses identified a common human genetic variant that increased LCN2 levels (rs13297295 “C” allele) and was also positively associated with module expression. Despite limited power to detect hypertrophic phenotype associations, further studies of this LCN2 eQTL in human cardiomyocytes is warranted.

In relation to increased Lcn2 expression in the HHR, evidence of underlying activation of inflammatory pathways is less clear. We could find no significant differential cardiac expression in neonatal HHR of the cytokines, tumor necrosis factor-α, interleukin-1β, or interleukin-6, or the acute-phase reactants that comprise the majority of GlycA (data not shown). Nor was there any significant association of Lcn2 with MMP9 expression. Given the polygenic nature of the HHR, other genetic factors are presumed to drive the higher Lcn2 expression. In a simple candidate gene approach, we identified 3 differences in DNA sequence in and around the Lcn2 gene between HHR and NHR, 1 of which (rs196968512) created a binding site for the transcriptional enhancer, RAR-related orphan receptor A. The significance of this SNP and other polymorphisms requires further investigation in cross-breeding linkage analyses.

The downstream effects of Lcn2 on cardiac expression pathways were reasonably consistent with changes in the pathways typically associated with hypertrophy observed in the HHR, the Lcn2 knockout mice, and in cultured cardiomyocytes. Of particular interest, in vitro Lcn2 overexpression downregulated the genes for dynamin 1 and thrombospondin 2 in cardiomyocytes. Mice with a mutation in the gene for dynamin 1 develop dilated cardiomyopathy through mitochondrial defect and thus energy deficiency in the heart.40 Thrombospondin 2-knockout mice also display dilated cardiomyopathy with progressive cardiomyocyte stress and death.41

Our findings add to the evidence supporting a role for LCN2 in CVD, likely through inflammatory processes. Previous studies have shown LCN2 to be associated with atherosclerosis and plaque instability, endothelial dysfunction, oxidative stress,52 interstitial fibrosis,53 myocarditis, cardiac remodeling, and heart failure.54,55 Baseline circulating LCN2 levels

Figure 8. Diagram showing summary of findings. LCN2 indicates lipocalin-2; SNP, single-nucleotide polymorphism.
have been independently associated with subsequent development of CVD in 1 population study and in a 4-year follow-up after cerebrovascular ischemia study.

**Perspectives**

The actions of LCN2 that we have observed on cardiomyocyte division and growth have implications for the development of cardiac hypertrophy and failure. The timing of the actions of LCN2 is also important. In the perinatal period, LCN2 could reduce the endowment of cardiomyocytes. In adulthood, LCN2 would tend to augment cardiomyocyte hypertrophy in response to any form of myocardial injury. The stimuli to LCN2 expression are both genetic—as in the HHR polygenic model in rats and possibly in the case of rs13297295 in man—and also environmental, as in the case of early-life deprivation and also environmental, as in the case of early-life deprivation or other inflammatory activators, such as obesity and type 2 diabetes mellitus. Unfortunately, there are no human data regarding LCN2 in the neonatal period, and it is yet to be determined whether adult human cardiac hypertrophy might have its origin in reduced numbers of cardiomyocytes, but this emerges as a fascinating and important possibility.

**Acknowledgments**

We thank the Ramaciotti Centre for Gene Function Analysis for the help with arrays and the Australian Genome Research Facility for the help with Sanger and next-generation sequencing. The authors thank Irina Lisinen for the expert technical assistance in the statistical analyses.

**Sources of Funding**

This work was supported by grants from the National Health & Medical Research Council of Australia (project grant APP1034371, APP509252), the National Heart Foundation (project grant G10M5155, GM6368), and the Federation University Australia “Self-sustaining Regions Research and Innovation Initiative,” an Australian Government Collaborative Research Network (CRN). Marques is supported by NHMRC (APP1052659) and National Heart Foundation (PF12M6785) co-shared Early Career Fellowships, and a National Heart Foundation Future Leader and Baker Fellowships. Prestes is supported by a Robert HT Smith Fellowship from the Federation University Australia. Patel is supported by a Melbourne University Career Development Fellowship. Inouye was supported by a Career Development Fellowship from the National Health & Medical Research Council of Australia and National Heart Foundation (APP1061435). Byars and Inouye were supported by an NHMRC Project Grant (APP1062227). Wlodek was supported by a National Health & Medical Research Council of Australia Project Grant (APP400003).

Salomaa was supported by the Finnish Foundation for Cardiovascular Research. Kettunen was supported by the Academy of Finland (grant 283045). Ala-Korpela receives funds from the University of Bristol and UK Medical Research Council (MC_UU_12013/1). Würitz was supported by the Novo Nordisk Foundation. The Young Finns Study was financially supported by the Academy of Finland: grants 286284, 134309 (Eye), 126925, 121584, 124282, 129378 (Salve), 117787 (Gendi), and 41071 (Skidi); the Social Insurance Institution of Finland; Kuopio, Tampere, and Turku University Hospital Medical Funds (grant X51001); Juho Vainio Foundation; Paavo Nurmi Foundation; Finnish Foundation of Cardiovascular Research; Finnish Cultural Foundation; Tampere Tuberculosis Foundation; Emil Aaltonen Foundation; Yrjö Jahnsson Foundation; and Signe and Ane Gyllenberg Foundation. The quantitative serum NMR metabolomics platform and its development was supported by the Academy of Finland, TEKES—the Finnish Funding Agency for Technology and Innovation, Sigrid Juselius Foundation, Novo Nordisk Foundation, Finnish Diabetes Research Foundation, Paavo Nurmi Foundation, and strategic and infrastructural research funding from the University of Oulu, Finland, British Heart Foundation, Wellcome Trust, and Medical Research Council, UK.

**Disclosures**

Würitz, Kangas, Soininen, and Ala-Korpela are shareholders of Brainstake Ltd. (www.brainshake.fi), a company offering NMR-based metabolic profiling. Würitz, Kangas, Soininen, and Kettunen report employment for Brainstake Ltd.

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Supplemental Material
Data S1.

Supplemental Methods

Animal Samples

We then studied the hypertrophic heart rat (HHR) as a normotensive inbred polygenetic model of adult cardiac hypertrophy and failure that begins life with a smaller heart and fewer cells.\textsuperscript{1,2} Neonatal male (day 2, \(n=11\) HHR, \(n=10\) NHR), adolescent (4-week old, \(n=4\) HHR and \(n=4\) NHR for cardiomyocyte isolation), young adult (13 weeks-old, \(n=7\) HHR, \(n=7\) NHR) and old adult (50 weeks-old, \(n=11\) HHR, \(n=10\) NHR) age-matched animals were euthanized by decapitation (neonatal) or with an overdose of pentobarbitone (Lethobarb) (adult animals). The heart was immediately removed, and the ventricles were dissected from the atriums. Cardiac weight index (CWI, mg/g) was calculated from the total heart weight (mg) relative to total body weight (g) of the animal. HHR samples presented hypertrophy since 4 weeks of age (data not shown). Plasma was also collected. An environmental model of left ventricular hypertrophy developed using Wistar Kyoto (WKY) rats by intra-uterine growth restriction induced by uteroplacental insufficiency on day 18 of pregnancy (term=22 days) was also investigated.\textsuperscript{3,4} Six month old male operated animals (\(n=3\)) were compared to WKY sham rats (\(n=7\)). For all samples, the tissues used were first preserved in liquid nitrogen and later transferred to a \(-80^\circ\text{C}\) freezer, and RNA was extracted using miRNeasy kit (Qiagen). Based on the means and standard deviation reported in the manuscript, the number of samples used resulted in power >95\% for both t-test and analysis of variance (ANOVA), as calculated using the software G*Power version 3.0.10 (http://www.gpower.hhu.de). The study was approved by the Animal Ethics Committee of the University of Melbourne and Deakin University, and ratified at Federation University Australia.

Microarray Experiments and Analyses

Total RNA was extracted from left ventricle of neonatal HHR (4 male, 4 female) and NHR (4 male, 4 female) using the miRNeasy Mini kit (Qiagen). Affymetrix GeneChip\textsuperscript{®} Rat Gene 1.0 ST Arrays were used for transcriptome-wide gene expression analysis. Each animal was considered an individual sample and no pooling was performed. Briefly, mRNA was converted to ssDNA, labelled and hybridized to GeneChip\textsuperscript{®} Rat Gene 1.0 ST Arrays, which analyse 27,342 gene transcripts using 722,254 probe sets (on average 26 probes per gene), according to the manufacturer’s instructions, and with the assistance of the Ramaciotti Centre for Gene Function Analysis (University of New South Wales, Sydney, Australia). The data set obtained has been deposited in the NCBI Gene Expression Omnibus (GEO) database according to MIAMI guidelines with series accession number GSE38607 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=bjsxbiaowmwozy&acc=GSE38607).

Results from arrays were normalized using robust-multi-array analysis (RMA). Differentially expressed genes were identified using a two-sample t-test in the Partek\textsuperscript{®} Genomics Suite\textsuperscript{™} (version 6.6). Such genes were selected based on their Bonferroni-adjusted \(P\)-value <0.05 and fold difference higher than 2. Hierarchical clustering using Euclidean distance with all genes with Bonferroni \(P\)-value<0.05 was performed with Partek\textsuperscript{®} Genomics Suite\textsuperscript{™}. Gene Ontology (GO), used to further interpret the differentially expressed gene data set and to identify over-represented functional groups of genes, and Gene Set Enrichment Analysis (GSEA), used to highlight pathways over- or under-represented as a set, were also performed in Partek. Molecular networks related to differentially expressed genes were built via the Ingenuity Pathway Analysis (IPA, Ingenuity\textsuperscript{®} Systems, www.ingenuity.com) application using the “Core Analysis” function. Briefly, a data set
containing differentially expressed genes and respective fold differences were first uploaded into the application. The genes were then correlated based on previous association between genes or proteins and known functional roles of genes. The biological relationship between two genes, represented as nodes, is shown as a line. Nodes with different shapes indicate different functional class.

Isolation of cardiomyocytes and other cells

Cardiomyocytes were isolated from whole NHR and HHR hearts as we previously described. After 10 min incubation, cardiomyocytes were deposited in the bottom of tubes. The supernatant was considered to contain other cells than cardiomyocytes. Cardiomyocytes or other cells were pelleted by centrifugation at 4,000 RCF for 5 min in a refrigerated bench top centrifuge. Total RNA was extracted from both cardiomyocytes and other cells for each sample using the miRNeasy kit (Qiagen) as described above. cDNA and qPCR were performed as described above.

Real-time Quantitative PCR (qPCR)

First-strand complementary synthesis reaction was performed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies). Primers were designed to flank an exon-exon junction using Primer3 and NCBI tool Primer Blast. Primers and conditions used for all qPCR are shown in Online Table 1. Amplification reactions used the SensiFast™ SYBR Low-ROX Kit qPCR reagent system (Bioline) in a Viia7 qPCR instrument (Life Technologies). Samples were run in duplicates. The specificity of the qPCR was ensured by melting curve analysis and electrophoresis in agarose gels (data not shown). The glyceraldehyde 3-phosphate dehydrogenase (Gapdh) was used as reference transcript. Significance was assessed by 2-ΔΔCt method.

Plasma measurement of LCN2

In animal samples, after euthanasia, blood was immediately collected in EDTA tubes and centrifuged. Plasma was stored separately. Lcn2 plasma and left ventricle levels were measured by enzyme-linked immunosorbent assay (ELISA) in duplicates in neonatal and adult HHR and NHR using the Lipocalin-2 Rat ELISA Kit (Abcam) according to the supplier. Human plasma collected in heparin tubes was used to measure LCN2 levels using the Quantikine ELISA Human Lipocalin-2 Immunoassay (R&D Systems) according to the supplier. Inter- and intra-assay coefficients of variability were calculated and only less than 15% variability was accepted (hence 7 human samples were excluded from further analyses). The overall intra-assay coefficient was 4.3% and the inter-coefficient was 4.5%.

Immunohistochemistry

Immunohistochemistry was performed using an anti-LCN2 Rabbit Polyclonal antibody (1:200 dilution, OriGene Technologies, TA322583), followed by the EnVision+System-HRP (Dako). In immunohistochemistry images, the positive staining (Lcn2) was the standard brown while the counterstain was haematoxylin (stains blue). Images were obtained in an EVOS® XL Cell Imaging System (Life Technologies) microscope using a 400x magnification.

Protein measurement of Lcn2 by Western Blots

Protein was extracted from left ventricle of 13 week old HHR and NHR by the use of RIPA buffer (Sigma-Aldrich) and 1% Halt Protease & Phosphatase Inhibitor Cocktail (Thermo Scientific). Fifty micrograms of extracted rat proteins were resolved by 4–15% Mini-PROTEAN® TGX™ Precast Gel (BioRad). Proteins were electroblotted on to a Nitrocellulose Membrane (Thermo Scientific). Membranes were blocked for two hours in 5%
skim milk, then incubated overnight at 4°C with Anti-LCN2 Rabbit Polyclonal antibody (1:2500 dilution, OriGene Technologies, TA322583) or β-actin (1:5000 dilution, Cell Signalling, 3700) in blocking solution, followed by several washing steps in PBS-Tween 20, 1 h at RT with secondary antibody (Lcn2: Anti-rabbit HRP-linked Antibody, 1:5000 dilution, Cell Signalling, 7074S; and β-actin: Anti-mouse HRP-linked Antibody, 1:5000 dilution, Cell Signalling, 7076S) in blocking solution, and several washing steps in PBS-Tween 20, before detection by enhanced chemiluminescence SuperSignal West Pico Substrate (Thermo Scientific) according to the manufacturer’s instructions. Images were captured with a UVITEC Alliance digital imaging system (Thermo Scientific). Bands were quantified using Image J software, and Lcn2 protein levels were calculated as a ratio relative to β-actin. Both 25 kDa monomer Lcn2 and Lcn2 complexed with matrix metallopeptidase 9 (Mmp9) were measured.

**DNA sequencing**

Sanger sequencing was used to sequence 10,000 base pairs (bp) before and 2,000 bp after the Lcn2 gene (primers and conditions in Online Table 1). Briefly we extracted DNA using PureLink® Genomic Extraction kit (Life Technologies), and then amplified it with IMMOLASE DNA Polymerase (Bioline) in a thermocycler (BioRad). PCR fragments were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) and send out for sequencing at AGRF. We sequenced the DNA of both HHR and NHR and align them to the sequence of both SHR and Fisher 344 (the two strains which originated the HHR and NHR), and the reference genome from the Rat Genome Database (version 5).

**Lcn2-knockout**

Whole body and heart size of adult (12-13 week-old) whole-body Lcn2 knockout (Lcn2-KO, n=6) and age-matched wild-type mice C57BL/6 (n=4) were measured upon death, and CWI was calculated as described above. Both Lcn2-KO and wild-type mice were sourced from Prof Alan Aderem (University of Washington). 7

**Vector containing Lcn2**

The pExpress vector containing the cDNA for the rat Lcn2 (Thermo Scientific, catalogue number ID MRN1768-98079404) was transformed into JM109 competent cells (Promega). Constructs were cultivated in plates of Luria-Bertani (LB) medium containing agar and ampicillin (50 μg/ml). Individual colonies were selected and the presence of the vector was confirmed by restriction enzyme and Sanger sequencing. The colonies containing the vector were cultured in LB medium overnight, and plasmids were extracted using a PureLink™ HiPure Plasmid Filter Kit (Life Technologies). To create an empty pExpress vector to be used as a control, Lcn2 sequence was cut out of the plasmid using the restriction enzymes NotI and SmaI (Promega). DNA Polymerase I Large (Klenow) Fragment (Promega) was used to generate blunted ends. The product was run in a 1% agarose gel, and the band of the correct size was purified from the gel using the Wizard SV Gel and PCR Clean-Up system (Promega). 163 ng of this purification was ligated using T4 DNA Ligase (Life Technologies), and this was transformed into competent cells as described above. The sequence of both plasmids has been confirmed by Sanger DNA sequencing using T7 and sp6 universal primers as a service at the Australian Genome Research Facility (AGRF) as a service.

**H9c2 Cell Transfections**

The rat embryonic ventricular myocardial cells (H9c2: ATCC® CRL1446™) were grown in Dulbecco’s modified Eagle’s medium (DMEM), containing 10% fetal bovine serum (FBS) (all from Life Technologies) at 37°C with 5% CO2. Cell culture medium was replaced every 48–72 hours. This cell line was specifically chosen because it has similar hypertrophic
properties as rat primary neonatal cardiomyocytes. The cells were purchased from ATCC, and short tandem repeats (STR) profiling was not available for this rat cell line. Moreover, cells were regularly tested for mycoplasma contamination using Hoechst staining (Life Technologies) and a Nikon C2 confocal microscope, with negative results during experiments. H9c2 cells were cultured in 6- to 24-well plates and transfected with 2ng/ml of Lcn2 or empty (pExpress only) plasmid using Lipofectamine 2000™ (Life Technologies) in cells at 50% confluence. Untransfected and mock controls were also included. Preliminary experiments were used to determine the best concentration of cells, reagents and vector to have similar levels of Lcn2 as in HHR, so copying physiological levels (Fig. S6). The minimum concentration of vector and transfection reagents, according to the manufacturer’s recommendations, were used. All in vitro experiments were independently repeated 3 times, each time in triplicates (i.e., total of 9 biological replicates). Experiments always included Lcn2 vector, empty plasmid, untransfected cells and a positive control.

**RNA-sequencing and molecular pathways**

Total RNA was extracted from whole snap-frozen hearts of Lcn2-KO mice and cells transfected with Lcn2 plasmid for 48h (and respective controls) using the miRNeasy Mini kit (Qiagen). The RNA from 3 samples of each group was sent to RNA-sequencing at AGRF according to the manufacturer’s instructions using the Illumina HiSeq platform (v3 chemistry 100bp paired-end sequencing). Each sample was considered an individual sample and no pooling was performed.

Read quality was assessed using the FastQC software version 0.10.1 (www.bioinformatics.babraham.ac.uk/projects/fastqc/), revealing high quality sequence and base scores (mean PHRED scores: overall=35.8, 1bp start=32.4, 100bp end=31.5), and thus read trimming was not applied. Analysis of differential expression was performed in the R statistical programming environment (version 3.1.0) using Rsubread (version 1.14.2) and edgeR (version 3.6.8) Bioconductor packages. Paired-end 100bp Illumina reads were aligned to mouse (UCSC mm10 assembly) and rat genomes (UCSC rn5 assembly) at the gene-level using Rsubread aligner with 82.4% and 92.1% of reads successfully mapped overall for mouse and rat samples, respectively. For individual sample and alignment characteristics (i.e. number of paired reads, % mapped, indels, etc) see Online Table 2.

Differential expression (DE) of genes was assessed with edgeR. Genes with 1-count-per-million mapped reads or less in at least one of the samples were filtered out due to unreliable data in any sample. Count data was then normalized by finding a set of scaling factors for the library sizes that adjust for compositional differences between samples and also help account for any biases due to changes in highly expressed genes. Scaling factors were calculated using the trimmed mean of M values (TMM) method, Biological coefficient of variation (BCV) was estimated using the common dispersion method (negative binomial dispersion by conditional maximum likelihood) for both mouse (BCV=0.250) and rat (BCV=0.023) samples. Both values were above the threshold (BCV=0.01) suggested for technical replicates in the edgeR User’s Guide indicating sufficient biological variability for analysis. BCV is derived from total CV, which is the sum of estimated true biological (BCV) and technical variation (i.e. measurement error) across libraries. Gene-specific dispersions were then estimated using the empirical Bayes method (tagwise negative binomial) where expression differences that are consistent between replicates are more highly weighted than those that are not, which is necessary so that DE is not driven by outliers. DE was calculated by computing genewise exact tests for differences in the means between both groups (wild-type and knockout in mouse, control and transfected in rat) of negative-binomially distributed counts in edgeR. P-values were adjusted for multiple testing using the Benjamini-Hochberg method.
correction with a FDR<0.05.

To check experimental data quality with sample clustering and heatmaps, variance stabilized expression values (i.e. normalized for variation library size/sequencing depth) were generated using the R package DESeq (version 1.16.0) 13. This data was entered for the top 100 (ranked by unadjusted $P$-value, all $P<0.01$) differentially expressed genes into principal component analysis (PCA), hierarchical cluster analysis (HCA), gene-gene heatmap and gene-sample heatmap analysis in R. PCA and HCA confirmed control and experimental group differences (data not shown) and also provided further insight into genes that are associated with the main experimental manipulations in both the rat and mouse studies.

GO enrichment analysis was performed on filtered lists (all genes with unadjusted edgeR $P$-value<0.05, including 529 and 721 rat and mouse genes, respectively) of differentially expressed genes using Database for Annotation, Visualization and Integrated Discovery (DAVID) 14,15 to ask which Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were enriched in genes differentially expressed in both mouse and rat samples. Circular plots were produced with the R package Rcircos (version 1.1.2) 16 and show the top 100 differentially expressed genes ranked by edgeR $P$-value and enriched pathways identified in KEGG.

**Number of cells and cell size**

We counted the number of cells after 48 hours of transfection by haemocytometry with the use of a Countess® Automated Cell Counter (Life Technologies). Wheat germ agglutinin (WGA) and Hoechst staining were performed as previously described to measure cell size 17. Cell size was measured in 10 different fields per slide obtained in a Nikon C2 confocal microscope using a 400x magnification. 823 cells in average were measured for each condition. Angiotensin II (Sigma-Aldrich) added to the cells for 48h at final concentration of 0.1 µM was used as a positive control.

**Cell cycle arrest**

To estimate cell proliferation, we counted phospho-histone H3 (pH3) stained cells after transfection with Lcn2 vector as described above. 500 nM of colcemid (Merck Biosciences), added 2 hours before fixing cells, was used as positive control. Briefly, cells on 12mm poly-D-lysine/laminin coated coverslips were stained with pH3 and DAPI for fluorescence imaging as described somewhere else 18. We used the primary pH3 anti-rabbit antibody (Millipore, Cat# 06-570, 1:100 dilution) and secondary goat anti-rabbit AlexaFluor-488 antibody (Invitrogen, 1:500 dilution). Ten images per slide were obtained in a Nikon C2 confocal microscope using a 200x magnification (Fig. S7).

**Apoptosis**

Cell death and apoptosis were measured by flow cytometry using an Annexin-V: FITC Apoptosis Detection Kit I (BD Pharmingen, Cat No. 556547). Cells were detached from plate by mild trypsinization (Life Technologies) and were washed twice with cold phosphate buffer saline (PBS). Cells were then resuspended in binding buffer and stained with annexin-V-FITC and propidium iodide as per manufacturer’s protocol. The stained cells were analysed using FACS Aria II (BD Biosciences). The data analysis was performed using FCS express (version 4) research edition analytical software. 100 nM staurosporine was used as a positive control.

**Human Samples**

Two independent cohorts were used (Online Tables 3 and 4). One hundred and twenty one subjects were selected from a prospective cohort of type-2 diabetes (Online Table 3) 19. Left
Ventricle (LV) structure and function was studied using transthoracic echocardiography – Acuson Sequoia 512 (Acuson, Mountain View, CA, USA) with 3.5 MHz scanning frequency phased-array transducer. From the ultrasound images, LV structure, systolic and diastolic function were measured following the guidelines of American Society of Echocardiography, as previously described. Cardiac hypertrophy was defined as LV mass indexed to the body surface of > 95 g/m² in women and > 115 g/m² in men. E/E' ratio was calculated using the average values of lateral and septal e' velocity. Other phenotypes, including blood pressure, were also available. Subjects with chronic kidney disease were excluded due to previous association with LCN2 in the literature. Plasma samples were collected in heparin tubes, and stored at –80 °C. All subjects gave informed consent and the study was approved by human ethics committee at the Austin Hospital, and ratified at Federation University Australia.

The Cardiovascular Risk in Young Finns Study (YFS) is an ongoing population-based prospective study started in 1980 to study the emergence and progression of cardiovascular disease risk factors from childhood. 4,320 individuals from six age groups (3, 6, 9, 12, 15, and 18 years of age) were randomly recruited from the five major population centres of Finland (Helsinki, Kuopio, Turku, Oulu, and Tampere). A total of 3,596 individuals participated in the original study and 2,064 individuals responded to the 2011 follow-up study. Transcriptome-wide microarray profiling, genome-wide genotyping, GlycA, and echocardiography measurements were available for subsets of these 2,064 individuals. Baseline cohort characteristics are provided in Online Table 3. Ethics were approved by the Joint Commission on Ethics of the Turku University and the Turku University Central Hospital.

Venous blood samples were collected after an overnight fast and serum samples were aliquoted and stored at –70°C. Samples were collected after an overnight fast for the YFS cohort.

**GlycA measurement**

A proton nuclear magnetic resonance platform (Bruker AVANCE III, 500 MHz spectrometer) was used to quantify the concentrations of 106 circulating lipids, proteins, and metabolites from serum (including GlycA and total triglycerides) in the YFS cohort. Detailed experimental protocols are described in.

**CRP measurement**

High-sensitivity C-reactive protein (CRP) was quantified from YFS serum samples using an automated analyser with a latex turbidimetric immunoassay kit.

**Coexpression networks and quantitative trait locus**

Transcriptome wide microarray profiling was performed on whole blood for 1,650 individuals in the YFS as previously described. Briefly, stabilised total RNA was obtained from whole blood for individuals in the YFS using the PAXgene Blood RNA System. An Eppendorf BioPhotometer was used to evaluate RNA concentrations and purity and the isolation process validated using an Agilent RNA 6000 Nano Chip Kit. RNA was hybridized to Illumina HT-12 (version 4) BeadChip arrays and raw probe data was exported with the Illumina BeadStudio software. Negative control probes were used to background correct the microarrays. Both positive and negative control probes were used to quantile normalised using the `limma` R package. Probe intensities were reported on a log₂ scale.

Coexpression was calculated for genes composing the previously described neutrophil module as the Spearman correlation coefficient between the gene expression microarray
data. The average probe intensity across samples was taken for genes with multiple probes (DEFA1B, OLFM4, COL17A1). Edges in the interaction network were defined as the absolute value of the coexpression exponentiated to the power 4 as in 30. The connectivity of each gene was calculated as the sum of edge weights to all other genes in the module then scaled by the connectivity of DEFA1B; the most connected gene. The scaled connectivity is a measure of biological importance to the network 31. A summary profile of module expression was calculated as the first eigenvector of a principal components analysis of the module’s gene expression data 32. The summary expression profile explained 57% of the variance the module’s gene expression matrix.

Genome-wide genotyping was carried out on whole blood samples for 2,442 individuals participating in the 2001 follow-up study of the YFS as previously described 25. Sample and genotype quality control was performed for these 2,442 individuals. Briefly, DNA was extracted and genotyped on a custom 670K Illumina BeadChip array sharing 562,643 SNPs with the Illumina 610 BeadChip array. The custom array removed poorly performing SNPs from the 610 array and improved copy number variation coverage 25. Genotypes were called using the Illuminus genotype calling algorithm 33. 2 individuals were removed after initial clustering (call rate < 90%) and 54 samples were subsequently removed following Sanger genotyping pipeline quality control (low call rate: < 90%, failing heterozygosity tests, duplicate samples: >98% concordance on pairwise comparison, previously unknown relationship: >70% concordance on pairwise comparison, or failing Sequenom genotype fingerprinting: < 90% concordance for > 10 genotypes). 546,770 SNPs passed quality control. 3 individuals were removed due to low genotyping success (< 95%) and 1 individual was removed for failing sex checks. 51 individuals were excluded due to previous unknown close relation to another sample (pairwise identity by descent pi-hat > 0.2) and 2 individuals were removed due to cryptic relatedness. Missing genotypes and un-typed SNPs were imputed using the 1000 Genomes Phase 1 (v3) reference panel. Imputed and genotyped SNPs were subsequently excluded where the genotype calling probability for any allele < 90%, information score < 0.4, minor allele frequency < 1% or Hardy Weinberg equilibrium P-value < 5 x 10^{-6}. A combined total of 6,721,082 directly genotyped and imputed SNPs passed quality control.

Module quantitative trait loci (QTLs) were identified for 1,386 individuals with matched genotype and gene expression data in the YFS through a genome-wide scan for SNPs associated with the summary expression profile using the 1.90 beta (version 3.32) software. Individual associations were tested using a linear model of minor allele dosage on neutrophil module summary expression. A SNP was considered a module QTL where $P < 5 \times 10^{-8}$ (genome-wide significance). Models were adjusted for age, sex, and the first two principal components of the genotype data. The module QTL rs13297295 on chromosome 9 was further tested for an association with expression levels LCN2 using the same model. Rs13297295 was also tested for association with GlycA and CRP in the 1,712 individuals with matched genotype and GlycA or CRP levels.

**LCN2 mRNA levels in human heart**

We used data in the repository GEO series GSE1145 to investigate the levels of LCN2 in human idiopathic dilated hearts (n=11 control hearts and n=15 idiopathic dilated hearts). We performed a whole-genome analysis using the GEO tools, including false discovery rate (FDR) adjustment for multiple comparisons, to determine whether LCN2 was over-expressed in human idiopathic dilated hearts.
| Official gene symbol | GenBank Accession # | Primer Sequence (5' → 3') | Concentration | Product length | Annealing temperature |
|----------------------|---------------------|-----------------------------|---------------|---------------|----------------------|
| **Rat Gapdh**        | NM_017008.3         | F: GGGGCTCTCTGCTCCTCCCTCG  | 200 nM        | 108 bp        | 58ºC                |
|                      |                     | R: ACGGCAAATCCGTTACACACC   |               |               |                      |
| **Rat Lcn2**         | NM_130741.1         | F: GGGCTGTCCGATGAACTGAA    | 200 nM        | 98 bp         | 58ºC                |
|                      |                     | R: CATTGGTCGGTGGGAACAGA    |               |               |                      |
| **Human ACTB**       | NM_001101           | F: CGCGAGAAGATGACCCAGAT    | 200 nM        | 119 bp        | 66 ºC               |
|                      |                     | R: GAGTCCATACGATGCCAGT     |               |               |                      |
| **Human LCN2**       | NM_005564.3         | F: CTACGGGAGAACAAAGGAGG    | 200 nM        | 114 bp        | 66ºC                |
|                      |                     | R: CACTGGTCAGATTGGGACAGG   |               |               |                      |
| **Sequencing primers** |                  |                             |               |               |                      |
| **Fragment 1**       | NM_130741.1         | F: GGGAGTTTGAGGCTACCTGG    | 500 nM        | 1144 bp       | 55ºC                |
|                      |                     | R: ATATTGTGCTTCCCTCCCG     |               |               |                      |
| **Fragment 2**       | NM_130741.1         | F: AGGTCAGGAGCAGAAACAG     | 500 nM        | 1339 bp       | 55ºC                |
|                      |                     | R: TACTAGTCGGATGTCGCC      |               |               |                      |
| **Fragment 3**       | NM_130741.1         | F: TCAACAGGATGTGCTGGCAA    | 200 nM        | 984 bp        | 58ºC                |
|                      |                     | R: CAGGTTTTCACCTGACAGG     |               |               |                      |
| **Fragment 4**       | NM_130741.1         | F: TCGGCTCCAGAAAGAAAGACA  | 200 nM        | 909 bp        | 58ºC                |
|                      |                     | R: GACACCCCTTCTCCAGCAAC    |               |               |                      |
| **Fragment 5**       | NM_130741.1         | F: AATGGACAGGGACTCGGGGAA   | 200 nM        | 976 bp        | 58ºC                |
|                      |                     | R: TCGTCCTTCATTCATCGG      |               |               |                      |
| **Fragment 6**       | NM_130741.1         | F: ACTTTATGGAGTGCCCTGG     | 200 nM        | 950 bp        | 58ºC                |
|                      |                     | R: AACACAGTGAGATGGGAGA     |               |               |                      |
| **Fragment 7**       | NM_130741.1         | F: CAAGTCTGAGGGTTGTTTGT    | 200 nM        | 982 bp        | 58ºC                |
|                      |                     | R: ACACAGTGAGATGGGAGA      |               |               |                      |
| **Fragment 8**       | NM_130741.1         | F: TGTCTGGCTTCATCCTTTC     | 200 nM        | 987 bp        | 58ºC                |
|                      |                     | R: ACCTCCCTGGGCTTCAGAGAT  |               |               |                      |

*F: forward primer, R: reverse primer, bp: base pairs.
Table S2. RNA-sequencing alignment characteristics of individual sample.

| Sample Name | Species | Treatment       | Paired Reads | Data Yield (bp) | # Mapped | % Mapped | Correctly paired | # Indels |
|-------------|---------|-----------------|--------------|----------------|----------|----------|------------------|---------|
| LCN2_KO_1   | mouse   | Lcn2 knockout   | 14,884,071   | 2.98 Gb        | 11,954,145 | 80.3     | 9,773,674        | 46,999  |
| LCN2_KO_2   | mouse   | Lcn2 knockout   | 18,268,306   | 3.65 Gb        | 15,479,156 | 84.7     | 12,730,249       | 61,344  |
| LCN2_KO_4   | mouse   | Lcn2 knockout   | 18,081,296   | 3.62 Gb        | 15,286,738 | 84.5     | 12,666,558       | 60,985  |
| WT_7        | mouse   | Wild-type       | 18,478,701   | 3.70 Gb        | 14,802,202 | 80.1     | 12,280,683       | 62,887  |
| WT_8        | mouse   | Wild-type       | 20,477,226   | 4.10 Gb        | 17,082,133 | 83.4     | 13,926,290       | 65,430  |
| WT_9        | mouse   | Wild-type       | 19,197,759   | 3.84 Gb        | 15,633,393 | 81.4     | 12,863,795       | 59,517  |
| P_1         | rat     | Empty plasmid   | 18,079,037   | 3.62 Gb        | 16,643,663 | 92.1     | 13,676,516       | 120,877 |
| P_2         | rat     | Empty plasmid   | 18,570,379   | 3.71 Gb        | 17,095,842 | 92.1     | 13,966,919       | 127,350 |
| P_3         | rat     | Empty plasmid   | 16,296,670   | 3.26 Gb        | 14,986,199 | 92.0     | 12,217,148       | 110,047 |
| L_1         | rat     | Lcn2 plasmid    | 17,256,951   | 3.45 Gb        | 15,898,212 | 92.1     | 13,017,963       | 117,624 |
| L_2         | rat     | Lcn2 plasmid    | 16,652,741   | 3.33 Gb        | 15,343,993 | 92.1     | 12,576,273       | 113,638 |
| L_3         | rat     | Lcn2 plasmid    | 17,646,480   | 3.53 Gb        | 16,252,059 | 92.1     | 13,287,420       | 117,522 |
| **Total**   |         |                 | **213,889,617** | **42.79 Gb** |          |          |          |         |
### Table S3. Characteristics of the participants in the type 2 diabetes cohort.

| Characteristic                              | Subjects without LVH (n=84) | Subjects with LVH (n=30) | P-value |
|---------------------------------------------|----------------------------|--------------------------|---------|
| Age (years)                                 | 61.2 ± 9.6                 | 65.6 ± 10.5              | 0.039   |
| Male sex, n (%)                             | 56 (67%)                   | 14 (47%)                 | 0.08    |
| BMI (kg/m²)                                 | 31.5 ± 6.6                 | 31.9 ± 4.9               | 0.77    |
| eGFR (ml/min/1.73cm²)                       | 86.4 ± 19.4                | 90.9 ± 25.5              | 0.32    |
| Lcn2 (ng/ml)*                               | 38.7 [35.2-41.7]           | 49.1 [39.8-58.5]         | **0.017** |
| Systolic BP (mmHg)                          | 141 ± 19                   | 146 ± 18                 | 0.22    |
| Hypertension, n (%)                         | 71 (85%)                   | 27 (90%)                 | 0.56    |
| **Structure**                               |                            |                          |         |
| Interventricular septum thickness (cm)      | 0.98 ± 0.16                | 1.20 ± 0.18              | <0.0001 |
| Posterior wall thickness (cm)               | 0.96 ± 0.16                | 1.15 ± 0.12              | <0.0001 |
| LV end diastolic dimension (cm)             | 4.8 ± 0.5                  | 5.3 ± 0.6                | <0.0001 |
| LV end systolic dimension (cm)              | 2.8 ± 0.5                  | 3.2 ± 0.6                | **0.001** |
| **Function**                                |                            |                          |         |
| LV ejection fraction (%)                    | 64 ± 9                     | 63 ± 9                   | 0.73    |
| s’ medial (cm/s)                            | 6.9 ± 1.4                  | 6.6 ± 0.5                | 0.28    |
| E (m/s)                                     | 0.75 ± 0.20                | 0.80 ± 0.19              | 0.16    |
| A (m/s)                                     | 0.81 ± 0.17                | 0.89 ± 0.18              | 0.040   |
| E/A ratio                                   | 0.93 ± 0.26                | 0.94 ± 0.31              | 0.97    |
| E’ (cm/s)                                   | 6.8 ± 1.9                  | 5.8 ± 1.7                | **0.016** |
| E/E’ ratio                                  | 11.4 ± 3.5                 | 15.2 ± 4.5               | <0.0001 |
| DT (ms)                                     | 215 ± 52                   | 228 ± 59                 | 0.26    |
| IVRT (ms)                                   | 92 ± 18                    | 92 ± 30                  | 0.97    |

Legend: Data presented as mean ± standard deviation and proportions n. *Lcn2 levels shown as the mean [95% CI]. The P-values represent the differences in parameters in those without LVH and with LVH. Legend: LVH, left ventricle hypertrophy; BMI, body mass index, eGFR, estimated glomerular filtration rate; Lcn2, lipocalin-2; BP, blood pressure; CI: confidence intervals.
Table S4. Characteristics of individuals from the YFS cohort.

| Characteristic                                           | Prevalence in YFS               |
|----------------------------------------------------------|---------------------------------|
| Mean age (and range) in years                            | 41.8 (34–49)                   |
| Number of individuals                                    | 2,064                           |
| Male sex, n (%)                                          | 936 (45%)                      |
| BMI (kg/m²)                                              | 26.5 ± 5.1                     |
| Body surface area (m²)                                   | 1.9 ± 0.2                      |
| GlycA (mmol/L)                                           | 1.6 ± 0.2                      |
| CRP (mg/L)                                               | 1.7 ± 3.2                      |
| Triglycerides (mmol/L)                                   | 1.4 ± 0.8                      |
| LCN2 microarray probe intensity                          | 10.0 ± 0.96                    |
| Systolic blood pressure (mmHg)                           | 119 ± 14                       |
| Use of antihypertensive therapy, n (%)                   | 197 (10%)                      |
| Use of lipid-lowering therapy                            | 75 (4.0%)                      |
| **Disease events, n (%)**                                |                                 |
| Prevalent CVD                                            | 20 (0.7%)                      |
| Prevalent diabetes                                       | 35 (1.3%)                      |
| Prevalent cancer                                          | 40 (1.4%)                      |
| Prevalent immunodeficiency                               | 10 (0.4%)                      |
| Recent febrile infection                                 | 93 (4.6%)                      |
| **Structure**                                            |                                 |
| Interventricular septum thickness (cm)                   | 0.73 ± 0.09                    |
| LV mass (g/m²)                                           | 135 ± 35                       |
| Relative wall thickness to LV diameter (%)               | 28.1 ± 3.8                     |
| **Function**                                             |                                 |
| Heart rate during echo (beats per minute)                | 62 ± 10                        |
| LV ejection fraction (%)                                 | 58.3 ± 3.6                     |
| LV cardiac output (L/minute)                             | 4.7 ± 1.3                      |
| LV volume during diastole (mL)                           | 131 ± 32                       |
| Ratio of LV mass to end diastolic volume                 | 1.04 ± 0.21                    |
| E/A ratio (log-scale)                                    | 0.41 ± 0.24                    |
| E/E' ratio (log-scale)                                   | 1.55 ± 0.21                    |
| Systolic myocardial velocity (s') (cm/s)                 |                                 |
| measured from lateral wall                               | 13.7 ± 3.3                     |
| measured from medial wall                                | 11.0 ± 1.9                     |
| Isovolumic relaxation time (IVRT) (ms)                   |                                 |
| measured from lateral wall                               | 104 ± 22                       |
| measured from medial wall                                | 123 ± 25                       |
| Mitral E-wave declaration time (DT) (ms)                 | 217 ± 38                       |

Legend: Data presented as mean ± standard deviation and proportions n (%). LV: left ventricle; BMI: body mass index; CRP: C-reactive protein; CVD: cardiovascular disease. Prevalent disease indicates events occurring prior to sample collection while incident disease indicates events occurring after sample collection.
Table S5. Linear regression of LV phenotypes on LCN2 expression in the YFS. Models were adjusted for age and sex. Effect size indicates standard deviation (SD) change in left ventricular (LV) phenotype per SD increase in LCN2 expression.

| LV Phenotype                                      | Effect size | 95% confidence interval | P-value   |
|---------------------------------------------------|-------------|--------------------------|-----------|
| LV cardiac output                                 | 0.11        | [0.066, 0.16]            | 3 x 10^{-6} |
| Heart rate during echo                            | 0.12        | [0.068, 0.17]            | 6 x 10^{-6} |
| LV mass                                           | 0.084       | [0.043, 0.12]            | 5 x 10^{-5} |
| E/A ratio                                         | -0.084      | [0.043, 0.12]            | 5 x 10^{-4} |
| Thickness of interventricular septum              | 0.077       | [0.032, 0.12]            | 8 x 10^{-4} |
| LV volume during diastole (end diastolic volume)  | 0.048       | [0.0073, 0.089]          | 0.02      |
| LV isovolumic relaxation time measured from the lateral wall | -0.057       | [-0.11, -0.0074]         | 0.02      |
| Ejection fraction                                 | -0.043      | [-0.093, 0.0077]         | 0.10      |
| E/E' ratio                                        | 0.034       | [-0.016, 0.083]          | 0.18      |
| Ratio of LV mass to end diastolic volume          | 0.035       | [-0.017, 0.087]          | 0.19      |
| LV isovolumic relaxation time measured from the medial wall | -0.020       | [-0.070, 0.030]          | 0.43      |
| Systolic myocardial velocity measured from the medial wall | 0.020       | [-0.030, 0.070]          | 0.43      |
| Relative LV wall thickness to diameter            | 0.018       | [-0.032, 0.069]          | 0.47      |
| Systolic myocardial velocity measured from the lateral wall | -0.014       | [-0.064, 0.036]          | 0.59      |
| Mitral E-wave declaration time                    | -0.00004    | [-0.50, 0.050]           | 1.00      |
### Table S6. Correlation of plasma LCN2 with echocardiogram parameters in the type 2 diabetes cohort.

| Structure                                      | Correlation coefficient (Spearman Rho) | P-value |
|-----------------------------------------------|----------------------------------------|---------|
| Interventricular septum thickness (cm)        | 0.077                                  | 0.418   |
| Posterior wall thickness (cm)                 | 0.165                                  | 0.079   |
| LV end diastolic dimension (cm)               | 0.159                                  | 0.091   |
| LV end systolic dimension (cm)                | 0.146                                  | 0.126   |
| **Function**                                  |                                        |         |
| LV ejection fraction (%)                      | -0.052                                 | 0.587   |
| S’ medial (cm/s)                              | -0.169                                 | 0.095   |
| E (m/s)                                       | **-0.258**                             | **0.006**|
| A (m/s)                                       | 0.081                                  | 0.399   |
| E/A ratio                                     | -0.185                                 | 0.051   |
| E’ (cm/s)                                     | -0.132                                 | 0.162   |
| E/E’ ratio                                    | 0.156                                  | 0.100   |
| DT (ms)                                       | 0.16                                   | 0.090   |
| IVRT (ms)                                     | 0.038                                  | 0.730   |
**Table S7.** Transcriptome-wide gene expression array results showing genes differentially expressed in the heart of neonatal HHR (n=8) and NHR (n=8) (only showing genes with \( P \)-value adjusted by Bonferroni <0.05 and fold difference >2).

| Gene Symbol | RefSeq     | Bonferroni (\( P \)-value) | Fold Change | QTL cardiac mass  |
|-------------|------------|-----------------------------|-------------|-------------------|
| Akr1b10     | NM_001013084 | 0.000003010000             | 2.81        |                   |
| Bphl        | NM_001037206 | 0.00027292800              | 2.10        |                   |
| Bnrl2       | ENSRNOT00000060366 | 0.00041218200       | 2.40        |                   |
| Car4        | NM_019174   | 0.00629238000              | –2.06       | Cm34, Cm55       |
| Endog       | NM_001034938 | 0.00000677000             | –2.33       | Cm73, Cm31, Cm78, Cm51, Cm33, Cm44, Cm75 |
| Hebp2       | NM_001107515 | 0.00069056300             | 2.01        |                   |
| Htr2a       | ENSRNOT00000013408 | 0.00001750000       | 3.18        |                   |
| Itgb1       | NM_001017505 | 0.00005030000             | 2.58        |                   |
| Lcn2        | ENSRNOT00000018776 | 0.00000000007       | 5.02        | Cm10, Cm43, Cm46, Cm48 |
| LOC100270669 | NM_001144957 | 0.00348216000             | –2.08       | Cm29, Cm49       |
| Med22       | NM_001077679 | 0.000000000009            | 3.07        |                   |
| Nt5c3a      | ENSRNOT00000058703 | 0.00020376200       | 2.59        |                   |
| Pex3        | ENSRNOT00000021524 | 0.00005840000       | 2.36        |                   |
| Pop5        | NM_001105752 | 0.01728910000             | –2.41       | Cm5, Cm72, Cm45, Cm76 |
| RGD1309362  | BC098065    | 0.00349021000             | 2.29        |                   |
| RGD1561200  | BC160898    | 0.000000009720            | 4.47        |                   |
| Serhl2      | NM_001130579 | 0.00000092500             | 2.28        | Cm27             |
| Trpm8       | NM_134371   | 0.00004230000             | –2.79       | Cm21, Cm53       |
| Vnn1        | NM_001025623 | 0.00002730000             | 2.03        |                   |
| Wdr46       | NM_212491   | 0.00000164000             | 2.54        |                   |
| Zfp347      | NM_133390   | 0.00934935000             | 2.43        |                   |

Positive values indicate higher expression in the HHR, and negative values indicate higher expression in the NHR. Legend: QTL, quantitative trait locus.
Table S8. Gene ontology analysis with all genes differentially expressed with Bonferroni-adjusted \( P \)-value <0.05.

| Function                                                                 | Type                     | Enrichment Score | Enrichment \( P \)-value |
|-------------------------------------------------------------------------|--------------------------|------------------|--------------------------|
| cell-cell adhesion                                                      | biological process       | 10.51            | 0.00003                  |
| antigen processing and presentation                                     | biological process       | 9.36             | 0.00009                  |
| antigen processing and presentation of exogenous peptide antigen via MHC class II cell surface | cellular component       | 7.73             | 0.00044                  |
| mitochondrion                                                           | cellular component       | 7.58             | 0.00051                  |
| intracellular membrane-bounded organelle myosin heavy chain binding    | cellular component       | 7.44             | 0.00059                  |
| response to interferon-alpha membrane                                   | cellular component       | 6.70             | 0.00123                  |
| neuromuscular process                                                   | biological process       | 6.62             | 0.00134                  |
| retinol metabolic process                                               | biological process       | 6.62             | 0.00134                  |
| retinal dehydrogenase activity                                          | molecular function       | 6.10             | 0.00225                  |
| LRR domain binding                                                      | molecular function       | 6.10             | 0.00225                  |
| synaptic transmission, glutamatergic homotypic cell-cell adhesion       | biological process       | 5.87             | 0.00281                  |
| secretion by cell                                                       | biological process       | 5.82             | 0.00298                  |
| cardiac right ventricle morphogenesis                                   | biological process       | 5.57             | 0.00380                  |
| heterotrimeric G-protein morphogen complex adherens junction            | cellular component       | 5.38             | 0.00459                  |
| NADPH binding                                                           | molecular function       | 5.36             | 0.00472                  |
| spectrin binding                                                        | molecular function       | 5.36             | 0.00472                  |
| water channel activity                                                  | molecular function       | 5.36             | 0.00472                  |
| plasma membrane                                                         | cellular component       | 5.33             | 0.00484                  |
| heparin binding                                                         | molecular function       | 5.32             | 0.00492                  |
| positive regulation of cell death                                       | biological process       | 5.21             | 0.00545                  |
| Term                                                                 | Category       | Score | P-value       |
|----------------------------------------------------------------------|----------------|-------|---------------|
| positive thymic T cell selection                                    | biological process | 5.16  | 0.00573       |
| generation of precursor metabolites and energy                      | biological process | 5.16  | 0.00573       |
| multicellular organismal response to stress                         | biological process | 5.16  | 0.00573       |
| response to cold                                                     | biological process | 5.05  | 0.00640       |
| MHC class II protein complex                                         | cellular component | 4.99  | 0.00683       |
| lysosome                                                             | cellular component | 4.86  | 0.00773       |
| cell adhesion molecule binding                                       | molecular function | 4.83  | 0.00801       |
| chaperone mediated protein folding requiring cofactor               | biological process | 4.83  | 0.00801       |
| fascia adherens                                                      | cellular component | 4.83  | 0.00801       |
| dorsal/ventral neural tube patterning                                | biological process | 4.54  | 0.01064       |
| myoblast differentiation                                             | biological process | 4.54  | 0.01064       |
| water transport                                                      | biological process | 4.54  | 0.01064       |
| laminin binding                                                      | molecular function | 4.42  | 0.01208       |
| anterior/posterior axis specification                                | biological process | 4.42  | 0.01208       |
| positive regulation of apoptotic process                             | biological process | 4.38  | 0.01251       |
| apoptotic process                                                    | biological process | 4.36  | 0.01281       |
| olfactory bulb development                                           | biological process | 4.30  | 0.01359       |
| fibroblast growth factor receptor binding                            | molecular function | 4.30  | 0.01359       |
| SNAP receptor activity                                               | molecular function | 4.30  | 0.01359       |
| immune response                                                      | biological process | 4.28  | 0.01385       |
| cytoplasmic membrane-bounded vesicle                                 | cellular component | 4.26  | 0.01410       |
| innate immune response                                               | biological process | 4.19  | 0.01520       |
| structural constituent of muscle                                     | molecular function | 4.08  | 0.01686       |
| response to drug                                                     | biological process | 4.03  | 0.01782       |
| protein homotetramerization                                          | biological process | 3.99  | 0.01844       |
| protein homotrimerization                                            | biological process | 3.98  | 0.01860       |
| positive regulation of calcium-mediated signalling                    | biological process | 3.98  | 0.01860       |
| positive regulation of cardiac muscle cell proliferation             | biological process | 3.98  | 0.01860       |
| Term                                                                 | Type               | Score  | P-value |
|----------------------------------------------------------------------|--------------------|--------|---------|
| amino acid transmembrane transporter activity                        | molecular function | 3.98   | 0.01860 |
| protein sumoylation                                                  | biological process | 3.98   | 0.01860 |
| trans-Golgi network                                                  | cellular component | 3.97   | 0.01883 |
| blood vessel development                                             | biological process | 3.94   | 0.01937 |
| oxidation-reduction process                                          | biological process | 3.91   | 0.02001 |
| cell-cell junction                                                   | cellular component | 3.87   | 0.02083 |
| basolateral plasma membrane                                          | cellular component | 3.86   | 0.02100 |
| cytoplasm                                                           | cellular component | 3.83   | 0.02170 |
| positive regulation of smooth muscle cell proliferation              | biological process | 3.76   | 0.02338 |
| integrin complex                                                     | cellular component | 3.72   | 0.02426 |
| negative regulation of fibroblast proliferation                      | biological process | 3.72   | 0.02426 |
| eukaryotic cell surface binding                                      | molecular function | 3.56   | 0.02838 |
| tubulin binding                                                      | molecular function | 3.56   | 0.02838 |
| lysosome organization                                                | biological process | 3.56   | 0.02838 |
| RNA polymerase II transcription cofactor activity                    | molecular function | 3.49   | 0.03053 |
| ATPase activity, coupled to transmembrane movement of substances     | molecular function | 3.49   | 0.03053 |
| positive regulation of BMP signalling pathway                         | biological process | 3.49   | 0.03053 |
| positive regulation of interleukin-4 biosynthetic process            | biological process | 3.46   | 0.03136 |
| antigen processing and presentation, exogenous lipid antigen via MHC class Ib | biological process | 3.46   | 0.03136 |
| medium-chain fatty acid transport                                    | biological process | 3.46   | 0.03136 |
| eye photoreceptor cell differentiation                                | biological process | 3.46   | 0.03136 |
| NADP-retinol dehydrogenase activity                                  | molecular function | 3.46   | 0.03136 |
| thermoception                                                        | biological process | 3.46   | 0.03136 |
| 5-phosphoribose 1-diphosphate biosynthetic process                   | biological process | 3.46   | 0.03136 |
| glycogen phosphorylase activity                                      | molecular function | 3.46   | 0.03136 |
| Term                                      | Type            | Score | p-value  |
|-------------------------------------------|-----------------|-------|----------|
| mRNA cis splicing, via spliceosome        | biological      | 3.46  | 0.03136  |
| platelet activating factor biosynthetic   | process         | 3.46  | 0.03136  |
| platelet activating factor biosynthetic   | biological      | 3.46  | 0.03136  |
| myosin filament assembly                  | process         | 3.46  | 0.03136  |
| ferrooxidase activity                     | molecular       | 3.46  | 0.03136  |
| coated vesicle                            | cellular        | 3.46  | 0.03136  |
| chemical homeostasis                      | biological      | 3.46  | 0.03136  |
| protein localization to adherens junction | biological      | 3.46  | 0.03136  |
| photoreceptor cell outer segment          | organization    | 3.46  | 0.03136  |
| death domain binding                      | molecular       | 3.46  | 0.03136  |
| thiol oxidase activity                    | molecular       | 3.46  | 0.03136  |
| cuticular plate                           | cellular        | 3.46  | 0.03136  |
| pulmonary myocardium development          | biological      | 3.46  | 0.03136  |
| antigen processing and presentation of    | process         | 3.46  | 0.03136  |
| exogenous peptide antigen via MHC class I| process         | 3.46  | 0.03136  |
| optic cup morphogenesis involved in       | camera-type     | 3.46  | 0.03136  |
| eye development                           | biological      | 3.46  | 0.03136  |
| double-stranded RNA adenosine deaminase   | activity        | 3.46  | 0.03136  |
| double-stranded RNA adenosine deaminase   | molecular       | 3.46  | 0.03136  |
| supraspliceosomal complex                 | cellular        | 3.46  | 0.03136  |
| ubiquitin homeostasis                     | biological      | 3.46  | 0.03136  |
| tangential migration from the subventricular zone to the olfactory bulb | biological process | 3.46 | 0.03136 |
| positive regulation of dopamine receptor  | biological      | 3.46  | 0.03136  |
| specification of organ position           | biological      | 3.46  | 0.03136  |
| blood vessel endothelial cell proliferation involved in sprouting angiogenesis | biological process | 3.46 | 0.03136 |
| BMP signalling pathway involved in heart induction | biological process | 3.46 | 0.03136 |
| deltoid tuberosity development            | biological      | 3.46  | 0.03136  |
| mesodermal cell differentiation            | biological      | 3.46  | 0.03136  |
| Term                                                                 | Category           | Score | P-value |
|----------------------------------------------------------------------|--------------------|-------|---------|
| cloacal septation                                                    | biological process | 3.46  | 0.03136 |
| regulation of pathway-restricted SMAD protein phosphorylation       | biological process | 3.46  | 0.03136 |
| trachea formation                                                    | biological process | 3.46  | 0.03136 |
| bud elongation involved in lung branching                            | biological process | 3.46  | 0.03136 |
| regulation of cartilage development                                  | biological process | 3.46  | 0.03136 |
| mesenchymal cell proliferation involved in ureteric bud development  | biological process | 3.46  | 0.03136 |
| ureter smooth muscle cell differentiation                            | biological process | 3.46  | 0.03136 |
| plasma cell differentiation                                          | biological process | 3.46  | 0.03136 |
| glycosphingolipid biosynthetic process                               | biological process | 3.46  | 0.03136 |
| connexin binding                                                     | molecular function | 3.46  | 0.03136 |
| nucleolar ribonuclease P complex                                     | cellular component | 3.46  | 0.03136 |
| homocysteine S-methyltransferase activity                            | molecular function | 3.46  | 0.03136 |
| inorganic diphosphate transport                                      | biological process | 3.46  | 0.03136 |
| sequestering of triglyceride                                         | biological process | 3.46  | 0.03136 |
| NADH pyrophosphatase activity                                        | molecular function | 3.46  | 0.03136 |
| response to iron(III) ion                                            | biological process | 3.46  | 0.03136 |
| response to anoxia                                                   | biological process | 3.46  | 0.03136 |
| virus-infected cell apoptotic process                                | biological process | 3.46  | 0.03136 |
| folic acid transporter activity                                       | molecular function | 3.46  | 0.03136 |
| creatine kinase activity                                             | molecular function | 3.46  | 0.03136 |
| regulation of mast cell activation                                   | biological process | 3.46  | 0.03136 |
| regulation of Fc receptor mediated stimulatory signalling pathway    | biological process | 3.46  | 0.03136 |
| ganglioside catabolic process                                        | biological process | 3.46  | 0.03136 |
| oligosaccharide catabolic process                                    | biological process | 3.46  | 0.03136 |
| glycerol channel activity                                            | molecular function | 3.46  | 0.03136 |
| forebrain cell migration                                             | biological process | 3.46  | 0.03136 |
| negative regulation of lymphocyte activation                         | biological process | 3.46  | 0.03136 |
| Biological Process                                                                 | Molecular Function                      | p-value  |
|-----------------------------------------------------------------------------------|-----------------------------------------|----------|
| axon midline choice point recognition                                             | biological process                      | 3.46     |
| olfactory bulb interneuron development                                            | biological process                      | 3.46     |
| Roundabout signalling pathway                                                     | biological process                      | 3.46     |
| regulation of epidermal growth factor receptor signalling pathway                 | biological process                      | 3.46     |
| localization within membrane                                                      | biological process                      | 3.46     |
| low-density lipoprotein particle mediated signalling                              | biological process                      | 3.46     |
| alanine-tRNA ligase activity                                                       | molecular function                      | 3.46     |
| alanyl-tRNA aminoacylation                                                        | biological process                      | 3.46     |
| transferrin transport                                                             | biological process                      | 3.46     |
| endocardial cushion to mesenchymal transition involved in heart valve formation    | biological process                      | 3.46     |
| ascending aorta morphogenesis                                                     | biological process                      | 3.46     |
| microsatellite binding                                                             | molecular function                      | 3.46     |
| arterial endothelial cell differentiation                                          | biological process                      | 3.46     |
| cardiac vascular smooth muscle cell development                                   | biological process                      | 3.46     |
| protease binding                                                                  | molecular function                      | 3.46     |
| activation of MAPKK activity                                                      | biological process                      | 3.42     |
| neuromuscular junction development                                                | biological process                      | 3.35     |
| response to retinoic acid                                                         | biological process                      | 3.35     |
| regulation of cell shape                                                           | biological process                      | 3.24     |
| cellular response to hydrogen peroxide                                            | biological process                      | 3.22     |
| protein kinase B signalling cascade                                               | biological process                      | 3.22     |
| cell adhesion                                                                     | biological process                      | 3.20     |
| positive regulation of cell projection organization                               | biological process                      | 3.18     |
| T cell receptor binding                                                           | molecular function                      | 3.18     |
| positive regulation of NK T cell activation                                        | biological process                      | 3.18     |
| neurotransmitter metabolic process                                                | biological process                      | 3.18     |
| smooth muscle cell migration                                                      | biological process                      | 3.18     |
| positive regulation of ovulation                                                  | biological process                      | 3.18     |
| Term                                                                 | Category          | Score | p-value |
|---------------------------------------------------------------------|------------------|-------|---------|
| peroxisome membrane biogenesis                                      | biological process| 3.18  | 0.04159 |
| vitamin binding                                                     | molecular function| 3.18  | 0.04159 |
| biotin binding                                                      | molecular function| 3.18  | 0.04159 |
| rhythmic synaptic transmission                                      | biological process| 3.18  | 0.04159 |
| sphingosine metabolic process                                       | biological process| 3.18  | 0.04159 |
| regulation of striated muscle contraction                            | biological process| 3.18  | 0.04159 |
| glycosaminoglycan catabolic process                                 | biological process| 3.18  | 0.04159 |
| morphogenesis of a polarized epithelium                             | biological process| 3.18  | 0.04159 |
| phospholipid dephosphorylation                                      | biological process| 3.18  | 0.04159 |
| AP-1 adaptor complex                                                | cellular component | 3.18  | 0.04159 |
| protein thiol-disulfide exchange                                    | biological process| 3.18  | 0.04159 |
| dichotomous subdivision of terminal units involved in salivary gland branching | biological process| 3.18  | 0.04159 |
| epsilon DNA polymerase complex                                       | cellular component | 3.18  | 0.04159 |
| phospholipid transporter activity                                   | molecular function| 3.18  | 0.04159 |
| telomerase activity                                                 | molecular function| 3.18  | 0.04159 |
| telomere maintenance via recombination                              | biological process| 3.18  | 0.04159 |
| ferric-chelate reductase activity                                    | molecular function| 3.18  | 0.04159 |
| fat pad development                                                 | biological process| 3.18  | 0.04159 |
| female meiosis I                                                    | biological process| 3.18  | 0.04159 |
| exploration behaviour                                               | biological process| 3.18  | 0.04159 |
| intracellular distribution of mitochondria                           | biological process| 3.18  | 0.04159 |
| negative regulation of dendritic spine morphogenesis                | biological process| 3.18  | 0.04159 |
| negative regulation of inclusion body assembly                      | biological process| 3.18  | 0.04159 |
| telencephalon regionalization                                        | biological process| 3.18  | 0.04159 |
| negative regulation of T cell differentiation in thymus              | biological process| 3.18  | 0.04159 |
| negative regulation of immature T cell proliferation in thymus       | biological process| 3.18  | 0.04159 |
| biological process                                           | score | p-value |
|-------------------------------------------------------------|-------|---------|
| tendon cell differentiation                                  | 3.18  | 0.04159 |
| trachea development                                          | 3.18  | 0.04159 |
| prostate gland morphogenesis                                  | 3.18  | 0.04159 |
| negative regulation of prostatic bud formation               | 3.18  | 0.04159 |
| regulation of morphogenesis of a branching structure          | 3.18  | 0.04159 |
| negative regulation of thymocyte apoptotic process           | 3.18  | 0.04159 |
| negative regulation of branching involved in ureteric bud morphogenesis | 3.18  | 0.04159 |
| epithelial structure maintenance                             | 3.18  | 0.04159 |
| cardiac muscle fibre development                              | 3.18  | 0.04159 |
| transepithelial transport                                    | 3.18  | 0.04159 |
| nucleotide diphosphatase activity                            | 3.18  | 0.04159 |
| nucleoside-triphosphate diphosphatase activity               | 3.18  | 0.04159 |
| nucleoside triphosphate catabolic process                    | 3.18  | 0.04159 |
| folic acid transport                                         | 3.18  | 0.04159 |
| water transmembrane transporter activity                      | 3.18  | 0.04159 |
| response to interferon-beta                                  | 3.18  | 0.04159 |
| phosphatidylerine biosynthetic process                       | 3.18  | 0.04159 |
| beta-N-acetylhexosaminidase activity                         | 3.18  | 0.04159 |
| selenocysteine incorporation                                  | 3.18  | 0.04159 |
| negative regulation of chemokine-mediated signalling pathway  | 3.18  | 0.04159 |
| regulation of translational fidelity                         | 3.18  | 0.04159 |
| negative regulation of vasodilation                          | 3.18  | 0.04159 |
| negative regulation of wound healing                         | 3.18  | 0.04159 |
| negative regulation of insulin secretion involved in cellular response to glucose stimulus | 3.18  | 0.04159 |
| muscular septum morphogenesis                                 | 3.18  | 0.04159 |
| dorsal aorta morphogenesis                                    | 3.18  | 0.04159 |
| Term                                                   | Type         | Score | P-value |
|--------------------------------------------------------|--------------|-------|---------|
| pulmonary artery morphogenesis                         | biological process | 3.18  | 0.04159 |
| vascular smooth muscle cell development                 | biological process | 3.18  | 0.04159 |
| serine-type carboxypeptidase activity                  | molecular function | 3.18  | 0.04159 |
| centrosome                                              | cellular component | 3.17  | 0.04214 |
| mediator complex                                        | cellular component | 3.16  | 0.04222 |
| ATP hydrolysis coupled proton transport                 | biological process | 3.16  | 0.04222 |
| peptidyl-tyrosine dephosphorylation                     | biological process | 3.16  | 0.04222 |
| intercalated disc                                       | cellular component | 3.16  | 0.04222 |
| embryonic hindlimb morphogenesis                        | biological process | 3.16  | 0.04222 |
| response to bacterium                                   | biological process | 3.11  | 0.04473 |
| positive regulation of proteasomal ubiquitin-dependent protein catabolic process | biological process | 3.11  | 0.04473 |
| odontogenesis                                           | biological process | 3.11  | 0.04473 |
| negative regulation of MAP kinase activity              | biological process | 3.11  | 0.04473 |
| adult walking behaviour                                 | biological process | 3.05  | 0.04729 |
| actin cytoskeleton reorganization                       | biological process | 3.05  | 0.04729 |
| metal ion binding                                       | molecular function | 3.04  | 0.04770 |
| receptor binding                                        | molecular function | 3.03  | 0.04816 |
| sarcolemma                                              | cellular component | 3.00  | 0.04956 |
| cation binding                                          | molecular function | 3.00  | 0.04991 |
| blood vessel remodeling                                 | biological process | 3.00  | 0.04991 |
Table S9. Gene set enrichment analysis, based on gene ontology, of the gene list for differentially expressed genes between HHR and NHR ($P<0.05$).

| Gene Set | Gene Set Description                                | Number of Markers | ES   | NES  | P-value       | HHR vs NHR |
|----------|----------------------------------------------------|-------------------|------|------|--------------|------------|
| 60048    | cardiac muscle contraction                        | 30                | -0.57| -1.99| <0.001       | Down       |
| 35335    | peptidyl-tyrosine dephosphorylation                | 38                | -0.56| -1.96| <0.001       | Down       |
| 97110    | scaffold protein binding                           | 25                | -0.55| -1.93| <0.001       | Down       |
| 16459    | myosin complex                                     | 42                | -0.60| -1.90| <0.001       | Down       |
| 30017    | sarcomere                                          | 26                | -0.59| -1.99| <0.001       | Down       |
| 3774     | motor activity                                     | 51                | -0.53| -1.87| <0.001       | Down       |
| 55010    | ventricular cardiac muscle tissue morphogenesis    | 26                | -0.58| -1.87| <0.001       | Down       |
| 5089     | Rho guanyl-nucleotide exchange factor activity     | 61                | -0.53| -1.84| <0.001       | Down       |
| 45859    | regulation of protein kinase activity              | 18                | -0.67| -1.82| <0.001       | Down       |
| 35108    | limb morphogenesis                                 | 24                | -0.54| -1.78| <0.001       | Down       |
| 3777     | microtubule motor activity                         | 68                | -0.48| -1.78| <0.001       | Down       |
| 45747    | positive regulation of Notch signalling pathway    | 17                | -0.66| -1.77| <0.001       | Down       |
| 32880    | regulation of protein localization                 | 40                | 0.49 | 1.81 | <0.001       | Up         |
| 5216     | ion channel activity                               | 51                | -0.51| -1.79| <0.001       | Down       |
| 45087    | innate immune response                             | 87                | 0.43 | 1.80 | <0.001       | Up         |
| 9635     | response to herbicide                              | 17                | 0.59 | 1.82 | <0.001       | Up         |
| 36094    | small molecule binding                             | 17                | 0.79 | 1.79 | <0.001       | Up         |
| 42098    | T cell proliferation                               | 18                | -0.66| -1.75| <0.001       | Down       |
| 16592    | mediator complex                                   | 30                | 0.53 | 1.78 | <0.001       | Up         |
| 30016    | myofibril                                          | 46                | -0.60| -1.79| <0.001       | Down       |
| 30506    | ankyrin binding                                    | 21                | -0.70| -1.75| <0.001       | Down       |
| 6730     | one-carbon metabolic process                       | 30                | 0.55 | 1.70 | <0.001       | Up         |
| 17134    | fibroblast growth factor binding                   | 28                | 0.61 | 1.83 | <0.001       | Up         |
| 8146     | sulfotransferase activity                           | 30                | 0.49 | 1.71 | <0.001       | Up         |
| 10765    | positive regulation of sodium ion transport        | 21                | 0.54 | 1.71 | <0.001       | Up         |
| 45471    | response to ethanol                                 | 139               | 0.43 | 1.71 | <0.001       | Up         |
| GeneID | Description                                                                 | FDR  | Log2FoldChange | p-value |
|--------|------------------------------------------------------------------------------|------|----------------|---------|
| 15171  | amino acid transmembrane transporter activity                               | 20   | 0.64           | 1.87    | <0.001  | Up      |
| 5758   | mitochondrial intermembrane space                                           | 43   | 0.56           | 1.72    | <0.001  | Up      |
| 1965   | G-protein alpha-subunit binding                                             | 22   | 0.54           | 1.67    | <0.001  | Up      |
| 16925  | protein sumoylation                                                         | 23   | 0.52           | 1.65    | <0.001  | Up      |
| 15807  | L-amino acid transport                                                      | 20   | 0.58           | 1.66    | <0.001  | Up      |
| 9636   | response to toxin                                                           | 95   | 0.43           | 1.75    | <0.001  | Up      |
| 19233  | sensory perception of pain                                                  | 68   | 0.42           | 1.66    | <0.001  | Up      |
| 6164   | purine nucleotide biosynthetic process                                      | 15   | 0.68           | 1.73    | <0.001  | Up      |
| 10862  | positive regulation of pathway-restricted SMAD protein phosphorylation       | 23   | 0.71           | 1.73    | <0.001  | Up      |
| 6979   | response to oxidative stress                                                | 126  | 0.39           | 1.62    | <0.001  | Up      |
| 71356  | cellular response to tumor necrosis factor                                  | 42   | 0.46           | 1.61    | <0.001  | Up      |
| 30838  | positive regulation of actin filament polymerization                        | 32   | 0.45           | 1.57    | <0.001  | Up      |
| 9725   | response to hormone stimulus                                                | 100  | 0.44           | 1.83    | <0.001  | Up      |
| 6103   | 2-oxoglutarate metabolic process                                            | 16   | 0.71           | 1.57    | <0.001  | Up      |
| 34446  | substrate adhesion-dependent cell spreading                                 | 23   | 0.54           | 1.63    | <0.001  | Up      |
| 118    | histone deacetylase complex                                                 | 24   | -0.47          | -1.71   | <0.001  | Down    |
| 70207  | protein homotrimization                                                     | 21   | 0.48           | 1.55    | <0.001  | Up      |
| 35265  | organ growth                                                                | 16   | -0.56          | -1.69   | <0.001  | Down    |
| 10165  | response to X-ray                                                           | 20   | 0.49           | 1.55    | <0.001  | Up      |
| 15908  | fatty acid transport                                                        | 18   | 0.56           | 1.57    | <0.001  | Up      |
| 7031   | peroxisome organization                                                     | 21   | 0.57           | 1.59    | <0.001  | Up      |
| 9966   | regulation of signal transduction                                           | 26   | -0.45          | -1.68   | <0.001  | Down    |
| 7266   | Rho protein signal transduction                                             | 31   | 0.42           | 1.63    | <0.001  | Up      |
| 5516   | calmodulin binding                                                          | 119  | -0.39          | -1.66   | <0.001  | Down    |
| 1968   | fibronectin binding                                                         | 19   | 0.66           | 1.83    | <0.001  | Up      |
| 30182  | neuron differentiation                                                     | 109  | 0.34           | 1.50    | <0.001  | Up      |
| Gene ID | Description                                                                 | Fold Change | p-value | Regulation |
|---------|------------------------------------------------------------------------------|-------------|---------|------------|
| 16874   | ligase activity                                                             | 0.46        | <0.001  | Up         |
| 1104    | RNA polymerase II transcription cofactor activity                            | 0.56        | <0.001  | Up         |
| 187     | activation of MAPK activity                                                  | -0.39       | <0.001  | Down       |
| 21549   | cerebellum development                                                       | -0.41       | <0.001  | Down       |
| 43292   | contractile fibre                                                           | -0.61       | <0.001  | Down       |
| 10524   | positive regulation of calcium ion transport into cytosol                    | -0.57       | <0.001  | Down       |
| 3333    | amino acid transmembrane transport                                          | 0.65        | <0.001  | Up         |
| 48513   | organ development                                                            | -0.65       | <0.001  | Down       |
| 43274   | phospholipase binding                                                        | -0.52       | <0.001  | Down       |
| 7050    | cell cycle arrest                                                           | -0.40       | <0.001  | Down       |
| 10628   | positive regulation of gene expression                                      | 0.33        | <0.001  | Up         |
| 45597   | positive regulation of cell differentiation                                  | 0.41        | <0.001  | Up         |
| 5080    | protein kinase C binding                                                     | -0.44       | <0.001  | Down       |
| 8289    | lipid binding                                                                | 0.33        | <0.001  | Up         |
| 7528    | neuromuscular junction development                                           | -0.49       | <0.001  | Down       |
| 5604    | basement membrane                                                            | -0.39       | <0.001  | Down       |
| 42391   | regulation of membrane potential                                             | -0.39       | <0.001  | Down       |
| 51091   | positive regulation of sequence-specific DNA binding transcription factor    | -0.42       | <0.001  | Down       |
| 30667   | secretory granule membrane                                                   | -0.50       | <0.001  | Down       |
| 50852   | T cell receptor signalling pathway                                           | -0.40       | <0.001  | Down       |
| 48485   | sympathetic nervous system development                                       | -0.51       | <0.001  | Down       |
| 8060    | ARF GTPase activator activity                                                | -0.50       | <0.001  | Down       |
| 51924   | regulation of calcium ion transport                                          | -0.40       | <0.001  | Down       |
| 10212   | response to ionizing radiation                                               | 0.36        | 0.018   | Up         |
| 2020    | protease binding                                                             | 0.41        | 0.019   | Up         |
| 5215    | transporter activity                                                         | 0.36        | 0.019   | Up         |
| 43200   | response to amino acid stimulus                                              | 0.47        | 0.019   | Up         |
| 45861   | negative regulation of proteolysis                                           | 0.53        | 0.019   | Up         |
| Gene ID | Description                                                                 | Fold Change (Up/Down) | Adjusted P-value | log2(abs(T)) | Sign | 
|---------|-----------------------------------------------------------------------------|-----------------------|------------------|----------------|------|
| 31667   | response to nutrient levels                                                 | 64                    | 0.019            | 1.58          | Up   |
| 9617    | response to bacterium                                                        | 32                    | 0.019            | 1.53          | Up   |
| 1937    | negative regulation of endothelial cell proliferation                      | 25                    | 0.019            | -1.55         | Down |
| 7219    | Notch signalling pathway                                                     | 59                    | 0.019            | -1.43         | Down |
| 33077   | T cell differentiation in thymus                                             | 31                    | 0.020            | 1.45          | Up   |
| 70301   | cellular response to hydrogen peroxide                                       | 30                    | 0.020            | 1.70          | Up   |
| 42573   | retinoic acid metabolic process                                             | 16                    | 0.020            | 1.70          | Up   |
| 50776   | regulation of immune response                                               | 20                    | 0.020            | 1.55          | Up   |
| 15450   | P-P-bond-hydrolysis-driven protein transmembrane transporter activity       | 17                    | 0.020            | 1.68          | Up   |
| 46686   | response to cadmium ion                                                      | 30                    | 0.020            | 1.62          | Up   |
| 6829    | zinc ion transport                                                          | 18                    | 0.020            | 1.58          | Up   |
| 5671    | Ada2/Gcn5/Ada3 transcription activator complex                              | 15                    | 0.020            | 1.56          | Up   |
| 7610    | behaviour                                                                    | 31                    | 0.021            | 1.60          | Up   |
| 8301    | DNA binding, bending                                                        | 58                    | 0.021            | -1.70         | Down |
| 19003   | GDP binding                                                                  | 33                    | 0.021            | 1.58          | Up   |
| 86091   | regulation of heart rate by cardiac conduction                              | 16                    | 0.021            | -1.66         | Down |
| 7417    | central nervous system development                                          | 71                    | 0.021            | 1.36          | Up   |
| 16023   | cytoplasmic membrane-bounded vesicle                                        | 89                    | 0.021            | -1.43         | Down |
| 45669   | positive regulation of osteoblast differentiation                           | 52                    | 0.021            | 1.54          | Up   |
| 48546   | digestive tract morphogenesis                                               | 17                    | 0.021            | 1.51          | Up   |
| 51287   | NAD binding                                                                  | 80                    | 0.022            | 1.74          | Up   |
| 35255   | ionotropic glutamate receptor binding                                       | 22                    | 0.022            | 1.54          | Up   |
| 1501    | skeletal system development                                                 | 73                    | 0.022            | 1.46          | Up   |
| 16581   | NuRD complex                                                                | 19                    | 0.022            | -1.51         | Down |
| 48812   | neuron projection morphogenesis                                             | 39                    | 0.022            | 1.34          | Up   |
| 8016    | regulation of heart contraction                                             | 22                    | 0.022            | -1.45         | Down |
| 6096    | glycolysis                                                                   | 77                    | 0.022            | 1.73          | Up   |
| 51015   | actin filament binding                                                      | 68                    | 0.022            | -1.68         | Down |
| ID     | Description                                                                 | Value1 | Value2 | Value3 | p-value | Regulation |
|--------|------------------------------------------------------------------------------|--------|--------|--------|---------|------------|
| 71805  | potassium ion transmembrane transport                                       | 18     | -0.58  | -1.59  | 0.022   | Down       |
| 42995  | cell projection                                                             | 68     | 0.36   | 1.39   | 0.022   | Up         |
| 8092   | cytoskeletal protein binding                                                 | 50     | -0.41  | -1.52  | 0.022   | Down       |
| 5506   | iron ion binding                                                            | 189    | 0.37   | 1.58   | 0.023   | Up         |
| 1836   | release of cytochrome c from mitochondria                                    | 21     | 0.60   | 1.55   | 0.023   | Up         |
| 46933  | hydrogen ion transporting ATP synthase activity, rotational mechanism        | 16     | 0.60   | 1.63   | 0.023   | Up         |
| 3007   | heart morphogenesis                                                          | 37     | -0.44  | -1.56  | 0.023   | Down       |
| 6897   | endocytosis                                                                  | 87     | -0.38  | -1.55  | 0.023   | Down       |
| 6099   | tricarboxylic acid cycle                                                     | 23     | 0.63   | 1.55   | 0.023   | Up         |
| 1078   | RNA polymerase II core promoter proximal region sequence-specific DNA binding transcription factor activity involved in negative regulation of transcription | 30     | -0.41  | -1.41  | 0.023   | Down       |
| 21772  | olfactory bulb development                                                   | 16     | -0.62  | -1.74  | 0.024   | Down       |
| 70588  | calcium ion transmembrane transport                                         | 50     | -0.47  | -1.71  | 0.024   | Down       |
| 6816   | calcium ion transport                                                        | 88     | -0.42  | -1.56  | 0.024   | Down       |
| 4190   | aspartic-type endopeptidase activity                                        | 18     | 0.54   | 1.46   | 0.024   | Up         |
| 35023  | regulation of Rho protein signal transduction                               | 59     | -0.49  | -1.69  | 0.025   | Down       |
| 32874  | positive regulation of stress-activated MAPK cascade                         | 18     | -0.60  | -1.76  | 0.026   | Down       |
| 16323  | basolateral plasma membrane                                                  | 154    | -0.39  | -1.57  | 0.026   | Down       |
| 3779   | actin binding                                                                | 220    | -0.39  | -1.52  | 0.028   | Down       |
| 1822   | kidney development                                                           | 116    | 0.30   | 1.32   | 0.036   | Up         |
| 43499  | eukaryotic cell surface binding                                              | 26     | 0.55   | 1.69   | 0.036   | Up         |
| 10243  | response to organic nitrogen                                                 | 63     | 0.40   | 1.52   | 0.036   | Up         |
| 16787  | hydrolase activity                                                           | 122    | 0.41   | 1.68   | 0.037   | Up         |
| 14823  | response to activity                                                         | 48     | 0.40   | 1.46   | 0.037   | Up         |
| 46328  | regulation of JNK cascade                                                     | 17     | -0.47  | -1.48  | 0.037   | Down       |
| 30279  | negative regulation of ossification                                          | 18     | 0.55   | 1.55   | 0.038   | Up         |
| GeneID | Description                                                                 | Fold Change | P-Value | Significance |
|--------|------------------------------------------------------------------------------|-------------|---------|--------------|
| 70371  | ERK1 and ERK2 cascade                                                        | 21          | 0.49    | Up           |
| 30100  | regulation of endocytosis                                                   | 20          | 0.48    | Up           |
| 1516   | prostaglandin biosynthetic process                                          | 16          | 0.46    | Up           |
| 45454  | cell redox homeostasis                                                      | 52          | 0.41    | Up           |
| 55114  | oxidation-reduction process                                                 | 259         | 0.37    | Up           |
| 45987  | positive regulation of smooth muscle contraction                            | 18          | 0.53    | Up           |
| 45907  | positive regulation of vasoconstriction                                     | 28          | 0.45    | Up           |
| 15459  | potassium channel regulator activity                                        | 21          | 0.49    | Up           |
| 51219  | phosphoprotein binding                                                      | 41          | -0.40   | Down         |
| 6633   | fatty acid biosynthetic process                                             | 42          | -0.40   | Down         |
| 1958   | endochondral ossification                                                   | 23          | 0.49    | Up           |
| 1974   | blood vessel remodeling                                                     | 35          | 0.45    | Up           |
| 4221   | ubiquitin thiolesterase activity                                            | 64          | -0.41   | Down         |
| 5525   | GTP binding                                                                | 355         | 0.44    | Up           |
| 8307   | structural constituent of muscle                                            | 18          | -0.63   | Down         |
| 60395  | SMAD protein signal transduction                                            | 18          | 0.56    | Up           |
| 43484  | regulation of RNA splicing                                                  | 21          | -0.47   | Down         |
| 5070   | SH3/SH2 adaptor activity                                                    | 17          | -0.56   | Down         |
| 30286  | dynein complex                                                             | 22          | -0.53   | Down         |
| 30217  | T cell differentiation                                                      | 33          | -0.43   | Down         |
| 1656   | metanephros development                                                     | 33          | 0.40    | Up           |
| 60359  | response to ammonium ion                                                    | 35          | 0.85    | Up           |
| 1657   | ureteric bud development                                                    | 44          | 0.45    | Up           |
| 4365   | glyceraldehyde-3-phosphate dehydrogenase (NAD+) (phosphorylating) activity | 34          | 0.84    | Up           |
| 71333  | cellular response to glucose stimulus                                       | 30          | 0.42    | Up           |
| 51056  | regulation of small GTPase mediated signal transduction                    | 59          | -0.49   | Down         |
| 32266  | phosphatidylinositol-3-phosphate binding                                    | 17          | -0.51   | Down         |
| 30889  | negative regulation of B cell proliferation                                 | 15          | 0.53    | Up           |
| ID     | Process                                                                 | ES  | NES   | NES  | Direction |
|--------|--------------------------------------------------------------------------|-----|-------|------|-----------|
| 7257   | activation of JUN kinase activity                                       | 25  | -0.45 | -1.52| Down      |
| 51018  | protein kinase A binding                                                | 16  | -0.51 | -1.42| Down      |
| 15991  | ATP hydrolysis coupled proton transport                                 | 34  | 0.48  | 1.61 | Up        |
| 50821  | protein stabilization                                                   | 99  | 0.56  | 1.63 | Up        |
| 31397  | negative regulation of protein ubiquitination                          | 24  | 0.53  | 1.51 | Up        |
| 2027   | regulation of heart rate                                                | 20  | -0.56 | -1.57| Down      |
| 32312  | regulation of ARF GTPase activity                                       | 21  | -0.49 | -1.47| Down      |
| 17048  | Rho GTPase binding                                                     | 24  | -0.58 | -1.71| Down      |
| 42383  | sarcolemma                                                              | 81  | -0.41 | -1.55| Down      |
| 71889  | 14-3-3 protein binding                                                 | 17  | -0.59 | -1.58| Down      |
| 7628   | adult walking behaviour                                                 | 34  | -0.37 | -1.38| Down      |
| 35267  | NuA4 histone acetyltransferase complex                                  | 16  | -0.55 | -1.70| Down      |
| 30032  | lamellipodium assembly                                                  | 25  | 0.51  | 1.54 | Up        |
| 10039  | response to iron ion                                                   | 21  | 0.58  | 1.50 | Up        |
| 5543   | phospholipid binding                                                   | 284 | -0.36 | -1.52| Down      |
| 4725   | protein tyrosine phosphatase activity                                   | 96  | -0.36 | -1.46| Down      |
| 45765  | regulation of angiogenesis                                             | 17  | 0.60  | 1.45 | Up        |
| 31175  | neuron projection development                                           | 105 | -0.33 | -1.37| Down      |
| 6874   | cellular calcium ion homeostasis                                       | 69  | -0.38 | -1.43| Down      |
| 48037  | cofactor binding                                                       | 18  | 0.42  | 1.45 | Up        |

Legend: ES; enrichment score; NES, normalized enrichment score.
Table S10. Genes differentially expressed between Lcn2-knockout compared to wild-type mice identified by RNA-sequencing (n=3/group). Here shown only genes with false discovery rate (FDR) q<0.05.

| GeneID | logFC | P-value       | q-value       |
|--------|-------|---------------|---------------|
| Hspa1a | –2.15 | 1.13E-10      | 1.00E-06      |
| Nr4a1  | –2.53 | 1.52E-10      | 1.00E-06      |
| Hspa1b | –2.50 | 2.35E-09      | 1.03E-05      |
| Zbtb16 | 2.93  | 7.95E-09      | 2.36E-05      |
| Hamp   | –4.70 | 1.78E-07      | 0.00047       |
| Hif3a  | 2.77  | 8.97E-07      | 0.0018        |
| Myl4   | –4.09 | 9.52E-07      | 0.0018        |
| Adipoq | –4.90 | 2.71E-06      | 0.0045        |
| Lcn2   | –2.25 | 1.06E-05      | 0.0155        |
| Slc4a8 | 2.16  | 1.47E-05      | 0.0195        |
| Fkbp5  | 1.38  | 1.72E-05      | 0.0195        |
| Cfd    | –3.38 | 1.78E-05      | 0.0195        |
| Sln    | –4.29 | 2.50E-05      | 0.0255        |
| Pdk4   | 1.68  | 3.17E-05      | 0.0299        |
| Serpine1 | 1.11 | 4.53E-05      | 0.0399        |
| Map3k6 | 1.31  | 5.30E-05      | 0.0437        |

*logFC: logarithmic fold change.
| Category | Term | Count | Total | %  | Involved Genes                                                                 | Fold Enrichment | P-Value      |
|----------|------|-------|-------|----|-------------------------------------------------------------------------------|-----------------|--------------|
| KEGG_PATHWAY | mmu04510: Focal adhesion | 31    | 198   | 15.7 | MYL7, XIAP, ACTG1, ARHGAP5, COL6A6, COMP, ITGB6, PPP1R12A, PDGFD, EGF, PIK3R1, FN1, COL4A4, COL4A2, COL4A1, FLT1, ROCK2, ITGA1, ITGA2, HGF, FLNB, COL5A1, KDR, LAMA2, LAMA3, LAMA5, ITGA8, PDGFRA, LAMC1, MYLK, ITGA2B | 4.36            | 7.98E-12     |
| KEGG_PATHWAY | mmu04512: ECM-receptor interaction | 17    | 83    | 20.5 | COL4A4, COL4A2, COL4A1, ITGA1, ITGA2, COL5A1, HMMR, LAMA2, LAMA3, COL6A6, LAMA5, COMP, ITGA8, ITGB6, LAMC1, ITGA2B, FN1 | 5.70            | 2.45E-08     |
| KEGG_PATHWAY | mmu05222: Small cell lung cancer | 14    | 85    | 16.5 | COL4A4, COL4A2, COL4A1, XIAP, ITGA2, CCNE2, LAMA2, LAMA3, CDKN2B, LAMA5, LAMC1, PIK3R1, FN1, ITGA2B | 4.59            | 8.06E-06     |
| KEGG_PATHWAY | mmu05200: Pathways in cancer | 29    | 323   | 9.0  | XIAP, FOXO1, FGF12, ZBTB16, CCNE2, CDKN2B, TPR, EGF, PIK3R1, FN1, APC, COL4A4, COL4A2, COL4A1, EPAS1, RUNXIT1, ITGA2, BRCA2, HGF, STAT1, CTNNA3, WNT2B, LAMA2, CBLC, LAMA3, LAMA5, PDGFRA, LAMC1, ITGA2B | 2.50            | 9.08E-06     |
| KEGG_PATHWAY | mmu04810: Regulation of actin cytoskeleton | 20    | 217   | 9.2  | MYL7, ROCK2, ITGAE, SSH2, ITGA1, ITGA2, FGF12, ACTG1, ITGA8, ITGB6, PPP1R12A, PDGFRA, | 2.57            | 2.44E-04     |
| KEGG_PATHWAY | mmu05412: Arrhythmogenic right ventricular cardiomyopathy (ARVC) | 11 | 75 | 14.7 | PDGFD, EGF, DIAP2, PIK3R1, MYLK, ITGA2B, APC, FN1, LAMA2, ACTG1, DMD, ITGA8, ITGB6, CACNG6, ITGA1, ITGA2, RYR2, CTNNA3, ITGA2B |
|----------------|-----------------------------------------------------------------|----|----|------|-------------------------------------------------------------------------------------------------------------------------------------|
| KEGG_PATHWAY | mmu05410: Hypertrophic cardiomyopathy (HCM) | 11 | 84 | 13.1 | LAMA2, ACTG1, DMD, ITGA8, ITGB6, CACNG6, ITGA1, ITGA2, RYR2, TTN, ITGA2B |
| KEGG_PATHWAY | mmu05414: Dilated cardiomyopathy | 11 | 92 | 12.0 | LAMA2, ACTG1, DMD, ITGA8, ITGB6, CACNG6, ITGA1, ITGA2, RYR2, TTN, ITGA2B |
| KEGG_PATHWAY | mmu04540: Gap junction | 9 | 86 | 10.5 | PLCB4, MAP3K2, GUCY1A2, PDGFR, PDGFD, PRKG1, EGF, ITPR1, ITPR2 |
| KEGG_PATHWAY | mmu04144: Endocytosis | 15 | 202 | 7.4 | FLT1, ERBB4, PSD3, ASAP1, HSPA1A, EEA1, HSPA1B, KDR, CBLC, RAB11FIP2, TFRC, HSPA2, PDGFR, H2-T24, EGF, AGAP2 |
| KEGG_PATHWAY | mmu04270: Vascular smooth muscle contraction | 10 | 120 | 8.3 | PLCB4, ROCK2, MYH11, PPP1R12A, GUCY1A2, ADRA1A, PRKG1, ITPR1, MYLK, ITPR2 |

**Footnote:** Count: number of genes from 721 differentially expressed genes (P<0.05) involved in that pathway; %: genes involved/total number of genes in pathway; P-value: modified Fisher exact P-value.
Table S12. Gene set enrichment analysis with the differentially expressed genes between cells transfected with Lcn2 or empty plasmid.

| Category | Term                              | Count | Total | %    | Involved genes                                                                 | Fold Enrichment | P-Value  |
|----------|-----------------------------------|-------|-------|------|--------------------------------------------------------------------------------|-----------------|----------|
| KEGG_PATHWAY | rno05222: Small cell lung cancer | 10    | 83    | 12.0 | E2F3, PTGS2, ITGA6, BCL2, BCL2L1, RB1, BIRC3, BIRC2, MYC, TRAF4                 | 4.46            | 3.43E-04 |
| KEGG_PATHWAY | rno04510: Focal adhesion          | 13    | 195   | 6.7  | CAV1, ROCK2, COL3A1, ITGA11, ITGB5, BIRC3, BIRC2, ITGA6, RASGRF1, BCL2, GSK3B, THBS2, MYLK | 2.47            | 0.006    |
| KEGG_PATHWAY | rno04110: Cell cycle             | 10    | 126   | 7.9  | E2F3, CCNH, PLK1, GSK3B, BUB1, CDC20, RB1, CDC25C, ABL1, MYC                 | 2.94            | 0.006    |
| KEGG_PATHWAY | rno04142: Lysosome               | 9     | 117   | 7.7  | TCIRG1, AP1S2, GUSB, HEXA, LGMN, CTSD, PPT1, FUCA1, MANBA                   | 2.85            | 0.013    |
| KEGG_PATHWAY | rno05200: Pathways in cancer     | 15    | 317   | 4.7  | E2F3, FGF7, PTGS2, BCL2L1, RB1, BIRC3, STAT1, BIRC2, FZD6, ITGA6, BCL2, GSK3B, ABL1, MYC, TRAF4 | 1.75            | 0.04     |
| KEGG_PATHWAY | rno00511: Other glycan degradation | 3   | 16    | 18.8 | HEXA, FUCA1, MANBA                                                             | 6.94            | 0.067    |
| KEGG_PATHWAY | rno05410: Hypertrophic cardiomyopathy (HCM) | 6    | 84    | 7.1  | ACTC1, ITGA6, ITGA11, ITGB5, CACNG1, TNN13                                  | 2.64            | 0.074    |
| KEGG_PATHWAY | rno04114: Oocyte meiosis         | 7     | 111   | 6.3  | REC8, SLK, PLK1, BUB1, CDC20, AURKA, CDC25C                                 | 2.33            | 0.077    |
| KEGG_PATHWAY | rno05414: Dilated cardiomyopathy | 6    | 90    | 6.7  | ACTC1, ITGA6, ITGA11, ITGB5, CACNG1, TNN13                                  | 2.47            | 0.09     |

Footnote: Count: number of genes from 529 differentially expressed genes (P<0.05) involved in that pathway; %: genes involved/total number of genes in pathway; P-value: modified Fisher exact P-value.
Figure S1. Hierarchical clustering showing that neonatal hypertrophic heart rat (HHR) (blue) and normal heart rat (NHR) (orange) form distinct groups based on gene expression (genes with Bonferroni adjustment <0.05). Numbers on the right are probeset numbers. Red depicts genes up-regulated and green those down-regulated.
**Figure S2.** Over-expression of *Lcn2* in vitro. *Lcn2* was over-expressed in H9c2 cells with the use of a plasmid. **A**, Optimisation of the concentration of *Lcn2* plasmid in vitro. **B**, There were higher *Lcn2* mRNA in cells transfected with LCN2-plasmid. **C**, Over-expression of in cells *Lcn2* visualised by immunofluorescence. **D**, There was no difference in apoptotic cells measured by flow cytometry in cells transfected with *Lcn2* compared to those transfected with empty plasmid. *** indicates \( P<0.001 \). Error bars represent standard error of mean.
Figure S3. Representative of confocal microscopy images after transfection with Lcn2 plasmid, which provided results for Figure 4 in the main paper. **A**, Wheat-germ agglutinin (WGA, red) and DAPI (blue) staining, used to estimate cell size (400x magnification, scale bar = 60µm). **B**, Phospho-histone H3 (pH3, green) and DAPI (blue), used to estimate cell proliferation and arrest (200x magnification, scale bar = 100µm).
Figure S4. There was no difference in apoptotic cells measured by flow cytometry in cells transfected with Lcn2 compared to those transfected with empty plasmid, demonstrated by annexin V bound to apoptotic cells determined by flow cytometry. Data shown as number of cells as a percentage of the total number of cells. Error bars represent standard error of mean.
Supplemental References:

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