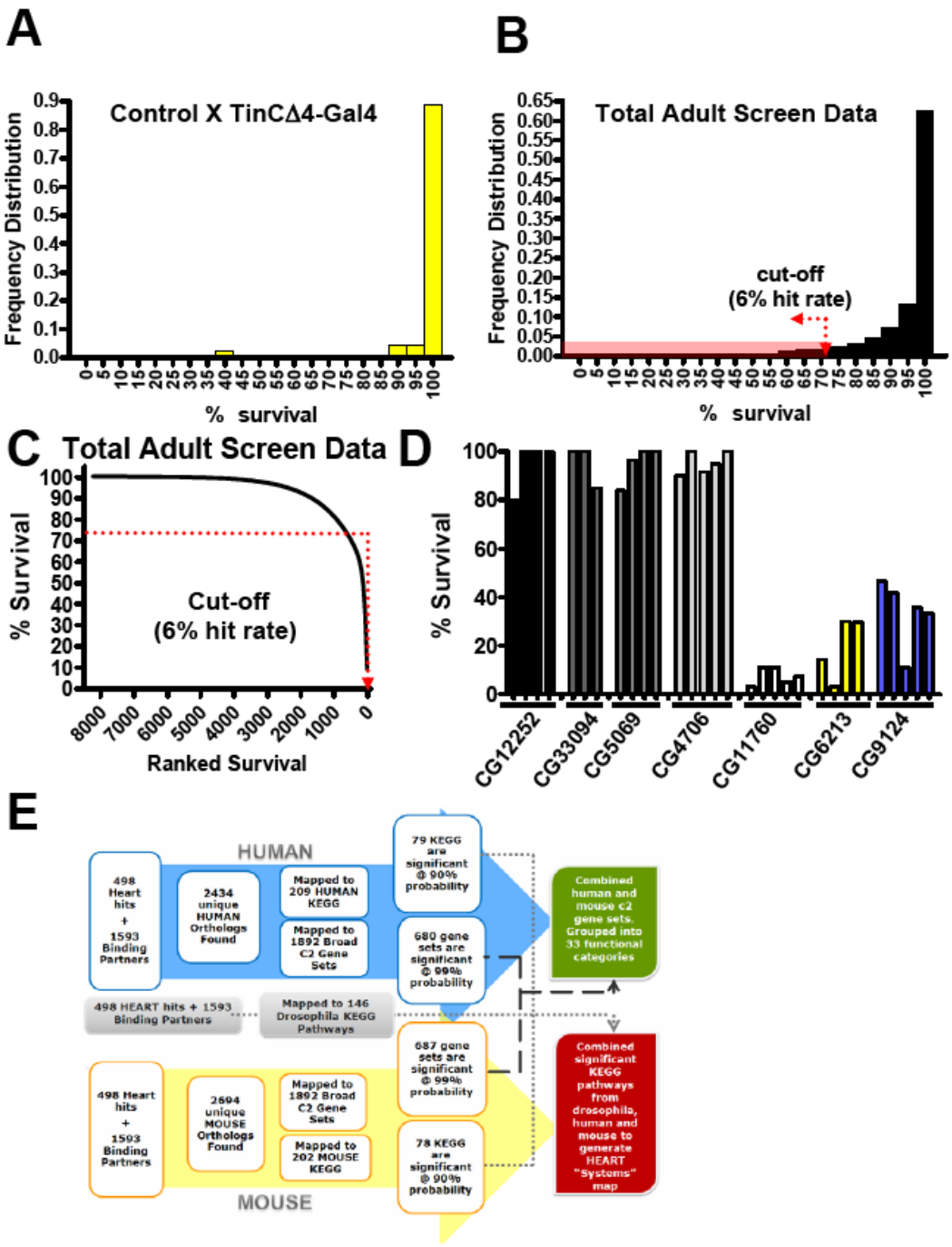
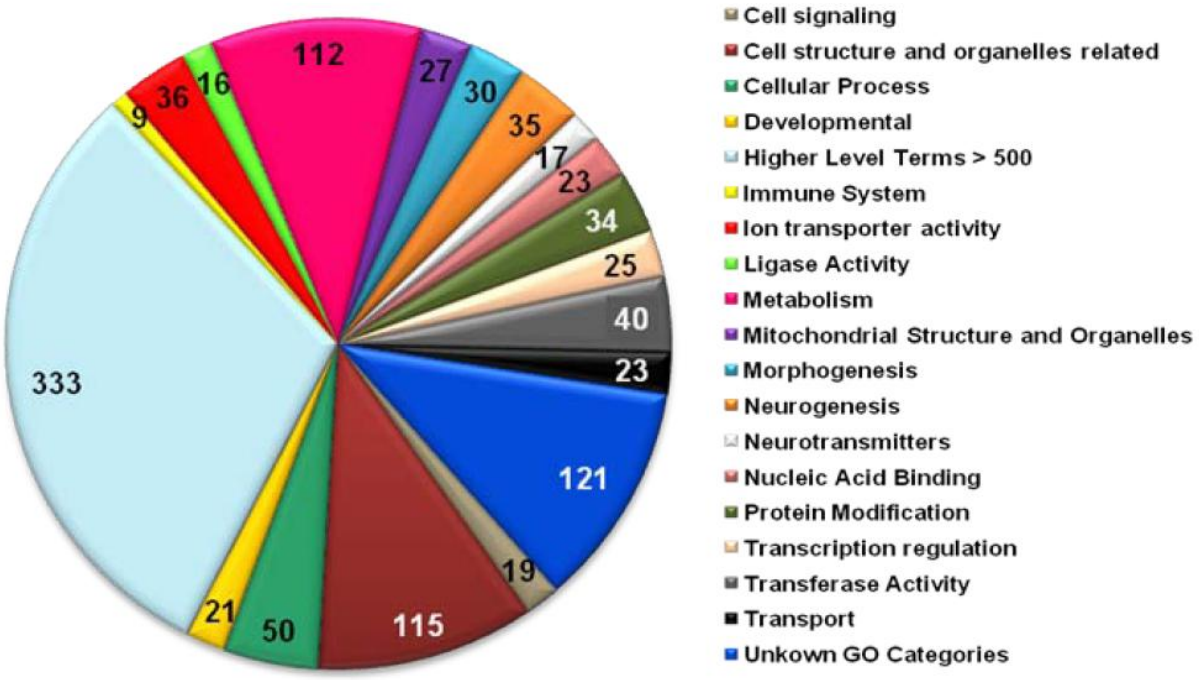


Supplemental Figure S1. Screen set-up (related to Figure 1).

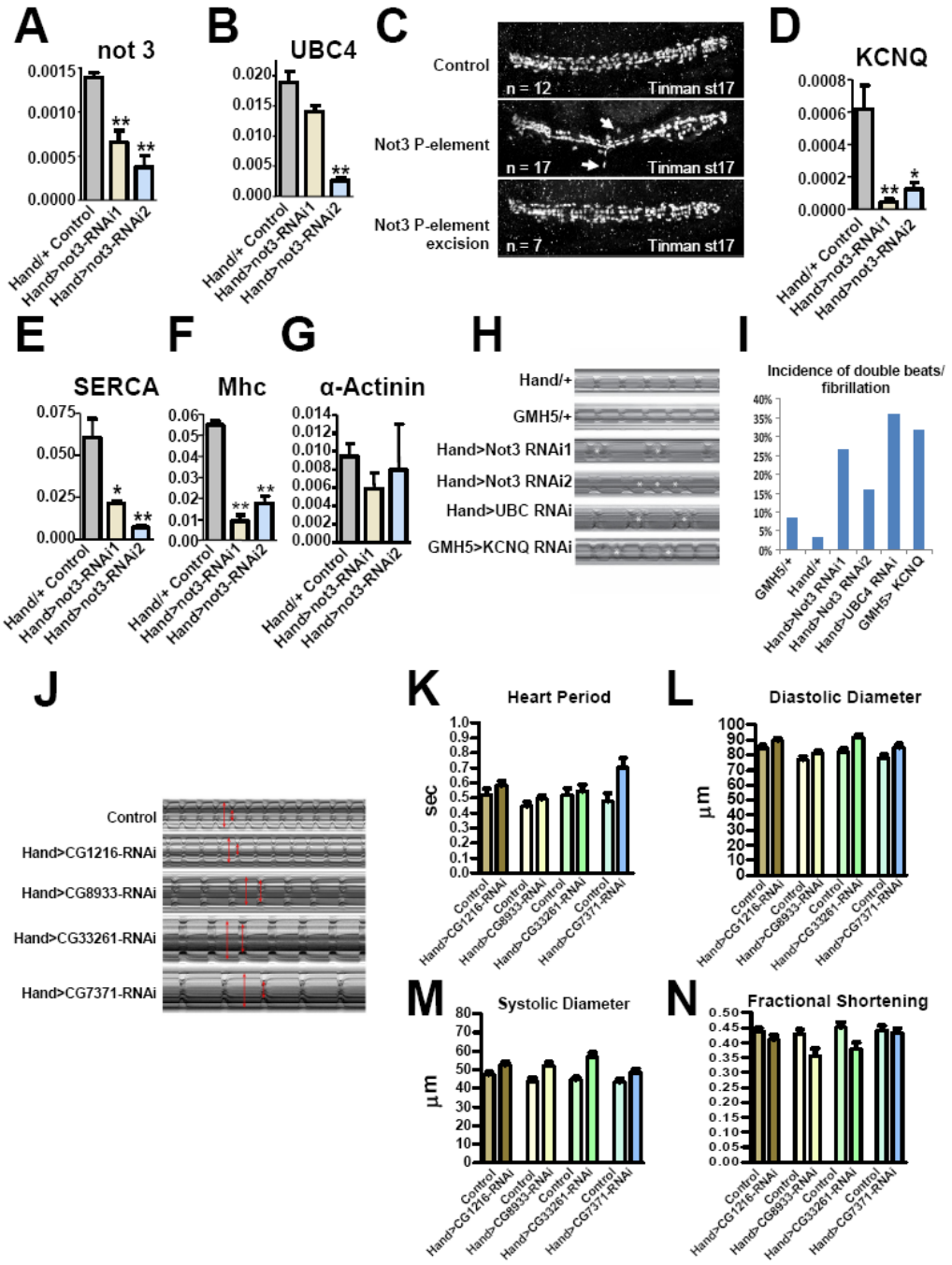
(A) Repeated testing of our background control (w^{1118}) X heart driver (TinCΔ4-Gal4) line on multiple days shows a consistent ability of these flies to pass the increased ambient temperature. The X-axis shows percent survival and the Y-axis the frequency of flies that passed (survival) or failed (death) the experimental challenge. n = 966 flies. **(B)** Histogram for the entire heart screen shows the distribution of survival for all tested TinCΔ4-Gal4_RNAi lines. The cut-off used for TinCΔ4-Gal4_RNAi lines to pass the threshold for further analyses is indicated (Z score >3 corresponding to 72% survival).

The X-axis shows percent survival and the Y-axis the frequency of flies that passed (survival) or failed (death) the experimental challenge. **(C)** Ranked Best Fit curve for the entire screen data set. The cut-off used for TinCΔ4-Gal4_RNAi lines to be included into our data-set for further analyses is indicated (Z score >3 corresponding to 72% survival. **(D)** Representative repeat experiments for select TinCΔ4-Gal4_RNAi lines that passed or failed (recorded as % survival on day 6 after a shift to 29^{o}C) to show reproducibility of the experimental model. Bar represent individual experiments for each RNAi line. **(E)** Flow chart indicating KEGG and C2 gene set analysis in *Drosophila*, mice, and humans. Data from all three organisms were pooled to generate a global network map. See experimental procedures for details.



Supplemental Figure S2. *Drosophila* GO and KEGG analysis (related to Figure 2).

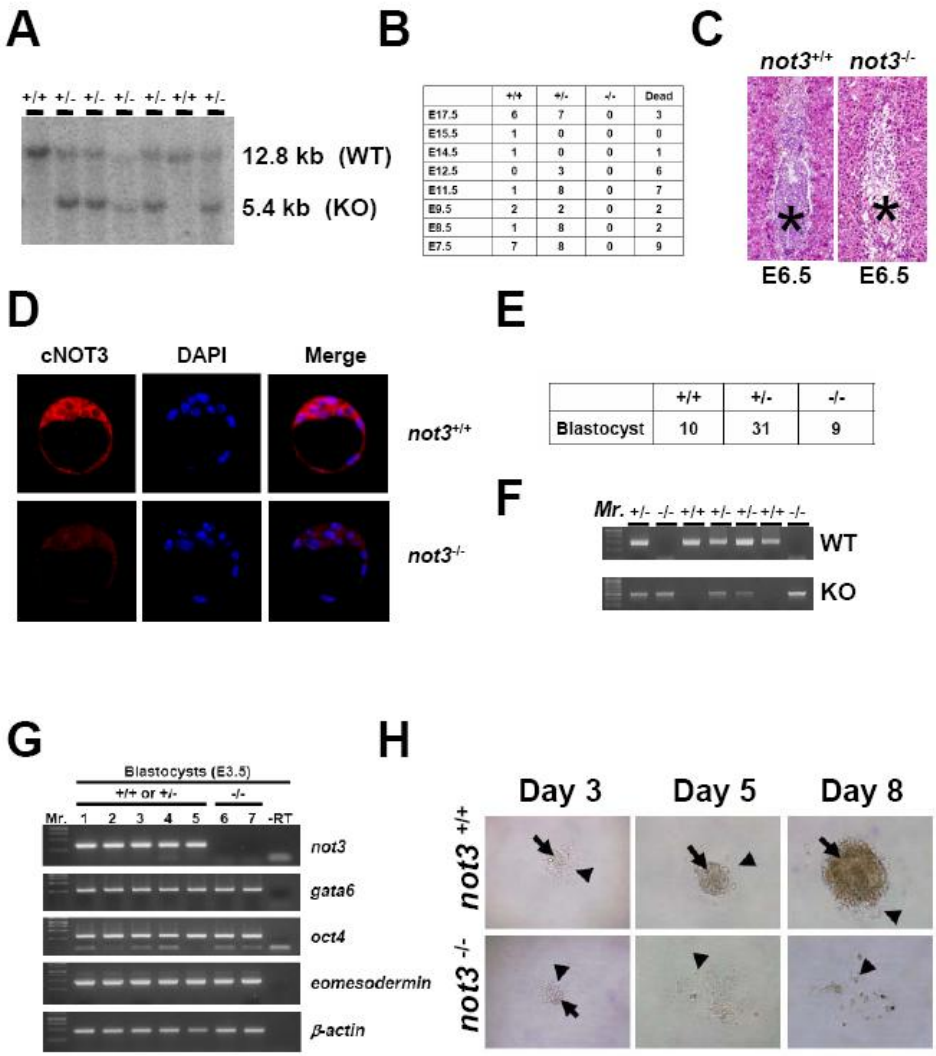
Functional classification of statistically enriched GO (gene ontology) terms for adult heart hits in *Drosophila*. Numbers indicate gene counts from all the GO terms included in each functional category. See Suppl. Table S4A contains the entire list of enriched GO terms and their classification into functional categories.



Supplemental Figure S3. Functional phenotyping of Not3 and other candidate heart genes (related to Figure 3).

Expression levels of **(A)** not3 and **(B)** UBC4 in Not3-knockdown hearts. (**C**) Wild-type embryonic heart at stage 17 (st17) stained with Tinman (Tin). Four cell rows can be identified: two inner cardioblast cell rows and two outer pericardial cell rows. In *not3*

mutants (P element; CG8426^{KG10496}) the inner cardioblast cell row is normal, whereas the pericardial cells are misarranged (arrows). Excision of the P-element completely rescues the phenotype observed in the *not3* mutant. Expression levels of **(D)** KCNQ, **(E)** SERCA (Calcium ATPase), **(F)** myosin heavy chain (Mhc) and **(G)** α-Actinin in Not3-knockdown hearts. mRNA transcripts were determined in 1-week-old adult fly hearts. 30 fly hearts were used for each sample. For statistical analysis, differences in mean values were calculated between w^{1118} x Hand-Gal4 control and each Not3-RNAi line. Student t-test: * = P < 0.05, ** = P < 0.01. **(H)** Heart specific knockdown of gene products in the Not3 complex results in an increase in the incidence of heart arrhythmia in one week old flies. M-modes made from movies of semi-intact fly heart preparations were examined for the presence of indications of developing arrhythmias (double beats and/or fibrillation episodes). M-modes from individual flies (4 sec) with examples of these heartbeat irregularities (asterisks) are shown for heart-specific Not3, UBC4, and KCNQ knockdown using Hand–Gal4 or GMH5-Gal4 (Wessells et al, 2004). **(I)** Quantification of fly heart records exhibiting one or more double beats and/or fibrillation episodes in a 30s M-mode per fly is presented as a percent of the total number of flies (indicated in parentheses). Heart-specific knockdown of Not3 components results in an increased incidence of these irregularities compared to control flies (GMH5/+ and Hand/+). Because the total number of these irregular beats is relatively small they do not have a large effect on the overall arrhythmia index. However, the pattern of these irregularities (**G**) and the percentage of flies that exhibit them (**H**) is similar to what is seen in response to heart-specific knockdown of the KCNQ K^+ channel at one week (GMH5>KCNQ RNAi) that reflects what has been reported previously for one week old KCNQ mutant flies (Ocorr et al, 2007). **(J)** M-modes reveal perturbations in normal wall movements induced by knock-down of the gene candidates CG1216, CG8933, CG33261 and CG7371. Distance is measured in the y- and time is measured in the x-direction. Note the differences in heart wall diameters (double-headed red arrows), the extent of wall movement and the heart periods (the amount of time required for a complete contraction and relaxation cycle) relative to those of the control heart. **(K-N)** The consequences of RNAi- mediated knockdown of CG1216, CG8933, CG33261 and CG7371 on cardiac dimensions and function. Knockdown of each gene appears to have a unique effect on overall cardiac performance since each line, relative to its control, exhibits a unique abnormal physiological signature. Unpaired t-tests were performed to compare each Hand-Gal4>UAS-RNAi line to the corresponding w^{1118} X UAS-RNAi control line. Mean values ± SEM are shown for each group (n =14 - 35). * p<0.05, ** p<0.01, *** p<0.001.



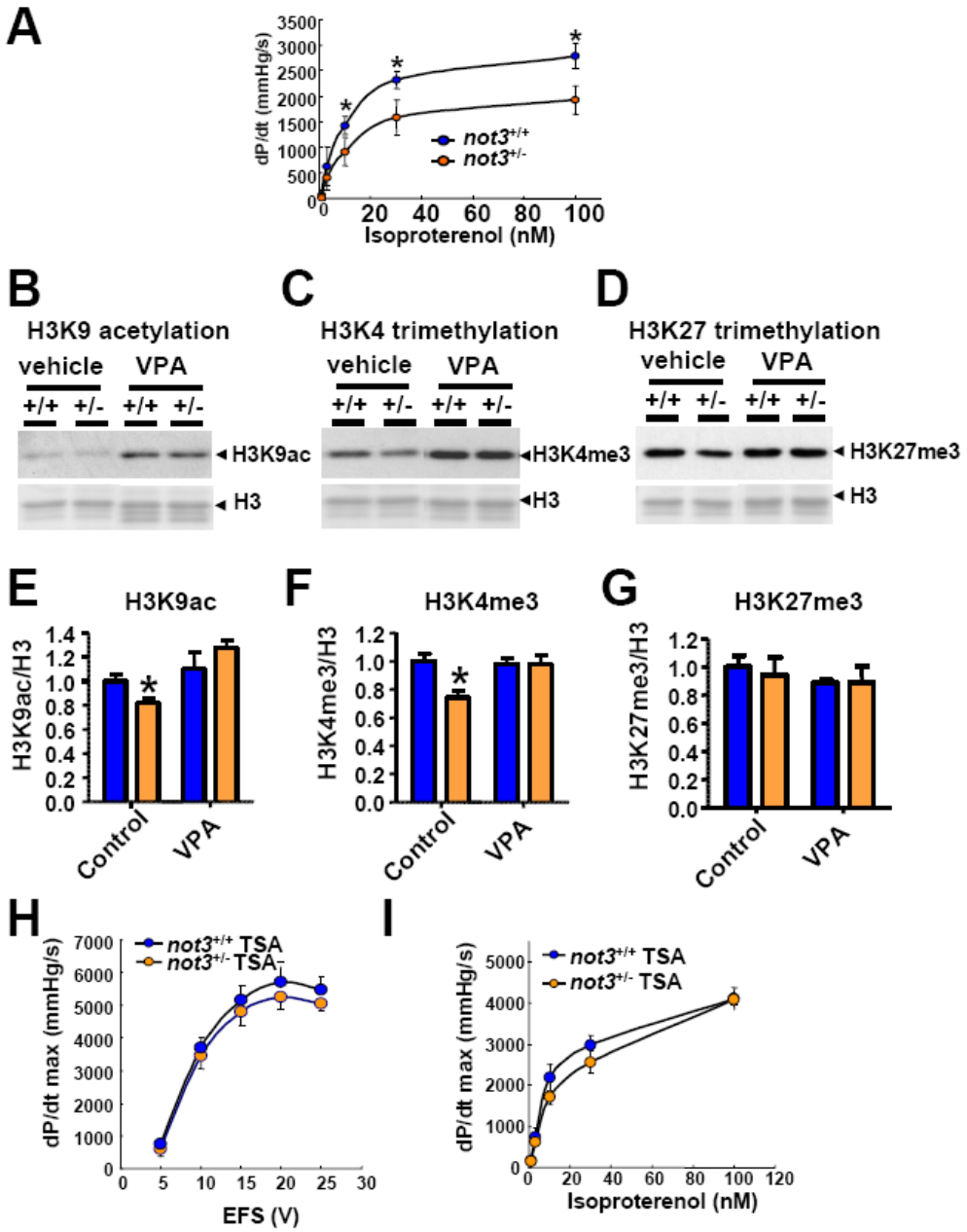
	+/+	+/-	-/-	Dead
E17.5	6	7	0	3
E15.5	1	0	0	0
E14.5	1	0	0	1
E12.5	0	3	0	6
E11.5	1	8	0	7
E9.5	2	2	0	2
E8.5	1	8	0	2
E7.5	7	8	0	9

	+/+	+/-	-/-
Blastocyst	10	31	9

Supplementary Figure S4. Generation and embryology of Not3 mutant mice (related to Figure 5).

(A) Southern Blot analyses for genomic DNAs of born off-spring from *not3* heterozygous matings. No homozygous null mice were obtained. **(B)** Genotypes of embryos from *not3* heterozygous intercrosses. No viable homozygous null embryos were obtained at the indicated embryonic days (E) using timed pregnancies. **(C)** Histological analysis of *not3*$^{-/-}$ mouse embryos. Longitudinal sections of E6.5 embryos in deciduas (asterix) are shown for wild-type and *not3*$^{-/-}$embryos. H&E staining. **(D)** Immunohistochemistry of Not3 (red) in E3.5 wild-type (top) and *not3*$^{-/-}$ (bottom) blastocyst-stage embryos. Nuclei were counterstained using DAPI (blue). Note that Not3 localizes to nuclei and the cytoplasm. **(E)** Frequency of *not3*$^{+/+}$, *not3*$^{+/-}$, and *not3*$^{-/-}$ E3.5 blastocysts from heterozygous intercrosses. **(F)** Representative genotypes for blastocysts obtained from heterozygous intercrosses using PCR. Mr, molecular weight marker. **(G)** RT-PCR

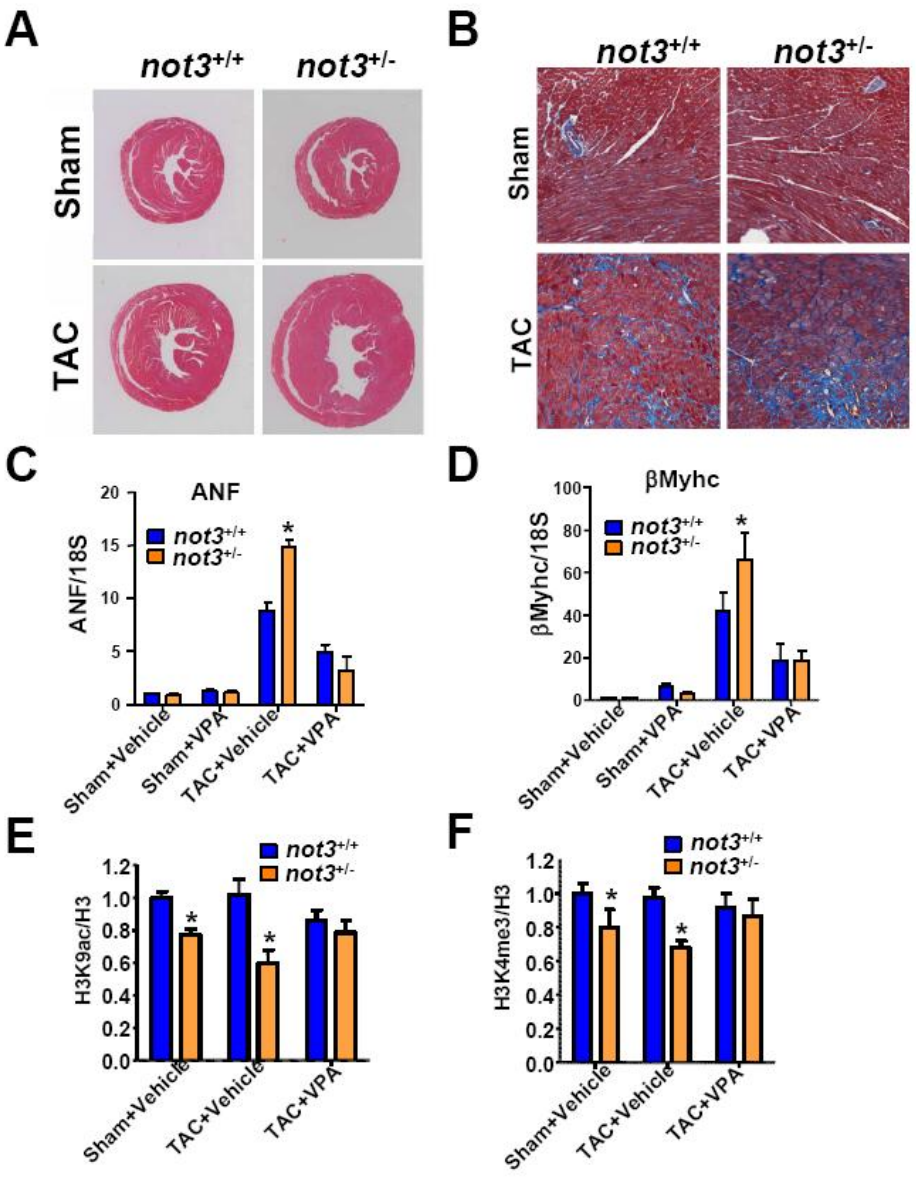
analyses for mRNA expression of prototypic early embryonic marker genes. The epiblast marker genes *gata6* and *oct4* and the trophoblast marker *eomesodermin* appear to be normally expressed in *not3* null embryos. **(H)** Blastocysts were recovered from heterozygous intercrosses, cultured individually, and genotyped by PCR. Cultures of representative wild-type and *not3*$^{-/-}$ epiblasts are shown for the indicated days. Arrows point at the ICM (inner cell mass). Arrowheads indicate the trophoblast.



Supplementary Figure S5. Impaired contractility and histone modifications in *ex vivo not3*$^{+/-}$ mouse hearts (related to Figure 5).

(**A**) Left ventricular pressure (LVP) measurements in isolated *ex vivo not3*$^{+/-}$ and control *not3*$^{+/+}$ hearts under different doses of isoproterenol perfusion. *not3*$^{+/-}$ hearts from 4 months old mice showed impaired contractile responses to isoproterenol perfusion in the retrograde Langendorff mode as compared to age matched controls. **(B, E)** H3K9 acetylation (H3K9ac) and **(C, F)** H3K4 trimethylation (H3K4me3) levels, but not **(D, G)** H3K27 trimethylation (H3K27me3) levels were decreased in *not3*$^{+/-}$ mouse hearts. Valproic acid (VPA) treatment reversed both **(B, E)** H3K9ac and **(C, F)** H3K4me3 levels of *not3*$^{+/-}$ hearts to those of littermate *not3*$^{+/+}$ hearts. Wild type and *not3*$^{+/-}$ mice are treated with vehicle or VPA (0.71% w/v in drinking water) for 1 week, and histones were

acid-extracted from whole heart ventricules. The histone extracts were separated by SDS-PAGE followed by immunoblotting with specific antibodies for each modified lysine residues of H3. Band intensities were quantified and normalized to total H3 levels. Representative immunoblot pictures (top) and corresponding ponceau S-stained membranes (bottom) are shown in **(B-D)**, and the respective quantified levels of H3K9ac, H3K4me3 and H3K27me3 are shown in **(E-G)**. n = 5-12 per group. Treatment with the HDAC inhibitor Trichostatin A (TSA) reversed the impaired contractile response of *ex vivo not3*$^{+/-}$ hearts to **(H)** electrical field stimulation (EFS) or (**I**) isoproterenol perfusion to wild type levels. Wild type and *not3*$^{+/-}$ mice were treated with vehicle or TSA (0.6 mg/kg/day, *i.p.*) for 1 week. Hearts were then excised and subjected to Langendorff perfusion. *Ex vivo* heart contractility was assayed in response to different EFS stimulations or different doses of isoproterenol perfusion. No statistically significant differences were detected at any dosage/stimualtion points between *not3*$^{+/+}$ and *not3*$^{+/-}$ mice. n = 6-8 per group. All values are mean +/- SEM. *****; $P < 0.05$.



Supplementary Figure S6. Severe cardiac hypertrophy and fibrosis in *not3*$^{+/-}$ mice under pressure overload and resuce of disease markers and histone modification by treatment with VPA (related to figure 7).
Representative sections of *not3*$^{+/+}$ and *not3*$^{+/-}$ hearts analyzed 3 weeks after sham or transverse aortic constriction (TAC) surgery. **(A)** Hematoxylin & eosin staining. **(B)** Masson-trichrome staining to visualize collagen deposits indicative of fibrotic changes. Note cardiac hypertrophy and ventricular dilation in *not3*$^{+/-}$ mice following TAC. **(C-D)** Real time PCR analyses for **(C)** ANF (atrial natriuretic factor) and **(D)** βMyhc (β-myosin heavy chain) mRNA expression. Total RNA was isolated from hearts 6 weeks after TAC or sham surgery in the presence of vehicle or the HDAC inhibitor VPA. ANF and βMyhc levels were normalized to 18S mRNA. **(E-F)** *In vivo* administration of the HDAC inhibitor Valproic acid (VPA) restores **(E)** reduced H3K9 acetylation (H3K9ac) and **(F)** H3K4 trimethylation (H3K4me3) in post-TAC *not3*$^{+/-}$ mouse hearts to that of wild type control

levels. Wild type and *not3*$^{+/-}$ mice were treated with vehicle or VPA (0.71% w/v in drinking water) for 6 weeks after TAC or sham surgery. Histones were acid-extracted from whole heart ventricules, separated by SDS-PAGE, and immunoblotted with antibodies specific to H3K9ac and H3K4me3. Band intensities were quantified and normalized to total H3 levels. All values are mean +/- SEM. *****; $P < 0.05$ n = 5-8 per group.

Legends to Supplemental Tables

Supplemental Table S1. Total Screen data (related to Figure 1).

(**A**) Predicted mammalian orthologues for *Drosophila* genes. To identify orthologues between *Drosophila* and mouse or *Drosophila* and human, we used pre-computed orthology predictions obtained from Compara r49, Homologene (03/08), Inparanoid v6.1, Orthomcl v2 (PMID: 18819722). One-to-one and one-to-many mappings were observed between the *Drosophila* and the mammalian genes. In case of one–to–many mappings, all the orthologue targets for a given gene were considered for further analysis. (**B**) Prescreen time-course. 80 "randomly" selected UAS-RNAi lines were crossed to TinCΔ4-Gal4 and survival at 29ºC was recorded over a 7 day time course. Percent survival at each day for each corresponding transformant ID is presented. (**C**) Total screening data. All experimental data for the entire list of lines screened are shown. TinCΔ4-Gal4_RNAi fly lines are listed from the strongest to weakest adult lethality. The mean developmental lethality score is presented with SEM and numbers of independent replicates performed for each line. Developmental lethality was assessed in a semi-quantitative fashion, with lethal crosses scored as 0, semi-lethal crosses scored as 0.5, and viable crosses scored as 1. Moreover, the Table shows percentage survival of adult flies on day 6 after a shift to 29^{o}C is presented with SEM, Z score, and number of independent replicates performed. Note that the Z score is presented as an absolute value. The total number of flies screened for adult survival is also indicated for each line. (**D**) Screening data minus potential off targets. All experimental data for transgenic TinCΔ4-Gal4_RNAi flies harboring specific hairpins with an S19 score ≥0.8 and CAN repeats ≤ 6 screened for adult heart function are shown. Survival of adult flies on day 6 after a shift to 29^{o}C is presented with SEM, Z score, and number of independent replicates performed. The Z score is presented as an absolute value. The total number of flies screened for adult survival, the respective S19 values, and CAN repeats are also indicated for each line. In addition, the mean developmental lethality score is presented with SEM and numbers of independent replicates performed for each line. Developmental lethality was assessed in a semi-quantitative fashion, with lethal crosses scored as 0, semi-lethal crosses scored as 0.5, and viable crosses scored as 1. (**E**) Developmentally lethal genes. The mean

developmental lethality score is presented with SEM and numbers of independent replicates performed for each line. Lines are sorted ascending by their lethality score. Lethality was scored for each cross (0 = lethal, 0.5 = semi-lethal, 1 = viable). A mean score of ≤0.6666 was considered lethal. Predicted mouse and human orthologues are included for each gene. Note that 7 transformant ids which map to genes that also show adult phenotypes are highlighted in yellow. (**F**) Staged lethality scores. The stage of lethality was scored for each cross: 0 = lethal (embryonic lethal or we never observed any offspring), 1 = larval lethal, 2 = pupal lethal, 3 = adult lethal, 4 = adult viable. Mean staged lethality scores are shown for each line together with SEM and numbers of independent replicates. Lines are sorted in ascending order. Predicted mouse and human orthologues are included for each gene.

Supplemental Table S2. Candidate *Drosophila* adult heart hits (related to figure 1).

Table shows percentage survival of adult TinCΔ4-Gal4_RNAi fly lines on day 6 after a shift to 29^{o}C. Mean % survival, SEM, mean absolute Z score, and number of independent replicates performed are shown. Only TinCΔ4-Gal4_RNAi fly lines with Z score ≥ 2 are shown. Lines are sorted by descending Z scores. Lines with a Z scores ≥ 3 are highlighted in orange. Lines with a Z score between 2 and 3 are shaded in grey. Predicted mouse and human orthologues are included for each gene.

Supplemental Table S3. Drosophila and mammalian pathway analysis (related to Figure 2).

(**A**) The systems network includes data from the significantly enriched *Drosophila* KEGG pathways. Pathways from the same biological process were grouped into common functional categories. Green nodes represent statistically enriched functional categories of pathways; red nodes represent direct primary fly RNAi hits; light red nodes represent their first degree binding partners; and blue nodes indicate genes that were scored as developmentally lethal in our *Drosophila* heart screen. Lines indicate associations of the genes to the appropriate functional category. For the entire data see

the Suppl. Tables S4E. Note the CCR4-Not complex. **(B).** A conserved network of heart function based on direct screen hits. (The systems network includes data from the significantly enriched *Drosophila* KEGG and mouse and human KEGG and C2 data sets when compared only to developmentally lethal and adult fly heart hits. Pathways and gene sets from the same biological processes were grouped into common functional categories. Yellow nodes represent statistically enriched functional categories of pathways; red nodes represent direct primary fly RNAi hits and blue nodes indicate genes that were scored as developmentally lethal in our *Drosophila* heart screen. Lines indicate associations of the genes to the appropriate functional category. All KEGG pathways and selected C2 gene sets have been represented in the systems map. For the entire data see Suppl. Table S4E-H.

Supplemental Table S4. Screen analysis (related to figure 2).

(**A**) Significantly enriched *Drosophila* GO terms. Gene Ontology (GO) analysis was performed on the candidate adult heart hits (Z score ≥3) using GOstat (http://gostat.wehi.edu.au/). Over- and under-represented ($p<0.1$) GO IDs and corresponding GO terms are listed. Significant GO terms were curated, pooled and organized into "manually annotated functional category". All terms were classified into their parent group of Biological Process (BP), Molecular Function (MF) and Cellular Component (CC). Heart hit genes and their corresponding CG IDs that correspond to the selected GO terms are listed. The "Count" of heart hits that map to a GO term as well as the "Total" number of genes in Flybase annotated with each GO term are shown. Corrected p-values for the enrichment (Yekutiali) of each GO term are given. (**B**) *Drosophila* GO gene-set enrichment analysis. Enrichment of selected GO gene sets in a rank ordered list of the genes from the entire screen data is shown. An enrichment score (Es) was calculated for each GO gene set, as described in Experimental Procedures. The enrichment score cut-offs (90^{th}, 95^{th} and 99^{th} quantiles) from 100 similar sized random gene sets, generated by bootstrapped permutations are given. If Es > Er, the GO term enrichment is marked as "TRUE". TRUE enrichment is indicated at each cut-off separately. BP, Biological Process. MF, Molecular Function. CC, Cellular Component. (**C**) Panther annotation of unknown genes. Panther

(www.pantherdb.org) functional annotations of 116 of 121 heart hit genes that did not have associated GO annotation. CG IDs of the genes and their assigned “Functional categories” are listed. (**D**) Interactions between candidate heart genes and their first degree binding partners in *Drosophila*. All interactions involving *Drosophila* heart hits and their first degree neighbors (binding partners) are listed. Each row corresponds to a biological interaction between two proteins. Every interaction has two participating nodes: Source and Target. Gene symbols and corresponding CG ids for all source and target nodes are listed. Classifications of all source and target nodes into heart hits (Heart), binding partners (BP) and developmental lethal hits (lethal) are provided. Note that in some cases FlyBase numbers (FBgn #) are provided. (**E**) Fly and mammalian KEGG pathway analyses. Enrichment of KEGG pathways in *Drosophila* heart hits and binding partners and their predicted mouse and human orthologues. The complete analysis for 146 Drosophila (top), 202 mouse (middle), and 209 human (bottom) KEGG pathways are shown. For each pathway, the KEGG database ID, pathway name, KEGG gene sets, CG/Entrez IDs for the mapped genes, numbers of genes assigned to each KEGG, numbers of hits and their binding partners that map to the KEGG pathways, percentages of genes mapped among the total KEGG gene sets, and hypergeometric probabilities are listed. *Drosophila* pathways that are over 90% enriched were manually annotated into functional groups for visual representation in Table S3. For constructing the systems map of heart function, significant KEGG pathways were combined from *Drosophila* (>70%), mouse (>85%) and humans (>85%). Pathways were manually grouped into functional categories. All relevant grouping information is provided at the bottom of the table. *Drosophila*, mouse, and human KEGG pathways that were used to construct the systems maps in Fig. 2 and Table S3 are highlighted in yellow. (**F**) Significant enrichment of mammalian GO terms for adult heart function hits. GO enrichment for all mouse and human orthologues corresponding to *Drosophila* heart hits. For all significant terms, the GO ids, GO terms, and all relevant information have been included. Since GO terms that lie at level 4 or below in the GO hierarchy tree convey more biological information, we excluded terms with more than 500 genes in them. Further, if both parent and several child terms are found significant, we manually curated the data and retained the term that contained the maximum overlapping genes

with the heart hit list. These terms have been highlighted in the mouse and the human lists separately. From these selected GO terms, those that denote related functions in biological systems were manually clubbed together and functionally annotated for pie chart visualization. The analysis is included in the column labeled “Manually assigned functional category”. BP, Biological Process. MF, Molecular Function. CC, Cellular Component. A P-value of 0.1 was considered significant. Count and Total indicate the numbers of hits among all genes assigned to a defined GO term. (**G**) Mammalian C2 gene set analysis. Analysis for 687 mouse (top) and 680 human (bottom) C2 gene sets. For each gene set, the C2 gene set name, gene symbols, counts for the total number of genes, heart hit orthologues, and percentages of genes mapped percentages of genes mapped among the total C2 gene sets are given. A hypergeometric probability of 99% (hyper geometric test) was considered significant. For constructing the systems map of heart function and uniform functional annotation as in the KEGG pathways, significant C2 gene sets were combined from mouse and humans and manually grouped into similar functional categories. (**H**) Combined systems map. This sheet represents a combined list of significant KEGG pathways and C2 gene sets from *Drosophila*, mouse, and human used to construct the combined systems map shown in Fig. 2. All significant KEGG pathways and C2 gene sets were manually grouped into uniform functional classes shown in the column “Gene set categories”. Gene symbols, Entrez IDs and *Drosophila* CG IDs were extracted for each pathway or gene set.

Supplemental Table S5. Fly hits that correspond to existing knock-out mice with cardiovascular phenotypes (related to figure 2).

Drosophila heart hits and the first degree binding partners (BP) whose mouse orthologues have reported knock-outs with “cardiovascular phenotypes” in MGI (www.informatics.jax.org) are listed. For each mouse knock-out gene with a heart defect reported, the corresponding *Drosophila* orthologue was extracted.

Supplemental Video S1. Visualization of control and Not3 RNAi heart function (Related to Figure 3). UAS-Not3 RNAi fly lines were crossed to Hand-

Gal4 (II) driver-flies and to w^{1118} wild type control flies. After dissection to expose the heart, the semi-intact preparations were visualized and assessed for heart function. Representative movies of roughly the central 1/3 of the cardiac tube for control (first movie) and Not3 RNAi hearts (second movie) are shown.

Supplemental experimental procedures

Fly stocks

All RNAi transgenic fly lines were obtained from the VDRC RNAi stocks. The whole genome VDCR library has been previously reported (Dietzl et al., 2007). The cardiac-tissue specific TinCΔ4 12a-Gal4 was kind gift from Manfred Frasch, (Lo and Frasch, 2001) and Hand-Gal4 was kind gift from Eric Olsen, (Han and Olson, 2005) driver lines have been previously described and used to study genes involved in *Drosophila* heart development and cardiac function in adult flies (Wolf et al., 2006). w^{1118} wild type control flies obtained from the VDRC were used when indicated since the *Drosophila melanogaster* w^{1118} background is isogenic to the VDRC RNAi library. The fly stock for the Not3 associated P-element ($CG8426^{KG10496}$) was obtained from Bloomington (#15271).

Screening system

Transgenic UAS-RNAi males were crossed to TinCΔ4 12a-Gal4 virgin females and 4 day old viable F1 adult progeny were transferred to test chambers containing 2 filter pads with 2 ml of a 50 mMol sucrose solution. The viable RNAi lines were then incubated at 29ºC for 6 days to expose flies to temperature stress as described previously (Paternostro et al., 2001) and the number of dead and surviving flies were counted. Initially a Z score cut-off of 2 (Mean control-test)/SD was used to select RNAi lines for re-testing. Developmental lethality was assessed in a semi-quantitative fashion, with lethal crosses scored as 0, semi-lethal crosses scored as 0.5, and viable crosses scored as 1. During re-testing, lethality was staged when apparent. In all cases, crosses were scored based on the furthest developmental stage reached: 0=lethal (embryonic lethal or no off-spring), 1= larval lethal, 2= pupal lethal, 3= adult lethal (die within 4 days after eclosion), 4= adult viable.

Drosophila cardiac analysis

UAS-RNAi fly lines obtained from the VDRC were crossed to Hand-Gal4 (II) driver-flies and to w^{1118} wild type control flies. As an additional control, Hand-Gal4 (II) driver-flies were crossed to w^{1118} wild type flies. The progeny were raised at 25˚C on standard

cornmeal-agar medium. At 1 week of age, 29-40 female offspring from each cross were anaesthetized and dissected. All procedures were done at room temperature (18-22°C) as previously described (Cammarato et al., 2008; Ocorr et al., 2007). Briefly, each head, ventral thorax and ventral abdominal cuticle was removed exposing the abdomen. All internal organs and abdominal fat were removed leaving only the heart and associated muscles for each fly. Dissections were performed in oxygenated artificial adult hemolymph. The semi-intact preparations were allowed to equilibrate with oxygenation for 20–30 min prior to filming. Analysis of gross heart morphology and physiology was performed using high speed movies. 30 sec movies were taken at rates of 100–200 frames/ sec using a Hamamatsu EM-CCD digital camera on a Leica DM LFSA microscope with a 10x immersion lens. All images were acquired and contrast enhanced using Simple PCI imaging software (Compix, Inc.). M-modes were generated and determination of cardiac parameters, including heart periods, diastolic and systolic diameters and fractional shortening for each group was performed using a MatLab-based image analysis program (Fink, 2009). ANOVA of genotype as a function of each measured cardiac parameter was employed to test for significant differences between w^{1118}>UAS-*Not3*-RNAi or w^{1118}>UAS-*UBC4*-RNAi control flies and both Hand-Gal4>UAS-*Not3*-RNAi or the Hand-Gal4>UAS-*UBC4*-RNAi mutants, respectively. Additionally, one-way ANOVAs with Bonferroni multiple comparison post tests were employed to determine if significant differences between w^{1118}>Hand-Gal4 and all w^{1118}>UAS-RNAi control lines were present.

Fluorescent Staining

Fluorescent staining and imaging of *Drosophila* heart tubes were performed as described (Alayari et al., 2009). Briefly, beating hearts of semi-intact *Drosophila* were placed in artificial adult *Drosophila* hemolymph containing 10mM EGTA. Cardiac tubes were examined to ensure contractions were inhibited. Hearts were fixed in 1xPBS containing 4% formaldehyde at room temperature for 20 minutes with gentle shaking. Washing of hearts was performed three times for 10 minutes with PBSTx (PBS containing 0.1% Triton-X-100) at room temperature with continual shaking. After washing, the hearts were incubated with Alexa584-phalloidin in PBSTx (1:1000) for 20

minutes with continual agitation. Washing of the hearts was carried out three times for 10 minutes with PBSTx at room temperature. The hearts were rinsed in 100 µl of PBS for 10 minutes. The specimens were mounted on microscope slides and viewed at 10X magnification using a Zeiss Imager Z1 fluorescent microscope equipped with an Apotome sliding module.

Q-RT-PCR of *Drosophila* cardiac associated transcripts

Quantitative RT-PCR was performed using standard procedures according to (Akasaka et al., 2006) with minor modifications. Total RNA was extracted from 30 hearts per genotype (all female flies). Quantitative Real Time-PCR was performed with the LightCycler FastStart DNA Master PLUS SYBR Green I kit (Roche) using single strand cDNA."

The primer sets were as follows:

Not3_05 CGCAAAGCAGTATCGGAAGT

Not3_06 CCGCAGGATTGTTACCAG

UBC4_01 CGCAGCTTTGTGTTTCGTTT

UBC4_02 GTTGGTGTCTCGCGTATT

SERCA_01 CAAGTCCTACTCGGGTCGTG

SERCA_02 CATAGCGGAGATTTCGTTCAT

KCNQ_11 ACCATCAAGGAGTACGAAGA

KCNQ_12 CGATGACCAGAGTCGAG

Mhc_01 AGTCCGAGCGTCGCGTCAAG

Mhc_02 TGAGGGCGGCGATTTC

rp49_01 GAC GCT TCA AGG GAC AGT ATC TG

rp49_02 AAA CGC GGT TCT GCA TGA G

actin_01 ATCCGCAAGGATCTGTATGC

actin_02 ACATCTGCTGGAAGGTGGAC

actinin_03 CCTGACCGCCAACGACATGA

actinin_04 CATTTGCCGCTCGATCCA

Whole mount fluorescent staining of *Drosophila* embryos

Following dechorionization in 7.5% bleach, embryos from overnight collections were devitellinized and fixed in heptane with 4% formaldehyde in 0.3% PBT buffer (1x PBS with 0.3% Triton) for 25 minutes. Dechorionisation and dehydration was accomplished by vortexing and washing in methanol. Primary antibodies used: anti-ßGal (mouse 1:1000; Cappel), anti-Tin (rabbit 1:800; R. Bodmer). The secondary antibodies used were donkey anti-mouse-Alexa488 and anti-rabbit-Alexa568 (1:500, Molecular Probes). Flat preparations of embryos were mounted in Vectashield mounting medium and 70% glycerol (1:1). Stained embryos were examined under a confocal microscope (Zeiss LSM510) and images were processed using Adobe Photoshop.

Identification of mouse and human orthologues

To identify orthologues between *Drosophila* and mouse and between *Drosophila* and human, we used pre-computed orthology predictions obtained from compara49, inparanoid, inparanoid6.1, inparanoid6.1 to ensembl, homologen08, and orthomclv2 (Kuzniar et al., 2008) databases. One-to-one and one-to-many mappings were observed between the *Drosophila* and the mammalian genes. In case of one–to–many mappings, all the orthologue targets for a given gene were considered for downstream analysis.

Functional annotation of "heart function" hits

Of the 490 RNAi hits (498 genes) with potential defects in heart function, we could annotate 377 genes by GO terms. To annotate the remaining 121 genes, we searched Panther db (http://www.pantherdb.org/). We were able to annotate an additional 116 genes with previously unknown functions.

Gene ontology (GO) classification and Gene Set Enrichment

GO analysis was performed using GOstat (http://gostat.wehi.edu.au/). GO analysis was run with *Drosophila* genes (and mouse or human orthologs) whose RNAi hits had a Z score >3. Over and under-represented ($p<0.1$) GO terms with corrections for multiple testing (Yekutieli correction), were identified in the above list, compared to the FlyBase (fb) for *Drosophila*, Mouse Genome Informatics (mgi) for mouse and goa_human for

human genes, respectively. Since terms that occur at a deeper level in the GO tree hierarchy and contain lesser number of genes are considered more biologically informative, we discarded terms containing more than 500 genes from further analysis. Significant GO terms were manually curated, pooled and organized into “functional groups”, for visual representation of the GO data. A complete list of significantly enriched GO terms pooled into functional groups is provided in Suppl. Table S4.

To remove any artificial bias in the gene list created by the ad-hoc Z score cut-off (>3) that we used previously in the GO analysis, we performed the Gene Set Analysis (GSA) to confirm enrichment of selected GO terms. GSA uses the entire list of genes corresponding to the selected biological terms and assesses if the process as a whole has been significantly altered in the entire dataset. A null hypothesis is that “Genes of a functionally irrelevant pathway are not clustered at the top of a rank ordered (based on Z score) list of all genes in the experiment”. Based upon the Z score of the RNAi hit phenotype, a rank order list of 6892 unique genes corresponding to 8291 (with reported Z score) RNAi transformants was created. Individual gene lists were constructed corresponding to 45 GO terms rendered significant by GO analysis (corrected $p<0.1$). An enrichment score (Es) for a gene list (s) was calculated based upon the “heart function” Z score using the formula:

$$Es = \frac{\text{sum of average z-scores of all genes in s}}{\sqrt{\text{(Number of genes in s)}}}$$

For every **s**, 100 bootstrap permutations were carried out to generate random functionally unrelated gene lists of the same size and their enrichment scores calculated as Er. A gene set s is considered enriched when Es > Er (90th quantile) and we reject the null hypothesis (p-value < 0.1). Enrichment of the GO term is also reported at p-value < 0.05 and 0.01.

Pathway analyses

Pathway analysis was performed using the *Drosophila* Pathway database in GeneSpring GX. Briefly, the Pathway database in GeneSpring GX contains molecular relations from two sources: (1) those reported in open-source public databases like

BIND and IntAct (IMEX consortium) or (2) extracted from PubMed abstracts using a proprietary natural language processing technique. The tool UI allows a query of the database to build networks of molecular relations (edges) amongst molecules (nodes) of interest. Relations are classified as binding, expression, transport, protein modification, general regulation, metabolism and promoter binding. . We constructed a network of “heart function” genes along with their first degree protein-protein interaction neighbours. Using filters in GeneSpring GX, we selected molecular relations reported by the IMEX consortium which represent “binding” interactions between the molecules. These interactions found the majority of binding partners for the heart hits. We also included the highest quality (Quality score ≥9) expression and promoter binding interactions using appropriate filter settings in GeneSpring GX. These interactions were manually curated and included in the final network.

Pathway Enrichment Analysis

A hypergeometric test, similar to the test used for GO enrichment analysis, was used to identify over-represented gene lists (C2 from Msigdb, BROAD Institute) and pathways (KEGG) amongst the “heart” hits. The hypergeometric test considers only the percentage representation of genes corresponding to a biological pathway in the pre-computed heart function gene list. This analysis was performed on the gene list identified as adult “heart” hits (Z score ≥ 3) in *Drosophila* and their corresponding mouse or human orthologues, respectively.

Pathway Systems map

For the combined systems map, all significant KEGG pathways and C2 gene sets were manually grouped into uniform functional categories as shown in Table S4 E-H. For uniformity, both lists have been manually annotated by the same functional classification terms. Functional classifications were assigned relevant to the biology of heart functions. For the systems map, we included all the functional categories assigned to the significant KEGG pathways. The information obtained from KEGG pathway analysis was supplemented with significant C2 gene sets from the appropriate functional categories: AKT/PI3K signaling activation, Calcium Signaling, cAMP

regulated signaling, Cardiac related disease/signaling, Cytoskeleton, Hematopoeisis related, Ion Channels, muscle contraction, NFAT transcription, PI3K signaling, Txn factors. For each functional category, the corresponding genes that mapped to the KEGG pathways or C2 gene set were extracted. *Drosophila* orthologues for these genes were found and assigned to the respective pathway in the visual representation of the systems map. For the *Drosophila* systems map, *Drosophila* pathways that were over 90% enriched were manually annotated into functional groups. Corresponding heart hit genes and their first degree binding partners that overlapped with the gene set of the pathway were extracted and assigned to each pathway node in the systems map. We found both primary heart hits as well as binding partners filtered into the systems map are significantly enriched ($p<0.01$) in cardiac genes of known function from independent studies, compared to any random list of the same size. We observe an almost equal enrichment of cardiac genes with known functions amongst the RNAi hits as well as binding partners included in the systems map.

Generation of *Not3* knockout mice

For gene targeting of *not3* in mice, a targeting vector was constructed to replace exons 2 and 9 of the murine *not3* gene. The linearized construct was electroporated into A9 embryonic stem (ES) cells derived from 129/Ola and C57BL/6J hybrids. Approximately 2-3 in 100 ES cell clones were identified as correctly targeted by genomic Southern blotting. Chimeric mice from two independent clones transmitted the mutant allele through the germ line, and the targeted allele was confirmed by Southern blot. F1 mice were backcrossed for 5 times onto a C57BL/6 background. F5-F7 intercrossed mice were used for all experiments reported in this study. Mice were genotyped by PCR and Southern blotting and maintained at the animal facilities of the Institute of Molecular Biotechnology (IMBA), Akita University Graduate School of Medicine and Tokyo Medical and Dental University. All animal experiments were performed in accordance with institutional guidelines.

Mouse embryo analyses

To analyze blastocyst-stage mouse emryos, embryos at 3.5 dpc were flushed out from the uteri of pregnant mice of heterozygous x heterozygous breeding. RNA and Genomic DNA were extracted from individual blastocysts with Trizol (Invitrogen). Genotypes were determined by nested PCR, and reverse transcription was performed with Primescript RT reagent kit (TAKARA) followed by RT-PCR with ExTaq PCR kit (TAKARA). For immunohistochemistry of whole embryos, blastocysts were fixed in 4% PFA for 20 min, permealized with 0.4% Triton X-100 in PBS for 20 min, and blocked with 0.1% Triton X-100, 1% BSA or 10% donkey serum in PBS for 30 min. After each step embryos were washed five times for 5 min in PBS, and anti-Not3 antibody (Protein Tech Group, 11135-1-AP) staining was performed at 4^{o}C overnight followed by Alexa543 secondary antibody for 1 h. Embryos were mounted with VECTASHIELD with DAPI (Vector Labs) and observed under LSM500 confocal microscopy (Carl Zeiss). Embryos were collected and genotyped by nested PCR. For *in vitro* culture of inner cell mass outgrowth, blastocysts were individually cultured onto gelatinized tissue culture plates (Nunc) in standard ES medium with leukemia inhibitory factor (Chemicon) and photographed every few days. After cultivation, embryos were harvested by trypsinization and genotyped by PCR.

mRNA and protein expression analyses in mice

For real-time PCR analysis of mouse Not3, 3 μg of DNase-treated total RNA was extracted from hearts and reverse transcribed using First-Strand Beads (GE Healthcare) with random primers. SYBR green real-time PCR reactions were carried out in 96-well plates using an iQ-Cycler (Bio-Rad). Immunohistochemistry to detect Not3 was carried out with antibodies to Not3 (Abcam 55681). Blastocysts were then counterstained DAPI. The primer sets were as follows:

NOT3-F: GTATAGCAAGGAGGGTCTGGGTCTGGCTCAG

NOT3-R: TCGGGGTCCTGGGATGAGTCAACGTAGTAC

ANF-F: GAGAGACGGCAGTGCTTCTAGGC

ANF-R: CGTGACACACCACAAGGGCTTAGG

βMyhc-F: GACGAGGCAGAGCAGATCGC

βMyhc-R: GGGCTTCACAGGCATCCTTAGGG

Kcnq1-F: ATCAGGCGCATGCAGTACTTTG
Kcnq1-R: TGACATCTCGCACGTCGTAGG
Kcne1-F: TAGTGAATGTCGCCTTGTTTGGAA
Kcne1-R: TGGCATCTCACGGTGCTCTC
Oct4-F: CCAATCAGCTTGGGCTAGAG
Oct4-R: TGTCTACCTCCCTTGCCTTG
GATA6-F: GAGCTGGTGCTACCAAGAGG
GATA6-R: TGCAAAAGCCCATCTCTTCT
Eomesodermin-F: GGCAAAGCGGACAATAACAT
Eomesodermin-R: GACCTCCAGGGACAATCTGA
18S-F: CTGCCGTCTGAGTGTATCGC
18S-R: GCTGGGGCTGAGGAAAGTG
GAPDH-F: GGCCAAGGTCATCCATGACAAC
GAPDH-R: GTTGTCATGGATGACCTTGGCC

Echocardiography

Echocardiographic measurements were performed as described previously (Kuba et al., 2007). Briefly, mice were anesthetized with isoflurane(1%)/oxygen, and echocardiography was performed using an Acuson Sequoia C256 equipped with a 15-MHz linear transducer. Fractional shortening (FS) was calculated as follows: FS = [(LVEDD – LVESD)/LVEDD] x 100.

Langendorff perfusion experiments

Not3 heterozygous and littermate control mice were anaesthetized with ketamine/xylazine and isolated hearts immediately placed in cold PBS. The isolated heart was mounted on a Langendorff apparatus and perfused at a constant hydrostatic pressure of 75 mm Hg with an oxygenated Tyrode solution (37 ^{o}C) composed of 136.9 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.33 mM NaH_2PO_4, 10.0 mM glucose, and 5.0 mM Hepes as described (Murakami et al., 2007). Atropine (5.5 μM) was added to the perfusate, and a fluid-filled balloon catheter connected to a pressure transducer (CD200; NihonKoden) was inserted into the left ventricle via the left atrium.

The sinoatrial node region was removed, and the heart was paced electrically at 400 beats/min (bpm). The electrical field stimulation (EFS) was applied in conjunction with the pacing stimulation (delay, 4 ms; duration, 1 ms for 5 s). Isoproterenol was perfused for 30 seconds using the indicated doses.

Transverse aorta constriction (TAC)

Eight to ten weeks old control wild type littermates and *not3* heterozygous mice were subjected to pressure overload by TAC through constriction of the thoracic aorta as described (Kuba et al., 2007). Heart function was determined by echocardiography. For histology, hearts were arrested with 1 M KCl, fixed with 10% formalin, and embedded in paraffin. 5 µm thick sections were then cut and stained with hematoxylin and eosin (H&E). For detection of fibrotic areas, sections were stained with Masson-Trichrome.

Treatment with HDAC inhibitors

For baseline measurements, wild type and *not3*$^{+/-}$ mice were treated with vehicle, Trichostatin A (TSA) (Sigma, 0.6 mg/kg/day, i.p.), or Valproic acid (VPA) (Na Valproate (WAKO) 0.71% w/v in drinking water) for 1 week. For TAC heart failure experiments, the mice were given vehicle or VPA (0.71% w/v in drinking water) for 6 weeks starting one day after TAC or sham surgery.

Western blot analysis for histone tail modifications

Acid-extracted histones were prepared from whole ventricles of mouse hearts as described (Cheung et al., 2000). Histones were resolved by SDS-PAGE (15%; 30:0.8) gels and transferred to nitrocellulose membranes for Western blotting. The amount of histone H3 was precalibrated with ponceau-S (Sigma) staining. Covalent modification status of H3 tails was analyzed using antibodies detecting acetylated H3K9 (Upstate), trimethylated H3K4 (Abcam), and anti-trimethylated H3K27 (kind gift from T. Jenuwein). Band intensities were quantified and normalized to total H3 levels.

Human QT interval association

Human QT interval association signals over the *NOT3* region (official gene name *CNOT3*) were obtained from data generated by the QTSCD Consortium as described in (Pfeufer et al., 2009).

Statistical analyses

Data are presented as mean values ± SEM and were analyzed for differences between wild type and *not3* heterozygous mice at discrete time points. *Drosophila* survival and heart function was analysed by *t*-test for single comparisons and ANOVA for multiple comparisons. Gene set enrichment is described above. GO enrichment was calculated using the online tool GOstat (http://gostat.wehi.edu.au/). For mouse heart function, normally distributed data were analyzed by *t*-test. Data not normally distributed were analyzed using the Mann-Whitney test. $P < 0.05$ was considered significant**.** GWAS association results are presented as mean values ± SD.

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