iScience, Volume *24*

Supplemental Information

Activation of amino acid metabolic program in cardiac HIF1-alpha-deficient mice

Ivan Menendez-Montes, Beatriz Escobar, Manuel J. Gomez, Teresa Albendea-Gomez, Beatriz Palacios, Elena Bonzon-Kulichenko, Jose Luis Izquierdo-Garcia, Ana Vanessa Alonso, Alessia Ferrarini, Luis Jesus Jimenez-Borreguero, Jesus Ruiz-Cabello, Jesus Vázquez, and Silvia Martin-Puig

TRANSPARENT METHODS

Animal care and housing

Hif1α$^{flox/flox}$ (Ryan et al., 2000)mice were maintained on the C57BL/6 background and crossed with mice carrying *Nkx2.5Cre* recombinase (Stanley et al., 2002) or *TnTCre* recombinase (Jiao et al., 2003) in heterozygosity. *Hif1α*$^{flox/flox}$ homozygous females were crossed with double heterozygous males and checked for plug formation. Mice were housed in SPF conditions at the CNIC Animal Facility. Welfare of animals used for experimental and other scientific purposes conformed to EU Directive 2010/63EU and Recommendation 2007/526/EC, enforced in Spanish law under Real Decreto 53/2013. Experiments with mice and embryos were approved by the authorized Environmental Department of Comunidad de Madrid, Spain, with reference numbers: PROEX 12/14 and PROEX 267/19.

Genotyping

Genotyping was performed using the following primers (Sigma Aldrich; USA) for *Hif1a floxed* alleles: 5' CGTGTGAGAAAACTTCTGGATG 3' and 5' AAAAGTATTGTGTTGGGGCAGT 3'. For *Hif1a* null allele: 5' GCCCATGGTAAGAGAGTAGGTGGG 3' and 5' 5' AAAAGTATTGTGTTGGGGCAGT 3'. For Cre alleles genotyping, *Nkx2.5*: 5' GCCCTGTCCCTCAGATTTCACACC 3', 5' GCGCACTCACTTTAATGGGAAGAG 3' and 5' GATGACTCTGGTCAGAGATACCTG 3' and *cTnT*: 5' TACTCAAGAACTACGGGCTGC 3' and 5' GCACTCCAGCTTGGTTCCCGA 3'.

Embryo extraction

Embryos at E12.5, E14.5 and E17.5 were extracted after pregnant female euthanasia by CO_2 inhalation and head and liver were removed. Equivalent proportion of male and female embryos have been included in all experiments. After dissection, embryos were snap frozen in liquid nitrogen for biochemical studies or fixed overnight at 4ºC in 4% PFA solution (RT15710, Electron Microscopy Sciences; USA). After fixation, embryos were dehydrated in ethanol series, embedded in paraffin and sectioned at 5µm for immunostaining and histological purposes and at 10µm for *in situ* hybridization.

Histological and immunohistochemical analysis

Histological sample processing and immunostaining was performed as described elsewhere (Menendez-Montes et al., 2016). Briefly, 5µm-thick paraffin sections were stained with hematoxylin &eosin (HE) following standard histological procedures at the CNIC Histopathology Facility. For adult cardiac tissue H&E and Masson Trichrome staining analysis, even distribution of male and female mice was used in each experimental group. No differences associated with sex were observed in structure or fibrosis. For immunostaining, sections were rehydrated, and antigens were retrieved by incubation in citrate buffer (10 mM sodium citrate, 0.05% Tween20, pH 6) in a pressure cooker. Sections were permeabilized with 0.5% Triton-X100 for 10 min and blocked with 10% goat serum (GS) (Cat. No. 16210-072, Life Technologies; NY; USA). Sections were incubated with primary antibodies in 10% GS overnight at 4ºC. After several washes with PBS-T, sections were incubated with secondary antibody (Life Technologies; NY; USA or Dako; Denmark) in 5% bovine serum albumin (BSA) for 1h at room temperature in the dark. When necessary, signal was amplified using fluorochrome-conjugated streptavidin (Life Technologies; NY; USA) for 1h at room temperature in the dark, or with the TSA System (Perkin Elmer; MA; USA). Finally, sections were incubated with DAPI (Millipore, MA; USA) and mounted in Fluorescent Mounting Medium (S3023, Dako, Denmark). Images were acquired with Zeiss

LSM700 (Zeiss; Germany) or Nikon A1R (Nikon; Japan) confocal microscopes. The primary antibodies used in this study were: HIF1α (NB100-479, Novus Biologicals; USA and GTX30647, Genetex, USA); cTnT (CT3, Developmental Studies Hybridoma Bank; USA); BrdU (347580, BD Biosciences; USA); Cy3-conjugated Smooth Muscle Actin (C6198, Sigma Aldrich; USA) and GLUT1 (Cat. No. 07-1401, Millipore, USA).

Quantification of histological and immunostained sections

HE staining was quantified as previously reported (Menendez-Montes et al., 2016) using ImageJ (Rasband, 2015). Briefly, images of HE-stained sections were acquired with a NanoZoomer-XR Digital slide scanner (Hamamatsu; Japan). Compact myocardium and IVS thickness and total width and height of ventricular chambers were measured using NDP View (Hamamatsu; Japan). Values of at least three independent litters were analyzed for significant statistical differences by Student's t test. For fluorescence intensity analysis in cardiomyocyte nuclei, our own pipeline for CellProfiler software was employed (Lamprecht et al., 2007). Briefly, cell nuclei were segmented and subsequently filtered by cTnT positive cytoplasmic staining. After filtering, HIF1α channel intensity was measured.

RNA extraction, cDNA synthesis and RT-qPCR

RNA extraction from embryonic hearts, cDNA synthesis and quantitative PCR were performed as previously described (Menendez-Montes et al., 2016). Primers are available under request. Briefly, total RNA was extracted using QiAzol Lysis Reagent (Qiagen; CA; USA) and the miRNeasy Mini Kit (Qiagen; CA; USA). Total amount of isolated RNA was retrotranscribed using the MultiScribe Reverse Transcriptase kit 8Applied Biosystems; CA; USA) and cDNA concentration was adjusted to 250ng/μL. All real-time qPCR reactions were performed in an AB7000 thermalcycler (Applied Biosystems; CA; USA) using SYBR Green PCR Master Mix (Applied Biosystems; CA; USA). Gene-specific primers were obtained from PrimerBank (http://pga.mgh.harvard.edu/primerbank/index.html) and checked for exon spanning using Primer3 (http://primer3.sourceforge.net/webif.php). Baseline normalization and thresholding were performed in automated mode with the SDS Software (Applied Biosystems; CA; USA). Cq values were analyzed using qBase (Biogazelle; Belgium) using 3 housekeeping genes (*GusB, Hprt* and *Rpl32*). Primer-specific efficiencies were tested with serial dilutions of control cDNA. Statistically significant differences between control and mutants were analyzed by Student's t test.

Probe synthesis and in situ hybridization

General probe synthesis, purification and in situ hybridization steps were followed according with our previous protocol (Menendez-Montes et al., 2016). Briefly, for anti-probe preparation, reverse primers carrying T7 polymerase promoter sequence at the 5' end were used. Amplified fragments were purified using QIAquick Gel Extraction Kit (Qiagen; CA; USA) and probes were transcribed using 100ng of the fragment and T7 polymerase in presence of DIG-labelled nucleotides (Roche, Switzerland) for 6h. Probes were treated with Dnase I and purified using illustra AutoSeq G50 Dye Terminator Removal kit (GE Healthcare, UK). For the probe synthesis, the following primers were used: *Glut1* 5' GGACTTTGATGGCTCCAGAA 3' and 5' GAGTGTCCGTGTCTTCAGCA 3', *Pdk1* 5' CTGGGTTTGGTTACGGATTG 3' and 5' GCCAGCTACTCCACGTTCTT 3' and *Ldha* 5' GGAAGGAGGTTCACAAGCAG 3' and 5' CTGCAGTTGGCAGTGTGTCT 3'.

For in situ hybridization staining, 10µm paraffin sections of embryos, stored at -20ºC, were rehydrated and subsequently post-fixed in 4% PFA. After this, mRNA was exposed by incubation with Proteinase K (0.01mg/mL) at 37ºC for 10 min, and masking proteins were denatured by incubation in 0.7N HCl for 15 min at room temperature. Sections were blocked with hybridization buffer (50% formamide, 25% SSC 20X pH 5.5, Denhardt's buffer 1X, 0.1% Tween20; Chaps 10% 0.01mL/L; 0.05g/L tRNA) and incubated overnight at 65ºC with DIG-labeled probe at a final concentration of 5µL/mL. After several washes in decreasingly stringent conditions, sections were incubated overnight at 4ºC with anti-DIG-AP Fab fragments (Roche; Switzerland). Finally, sections were conditioned in alkaline phosphatase buffer (NaCl 0.1M; MgCl2 0.05M; Tris-HCl 0.1M pH 9.5; 0.1% Tween20) and developed with BM Purple (Roche; Switzerland) at 37ºC for 1-4 days, until the signal was clear

Electron microscopy and micrograph quantification

Embryonic hearts were processed for transmission electron microscopy following the standard procedures. Briefly, after overnight fixation in 3% glutaraldehyde/4%PFA, samples were refixed in 1% osmium tetroxide and embedded in epoxy resin. 60nm sections were counterstained with uranyl acetate and lead citrate and imaged using a JEOL JEM1010 (100 KV) transmission electron microscope. Control and mutant embryonic hearts from three independent litters were analyzed. For quantification of mitochondria and lipid droplets, ten images of compact myocardium and ten of trabeculae were taken at 5000x magnification. Mitochondria and droplets were counted manually by blinded observers using the ImageJ CellCounter plugin. Values were normalized to the total tissue area, in pixels, excluding extracellular areas in the image.

Protein extraction and Western Blot

Embryonic hearts were homogenized using RIPA buffer and a TissueLyser in presence of protease and phosphatase inhibitors (Inhibitor cocktail (Roche, Switzerland) and 1µM sodium ortovanadate). After clarification by centrifugation, protein concentration was measured using Pierce BA Protein Assay kit (23227, Thermo Scientific; USA) following manufacturer instructions. 30µg of protein were denaturized at 95ºC for 5 min, loaded on an 8% polyacrylamide SDS-PAGE gel and run at 120V for 90min. Subsequently, samples were transferred to a nitrocellulose membrane by wet transfer at 400mA for 2h. Membranes were blocked with 5% BSA for 1h and incubated with primary antibodies O/N at 4ºC. Next day, membranes were washed in TBS-T buffer and incubated with the corresponding HRP-conjugated secondary antibodies (Dako, Denmark) at 1:5000 dilution for 1h at RT. After washing, signal was developed using ECL Primer Western Blotting Detection Reagent (Amersham; UK) and detected by a LAS-3000 imaging system (Fujifilm; USA). The primary antibodies used in this study were: anti-HIF2α[ep190b] (NB100-132, Novus Biologicals) dilution 1:200, anti-HIF1α (10006421, Cayman; USA) dilution 1:200, anti-PAI-1 (sc-5297, Santa Cruz) dilution1:500, anti-ATF4 (11815, Cell Signalling; USA) dilution 1:500, anti-vinculin (V4505, Sigma-Aldrich; USA) dilution1:5000 , anti-αtubulin[DM1A] (ab7291,Abcam; UK) dilution 1:1000 and anti-SMA cy3 (C6198, Sigma-Aldrich; USA) dilution 1:1000.

RNASeq and bioinformatics analysis of gene expression

RNASeq data processing and differential expression analyses (n=2 per group) were performed as previously described (Menendez-Montes et al., 2016): E12.5 ventricles were collected and total RNA was extracted as detailed above. RNA integrity was verified using Agilent 20100 Bioanalyzer (Agilent Technologies; CA; USA). Index-tagged cDNA libraries were constructed from 500ng total RNA using TruSeq RNA Sample Preparation v2 kit (Illumina; CA; USA). Libraries were quantified in a Q-bit fluorometer (Life Technologies; CA; USA). After normalization, libraries

were applied to an Illumina flow cell for cluster generation and sequencing-by-synthesis. Single reads of 75bp were generated following the standard RNA sequencing protocol. To produce fastq files, reads were processed using CASAVA package (Illumina; CA; USA).

For differential expression analysis, only genes expressed with at least at 1 count per million in at least in 2 samples were considered. Changes in gene expression were considered significant if associated with Benjamini-Hochberg adjusted P value < 0.055. Functional enrichment analyses were performed with Gorilla (Eden et al., 2009), Panther (Thomas et al., 2003), IPA (Qiagen, USA), REVIGO (Supek et al., 2011) and GSEA (Subramanian et al., 2005). Enriched functional terms were filtered by applying P value thresholds described in the corresponding Table or Figure captions. Circular plots summarizing logFC values for genes, and their association to enriched functional terms were generated with GO plot (Walter et al., 2015).

Magnetic resonance spectroscopy and data processing

E12.5 snap-frozen embryonic ventricles were processed and analyzed by High Resolution Magic-Angle Spinning (HR-MAS) Nuclear Magnetic Resonance Spectroscopy (^{1}H-NMR as previously described (Menendez-Montes et al., 2016). Briefly, intact heart tissue was placed in zirconium oxide rotor in the presence of 0.1mM Trimethylsilyl propanoic acid in deuterium water. Samples were acquired at 500.13 MHz using a Bruker AVIII 500 spectrometer at 11.7 T over 4 hours using a CPMG pulse sequence. Data processing was performed using the Metabonomic R package (Izquierdo-García et al., 2009, Röst et al., 2016, Gil-De-La-Fuente et al., 2019). Spectra were referenced to the TSP singlet at 0 ppm (parts per million) chemical shift.

Proteomics analysis

Proteins from pellets after metabolite extraction were pooled in groups of four, treated with 50mM iodoacetamide (IAM) and digested with trypsin using the Filter Aided Sample Preparation (FASP) digestion kit (Expedeon) (Wiśniewski et al., 2011) according to manufacturer's instructions. Dried peptides were labeled with iTRAQ-8plex according to manufacturer's instructions, desalted on OASIS HLB extraction cartidges (Waters Corp.), separated into 4 fractions using the high pH reversed-phase peptide fractionation kit (Thermo) and dried-down before MS analysis on an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) (García-Marqués et al., 2016). Peptide identification, quantification and systems biology analysis was performed as in (García-Marqués et al., 2016) Significant abundance changes of proteins or homogeneous categories of KO mice compared to controls were detected at 1% FDR.

Adult mice echocardiography and analysis

5 months-old mice were anesthetized using 1.5% isoflurane at a flow rate of 1L/min. Once anesthetic plane was reached, cardiac images were acquired using a MS400 probe, at 30MHz for 2D and M mode images and 24MHz for Color and Pulsed Doppler modes, using an ultrasound scanner VEVO2100 (Visualsonics, Canada). Equal male and female mice were used in each experimental group. No differences associated with sex were observed for cardiac functional parameters.

Statistical analysis and data representation

For histological, immunohistochemical quantifications, electron microscopy and RT-qPCR, values were pooled for embryos with the same genotype from independent litters and analyzed by the indicated statistical test using SPSS software (IBM; USA), with statistical significance

assigned at P ≤0.05. Values were represented as mean±SEM using GraphPad Prism (GraphPad; USA).

SUPPLEMENTAL REFERENCES

Eden, E., Navon, R., Steinfeld, I., Lipson, D., Yakhini, Z., 2009. GOrilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists. BMC Bioinformatics 10, 48.

García-Marqués, F., Trevisan-Herraz, M., Martínez-Martínez, S., Camafeita, E., Jorge, I., Lopez, J.A., Méndez-Barbero, N., Méndez-Ferrer, S., del Pozo, M.A., Ibáñez, B., Andrés, V., Sánchez-Madrid, F., Redondo, J.M., Bonzon-Kulichenko, E., Vázquez, J., 2016a. A Novel Systems-Biology Algorithm for the Analysis of Coordinated Protein Responses Using Quantitative Proteomics. Mol. Cell. Proteomics 15, 1740–1760.

Izquierdo-García, J.L., Rodríguez, I., Kyriazis, A., Villa, P., Barreiro, P., Desco, M., Ruiz-Cabello, J., 2009. A novel R-package graphic user interface for the analysis of metabonomic profiles. BMC Bioinformatics 10, 363.

Jiao, K., Kulessa, H., Tompkins, K., Zhou, Y., Batts, L., Baldwin, H.S., Hogan, B.L.M., 2003. An essential role of Bmp4 in the atrioventricular septation of the mouse heart service. Genes Dev. 17, 2362–2367.

Lamprecht, M.R., Sabatini, D.M., Carpenter, A.E., 2007. CellProfiler???: Free, versatile software for automated biological image analysis. Biotechniques 42, 71–75.

Menendez-Montes, I., Escobar, B., Palacios, B., Gómez, M.J., Izquierdo-Garcia, J.L., Flores, L., Jiménez-Borreguero, L.J., Aragones, J., Ruiz-Cabello, J., Torres, M., Martin-Puig, S., 2016. Myocardial VHL-HIF Signaling Controls an Embryonic Metabolic Switch Essential for Cardiac Maturation. Dev. Cell 39.

Rasband, W., 2015. ImageJ. U. S. Natl. Institutes Heal. Bethesda, Maryland, USA //imagej.nih.gov/ij/.

Ryan, H.E., Poloni, M., McNulty, W., Elson, D., Gassmann, M., Arbeit, J.M., Johnson, R.S., 2000. Hypoxia-inducible factor-1α is a positive factor in solid tumor growth. Cancer Res. 60, 4010–4015.

Stanley, E.G., Biben, C., Elefanty, A., Barnett, L., Koentgen, F., Robb, L., Harvey, R.P., 2002. Efficient Cre-mediated deletion in cardiac progenitor cells conferred by a 3'UTR-ires-Cre allele of the homeobox gene Nkx2-5. Int J Dev Biol 46, 431–439.

Subramanian, A., Tamayo, P., Mootha, V.K., Mukherjee, S., Ebert, B.L., Gillette, M.A., Paulovich, A., Pomeroy, S.L., Golub, T.R., Lander, E.S., Mesirov, J.P., 2005. Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl. Acad. Sci. U. S. A.

Supek, F., Bošnjak, M., Škunca, N., Šmuc, T., 2011. Revigo summarizes and visualizes long lists of gene ontology terms. PLoS One.

Thomas, P.D., Campbell, M.J., Kejariwal, A., Mi, H., Karlak, B., Daverman, R., Diemer, K., Muruganujan, A., Narechania, A., 2003. PANTHER: A library of protein families and subfamilies indexed by function. Genome Res.

Walter, W., Sánchez-Cabo, F., Ricote, M., 2015. GOplot: an R package for visually combining

expression data with functional analysis. Bioinformatics 31, 2912–4.

Wiśniewski, J.R., Ostasiewicz, P., Mann, M., 2011. High recovery FASP applied to the proteomic analysis of microdissected formalin fixed paraffin embedded cancer tissues retrieves known colon cancer markers. J. Proteome Res. 10, 3040–3049.

SUPPLEMENTAL FIGURES

Supplemental Figure S1

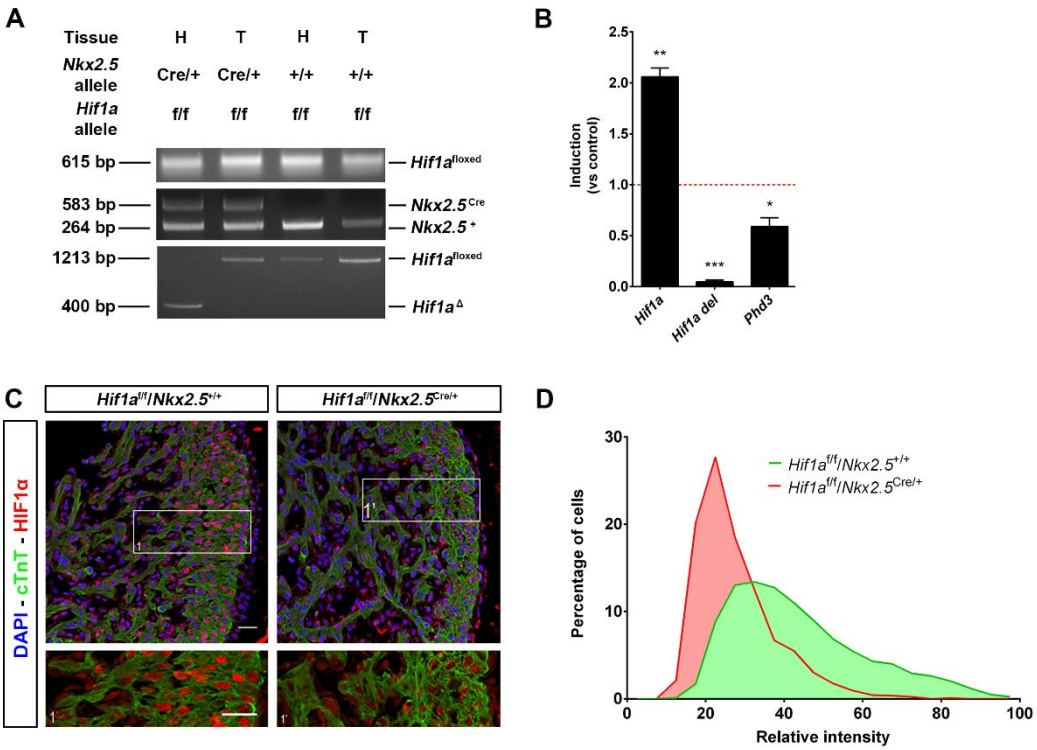

Figure S1. Deletion efficiency of *Hif1a*/*Nkx2.5* mutants.

Related to Figure 1.

A) Agarose electrophoresis showing PCR products from heart (H) and tail (T) tissue of E12.5 mutant embryos (*Hif1a*$^{f/f}$/*Nkx2*$^{rCre/+}$, lanes 1 and 2) and controls (*Hif1a*$^{f/f}$/*Nkx2.5*$^{+/+}$, lanes 3 and 4). Top gel: floxed (615 bp) allele of the *Hif1a* gene. Middle gel: wild-type (264 pb) and Cre (583 bp) alleles of the Nkx2.5 gene. Bottom gel: processed *Hif1a* allele after Cre-mediated recombination (400bp) and unprocessed allele (1213bp). **B)** RT-qPCR quantification of *Hif1a,* exon 2 (floxed) from *Hif1a* and *Phd3* transcripts in E14.5 *Hif1a*-mutant hearts. Bars (mean±SEM, n=3-6) represent fold induction relative to baseline expression in littermate controls (red line). *p-value<0.05; **0.01<p-value<0.05; ***p-value<0.005, Student's t test. **C)** HIF1α immunofluorescence at E12.5 in control and mutant embryos (Dapi staining shows nuclei in blue, Troponin T in green and HIF1α in red). Scale bars, 20µm. **D)** Representative analysis of cardiomyocyte HIF1α nuclear protein expression intensity, quantified by immunohistochemical staining of heart

sections from an E12.5 control embryo (green curve) and a *Hif1a*-null littermate (red curve).

Supplemental Figure S2

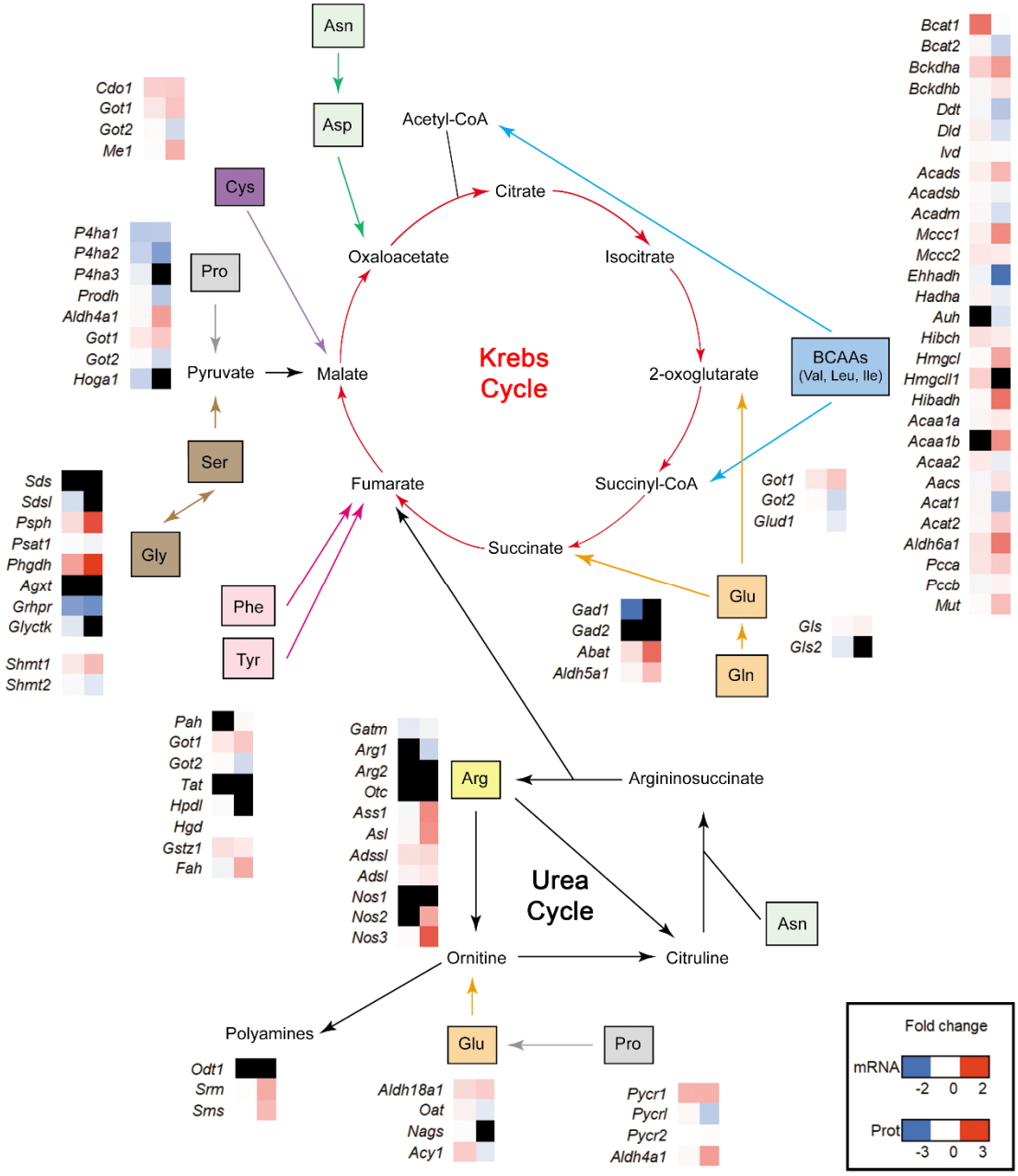

Figure S2. Schematic representation of amino acid contributions to Krebs and Urea Cycles. Related to Figure 5.

Schematic overview of the transcriptomics (mRNA expression, left column) and proteomics data (standardized protein quantifications, right column) of the re-wired metabolic pathways in the heart of *Hif1a*/*Nkx2.5* mutants (*Hif1a*$^{f/f}$/*Nkx2.5*$^{Cre/+}$) over control embryos (*Hif1a*$^{f/f}$/*Nkx2*$^{r+/+}$) at E12.5. Data are represented as individual heat maps for the transcript/protein of each pathway calculated as logarithmic Fold Change (logFC) and coded by color intensity following the scale at the bottom. ND indicates no detection. All 14406 expressed genes and 4276 quantified proteins were considered.