Supplemental Methods and Data

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Supplemental Experimental Methods

Mouse population overview and handling

BXD strains were sourced from the University of Tennessee Health Science Center (Memphis, TN, USA) and bred at the EPFL animal facility for 2+ generations prior to incorporation into the study. 3 to 5 males from 55 strains of the BXD mouse population, plus the two parental strains DBA/2J and C57BL/6J, were phenotyped for metabolic traits over a period of 29 weeks, with staggered entry over 6 years (February 2010 through February 2016). All individuals were fed Harlan chow diet Teklad 2018 from weaning until sacrifice. The related phenotype data were published previously [1]. The specific order of strain phenotyping, the number of individuals, and the times can be found on GeneNetwork.org by searching under Species: Mouse; Group: BXD, Type=Phenotypes for Record ID **17555**. Count is in weeks from the start of the study (BXD44). The 8 strains analyzed for this study were all phenotyped between February 2010 and February 2012 and are known to vary in their mitochondrial phenotypes [1]. One individual mouse from each of the eight strains was selected for all analyses. Animals were previously

phenotyped for molecular characteristics including respiration, blood pressure, cold response, and exercise capacity from 16 weeks until 25 weeks of age [1]. Animals were rested in individual isolator cages for the 4 weeks between the end of phenotyping and sacrifice. The transcript data used for this study have been published previously for quadriceps, liver, and BAT [2] and for heart [3], and were collected and run from the same individuals (and cohorts). All cohorts were sacrificed by perfusion under isoflurane anesthesia. Liver and heart were collected and frozen ~1–2 minutes after the complete onset of anesthesia, ~4–6 minutes for brain and BAT, and ~6–8 minutes for quadriceps. All cohorts were maintained in the same housing facility. Further details on these phenotyping experiments can be found in our prior publication [1] or under the EMPReSS protocols (http://empress.har.mrc.ac.uk/browser/). All phenotyping and handling was approved by the Swiss cantonal veterinary authority under licenses 2257 and 2257.1. The BXD population was selected for several reasons, particularly as it was the largest and most readily-available recombinant inbred mouse population at the time the study was designed. Further details about different mouse genetic reference populations have been covered elsewhere [4].

Sample size

A minimum of 8 genotypes are needed for quantitative trait locus (QTL) mapping in R/qtl [5] and GeneNetwork [6] for 2 parental recombinant inbred crosses (like the BXDs). We have previously identified several dozen strong cis-pQTLs in the liver of BXD strains—i.e. proteins whose variation in expression is driven by variants within the gene itself [1]. To serve as a positive control for genotyping and for protein assignment and quantifications, we mapped QTLs for 4 proteins which are known to have *cis*-acting sequence variants affecting the protein level in the BXDs (COX7A2L [1], HAO1 [1], GLO1 [7], and NNT [8]). This small number were selected in order to reduce the effects of multiple testing. *cis*-pQTLs were calculated in GeneNetwork by searching for locally-significant QTLs within 15 megabases of the proteins' gene locations. COX7A2L yielded a locally-significant cis-pQTL in all tissues, HAO1 in 2 of 5 (in BAT and liver), GLO1 in 4 of 5 (not quadriceps), and NNT in 3 of the 5 tissues (not in BAT or quadriceps), indicating the proteins are being correctly identified and assigned to their genotypes. Notably, all four cis-pQTLs were detected in liver, which is currently the primary reference tissue for proteome data in the BXDs [1, 9, 10] (note that some BXDs are included in the HMDP population). This initial validation confirmed that the links between genotype to expression level were as expected, and that while some findings will replicate what has been previously observed in liver, in other cases, the same gene will have different primary regulatory mechanisms across tissues.

BXD mitochondria isolation procedure

For each tissue, 50–80 mg of frozen sample was collected for both SWATH-MS proteomics and for mitochondrial enrichment. We used a recently developed protocol [11], which evolved from an older common method [12]. In brief, tissues were first pulverized in liquid nitrogen with a mortar and pestle. The fragments were then transferred to a cooled glass vial, where they were homogenized in 2 mL cold isolation buffer. Homogenization was achieved at maximum speed after 20 strokes of a motorized dounce-type ceramic tissue grinder (Wheaton Science Products). Following two rounds of centrifugation at 600 x g for 10 min, the mitochondrial fraction was washed and pelleted at 7000 x g for 10 min. For brain and quadriceps, an additional round of centrifugation was performed at 600 x g for 10 min. The pellet was resuspended in 100–150 µl of cold isolation buffer and stored at -80°C for later SWATH extraction. For four samples—BAT BXD45, BAT BXD101, heart BXD66, and quadriceps BXD66—the tissue remaining after mRNA and protein isolation turned out to be insufficient for mitochondrial isolation followed by protein extraction and peptide digestion and cleanup, and no sample was remaining at the end of the process for SWATH-MS measurement. Consequently, these samples are absent for the mitochondrial enriched fraction. For WB and ICC validation we used a more technically challenging protocol that requires fresh (i.e. never frozen) samples an order of magnitude larger. That is, 1 gram of tissue yields approximately 4 mg of crudely-purified mitochondrial protein, but only 0.3 mg of ultrapure mitochondrial protein. This yields highly purified mitochondria samples that contain nearly no trace of nucleus or cytosol, and which also separates the mitochondria associated membrane (MAM) from the outer mitochondrial membrane [13].

Proteomics: technical considerations

There currently exist several mass spectrometry (MS)-based techniques for the large-scale identification and quantification of complex protein samples. All current techniques which can quantify hundreds-to-thousands of proteins at a time rely on bottom-up proteomics techniques, i.e. meaning that proteins are digested into smaller peptide fragments which are then injected into the MS, fragmented, and quantified. The peptide quantifications are then collated to provide estimates of the overall original proteins' levels. There are several alternative bottom-up techniques, most notably using data-dependent acquisition (DDA, often called shotgun proteomics), targeted proteomics, and data-independent acquisition (DIA, to which SWATH belongs). There are many technical differences between these approaches which have been detailed elsewhere [14-16]. In brief, we elected to use SWATH for this study after considering the numerous tradeoffs between techniques, including considerations of MS time/availability, study cost, labeling requirements, fractionation requirements, proteome coverage, and accuracy. No other study has yet examined subcellular localization using unlabeled DIA techniques, so in large part we also selected SWATH to compare how its results compare to

other state-of-the-art techniques. Unlabeled and unfractionated samples, as in the case for SWATH, provides a simplified and, based on the outcome of this study, appears to provide similar coverage and accuracy as compared to gold-standard MS techniques used to determine protein localization, e.g. the isotopically labeled and DDA-based approach hyperLOPIT [17]. In this context, protein fractionation means to take an input protein mixture and reduce its complexity by separating it into several different samples, which will be injected in the MS either in separate runs, or together but tagged separately by tandem mass tags (e.g. [18]). This concept is distinct from the fractionation discussed in this study, referring to the division of cells into different organelles.

Proteomics: sample preparation

Our current protein preparation protocol has been published separately [19]. In brief: whole tissues were collected and snap-frozen in liquid nitrogen, then at a later date tissues were shattered in liquid nitrogen for proteomics and transcriptomics preparations. For total protein, around 50 mg of tissue was homogenized with 4 mL of radioimmune precipitation assay-modified buffer (1% Nonidet P-40, 0.1% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 50 mM Tris, pH 7.5, protease inhibitors EDTA-free, then phosphatase inhibitors 10 mM NaF, 10 mM sodium pyrophosphate, and 5 mM 2-glycerophosphate) in a glass-glass ceramic dounce homogenizer (Wheaton Science Products) at 4°C. After the homogenates were centrifuged (20,000 g at 4°C for 15 min), the supernatant was collected and kept at 4°C. Next, both total protein pellets and isolated mitochondrial pellets were resuspended with UREA-Tris buffer (50 mM Tris, pH 8.1, 75 mM NaCl, 8 M urea, EDTA-free protease inhibitors, 10 mM NaF, 10 mM sodium pyrophosphate, 5 mM 2-glycerophosphate), sonicated for 5 min, then centrifuged at 20,000 g for 15 min at 4°C. The protein concentrations were then determined with the BCA protein assay (Thermo Fisher Scientific). For the precipitation of proteins in each sample, 200 µg of protein was precipitated with 6 volumes of ice-cooled acetone and kept 16 hours at -20°C. Then protein were re-suspended in a buffer of 8 M urea/0.1 M NH_4HCO_3, reduced with 12 mM dithiotreitol for 30 min at 37 °C, then alkylated in the dark with 40 mM iodoacetamide for 45 min at 25°C. Samples were diluted with 0.1 M NH_4HCO_3 to a final concentration of 1.5 M urea and for digestion, sequencing grade modified porcine trypsin (Promega catalogue number V5111) was added for 1 µg of trypsin for every 100 µg of protein, incubated for 16 h at 37°C. Trypsin cleaves specifically at the carboxyl side of lysine (K) and arginine (R) amino acids except when either is immediately followed by proline (P). Peptide mixtures were cleaned by Sep-Pak C18 columns (Waters, Milford, MA, USA) and eluted with 40% acetonitrile. The resulting peptide samples were evaporated on a vacuum centrifuge to dryness, re-solubilized in 2% acetonitrile/0.1% formic acid to 1µg/µL concentration. Samples were spiked with indexed

retention time peptides (iRT, from Biognosys) and run in a SCIEX TripleTOF 5600 (described further in the following two sections).

Proteomics: workflow overview

After proteins were extracted, denatured, and digested with trypsin, the generated peptides were desalted using C18 column and measured in shotgun-MS and SWATH-MS mode on a SCIEX TripleTOF 5600. In the SWATH-MS workflow, we first built an assay library of the peptidome from compendia of fragment ion spectra by performing the MS in shotgun mode, described further under the next subheading. A complete digital map of all ionized species was recorded from all five tissues in both total and mitochondrial fractions, then combined with a detailed liver proteome library obtained by off-gel electrophoresis (OGE) as previously described [1]. The identification and quantification of peptides in these fragment ion maps was performed using targeted data analysis strategies. Hence, the number and type of proteins quantified are determined by the available assay libraries supporting targeted data extraction, and by signal intensities of the respective fragment ions in the fragment ion map. After these libraries are generated—which is not necessary to do *de novo* for every study but were created *de novo* in this case—SWATH-MS is performed on the sample of interest. Targeted data extraction of the SWATH-MS maps, retention time alignment and false discovery rate (FDR) estimation were performed in the OpenSWATH workflow [20]. For each protein, the top 3 intense transitions of the top 3 most intensive peptides were selected to estimate the abundance of the protein using imsbinfer (https://github.com/wolski/imsbInfer, April 2016 release).

Proteomics: assay library generation

Full details on SWATH library generation have been previously published [21], but we include a summary of the method as follows. We generated our SWATH assay FASTA library against the 16,860 reviewed, canonical proteins in the UniProt database for *Mus musculus* (taxonomy ID: 10090, downloaded May 2016) considering one protein per gene. The complete UniProt protein isoform database includes 82,868 reviewed proteins stemming from the same 16,860 genes, i.e. an average of 4.9 isoforms per protein. However, we only considered the smaller canonical protein set to (1) limit the large increase in FDR from using the full isoform set, and (2) the loss of unique proteotypic peptides when multiple protein isoforms are considered as separate proteins. We then ran the Trans-Proteomic Pipeline (TPP; [22]) on the 16,860 protein FASTA file to generate the theoretical peptide search space for the SWATH library. In the TPP pipeline, a decoy database was generated by reversing the amino acid sequences and appended to the target database. Cysteine carboxy-amido-methylation was set as the static modification, and methionine oxidation was set as the variable modification. Peptides with up

to 1 missed cleavage site were allowed. This library was then used as the search space for the shotgun-MS runs which will generate the SWATH search space.

To construct a peptide assay library with maximum coverage of mitochondrial proteins, we combined shotgun MS runs (also called DDA) from 10 OGE-fractionated mouse liver samples, as well as both whole tissue lysate and purified mitochondria from all five tissues. First, we selected 58 mice cohorts, and for each sample 100 µg of protein lysate was digested as described above. The resulting peptides were mixed and loaded for OGE fractionation as previously described [23]. The 24 fractions were combined into 10 fractions and cleaned up with C18 columns. Each fraction was analyzed with classical DDA acquisition-mode with a SCIEX TripleTOF 5600 mass spectrometer interfaced to an Eksigent NanoLC Ultra 2D Plus HPLC system. Samples were loaded on to a PicoFrit emitter coupled with an analytical column (75 µm diameter) with buffer A (2% Acetonitrile, 0.1% formic acid) and eluted with a 135 minutes linear gradient of 2-35% Buffer B (90% Acetonitrile, 0.1% formic acid) with a flow rate of 500 nL/min. The 20 most intense precursors with charge state 2-5 were selected for fragmentation and the MS2 spectra were acquired in the range 50-2000 m/z for 100 ms, and precursor ions were excluded from reselection for 15 seconds.

Profile mode .wiff files from shotgun data acquisition were transformed to centroid mode mzXML files using msconvert as part of Proteowizard 3.0.5533. The mzXML files were searched against the canonical UniProt database generated earlier using Comet 2013.02 rev 2. These four searches were combined by (and are part of) TPP version 4.7.0. Mass tolerance was set to 25 ppm for precursor ions and 0.4 Da for fragment ions. The pepXML files were combined using iProphet [24] (part of TPP 4.7.0) at 1% protein FDR, and the integrated pepXML file was used to generate the redundant spectra library containing all peptide spectra matches using SpectraST version 5.0 [25]. Retention time of peptide identification was transformed to iRT values based on the linear regression calibrated for each shotgun runs using the information of the spiked iRT peptides. The median of iRT values of each peptide were calculated using in-house script, and the consensus library was constructed using SpectraST version 5.0. Library spectrum of proteins identified by single proteotypic peptides were checked by using SpectraST (available on PRIDE). We then selected the top five most abundant b and y fragment ions of each peptide to generate the assays for SWATH-MS targeted extraction. The target assay library contains the precursor and fragment mass to charge (m/z) values and the relative intensities of the fragment ions, as well as the average iRT value of each precursor. Decoy assays were appended to the target assay library for error rate estimation.

Proteomics: SWATH-MS and targeted data extraction

The SWATH-MS was performed on the SCIEX 5600 TripleTOF mass spectrometer as previously described [26]. The chromatographic parameters were as described above. For SWATH-MS-based experiments, the mass spectrometer was operated in a looped product ion mode, and specifically tuned to allow a quadrupole resolution of 25 Da mass selection. Using dynamic isolation width ranging from 6 to 91 Da (containing 1 Da for the window overlap), a set of 64 overlapping windows was consecutively constructed covering the 400–1200 m/z precursor range. The collision energy (CE) for each window was determined based on appropriate collision energy for a charge 2+ ion centered upon the window with a spread of 15 eV. An accumulation time (dwell time) of 100 ms was used for all fragment ion scans in high sensitivity mode. The survey scans were acquired in high-resolution mode at the beginning of each SWATH-MS cycle, resulting in a duty cycle of 3.4 seconds.

The SWATH-MS were first converted to profile mzXML files using ProteoWizard 3.0.5533 [25]. The SWATH-MS targeted data extraction was performed using OpenSWATH version 1.10 [20], which applies target-decoy scoring model to estimate the false discovery rate (FDR) by the mProphet algorithm v2.0.3 ("Frog Fish") [27]. Retention alignment between SWATH-MS maps were performed based on the clustering of reference peptides using a nonlinear alignment algorithm [28]. Fragment ion chromatograms were extracted according to the target-decoy assay library with a width of 0.05 m/z, and peak groups were scored based on the elution profile of fragment ions, similarity of elution time and relative intensities with the assay library, and the properties of the MS/MS spectrum extracted at the chromatographic peak apex. Peptide FDR was estimated according to the score distribution of target and decoy assays. In the final SWATH results, 737 proteins were inferred on the basis of one proteotypic peptide. The MS2 spectra of these 737 were manually annotated in Skyline 4.1 [29]. First, query parameters from the sptxt file from the library were imported into Skyline. Then, all raw files acquired in SWATH mode and their OpenSWATH results were imported, and peak boundaries defined by OpenSWATH were manually checked. These results in Skyline format (a free download, but Windows-only) are available in PRIDE (filename annotated_single_peptide_ids_swath_skyline.zip).

Data normalization

The proteomics output data from OpenSWATH are subsequently $\log_2$ normalized and centered at zero using imsbInfer (described earlier). EF_{mito} is calculated as the difference between the mitochondrial and total fractions, normalized to the average expression of the 774 measured MitoCarta proteins. All EF_{mito} values can be found in the second and third sheets included in Table S1. All microarray data are $\log_2$ normalized using RMAExpress v1.2.0 (http://rmaexpress.bmbolstad.com) and are centered at 8.0 units.

Annotation

In line with the nomenclature standards of the Mouse Genome Informatics (MGI), we report genes and transcripts in italics with the first letter capitalized (e.g. *Cox7a2l*), while proteins are in normal script and all uppercase (e.g. COX7A2L). Mouse gene symbols from NCBI and mouse protein identifiers from UniProt were downloaded in May 2016 and fixed (e.g. Q61387 is COX7A2L) using the canonical reviewed entries. Take note that some gene symbols are, unfortunately, not uniquely attributed. In particular, the gene *sterol O-acyltransferase 2* is currently given the gene symbol *Soat2*, but it has a deprecated gene alias *Acat2*, which is currently used by the gene *acetyl-Coenzyme A acetyltransferase 2*. Note that the validation experiment in this study is on *Soat2*. Unique UniProt identifiers are listed in supplemental Table 1 if any ambiguities are noted for gene symbols. The mouse genome assembly mm10 was used for all reference gene locations. For comparisons with HPA, proteins were aligned based on exact gene symbols matching between mouse and human annotations. Strong orthologs overwhelmingly share the exact symbol between mouse and human; only 22 of the 283 tissue-specific proteins checked across datasets do not have an exact alignment.

Resource comparisons for tissue localization

We considered several different resources to perform *post hoc* validation of both the results from EF_{mito} and for the general protein quantifications. Our first examination was to test whether the proteins we localized in each tissue were broadly in agreement with literature. Although HPA is generated in humans, we took it as the reference, as it is the most comprehensive resource with protein quantifications across many tissues and it was developed using a non-MS-based technology (antibodies). BAT was not considered as it is not included among the tissues analyzed in HPA. Using the whole cell fractions, we took the subset of proteins uniquely identified in brain, liver, or heart+muscle, as well as the subset of proteins identified in all 4 tissues, similar to the outermost and innermost Venn diagrams in Fig. 1B. However, we used a higher significance threshold to attribute proteins as specifically expressed in one or all tissues. To determine proteins uniquely localized to a single tissue, we required of an m-score ≤ 0.001 in ≥ 75% of strains for a given tissue and detected at m-score > 0.001 for all other tissues. For instance, synuclein beta (SNCB) was detected at m < 0.001 in all 8 strains' brains, and was always detected at m > 0.001 for all other tissues. To determine proteins certainly localized in all four tissues, we required m-score ≤ 0.001 for at least 75% of all tissues, taken independently (e.g. a protein confidently identified in 5 of 8 brains but 8 of 8 of the other three tissues would not be considered). For muscle, proteins were considered as localized if they were in ≥ 75% of both tissues combined (i.e. 12+ of 16), or in ≥ 75% of either heart or quadriceps (i.e. 6+ of 8). These cutoffs provided us with 757 proteins with confident tissue localizations based on SWATH data alone—310 only in brain, 185 in liver, 90 in muscle, and

172 observed in all four tissues. These lists are identified in Table S1 under the column titled "Tissue Localization". Of these 757 proteins, 251 also had high-confidence, validated data from the HPA (a reliability score of "Supported")—94 from brain, 57 liver, 41 muscle, and 59 ubiquitous. Of these, 161 proteins' localizations precisely overlapped (64%, e.g. either uniquely expressed to the same tissue in HPA and SWATH, or ubiquitously expressed in all three tissue types in both). 19 had similar overlap (8%, e.g. we observed the protein solely in one tissue, and while HPA observed it in another tissue as well, it is predominantly expressed in the same tissue to which we localized the protein). 51 were somewhat different (20%, e.g. we observed the protein solely in one tissue, while HPA observed it equally or even more highly expressed in one of the other tissues), while 20 were very different (8%, e.g. we detected a protein in a tissue where HPA did not detect it at all). This full list can be found in the column "HPA Overlap" in Table S1 and representative examples are highlighted in Figs. 2A and 2B.

Mitochondria: gene selection & localizations

Several studies have been performed examining the set of proteins localized in the mitochondria, including resources which have determined mitochondrial proteins using multiple technologies like the Human Protein Atlas (HPA; [30]) and MitoCarta 2.0 [31], resources which have collated the results of many primary research manuscripts such as AmiGO [32] and UniProt [36], and resources which have examined mitochondria as a way to validating new technologies or methods such as by hyperLOPIT [33], our own study here using SWATH, MitoFates [34], or TargetP/MTS [44]. There are now a number of different technologies available for reasonably-high throughput and coverage (e.g. thousands of proteins). Studies such MitoCarta have combined these newer approaches, e.g. APEX [35, 36] and MS^2 proteomics, together with traditional methods like GFP and with analytical approaches like coexpression analysis. In the manuscript, we have considered MitoCarta 2.0 as the “definitive” list for mitochondrial localization which we compared against, as it is the most comprehensive resource specifically generated in mice (across 14 tissues). We have considered HPA as a secondary reference list for localization and expression and in the manuscript we have considered all proteins on the MitoCarta 2.0 list as "mitochondrial", although MitoCarta provides several different measures of confidence in assignment based on literature, targetP, literature, etc. At the broadest sense, we observe 774 MitoCarta-reported proteins in any of the five tissues and fractions, or 67% coverage. This coverage is skewed substantially according to MitoCarta's FDR-based confidence assignment (also reported in Table S1): among the 615 MitoCarta proteins with FDR ≤ 1%, we observe 479 (78%), while among the 159 MitoCarta proteins with FDR ≥ 10%, we observe only 65 (41%). Across the entire UniProt-reported canonical mouse proteome, we quantify roughly 22% (3648 proteins quantified out of 16,916 canonical entries), while for mitochondria, 67–80% of the expected

proteome is quantified. Other resources' indications of proteins' mitochondrial localizations are listed in Table S1.

Next, we examined whether the subcellular localization of proteins to the mitochondria as calculated by EF_{mito} broadly overlapped with literature using the resources described earlier (HPA, UniProt, hyperLOPIT, AmiGO, MitoCarta, MitoFates). The localization assignments of proteins to the mitochondria from other resources are in Table S1 (columns labeled with the name of the resource, e.g. "In_MitoCarta2", "In HPA v16", "UniProt", "hyperLOPIT") along with our sliding probability predictor of localization ("EFmito" column). The resources all broadly agree on which proteins are mitochondrial, e.g. of the 55 mitochondrial ribosome proteins (MRPs) identified in our dataset, 52 of them have enriched EF_{mito} values, and only one is substantially un-enriched (MRPS7). All 55 MRPs that also have records in AmiGO, hyperLOPIT, and UniProt are also classified as mitochondrial, as are the strong majority in HPA (of the 43 with records in HPA, 36 are classified as mitochondrial). As canonical mitochondrial proteins have substantially higher EF_{mito} values than non-canonical mitochondrial proteins (e.g. Fig. 4*B*), we could be certain that the purifications and quantifications worked broadly as expected, providing us with the confidence to look at outliers as potentially-novel mitochondrial proteins.

To select putative novel mitoproteins for validation, we devised a ranking system built off of EF_{mito}, MTS scores, and literature known about each gene. First, all genes that were repoted as mitochondrial in any of the reference databases were removed from consideration, as validating a validated mitoprotein is of limited interest. Next, proteins were rated in a binary fashion earning 1 'point' per tissue in which they were expressed at or above the average EF_{mito} for a mitochondrial protein (Figure S1A), and were given a second continuous ranking based on the average cross-tissue EF_{mito}. Next, proteins which have putative MTS were flagged as more likely potential candidates, and proteins reported as part of the endoplasmic reticulum were flagged as less likely potential candidates (as the endoplasmic reticulum is partially enriched with mitochondria). ABCC6 was the top novel candidate, being enriched in all 5 tissues, having a putative MTS, and—although not reported as mitochondrial in any standard database—we uncovered two conflicting reports in literature as to whether it is part of the MAM [38, 39]. ABCC6 was also selected due to a known major sequence variant between the BXDs which leads to large expression differences depending on the parental allele [40], which serves as a positive control for the antibody. ABCC6 was then validated both as part of the MAM, and the decreased expression in the DBA/2 strain. Rather than select only the top candidates, we elected to examine less certain candidates with good (MTAP, SOAT2) and moderate (IMPDH2) selection criteria to assess the robustness of the enrichment scoring. Candidates were then screened for variability in their selection criteria, e.g. MTAP is relatively evenly expressed across tissues, while SOAT2 is more predominantly expressed in liver, and

IMPDH2 had a large difference between EF_{mito} across tissues (highly enriched in heart, unenriched in brain). Several dozen candidates met these criteria, and ultimately the three selected were chosen due to having good commercial antibody availability. The results from the Western blot largely mirrored the values expected from the SWATH, e.g. that SOAT2 is most highly expressed in liver, and that IMPDH2 is not mitochondrially localized in brain (or at least, the larger isoform is not).

Protein localization: Western blots and immunocytochemistry

For validation of localization for SOAT2, MTAP, and IMPDH2, we examined five tissues from a C57BL/6 mouse (Fig. 3*F*–*G*), while for ABCC6 we examined the livers from both C57BL/6 and DBA/2J mice due to the presence of a genetic sequence variant between C57BL/6 and C3H known to affect its expression [38] (DBA/2 has the same allele of ABCC6 as C3H and *Abcc6* in the BXDs also yields strong and consistent cis-QTLs [1]). ~50 mg fragments were broken into two pieces: one for proteome analysis and the other for nuclear+cytosol fraction (NC) and mitochondrial fractions. For ABCC6, we retained the MAM fraction. (The NC and the mito fraction for SOAT2, MTAP, and IMPDH blots does not include MAM.) The fractions in Fig. 4 and S1 were isolated according to published methods which gives high purity mitochondria [13]. Briefly: fractions were boiled at 70°C for 10 minutes with LDS buffer (Invitrogen), then subjected to Western blots in NuPAGE Novex Bis-Tris 4–12% gradient gel (Invitrogen). Blots were transferred to PVDF membranes, blocked by 5% skim milk for 20 minutes, treated for 20 hours with each primary antibody, then treated for 1 hour with the appropriate secondary antibody. Blots were detected using ECL treatment and an Azure c300 imaging machine. Western blots were run to determine localization with LONP1, tubulin, calnexin, caspase 3, and NUP62 serving as controls (as labeled in the figures).

Cell line information

The C2C12 (CRL-1772) and AML12 (CRL-2254) cell lines were obtained from ATCC (CRL-1772). C2C12 myoblasts were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) including glucose 25 mM, 10% FCS and 50 µg/ml gentamicin (Invitrogen). Differentiation was induced for 4 d in Dulbecco's modified Eagle's medium (DMEM, Gibco) including 25 mM glucose, 2% horse serum and 50 µg/ml gentamicin. AML12 murine hepatocytes were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 medium (DMEM/F12, Gibco) including glucose 25 mM, 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 5 ng/ml selenium, and 40 ng/ml dexamethasone, 20% FCS and 50 µg/ml gentamicin (Invitrogen). All cells were cultured at 37°C under a 5% CO2 atmosphere and tested regularly for mycoplasma using Mycoprobe (#CUL001B, R&D systems) following the manufacturer's instructions.

Other statistical considerations

All 76 proteomics measurements stem from the same 8 individual mice, with the same mouse's 5 organs used for both total protein and mitochondrial fraction. Two individuals did not have sufficient BAT for mitochondrial enrichment, and 1 individual each did not have sufficient quadriceps and heart. Please note that BAT only has 6 enriched samples, quadriceps and heart have 7, while brain and liver have 8 samples. Moreover, the enrichment technique varies in efficiency across tissue. For instance, the average ETC protein has EFmito of +1.17 in BAT, 1.09 in quadriceps, 0.85 in heart, 0.67 in brain, and 0.31 in liver (Supplemental Table 1, and summarized in Figure 4B-C and S1A). While we have considered EF_{mito} on a per-tissue basis, those who wish to assess the pan-tissue enrichment of a protein will need to account for differences between tissues' enrichment capacities and may instead calculate p-values for each tissue. In this case, the difference in *n* between tissues should be kept in mind.

X-Y correlation plots in Figure 2 are shown using Pearson correlation in order to permit visualization of relative expression differences, as Spearman plots obscure relevant information regarding the relationship between variance and mRNA–protein correlation (Figure 2*D*, 2*G*). For summary histograms, Spearman is preferred due to the large variability in the data and presence of outliers, although the averages are little changed. For instance, across all 3574 mRNA–protein pairs (Figure 2*E*) the average correlation is Pearson r = |0.47| with 60% nominally-significant correlations, compared to the average Spearman rho = |0.42| with 57% nominally-significant correlations. The association network in Fig. 3*E* was constructed from significantly nonzero ($p < 0.01$, multiple testing correction) and strong positive associations among proteins ($r[XY] > 0.6$) using Spearman rank correlation. All Affymetrix data were normalized from the .CEL files using RMAExpress. Proteomics data are all normalized with imsbInfer, as described above.

Statistical tests were used as follows: (1) hierarchical clustering in figure panels 1*F*, 3*A*, 3*C,* and 4*A* were performed using GENE-E (Broad Institute) and columns were clustered by the "one minus spearman rank correlation" metric using average linkage. While the y-axes are trimmed for brevity in the visualization, the full dataset was used to generate the clusters, i.e. the dendograms displayed at the top of both figure panels were calculated using all proteins (Fig. 1F) and EF_{mitos} (Fig. 4*A*); (2) Spearman correlation in Fig. panels 2*D* and 2*G*; (3) Spearman correlation in Fig. 2*E*, 2*F*, 3*D*; (4) Welch's two-sided *t*-test in Fig. 3*C* comparing each complex between heart and BAT; (5) one-way ANOVA in Fig. 3*A* for Complex V, with Dunnett post hoc tests to compare BAT CV with the other four tissues; and (6) one-way ANOVA with Prism v6 in Fig. 3*B*, with Tukey post hoc tests to compare all means across groups for CI, CIV, and CV, with multiplicity adjusted p-values used to test significance ($p <$

0.005 represented by * on the graph, and each complex was run as a separate ANOVA. For all dot plots, horizontal bars represent median value.

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SUPPLEMENTAL FIGURE LEGEND

Supplemental FIG. 1. **EF_{mito} across tissues and genes.**

A Histogram of EF_{mito} frequencies across heart, liver, quadriceps, and BAT (brain is shown in Fig. 4*B*). All tissues show enrichment of mitochondrial proteins in the mitochondrial fraction, though with slightly varying patterns. Note that EF_{mito} was calculated across all tissues, while here each histogram only includes analysis of proteins detected significantly

in that specific tissue, hence all histograms do not have the same number or specific protein composition for each category.

B AKAP1 and MAVS expression across tissue and their relative EF_{mito} values. As with ATAD1, these proteins have been observed as differentially localized to multiple cellular compartments.

C ABCC6 expression in both fractions, along with the EF_{mito}. Unlike the other three proteins, ABCC6 had significant enrichment in all five tissues.

D Longer exposure of ABCC6 gel from Fig. 4*G*, showing the dual bands and cytosolic localization more clearly. (The other exposures are identical to Fig. 4*G*—retained here for reference).

SUPPLEMENTAL TABLE LEGENDS

Further details on the datasets are available in a separate text file included with the manuscript.

Supplemental Table 1. Protein Enrichment Factors

Sheet 1: Table of the EF_{mito} calculations for each tissue, including (separately) the BAT from the additional validation strains along with a comparison with the localization of proteins as calculated by hyperLOPIT, Gene Ontology, or MitoCarta.

Sheet 2: Table of the EF_{mito} calculations for the 5 tissues.

Supplemental Table 2. Peptide-Level Data

All of the peptide-level data for (a) the total cell fractions and (b) the mitochondrial fractions, including the identified retention times and m-scores. M-scores of "2" are used to denote not-even-suggestive, and data were suppressed for purposes of localization identifications if all m-scores for a given peptide in a given tissue were 2. The library and raw spectral data are available online at the ProteomeXchange Consortium [41] under the identifier PXD005044.

Supplemental Table 3. Protein-Level Data

Normalized, log_2-scaled protein level data for the 3648 proteins quantified in this study (including the indexed retention time, or iRT, protein—a synthetic peptide spiked in to each sample used to adjust for small changes in measured mass-to-charge ratios between runs).

Sheet 1: Annotation notes

Sheet 2: Protein quantifications for all samples, both total and mitochondrial fractions.

Supplemental Table 4. Transcript-Level Data

Normalized, log-scaled transcript level data for the 35511 probesests quantified in this study, separated into tables for the liver and quadriceps (run on Affymetrix 1.0 ST arrays) and BAT

and heart (run on Affymetrix 2.0 ST arrays). Raw CEL files are available online at GEO [42] under the identifiers GSE60149, GSE60150, GSE60151, and GSE60489.

Sheet 1: Annotation notes

Sheet 2: Liver and quadriceps data for the 8 strains analyzed, using Affymetrix Mouse Gene 1.0 ST arrays.

Sheet 3: BAT and heart data for the 8 strains analyzed, using Affymetrix Mouse Gene 2.0 ST arrays. Note, the same individuals and cohorts are used for the data across all tissues.

Supplemental Figure 1. EF_{Mito} Across Tissues and Genes