APEX2 proximity labeling in NRCMs

NRCMs are isolated from 0- to 3-day-old Wistar rats (details described below), and 5×10^5 cells per well seeded on six-well plates. NRCMs are cultivated in heavy, medium, and light (Eurisotop) SILAC (stable isotope labeling by amino acids in cell culture) medium (Thermo Fisher Scientific) under humidified conditions at 37°C. After 13 days, NRCMs are transduced with adenoviral vectors [V5-APEX2–PLN, V5-APEX2–PLN, or eGFP (enhanced GFP)] with a multiplicity of infection of 10 for 48 hours. The proximity labeling is conducted as described in detail below. Briefly, the cells are incubated for 30 min with 0.5 mM biotin-phenol at 37°C followed by 1 mM H_2O_2 for 1 min at room temperature. Cells are washed with quenching buffer [5 mM Trolox, 10 mM NaN3, and 10 mM Na-ascorbate in phosphate-buffered saline (PBS)] and harvested in radioimmunoprecipitation assay buffer [0.5% Na-deoxycholate, 50 mM tris-HCl (pH 7.5), 150 mM NaCl, 0.2% SDS, 1% Triton, 10 mM NaN3, 5 mM Trolox, 10 mM Na-ascorbate, 1 mM PMSF, and complete mini]. Cells are lysed by passing 10 times through a 27-gauge needle and centrifuged at 13,000g for 10 min. Protein concentrations are determined with the Pierce 660nm Protein Assay Kit (Thermo Fisher Scientific). The lysates of heavy-, medium-, and light-labeled NRCM are mixed in a ratio of 1:1:1 for a total of 250 μg of protein. Biotinylated proteins are enriched by avidin pull-down assay (Thermo Fisher Scientific; see below for details). Eluted proteins are analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) and MaxQuant (see below). For quantification, log2 fold changes of the ratios of PLN/eGFP and PLN/PLN(Δ1–29) are calculated and proteins that differ positively from 0 according to a one-sample t test ($P < 0.05$) are plotted. The experiment is conducted in three biological and two technical replicates with systematic label switching.

Neonatal Rat Cardiomyocyte (NRCM) Isolation and Purification

Hearts from 40 Wistar rat pups (P0-P3) are collected on ice in CBFHH buffer (Table I). The atria are excised with scissors (914012-12, FST) under magnification, the ventricles harvested for enzymatic digestion (Enzyme D, Neonatal Heart Dissociation Kit, Miltenyi Biotech), and NRCMs dissociated (gentleMACS Dissociator, Miltenyi Biotech) according to the manufacturer's instructions (Neonatal Heart Dissociation Kit, Miltenyi Biotech). To enrich isolated ventricular NRCMs, the raw cell suspension is filtered by gravity through a stainless steel mesh (grid size 250 µm, Thermo Fisher Scientific). The NRCMs are pelleted by centrifugation (60 x g for 20 min at 4 °C), resuspended in 5 mmol/L ice-cold PBS (PBS, pH 7.4, without Ca2+ and Mg2+, Gibco), and 2.5 mmol/L of the suspension layered on top of a Percoll density gradient (63 %, 40.5 % Centrifugation Media, pH 8.5 to 9.5, GE-Healthcare), and centrifuged at 3,000 x g for 30 min at RT (acceleration speed 9; deceleration speed 0; Heraeus Multifuge X1R, Thermo Fisher Scientific). NRCM enriched at the Percoll layer interface are collected with a 10 mL glass pipette (10 mL wide tip, Ratiolab) and suspended in 10 mL of 37 ºC NRCM cultivation medium (Table I).

We used flow cytometry to quantify the purity of isolated NRCMs. For this, freshly isolated NRCMs were fixed in 4% paraformaldehyde (PFA, Sigma-Aldrich) for 10 min at RT and permeabilized with chilled 90% methanol (Methanol, Sigma-Aldrich) for 15 min followed by a blocking step with 0.5 % BSA (BSA; Sigma-Aldrich) diluted in PBS (PBS, pH 7.4, without Ca2+ and Mg2+, Gibco) for 20 min at RT. Cells were stained with a mouse cardiac Troponin T (cTnT) antibody at a dilution of 1:50 and with mouse IgG1 at a dilution of 1:50 as isotype control for 1 h at RT. Donkey anti-mouse IgG-cy3 was used at a dilution of 1:100 for 1 h at RT as secondary antibody. Cy3 stained cells were counted by flow cytometry (BD Accuri C6 plus, BD Biosciences) and the amount of cTnT positive cells analyzed with FlowJo Software (Tree Star Inc) resulting in 95.3 % cTnT positive cells.

3-State SILAC Cardiomyocyte Culture Conditions

SILAC containing DMEM (Flex Media, Gibco) without L-arginine and L-lysine is supplemented with penicillin-streptomycin (100 U/mL), D-glucose (1 g/L), Na-pyruvate (100 mmol/L), and BRDU (10 mmol/L) containing either heavy, medium, or light isotope lysine and arginine as follows: for heavy SILAC labeling, L-lysine [13C6,15N2]HCl (Lys-8) and L-arginine [13C6,15N4]HCl (Arg-10) are added; for medium SILAC labeling, L-lysine-4,4,5,5-d4 (Lys-4) and L-arginine [13C6]HCl (Arg-6); and for light SILAC labeling, DMEM liquid medium with 1 g/L D-glucose is used. All solutions are vacuum-filtered (Steritop, Merck). For NRCM culture, 10% (vol/vol) heat inactivated FBS (Gibco) is added to the medium. NRCMs are seeded at a density of 500,000 cells on 35 mm dishes (CELLSTAR 6-well plate, Greiner) coated with collagen (13.96 mg/mL Collagen I rat tail, Corning) diluted 1:100 in PBS (PBS, pH 7.4, without Ca2+ and Mg2+, Gibco) and cultivated for 13 days in 2 mL light (non-labeled), medium, or heavy SILAC medium in 5% CO2 / 21% O2 at 37 °C (Heracell VIOS, Thermo Fisher Scientific). SILAC media are completely exchanged every second day. Mass spectrometry is used to detect SILAC incorporation (%), reaching a plateau (>95%) after 13 days culture. For proximity proteomic analysis an experimental design for systematic label switching with three biological replicates is applied.

Ratiometric APEX2 Mediated Biotinylation in NRCMs

For ratiometric APEX2 mediated biotinylation of endogenous NRCM proteins, SILAC labeled NRCM are transfected with recombinant adenoviral vectors expressing V5-APEX2-PLN for 48h using a minimal MOI 1 between day 11 and 13 in SILAC culture (higher MOI doses can be compared). In parallel, adenoviral vectors expressing soluble V5-APEX2 or eGFP are used as controls. Based on protocols published previously for ratiometric APEX biotinylation in heterologous cell systems, 1 mL of each SILAC medium (chapter above) is exchanged by the same SILAC medium containing 500 µmol/L biotin-phenol. After 30 min equilibration, a final concentration of 1 mmol/L H2O2 is added and the medium gently mixed for 1 minute. After 1 min, the biotinylation reaction is quenched by replacing the medium with 1 mL quenching buffer (Table II). NRCMs are washed thrice with quenching buffer, scraped (Cell Scraper 25 cm, Sarstedt), and collected in 250 µL RIPA quenching buffer (Table II). The NRCM suspension is passed 15 times through a 27 gauge syringe on ice and centrifuged at 13,000 x g for 10 min at 4 °C to collect the solubilized proteins in the supernatant. The protein concentration is determined by absorption measurement (Pierce 660 nm protein assay, Thermo Fisher Scientific). Heavy, medium and light labeled NRCM lysates are mixed 1:1:1.

Avidin Capture and Elution of Biotinylated Proteins

Avidin beads (Pierce Monomeric Avidin Agarose, Thermo Fisher Scientific) are equilibrated with RIPA quenching buffer (Table II) and 40 µL avidin beads added to 250 µg of NRCM lysate. The suspension is gently rotated for 1 h at 4 °C in a spin column (Pierce Spin Columns Screw Cap, Thermo Fisher Scientific). Next, beads are washed twice with 500 µL RIPA quenching buffer, once with 500 µL Tris/HCl buffer containing 2 mml/L urea (pH 8.0), and again twice with 500 µL RIPA quenching buffer (Table II). Two centrifugation steps at 100 x g for each 30 s and 2 min at 2000 x g (Heraeus, Fresco 21 centrifuge, Thermo Fisher Scientific) are used to harvest the beads, while the supernatant is discarded. Biotinylated proteins are eluted in 75 µL biotin buffer (Table II) for 15 min at RT, followed by 15 min at 70 °C. Beads are pelleted by centrifugation for 1 min at 1000 x g and the supernatant containing the eluted proteins is collected. The eluted proteins are analyzed by mass spectrometry as described below.

Sample Preparation for NanoLC-MS/MS Analysis of SILAC Labeled Samples

Eluted protein samples are fractionated on 4-12 % Bis-Tris minigels (NuPAGE Novex, Invitrogen). Gels are stained with Coomassie Blue overnight (Coomassie Brilliant Blue R-250 Staining Solution, BioRad) for protein visualization, and each lane sliced into 11 equal-sized gel pieces. After washing the gel pieces with 50 mmol/L ammonium bicarbonate (TEAB, Sigma Aldrich), gel slices are reduced with 10 mmol/L dithiothreitol (1,4-dithiothreitol, Sigma-Aldrich), alkylated with 55 mmol/L 2-iodoacetamide (Sigma-Aldrich), and digested with endopeptidase trypsin (sequencing grade, Promega) diluted 1:50 in 55 mmol/L iodoacetamide overnight. Post-trypsinization the peptides are solubilized in MS loading buffer (Table III), dried (SpeedVac, Thermo Fisher Scientific), reconstituted in MS loading buffer and prepared for NanoLC-MS/MS analysis.

NanoLC-MS/MS Analysis of SILAC Labeled Samples

For mass spectrometric analysis of solubilized peptides, samples are enriched on a self-packed reversed phase-C18 precolumn (0.15 mm ID x 20 mm, Reprosil-Pur120 C18-AQ 5 µm, Dr. Maisch, Ammerbuch-Entringen, Germany) and separated on an analytical reversed phase-C18 column (0.075 mm ID x 200 mm, Reprosil-Pur 120 C18-AQ, 3 µm, Dr. Maisch, Ammerbuch-Entringen, Germany) using a 30 min linear gradient of 5-35% acetonitrile/0.1% formic acid (v/v) at 300 nl min-1. The eluent is analyzed on a hybrid quadrupole/orbitrap mass spectrometer (Q Exactive, Thermo Fisher Scientific) equipped with a FlexIon nanoSpray source and operated under Excalibur 2.5 software using a data-dependent acquisition method. Each experimental cycle is of the following form: one full MS scan across the 350-1600 m/z range is acquired at a resolution setting of 70,000 full width at half maximum (FWHM), and AGC target of 1*10e6 and a maximum fill time of 60 ms. Up to 12 most abundant peptide precursors of charge states 2 to 5 above a 2*10e4 intensity threshold are then sequentially isolated at 2.0 FWHM isolation width, fragmented with nitrogen at a normalized collision energy setting of 25%, and the resulting product ion spectra recorded at a resolution setting of 17,500 FWHM, and AGC target of 2*10e5 and a maximum fill time of 60 ms. Selected precursor m/z values are then excluded for the following 15 s. Two technical replicates per sample are acquired.

APEX2 Assay Data Processing

Raw data are processed using quantitative proteomic software (MaxQuant Software version 1.5.7.4, Max Planck Institute for Biochemistry). Proteins are identified against a UniProtKB-derived rattus norvegicus protein sequence database (v2018.02, 37830 protein entries) along with a set of common lab contaminants. The search is performed with trypsin as enzyme and iodoacetamide as cysteine blocking agent. Up to two missed tryptic cleavages and methionine oxidation as a variable modification are allowed for. Instrument type 'Orbitrap' is selected to adjust for MS acquisition specifics. The Arginine Arg-10, Arg-6 and Lysine Lys-8, Lys-6 labels including the 'Re-quantify' option are specified for relative protein quantitation. For identification of APEX2 biotinylated proteins, the ratios of V5-APEX2-PLN versus V5-APEX2 or eGFP are calculated and log2 transformed. The V5-APEX2-PLN/V5-APEX2 ratio is plotted on the X-axis and the V5-APEX2-PLN/eGFP ratio on the Y-axis. Scatter plots are generated with Prism version 7.03 (GraphPad). Enriched biotinylated proteins are tested for statistical significance ($p<0.05$) by one sample z-test (Excel, Microsoft Office) and visualized as 'positive' or 'negative' hits, including previously established proteins-of-interest (POIs).

Online Table I. NRCM isolation buffer and cultivation medium

CBFHH buffer

	MW (g/mol)	Final concentration
NaCl	58.44	37 mmol/L
KCl	74.56	5.4 mmol/L
KH_2PO_4	136.09	0.44 mmol/L
$Na_2HPO_4 \cdot 2\ H_2O$	177.99	33.5 mmol/L
Glucose	180.16	5.6 mmol/L
HEPES	238.31	20 mmol/L
$MgSO_4$	120.37	0.8 mmol/L
in 500 mL ddH_2O, pH 7.4		

NRCM cultivation medium

	MW (g/mol)	Final concentration
FBS	-	10% (v/v)
5-Bromo-2'-deoxyuridine	307.1	10 mmol/L
Penicillin/streptomycin	647	1% (v/v)
in 500 mL cell culture medium		
(DMEM-1 g/L D-glucose, Thermo Fisher Scientific)		

Online Table II. APEX2 biotinylation buffer compositions

Quenching buffer		
	MW (g/mol)	Final concentration (mmol/L)
6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic-acid (Trolox)	250.29	5
Sodium azide	65	10
Sodium ascorbate	136.09	10
in 50 mL PBS (PBS, pH 7.4, without Ca^{2+} and Mg^{2+}, Gibco)		

RIPA quenching buffer		
	MW (g/mol)	Final concentration
Tris HCl, pH 7.4	157.60	50 mmol/L
NaCl	58.44	150 mmol/L
Triton-X-100	647	1% (v/v)
Sodium deoxycholate	414.55	0.5% (w/v)
Sodium dodecyl sulfate	288.37	0.2% (v/v)
6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic-acid (Trolox)	250.29	5 mmol/L
Sodium azide	65	10 mmol/L
Sodium ascorbate	136.09	10 mmol/L
in 10 mL ddH_2O, pH 7.4		
1 tablet of protease inhibitors (Complete Mini EDTA free, Sigma Aldrich)		

Tris/HCl buffer containing urea		
	MW (g/mol)	Final concentration
Tris HCl, pH 8	157.60	50 mmol/L
Urea	60.06	2 mmol/L
in 1 L ddH_2O, pH 8		

Biotin buffer		
	MW (g/mol)	Final concentration
Biotin	244.31	2 mmol/L
Sodium dodecyl sulfate	288.37	2% (v/v)
in 1 mL ddH_2O, pH 8		

Online Table III. Mass spectrometry loading buffer

MS loading buffer		
	MW (g/mol)	Final concentration
Acetonitrile	41.05	2% (w/v)
Formic acid	46.03	0.1% (w/v)
in 50 mL ddH_2O		

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