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A COMPARATIVE PROTEOMIC ANALYSIS OF THE K_{ATP} CHANNEL COMPLEX IN DIFFERENT TISSUE TYPES

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ABSTRACT

ATP-sensitive K^+ (K_{ATP}) channels are expressed ubiquitously, but have diverse roles in various organs and cells. Their diversity can partly be explained by distinct tissue-specific compositions of four copies of the pore-forming inward rectifier potassium channel subunits (Kir6.1 and/or Kir6.2) and four regulatory sulfonylurea receptor subunits (SUR1 and/or SUR2). Channel function and/or subcellular localization also can be modified by the proteins with which they transiently or permanently interact to generate even more diversity. We performed a quantitative proteomic analysis of K_{ATP} channel complexes in the heart, endothelium, insulin-secreting min6 cells (pancreatic β-cell like) and the hypothalamus to identify proteins with which they interact in different tissues. Glycolysis is an over-represented pathway in identified proteins of the heart, min6 cells and the endothelium. Proteins with other energy metabolic functions were identified in the hypothalamic samples. These data suggest that the metabolo-electrical coupling conferred by K_{ATP} channels is conferred partly by proteins with which they interact. A large number of identified cytoskeletal and trafficking proteins suggests endocytic recycling may help control K_{ATP} channel surface density and/or subcellular localization. Overall, our data demonstrate that K_{ATP} channels in different tissues may assemble with proteins having common functions, but that tissue-specific complex organization also occurs.

INTRODUCTION

The ATP-sensitive K^+ channel is a key element of metabolo-electrical coupling, defined as the functional relationship between intracellular energy metabolism and membrane excitability. The channel is composed of subunits of the Kir6 inward rectifier potassium channel subfamily in association with sulfonylurea receptor (SUR) subunits [1, 2]. In mammals, two genes (KCNJ11 and KCN8) give rise to the expression of two Kir6 subunits (respectively, Kir6.1 and Kir6.2). Similarly, two genes (ABCC8 and ABCC9) are respectively responsible for two SUR subunits (SUR1 and SUR2). Various SUR splice variants have been described [3], but the most commonly studied are SUR1, SUR2A and SUR2B, the latter two being alternatively spliced variants of the ABCC9 gene that differ from each other in the distal C-terminus. K_{ATP} channel biophysical and pharmacological properties have significant diversity amongst different tissues [4] and these differences originate in part from the specific Kir6.x and SURx subunit combinations. Since the K_{ATP} channel unitary conductance is largely determined by the Kir6.x subunit, and its nucleotide sensitivity and pharmacological properties are strongly regulated by the SURx subunit, a rich diversity is possible for K_{ATP} channels with these various subunit combinations. The pancreatic β-cell K_{ATP} channel, responsible for triggering insulin release, is composed of a Kir6.2/SUR1 subunit combination, whereas the cardiac K_{ATP} channel is typically described as consisting of Kir6.2/SUR2A subunits [2]. Even within a single organ such as the heart there can be significant variation. The mouse atrial K_{ATP} channel, for example, contains the SUR1 subunit [5], whereas K_{ATP} channels in the specialized cardiac conduction system consist of SUR2B/Kir6.1/Kir6.2 subunits [6].

The function of an ion channel is not only determined by its primary subunit composition, but also by processes such as subunit post-translational modification (phosphorylation, palmitoylation, etc) as well as their interaction with other proteins. An emerging theme is that ion channels form macromolecular complexes, and that the proteins associating with ion channel subunits assist to regulate the subcellular localization and/or overall function of the channel. For example, interaction of ion channels with various kinases and phosphatases (often mediated by specific scaffolding proteins) directly regulates channels such as Ca^{2+} channels and delayed rectifier K^+ channels [7]. Defects in proteins comprising the ion channel macromolecular complex of the ryanodine receptor give rise to abnormalities in Ca^{2+} regulation [8]. Similarly, macromolecular assemblies of cystic fibrosis transmembrane conductance regulator (CFTR) and the cardiac Na^+ channel, respectively, affect cystic

fibrosis [9] and cardiac arrhythmias [10, 11]. K_{ATP} channels are also megadalton macromolecular complexes, as evidenced by published reports of their interaction with proteins such as lactate dehydrogenase, creatine kinase and adenylate cyclase in the heart [12]. Other proteins described to interact with K_{ATP} channels in heart and/or pancreatic β-cells include syntaxin 1A [13], the cAMP sensor Epac1/2 [14, 15] and the membrane targeting protein ankyrin B [16]. We found that the cardiac K_{ATP} channel directly interacts with the glycolytic enzymes glyceraldehyde 3-phosphate dehydrogenase, pyruvate kinase and triose phosphate isomerase [17], which provides a physical basis for the long-recognized preferential regulation of cardiac K_{ATP} channel activity by glycolytically derived ATP [18]. In a subsequent proteomic analysis of the cardiac K_{ATP} channel macromolecular complex, we found glycolysis to be the most enriched biological process carried out by proteins in immunoprecipitates obtained with antibodies directed against K_{ATP} channel subunits [19]. It is unclear whether other K_{ATP} channels (e.g. in the pancreatic β-cell and the hypothalamus) similarly interact with these metabolic enzymes [20]. Given the differences in K_{ATP} channel function and regulation in different tissue types, we postulated that there may be tissue-specific differences in the macromolecular assemblies of the various types of K_{ATP} channels. To examine this hypothesis, we performed a quantitative proteomic comparison of the K_{ATP} channel macromolecular complexes in the cardiac ventricle, pancreatic β-cell insulin secreting cells, vascular endothelial cells and the hypothalamus of the brain. Our data demonstrate that K_{ATP} channels in these different tissues interact essentially with non-overlapping pools of proteins, suggesting unique and tissue-specific modes of regulation.

MATERIALS AND METHODS

Additional details are provided in Supplemental Materials and Methods.

Animals

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Animal procedures were approved by the New York University School of Medicine Institutional Animal Care and Use Committee.

Cell culture and Cardiac Myocyte isolation

Human coronary artery endothelial cells (HCAEC, from Lonza) and Min6 pancreatic β-cells (kindly provided by Dr. Michael Rindler, NYU School of Medicine, New York) were used at 80% confluency. Ventricular myocytes were isolated from rat hearts as described previously [24] and used for membrane preparations.

Membrane Preparations and co-immunoprecipitation Assays

Membrane fractions from cardiomyocytes, HCAECs and Min6 cells were prepared using a modification of a previously described method [24]. Membrane fractions from rat hypothalamus were prepared using a modification of a protocol by Neuromab (http://neuromab.ucdavis.edu/files/RBM.pdf). Protein concentration was determined (Bradford assay, Biorad) and samples were immediately used for immunoprecipitation experiments. Co-immunoprecipitation assays for mass spectrometry analysis were performed using a commercial system (Thermo Scientific Pierce). Eluted proteins were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and western blotting. Some immunoprecipitates prepared for western blot analysis (as indicated in the figure legends) were produced with Protein A/G magnetic beads (Dynabeads, Invitrogen). Details on the antibodies used are given in the Supplemental Methods section.

Tryptic Digestion

Eluted proteins were run 1 cm into the resolving gel of a pre-cast 7.5% TRIS-HCl gel (Bio-Rad, Richmond, CA). The protein samples were cut into 2 slices for in-gel digestion with trypsin as described previously [21]. Dried peptides were resuspended in 0.1% formic acid/3% acetonitrile with 10 fmol/µl trypsin-digested bovine serum albumin as internal quantitation standard (BSA, Bruker-Michrom, Auburn, CA).

Protein Identification and Quantification by LC-MS^E and LC-MS/MS

For all LC-MS^E experiments, a QTof-Premier (Waters) coupled to a nanoAcquity Ultra Performance LC (Waters) with a 5 µm Acclaim PepMap100 C18 µ-precolumn cartridge (300 µm x 5 mm; LC Packings) and a 1.7 µm BEH130 C18 analytical column (100 µm x 100 µm; Waters).

For other LC-MS/MS experiments, an LTQ-Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific) with a nanoelectrospray ionization source (Jamie Hill Instrument Services) coupled to an Eksigent nanoLC system (Eksigent Technologies) with a self-packed 100 mm x 15 cm reverse phase column (Reprosil C18, 3 mm, Dr. Maisch GmbH, Germany) was used.

Data acquired via LC-MS^E were processed using ProteinLynx Global Server (PLGS) software (version 2.4, Waters). Final results were exported into Scaffold, where gel slices were combined and the protein/peptide percent probability cut-off was set to 80% (corresponding to <1% protein FDR). Data acquired via LC-MS/MS (Orbitrap) were processed using Mascot Distiller (version 2.4, Matrix Science)

Western blotting

Unboiled proteins were analyzed by western blotting using standard techniques. Chemiluminescent Western blotting substrate (Pierce) was used for peroxidase detection and Kodak Biomax light films for signal detection. Details on the antibodies used are given in the Supplemental Materials and Methods section.

RESULTS

Characterization of the antibodies and detergents

We used an immunoprecipitation approach to identify novel subunit components of the K_{ATP} channel complex in the different cell types. It was therefore necessary to characterize the antibodies used for their ability to detect and immunoprecipitate the K_{ATP} channel protein subunits found in each tissue (the antibodies used are listed in Table 1). The SUR1 protein was readily detected in Western blots of Min6 membrane preparations with two anti-SUR1 antibodies (RSUR1 and CSUR1) as a ~150kDa band in Western blotting (Figure S1). Western blotting of Min6 or cardiomyocyte membranes with an anti-Kir6.2 antibody (W62b) demonstrates detection of two bands (~37 and 45kDa, respectively). Since a 37kDa band is observed in Western blots of lysates from cells transiently transfected with Kir6.2 cDNA (not shown) as well as in immunoprecipitates of different K_{ATP} channel subunits (see later), we assume that the 45kDa band represents a nonspecific band of unknown origin (though pyruvate dehydrogenase has a predicted mass of 43 kDa, and contains the sequence YRSRE which is similar to a sequence in the peptide EDPAEPRYRTRERRARFVSKKC from mouse Kir6.2 used to generate the W62b antibody). We also optimized the type and concentration of detergent to solubilize the K_{ATP} channel subunit (Figure S2A).

The ability of the antibodies to specifically immunoprecipitate the respective proteins was confirmed next. We have previously demonstrated the suitability of the W62 antibody in immunoprecipitation assays [22, 23]. For the other antibodies, we performed an immunoprecipitation reaction and then ensured that we could identify K_{ATP} channel subunits in immunoblots using a different antibody against a K_{ATP} channel subunit (Figure S2B).

Mass spectrometry of immunoprecipitates obtained with antibodies against K_{ATP} channel subunits

We next analyzed immunoprecipitates obtained with antibodies against K_{ATP} channel subunits by mass spectrometry in order to identify possible interacting proteins. We performed immunoprecipitation reactions using membranes obtained from isolated rat cardiac ventricles, Min6 insulin-secreting pancreatic β-cells and the rat hypothalamic brain region. For each of these tissues, we used 3-4 different antibodies in separate reactions, either against different subunits of the K_{ATP} channel (e.g. Kir6.2 and SUR1) and/or antibodies designed to different epitopes of the same subunit (e.g. W62b or C62 against Kir6.2). Multiple immunoprecipitations with each antibody (experimental numbers are in

Table 1) were performed using membrane preparations isolated on different days (biological replicates) and each was subjected to mass spectrometry in triplicate (technical replicates). Following in-gel trypsin digestion, we used a variant of the quantitative label-free approach in which mass spectra are recorded alternately at low (precursor) and high (product) fragmentation voltages (MS^E [24]). Proteins were identified by database searches of peptide spectra and quantified based on the mass spectrometric signal intensity of the three most intense peptides for each protein compared to the BSA digest internal standard. We applied stringent criteria to select proteins as potential channel-interacting proteins. First, they must have been significantly enriched over (or not present in) negative IgG control reactions based on our data modeling experiments. The list of proteins obtained using these criteria are shown in Table S1. A high-confidence subset of these proteins, which were immunoprecipitated by at least two separate antibodies in a given tissue, and which were present in amount ten times or less than the immunoprecipitated K_{ATP} channel subunit itself, is listed in Table 2.

Verification of interaction of selected proteins

We performed independent co-immunoprecipitation experiments to verify that some of the identified proteins are indeed in complex with the K_{ATP} channel by reciprocal immunoprecipitation followed by Western blotting. We selected two protein subunits for which suitable precipitating antibodies were readily available: the α1-subunit of sodium/potassium ATPase (NKA) and actin. The antibodies were coupled to resin or magnetic beads and used in co-immunoprecipitation reactions performed with membranes from ventricular myocytes or Min6 cells respectively. As positive controls we used antibodies against K_{ATP} channel subunits. Indeed, when immunoprecipitates obtained with antibodies against the Na^+/K^+ pump α-subunit or actin were visualized by Western blotting, the K_{ATP} channel subunits were readily detected (Figure 1). This reciprocal immunoprecipitation experiment raises confidence that the identified proteins represent de-facto interacting proteins within the K_{ATP} channel complex.

Functional grouping of putative interacting proteins

A web-based functional annotation tool for enrichment analysis at the DAVID knowledgebase [25] was used to determine functional categories to which proteins listed in Table S1 belong. The result is depicted as a heat map in Figure 2, which shows that the putative K_{ATP} channel interacting proteins in the different tissues groups both in overlapping as well as unique, tissue-specific functional classes. Metabolic pathways were well represented for identified proteins in the cardiac, endothelial and Min6 cell groups, but not

for the hypothalamic proteins. Proteins with common functions associated with cytoskeletal organization (actin, myosins and microtubules) were commonly found in all four tissues. Similarly, proteins with the common function of subcellular protein localization and those involved in intracellular vesicular transport were well represented across groups. Another common pathway represented in all tissues is broadly categorized as “morphogenesis”. A hypothalamus-specific functional category included “regulation of synaptic transmission” and a cardiac-specific category included “monocarboxylic acid transport”.

DISCUSSION

A picture emerging from recently published data is that the molecular composition of the K_{ATP} channel is complex and contains proteins other than the Kir6 and SUR subunits. These associated proteins may be important for determining the function of the channel under physiological and pathophysiological settings. Considering the diverse roles that have been reported for this channel in different tissues and disease conditions, we hypothesized that the K_{ATP} channel macromolecular complex comprises different partners in different cell types. To test this hypothesis we performed co-immunoprecipitation experiments using subunit-specific K_{ATP} channel antibodies and different types of cells and analyzed the immunoprecipitates by tandem mass spectrometry. Moreover, we searched for biological pathways statistically enriched within the groups of putative interacting proteins. Although we found only a few specific proteins to overlap between different tissue immunoprecipitates, several functional pathways emerged that were well-represented across groups. Moreover, there were some tissue-specific complements of auxiliary proteins, which may point to unique functions/roles for the K_{ATP} channel complex in the different tissues. It should be noted that the absence of a protein from our data should not be taken as evidence that interaction does not occur, as some proteins may have been missed for technical or biological reasons. For example, due to the specific experimental conditions used (homogenization methods, detergents and their concentrations) we may have disrupted certain protein interactions. We may also have missed weak or transient interactions. It should be further noted that, even though we used stringent inclusion criteria, the presence of a protein in our immunoprecipitates does not necessarily imply direct interactions. The interactions may be indirect (via another protein or complex) and we hoped to capture this information in the pathway analysis and functional classification analysis. It may also be possible that some proteins were identified non-specifically due to antibody cross-reactivity, and the data should be interpreted with this caveat in mind. We grouped the proteins in which we have the highest confidence (identified by at least two separate K_{ATP} channel antibodies) in Table 2.

K_{ATP} channels and energy metabolism

The most prominent functions of putative K_{ATP} channel interacting proteins was cellular energy metabolism, ranging from glycolysis and glucose oxidation to lipid metabolism. In particular, glycolytic enzymes, previously described as components of the K_{ATP} channel complex [17, 19, 26, 27], were found in K_{ATP} channel subunit immunoprecipitates from

heart, endothelium and pancreatic origin, suggesting that the K_{ATP} channel opening may be fine-tuned by glycolytic ATP production in these tissues [28]. Curiously, few proteins with metabolic functions were found in the hypothalamic immunoprecipitates. A role for hypothalamic K_{ATP} channels in glucose homeostasis has been demonstrated [29] and future research should be directed to determine whether K_{ATP} channels in these cells (neurons and/or glial cells) are subject to direct regulation by glycolysis, as they are in cardiac cells [18]. A novel and potentially interesting finding is that the cardiac K_{ATP} channel may associate with proteins involved with fatty acid transport and metabolism, including carnitine O-palmitoyltransferase (CPT2_RAT), 2,4-dienoyl-CoA reductase (DECR_RAT), trifunctional enzyme subunit alpha (ECHA_RAT), fatty acid transport protein (CD36_RAT) and monocarboxylate transporter 1 (MOT1_RAT). This finding is of potential interest, given that under normoxic conditions the heart is preferentially fueled by fatty acid oxidation [30], and that cardiac K_{ATP} channel function has been described to be regulated by monocarboxylate transport [31]. Although the role of fatty acid metabolism in the metabolo-electrical coupling function of K_{ATP} channels remains to be elucidated, these findings are consistent with prior reports regarding the regulation of the cardiac K_{ATP} channel by long-chain acyl-coenzyme A esters and fatty acids [32] and the modulation of the energetic status (glycogen content, lactate, and amino acids) by K_{ATP} channels in the ischemic heart [33].

K_{ATP} channels, subcellular trafficking and localization

Major classes of identified proteins are involved in cytoskeletal organization and subcellular trafficking events. These included cardiac and endothelial proteins functionally related to actin organization and assembly, such as F-actin-capping proteins (CAZA2_RAT and CAZA1_HUMAN), actin-related protein (Arp) 2/3 complex subunits and the Ras homolog gene family member A (RhoA). Other proteins related to microtubule organization (several tubulin subunits and dynactin identified in heart, hypothalamus and/or endothelium) as well as motor proteins (Dynein) and some non-muscle myosin (e.g. Myh1, Myh3, Myh4, Myh6, Myh8 and Myh9) were found. Of particular interest are proteins with functions involved in the intracellular endocytic recycling pathway, which were present in each tissue studied. Examples of such proteins include Ras-related proteins Rab1A, Rab-1B, Rab2A, Rab7A, Rab8A, Rab11A, Rab14, Rab18, the Ras-related protein Ral-A, AP-1 and AP-2 complex subunits, several trafficking protein particle complex subunits, vacuolar protein sorting-associated protein 37B, ADP-ribosylation factors (Arf-1 and Arl8B), Sec23B, and members of the C-terminal EHD-domain containing protein family (EHD2 and EHD4). There is an

increasing realization that K_{ATP} channels are subject to constitutive endocytic recycling events [34, 35] and that endocytic recycling is responsible for increased K_{ATP} channel surface expression after cardiac ischemia as a result of translocation from endosomal compartments [36]. Since trafficking proteins have distinct functions in specific steps of the endocytic recycling pathway [37], the identification of these proteins may highlight the specific processes involved in K_{ATP} channel trafficking and may offer therapeutic opportunities for altering the steady-state density of plasmalemmal K_{ATP} channel density. Finally, the presence of junction plakoglobin in cardiac immunoprecipitates raises the possibility of polarized and uneven subcellular expression of K_{ATP} channels in this cell type, akin to the higher expression of other channels, such as Nav1.5 [38], at the cardiac intercalated disk. If confirmed, then this observation may have important clinical considerations regarding the role of K_{ATP} channels in arrhythmogenic disease-causing mutations in the plakoglobin gene [39].

Regulation of ion transport

It is of interest that, in addition to the K_{ATP} channel subunits themselves, the putative interacting proteins in all tissues included those with a common function of ion transport. For example, α subunits of the Na^+/K^+-transporting ATPase were present both in cardiac and hypothalamic immunoprecipitates (Tables 2 and S1). A physical interaction of the Kir6.2 K_{ATP} channel subunit and the α-subunit of the Na^+/K^+ ATPase, mediated by binding to ankyrin B, previously has also been reported in the heart [41]. Their physical interaction might provide mechanistic clues into the functional interaction between the activities of the electrogenic Na^+/K^+ pump and K_{ATP} channel that has been noted in several tissues, including the heart [42], neurons [43], smooth muscle [44] and kidney [45]; for example, by competing for the same local pool of submembrane ATP/ADP.

Pathways represented

We next analyzed the putative K_{ATP} channel interacting proteins in terms of enriched pathways in the different tissues by performing an unclustered functional annotation of proteins in Table S1. As reported previously [19], glycolysis was the best represented pathway for putative associated proteins of the cardiac K_{ATP} channel (Table S2). Glycolysis was also a well represented pathway in the pancreatic β cell-like min6 cells, and among the top of the list for the endothelial samples. The hypothalamic data did not lend themselves well to pathway enrichment analysis due to the relatively small number of proteins in the dataset. Instead, hypothalamic K_{ATP} channels appear to associate with enzymes driving

intermediate metabolism (Table 2). Surprisingly, however, glycolytic enzymes were not among them, despite evidence supporting a role for glycolysis in hypothalamic glucose-sensing [40]. Interestingly, hypothalamic K_{ATP} channels associated with the excitatory amino acid transporter 1, which is expressed in glial cells [41]. This observation is consistent with recent studies implicating K_{ATP} channels in mediating effects of glucose on glucose-sensing hypothalamic tanycytes, a specialized type of glial cell implicated in nutrient sensing [42].

Perspectives and future directions

Many of the proteins previously described to be components of K_{ATP} channel complexes were identified in our screen, including glycolytic enzymes [17, 19, 26, 27], creatine kinase [43], the Na^+/K^+ pump α subunit [44] and heat-shock proteins [45]. However, there are reports of several other K_{ATP} channel interacting proteins that were not identified here, including adenylate kinase [46], syntaxin 1A [13, 47], caveolin-3 [48], AMPK [19, 23], Epacs [15], Rim, piccolo and calcium channels [49]. Although veracity remains to be demonstrated for the putative interacting proteins identified here, their large number (even if only considering the “high confidence” list in Table 2) suggests it is unlikely that all of these proteins assemble with the K_{ATP} channel at the same time, e.g. due to geometrical constrains. It may be possible that some interactions are transient. Alternatively, diverse populations of K_{ATP} channels may exist, even within a given tissue such as the heart [5, 6], or possibly even within the same cell [36]. If separate pools of K_{ATP} channel complexes have distinct molecular compositions, then it raises the possibility that unique modes of regulation by their associated proteins may exist. For example, channels undergoing endocytic recycling [35, 36] may associate with different Rab proteins, depending on their location within the trafficking pathway [37]. A challenge for future studies is to verify the nature of the putative K_{ATP} channel interacting proteins identified in this study and to identify how they regulate K_{ATP} channel function, trafficking or their subcellular localization.

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TABLES

Table 1: Subunit composition of K_{ATP} channels and the antibodies used in the immunoprecipitation reactions.

Cell type	Subunit	Antibodies used
Ventricular myocytes	Kir6.2	W62b (n=2), G16 (n=2)
	SUR2A	M19 (n=2)
HCAEC	Kir6.1	C16 (n=1)
	Kir6.2	W62b (n=2)
	SUR2B	C15 (n=1)
Min6	Kir6.2	W62b (n=2), C62 (n=1), G16 (n=1)
	SUR1	RSUR1 (n=2)
Hypothalamic	Kir6.2	W62b (n=2), C62 (n=2)
	SUR1	RSUR1 (n=2)

Shown in brackets is the number of separate immunoprecipitation reactions performed (biological replicates). Negative control reactions consisted of immunoprecipitation reactions performed with unrelated IgG (n=2 for each of the four tissue types listed).

Table 2 – Candidate K_{ATP} channel interacting proteins

Ventricular myocytes

Accession	Description	Ratio Channel/Protein	Peptides	Coverage	Amount (fmol)	Antibody
ACSF2_RAT	Acyl CoA synthetase family member 2 mitochondrial	3.13	2	5.89%	2.76	W62, G16, M19, C62
ACSL1_RAT	Long chain fatty acid CoA ligase 1	1.41	5	10.01%	6.11	W62, G16, M19
ALDOC_RAT	Fructose bisphosphate aldolase C	1.26	2	14.53%	6.86	W62, G16, M19
ANXA2_RAT	Annexin A2	2.76	2	6.88%	3.13	W62, G16, M19
AT1A2_RAT	Sodium potassium transporting ATPase subunit alpha 2	0.74	2	15.14%	11.64	W62, G16, C62
AT1A3_RAT	Sodium potassium transporting ATPase subunit alpha 3	1.2	5	11.78%	7.18	W62, G16, M19
CAZA2_RAT	F-actin-capping protein subunit alpha-2	1.44	2	8.60%	5.98	W62, C62
DECR_RAT	2,4-dienoyl-CoA reductase, mitochondrial	2.17	4	18.93%	3.98	W62, C62, M19
ECHA_RAT	Trifunctional enzyme subunit alpha mitochondrial	0.24	47	66.00%	36.39	W62, G16
EF1A2_RAT	Elongation factor 1 alpha 2	0.69	3	9.24%	12.51	W62, G16, M19
ENOA_RAT	Alpha enolase	4.06	2	7.19%	2.12	W62, G16, M19
ENOB_RAT	Beta enolase	5.31	1	7.07%	1.62	W62, G16, M19
G3PT_RAT	Glyceraldehyde-3-phosphate dehydrogenase	3.9	2	13.43%	2.21	W62, M19
G6PI_RAT	Glucose 6 phosphate isomerase	2.55	2	4.35%	3.38	W62, G16
GBB1_RAT	Guanine nucleotide-binding protein G(I)/G(S)/G(T)	3.72	3	7.95%	2.32	W62, C62, M19
GRP75_RAT	Stress 70 protein mitochondrial	1.18	10	19.87%	7.32	W62, G16, M19
GYS1_RAT	Glycogen starch synthase muscle	0.92	12	23.11%	9.42	W62, G16, M19
HPRT_RAT	Hypoxanthine guanine phosphoribosyltransferase	2.2	4	21.11%	3.92	W62, G16, M19
HSP7C_RAT	Heat shock cognate 71 kDa protein	2.81	6	15.24%	3.07	W62, G16, M19
HYOU1_RAT	Hypoxia up regulated protein 1	2.36	1	1.30%	3.66	W62, G16, M19
IRK11_RAT	ATP-sensitive inward rectifier potassium channel 11	1.18	6	11.48%	7.29	W62, C62
KCRU_RAT	Creatine kinase U type mitochondrial	0.77	1	14.33%	11.14	W62, G16
MCCB_RAT	Methylcrotonoyl CoA carboxylase beta chain mitochondrial	1.28	1	2.40%	6.75	W62, G16, M19
MOT1_RAT	Monocarboxylate transporter 1	1.75	3	7.88%	4.93	W62, M19
MYG_RAT	Myoglobin	1.4	5	41.50%	6.15	G16, W62
ODBA_RAT	2-oxoisovalerate dehydrogenase subunit alpha	2.96	2	8.55%	2.91	W62, M19
P4HA1_RAT	Prolyl 4 hydroxylase subunit alpha 1	1.32	10	27.33%	6.55	G16, M19
PCCB_RAT	Propionyl CoA carboxylase beta chain mitochondrial	3.51	1	3.93%	2.46	W62, G16, M19
PDIA1_RAT	Protein disulfide isomerase	1.47	11	30.50%	5.88	G16, M19
PDIA6_RAT	Protein disulfide isomerase A6	1.62	2	8.38%	5.33	W62, G16, M19
PLAK_RAT	Junction plakoglobin	3.66	1	2.10%	2.36	W62, G16, M19
PPIB_RAT	Peptidyl prolyl cis trans isomerase B	1.61	4	19.95%	5.35	G16, M19
PYGB_RAT	Glycogen phosphorylase brain form Fragment	1.29	3	9.36%	6.68	W62, G16, M19
PYGM_RAT	Glycogen phosphorylase muscle form	0.72	14	19.68%	11.92	W62, G16, M19
RAB1B_RAT	Ras related protein Rab 1B	3.3	2	9.25%	2.62	G16, M19
RALA_RAT	Ras-related protein Ral-A	2.98	3	16.00%	2.9	W62, C62
SCPDH_RAT	Probable saccharopine dehydrogenase	0.49	3	8.19%	17.75	W62, G16, M19, C62
SERPH_RAT	Serpin H1	0.25	18	50.17%	34.2	G16, M19
TBB2A_RAT	Tubulin beta-2A chain	10.88	2	17.00%	0.79	W62, C62, M19
TBB2C_RAT	Tubulin beta 2C chain	2.81	4	11.35%	3.07	W62, G16, M19
TBB5_RAT	Tubulin beta-5 chain	4.37	5	15.77%	1.98	W62, C62, M19
TNNI3_RAT	Troponin I cardiac muscle	1.54	2	13.29%	5.6	W62, G16, M19, W62
TRI72_RAT	Tripartite motif containing protein 72	2.26	5	18.79%	3.82	W62, G16, M19

Hypothalamic

Accession	Description	Ratio Channel/Protein	Peptides	Coverage	Amount (fmol)	Antibody
ACADL_RAT	Long chain specific acyl CoA dehydrogenase mitochondrial	0.74	5	12.65%	3.73	W62, RSUR1
AT2B2_RAT	Plasma membrane calcium transporting ATPase 2	0.72	2	2.42%	3.82	W62, RSUR1
BACH_RAT	Cytosolic acyl coenzyme A thioester hydrolase	1.56	2	5.90%	1.77	C62, RSUR1
D3D2_RAT	3 2 trans enoyl CoA isomerase mitochondrial	1.7	2		1.61	W62, C62, RSUR1
DCTN2_RAT	Dynactin subunit 2	0.79	4	13.71%	3.49	W62, C62
DHB12_RAT	Estradiol 17 beta dehydrogenase 12	1.3	2	6.95%	2.11	C62, RSUR1
EAA1_RAT	Excitatory amino acid transporter 1	0.19	11	19.50%	14.43	W62, RSUR1
EFTU_RAT	Elongation factor Tu mitochondrial	1.01	2	6.30%	2.72	W62, RSUR1
IDH3A_RAT	Isocitrate dehydrogenase NAD subunit alpha mitochondrial	1.12	2	6.35%	2.46	C62, RSUR1

MIF_RAT	Macrophage migration inhibitory factor	0.84	3	18.50%	3.27	W62, RSUR1
SEPT2_RAT	Septin 2	0.94	4	15.50%	2.92	W62, C62
SEPT3_RAT	Neuronal specific septin 3	0.49	8	26.67%	5.56	W62, RSUR1
TBA3_RAT	Tubulin alpha 3 chain	0.85	2	42.67%	3.24	W62, C62
TCPD_RAT	T complex protein 1 subunit delta	1.11	2	5.40%	2.49	W62, RSUR1

HCAEC

Accession	Description	Ratio Channel/Protein	Peptides	Coverage	Amount (fmol)	Antibody
ALDOA_HUMAN	Fructose-bisphosphate aldolase A	0.57	9	26.83%	5.19	W62, C16
BASI_HUMAN	Basigin	1.38	3	10.14%	2.16	W62, C16
CAV1_HUMAN	Caveolin-1	0.46	2	12.27%	6.42	W62, C16
CISY_HUMAN	Citrate synthase, mitochondrial	1.45	3	6.38%	2.05	W62, C16
EF1A1_HUMAN	Elongation factor 1-alpha 1	0.32	7	19.58%	9.19	W62, C16
EHD2_HUMAN	EH domain-containing protein 2	1.31	5	11.43%	2.28	W62, C16
EHD4_HUMAN	EH domain-containing protein 4	1.64	2	5.13%	1.82	W62, C16
ENOA_HUMAN	Alpha-enolase	1.14	3	9.78%	2.62	W62, C16
ENPL_HUMAN	Endoplasmin	0.92	5	7.73%	3.24	W62, C16
HSP7C_HUMAN	Heat shock cognate 71 kDa protein	9.93	9	20.77%	0.3	W62, C16
LEG1_HUMAN	Galectin-1	0.68	2	19.25%	4.38	C16, C15
LRC59_HUMAN	Leucine-rich repeat-containing protein 59	0.92	6	25.08%	3.25	W62, C16
MDHM_HUMAN	Malate dehydrogenase, mitochondrial	1	6	23.88%	2.97	W62, C16
MOES_HUMAN	Moesin OS=Homo sapiens GN=MSN PE=1 SV=3	0.93	6	9.95%	3.21	W62, C16
MYH9_HUMAN	Myosin-9	0.3	6	3.82%	9.95	W62, C16
MYL6_HUMAN	Myosin light polypeptide 6	0.16	7	37.44%	19.2	W62, C15
MYL9_HUMAN	Myosin regulatory light polypeptide 9	2.38	2	32.33%	1.25	W62, C16
NDKB_HUMAN	Nucleoside diphosphate kinase B	1.06	3	23.48%	2.82	W62, C16
P3H1_HUMAN	Prolyl 3-hydroxylase 1	0.12	28	43.50%	24.57	C16, C15
P4HA1_HUMAN	Prolyl 4-hydroxylase subunit alpha-1	0.18	16	32.83%	16.31	C16, C15
PDIA1_HUMAN	Protein disulfide-isomerase	0.14	24	44.90%	20.81	W62, C16, C15
PLOD1_HUMAN	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1	0.5	15	24.83%	6	C16, C15
POTEI_HUMAN	POTE ankyrin domain family member I	0.5	1	10.55%	5.93	W62, C16
PPIB_HUMAN	Peptidyl-prolyl cis-trans isomerase B	0.15	12	37.89%	20.07	W62, C16, C15
STOM_HUMAN	Erythrocyte band 7 integral membrane protein	0.21	2	7.90%	14.45	W62, C16
TBA1A_HUMAN	Tubulin alpha-1A chain	1.99	2	7.00%	1.5	W62, C16
TGM2_HUMAN	Protein-glutamine gamma-glutamyltransferase 2	0.12	17	28.85%	25.56	C16, C15

Min6

Accession	Description	Ratio Channel/Protein	Peptides	Coverage	Amount (fmol)	Antibody
ARL8B_MOUSE	ADP-ribosylation factor-like protein 8B	0.26	3	17.70%	8.27	W62, C62
ASPG_MOUSE	N(4)-(beta-N-acetylglucosaminyl)-L-asparaginase	0.79	3	15.50%	2.77	W62, C62
CHCH3_MOUSE	Coiled-coil-helix-coiled-coil-helix domain-containing protein 3	15.56	3	14.75%	0.14	W62, RSUR1
DLDH_MOUSE	Dihydrolipoyl dehydrogenase, mitochondrial	0.1	10	25.64%	20.75	W62, C62
FAF2_MOUSE	FAS-associated factor 2	6.39	4	11.10%	0.34	W62, RSUR1
GSTM1_MOUSE	Glutathione S-transferase Mu 1	0.69	2	11.95%	3.16	W62, C62
H2A1F_MOUSE	Histone H2A type 1-F	0.26	3	30.00%	8.41	W62, G16, RSUR1
HS71L_MOUSE	Heat shock 70 kDa protein 1-like	1.4	3	13.22%	1.55	C62, W62
ML12B_MOUSE	Myosin regulatory light chain 12B	2.05	4	24.70%	1.06	W62, RSUR1
MOGS_MOUS	Mannosyl-oligosaccharide glucosidase	1.67	3	6.32%	1.3	W62, G16, RSUR1
NIPS2_MOUSE	Protein NipSnap homolog 2	8.67	3	10.15%	0.25	W62, RSUR1
PAIRB_MOUSE	Plasminogen activator inhibitor 1 RNA-binding protein	2.62	3	10.60%	0.83	G16, RSUR1
RAB14_MOUSE	Ras-related protein Rab-14	1.85	4	16.82%	1.17	W62, G16, RSUR1
RAB18_MOUSE	Ras-related protein Rab-18	1.61	3	14.84%	1.35	W62, C62, RSUR1
RAB7A_MOUSE	Ras-related protein Rab-7a	2.11	4	20.02%	1.03	C62, W62, G16
RAB8A_MOUSE	Ras-related protein Rab-8A	1.13	3	20.36%	1.92	W62, G16, RSUR1
RAN_MOUSE	GTP-binding nuclear protein Ran	1.04	4	23.25%	2.09	W62, C62
RHOA_MOUSE	Transforming protein RhoA	0.51	3	24.00%	4.23	C62, RSUR1
SDF2_MOUSE	Stromal cell-derived factor 2	0.76	3	19.50%	2.88	W62, C62
SMD2_MOUSE	Small nuclear ribonucleoprotein Sm D2	0.86	2	20.88%	2.54	W62, RSUR1

SURF4_MOUSE	Surfeit locus protein 4	5.45	3	15.13%	0.4	W62, RSUR1
THOC4_MOUSE	THO complex subunit 4	0.59	2	14.27%	3.68	W62, C62, RSUR1
TIM16_MOUSE	Mitochondrial import inner membrane translocase subunit	5.41	3	43.20%	0.4	W62, RSUR1
TMED9_MOUSE	Transmembrane emp24 domain-containing protein 9	0.86	3	18.22%	2.52	W62, C62, RSUR1
TPIS_MOUSE	Triosephosphate isomerase	7.71	4	22.65%	0.28	W62, RSUR1
VATE1_MOUSE	V-type proton ATPase subunit E 1	3.39	3	15.92%	0.64	W62, G16, RSUR1
VPP1_MOUSE	V-type proton ATPase 116 kDa subunit a isoform 1	2.54	3	5.15%	0.86	W62, RSUR1

The table lists proteins found by mass spectrometry. Proteins are only included if they were present in immunoprecipitates obtained with at least two separate antibodies against K_{ATP} channel subunits, if they were not present (or enriched with a false discovery rate of 5% or less as determined by data simulation calculations as described in Supplemental Methods) in a negative control immunoprecipitation reaction obtained with IgG, and if they are present at 10-fold or less in abundance relative to the immunoprecipitated channel. For a full list of proteins, please refer to Table S1. The ratio (Channel/Protein) refers to the protein enrichment relative to the average amount of KATP channel (fmol) from all technical replicates.

FIGURE LEGENDS

Figure 1: Verification of interaction for selected proteins. Immunoprecipitates of canine ventricular tissue (left panels) or Min6 membranes (right panels) obtained with antibodies against the α-subunit of Sodium/potassium ATPase (NKA), actin, Kir6.2 (W62b or G16), SUR1 (RSUR1) or with an unrelated IgG (as a negative control) were immunoblotted with NKA, C62, CSUR1 or G16 antibodies. Input lanes containing the membranes before the immunoprecipitations are also shown. In contrast with the mouse (Figure S2, right panel) and rat protein (Figure S2, middle and left panels), the canine ventricular Kir6.2 (bottom left panel) runs as a 42 kD band (long arrows). Nonspecific antibody bands are detected when the Protein A/G beads are used for the immunoprecipitation (marked as IgG, bottom right panel). Note that the RSUR1 antibody immunoprecipitated both the glycosylated (top band) and non-glycosylated (bottom band) forms of SUR1 [50] (top right panel, left lane).

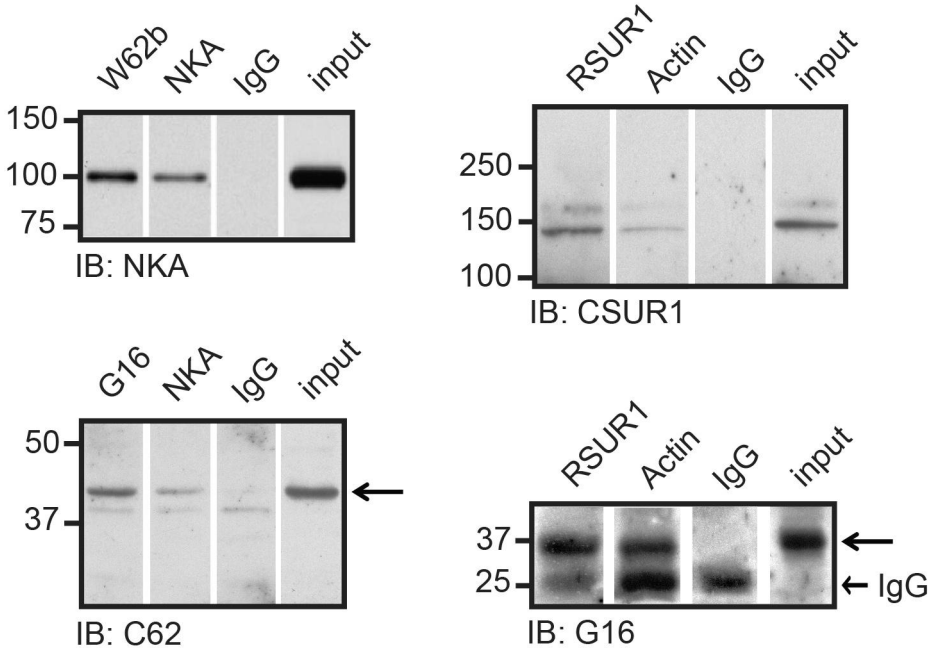

Figure 2: A heat map representing functional classes of putative interacting proteins. Identified proteins listed in Table S1 were submitted to the web-based DAVID Functional Annotation Clustering Tool (http://david.abcc.ncifcrf.gov) with a species-specific background, using the default options. A Gene Ontology (GO) Consortium classification was performed at the BP_FAT term specificity level. Representative entries from the functional categories were used, along with their associated enrichment scores, for comparative purposes. Following log(2) transformation of the DAVID Fold Enrichment score, the represented pathways were plotted as a heat map using Multiple Experiment Viewer (MEV 4.8.1) software.