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Mitochondrial phosphoproteomes are functionally specialized across tissues

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Abstract

Mitochondria are essential organelles involved in critical biological processes such as energy metabolism and cell survival. Their dysfunction is linked to numerous human pathologies that often manifest in a tissue-specific manner. Accordingly, mitochondrial fitness depends on versatile proteomes specialized to meet diverse tissue-specific requirements. Furthermore, increasing evidence suggests that phosphorylation may also play an important role in regulating tissue-specific mitochondrial functions and pathophysiology. We hypothesized that recent advances in mass spectrometry (MS)-based proteomics would now enable in-depth measurement to quantitatively profile mitochondrial proteomes along with their matching phosphoproteomes across tissues. We isolated mitochondria from mouse heart, skeletal muscle, brown adipose tissue, kidney, liver, brain, and spleen by differential centrifugation followed by separation on Percoll gradients and high-resolution MS analysis of the proteomes and phosphoproteomes. This in-depth map substantially quantifies known and predicted mitochondrial proteins and provides a resource of core and tissue

27 modulated mitochondrial proteins ([mitophos.de](#)). We also uncover tissue-specific repertoires of dozens
28 of kinases and phosphatases. Predicting kinase substrate associations for different mitochondrial
29 compartments indicates tissue-specific regulation at the phosphoproteome level. Illustrating the
30 functional value of our resource, we reproduce mitochondrial phosphorylation events on DRP1
31 responsible for its mitochondrial recruitment and fission initiation and describe phosphorylation
32 clusters on MIGA2 linked to mitochondrial fusion.

33 Introduction

34 Mitochondria are double-membrane-bound organelles with an essential role in homeostasis of eukaryotic
35 cells. They are often referred to as the “powerhouse of the cell” due to their prominent function in
36 bioenergetics. Among many other processes, they are also involved in several biosynthetic processes such
37 as balancing redox systems, the regulation of metabolic by-products like reactive oxygen species (ROS)
38 (Spinelli and Haigis, 2018) and hold a central role in cell death (Bock and Tait, 2020). The function and
39 stability of mitochondria depend on their intrinsic bioenergetics regulation and finely orchestrated
40 interaction with the cellular microenvironment. Energy conversion via the oxidative phosphorylation
41 system (OXPHOS) plays an essential role in harvesting energy from ingested nutrients. Moreover, the
42 morphology of mitochondria within an eukaryotic cell is actively regulated by fusion and fission events
43 which dynamically modulate their number, size, and localization (Liesa et al., 2009). Regulation of
44 mitochondrial dynamics also affects the interplay of mitochondria with other cellular structures, such as
45 the cytoskeleton for active regulation of their localization (Moore and Holzbaur, 2018), and organelles like
46 the endoplasmic reticulum (ER) and lipid droplets to regulate many physiological processes such as energy
47 metabolism and ion buffering. The mitochondria-associated membrane (MAM), which is the contact site
48 of the outer mitochondrial membrane with the ER, comprises a unique set of proteins mediating this
49 interaction and fine-tune mitochondrial functions with the cellular microenvironment (Kwak et al., 2020;
50 Nunnari and Suomalainen, 2012). Dysregulation of any of these intricate processes can lead to severe
51 mitochondrial dysfunctions and diseases, including neurodegenerative diseases, cardiovascular disorders,
52 myopathies, obesity, and cancers, which can manifest in a cell type- and tissue-specific manner
53 (Suomalainen and Battersby, 2018).

54 The fitness of mitochondria depends on the production and maintenance of functional as well as versatile
55 proteomes specialized to carry out a variety of functions within the eukaryotic metabolism and meet

56 diverse cellular and tissue-specific requirements (Kuznetsov et al., 2009). The mitochondrial proteome
57 includes over a thousand proteins (see below), but only a small fraction of 13 proteins are encoded on the
58 circular mitochondrial DNA molecule (Anderson et al., 1981). Thus, the majority of mitochondrial proteins
59 are encoded by the nuclear genome, synthesized outside of mitochondria and subsequently imported into
60 the organelle, implying that mechanisms controlling mitochondrial protein quality (e.g., correct protein
61 folding and import) are essential for health and integrity of mitochondria (Jadiya and Tomar, 2020).
62 Furthermore, investigations of mitochondrial dynamics and functional plasticity have revealed regulatory
63 roles for post-translational modifications (PTMs), including phosphorylation (Niemi and Pagliarini, 2021).
64 Studies have shown that phosphorylation of several mitochondrial proteins is involved in the regulation
65 of central processes such as metabolic function, for instance through phosphorylation of the E1alpha
66 subunit of PDH (Patel et al., 2014), mitophagy (Kolitsida et al., 2019) and fission (Cribbs and Strack, 2007;
67 Ducommun et al., 2015; Lewis et al., 2018; Taguchi et al., 2007; Toyama et al., 2016). Thus, deregulation
68 of protein phosphorylation might be an important underlying feature of mitochondrial physiology and
69 pathophysiology. Currently, there is a significant knowledge gap of the mitochondrial variable proteomic
70 composition and to what extent it is phosphorylated in a tissue-specific manner and how post-
71 translational regulation influences organelle function. A detailed understanding of the functional
72 specialization of mitochondria at the protein and phosphorylation levels is needed to elucidate the
73 contribution of mitochondria to health and disease.

74 Large-scale mass spectrometry (MS)-based quantitative proteomics studies from our and other groups
75 have already shed light on the proteomic composition of mitochondria of various mammalian tissues and
76 cell types, mostly highlighting that the majority of proteins are shared between mitochondria of different
77 tissues (Forner et al., 2006; Mootha et al., 2003; Pagliarini et al., 2008). The breadth and depth of such
78 studies has been largely driven by technological advances in the field in combination with improvements
79 of mitochondria isolation procedures, such as differential centrifugation (DC), DC in conjunction with

80 ultracentrifugation on e.g. Percoll gradients, magnetic bead-assisted methods (MACS) (Kappler et al.,
81 2016) or MitoTags (Bayraktar et al., 2019). Efforts in defining the mitochondrial proteome lead to
82 databases like MitoCarta2.0 and IMPI (<http://impi.mrc-mbu.cam.ac.uk/>), both integrated in Mitominer4.0
83 (Smith and Robinson, 2019). A recent quantitative and high confidence proteome of human mitochondria
84 identified 1134 different proteins that vary over six orders in magnitude in abundance (Morgenstern et
85 al., 2021). Similarly, efforts have been undertaken to map the mitochondrial phosphoproteome, and
86 identified dozens to hundreds of phosphorylation sites on mitochondrial proteins (listed in Supplementary
87 Table 1). However, there has been a dramatic improvement in the technology of phosphoproteomics
88 workflows during the last years, leading to the routine identification and quantification of thousands of
89 phosphorylation sites in cell culture and in vivo systems (Bekker-Jensen et al., 2020; Humphrey et al.,
90 2018) which had not been available in earlier studies. Furthermore, comparisons between mitochondrial
91 phosphoproteomes have been difficult because only a single or a few tissues were analyzed. This
92 complicates the combination and comparison of data sets across studies to obtain a clear view of
93 mitochondrial diversity on proteome and phosphoproteome levels. Thus, a concerted effort is needed to
94 systematically and quantitatively profile mitochondrial proteomes together with their matching
95 phosphoproteomes from the same biological source. This would further help to investigate the dynamic
96 composition of mitochondria and help identify the tissue-specific repertoire of mitochondria-resident
97 kinases and phosphatases and their substrate associations.

98 Here we performed a systematic analysis of the mitochondrial composition at the level of proteins, major
99 functional entities, and phosphorylation in seven mouse tissues – brain, brown adipose tissue (BAT), heart,
100 kidney, liver, skeletal muscle (SKM), and spleen. Our study employs state of the art MS-based proteomics
101 technology to systematically map divergent composition and phosphorylation of mitochondria between
102 tissues and provides functionally valuable insights into their proteome and post-translational regulations.
103 This study contributes to our understanding of tissue-specific mitochondrial processes controlled by

104 protein abundance and phosphorylation and helps to manipulate these in health and disease. Our
105 mitochondrial (phospho)proteomes are composed into an extensive resource and made freely accessible
106 via mitophos.de.

107 Results

108 Comprehensive mitochondria proteome coverage across various mouse tissues

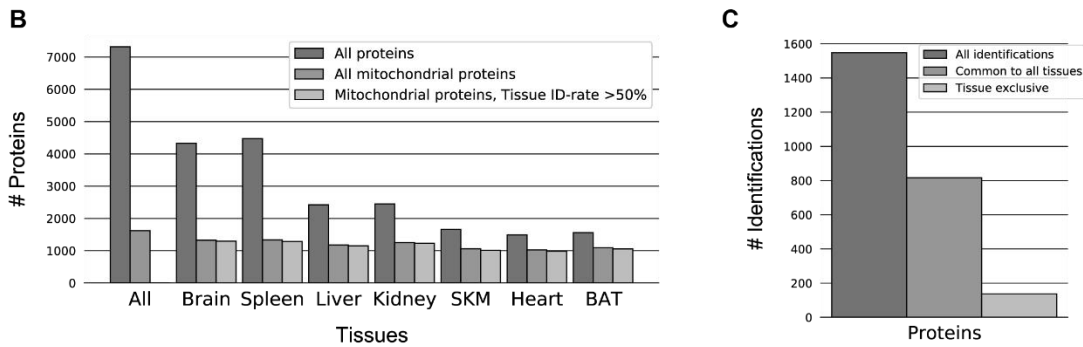
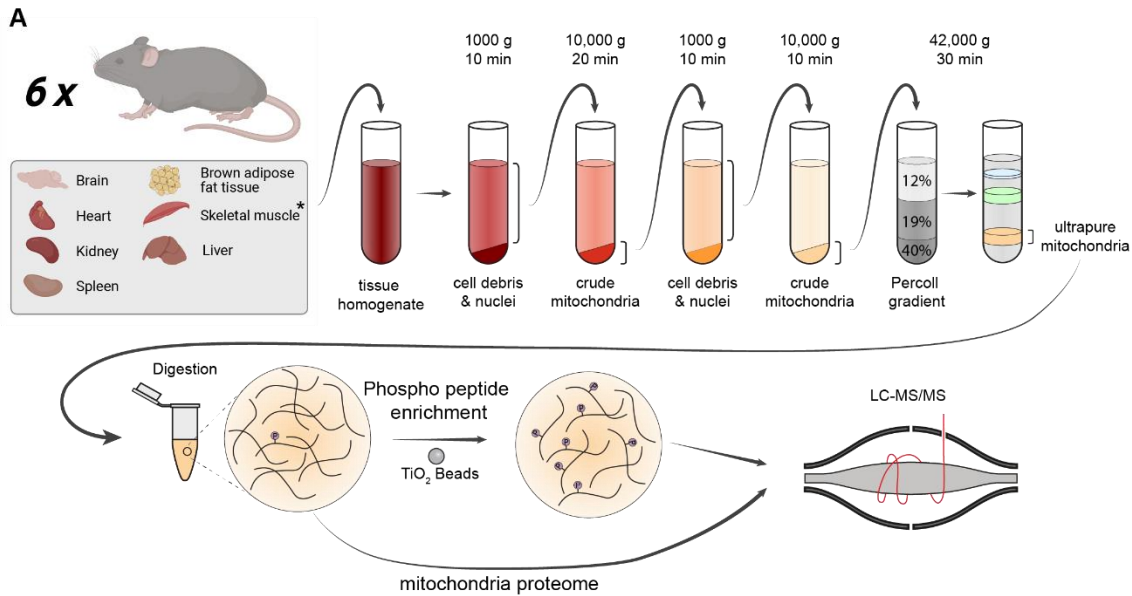
109 To advance our understanding of tissue-specific functional specializations of mitochondria at the protein
110 level, we set out to characterized proteomes of mitochondria collected from various mouse tissues by LC-
111 MS/MS analysis. To this end, we first isolated mitochondria from seven tissues – brain, brown adipose
112 tissue (BAT), heart, kidney, liver, skeletal muscle (SKM), spleen - from six 18-21 weeks old C57BL/6N mice
113 (three females and three males). Mouse tissues were homogenized with a Dounce homogenizer, and
114 crude mitochondria were isolated via differential centrifugation and subsequently purified on a Percoll
115 density gradient to obtain ultra-pure mitochondria isolates (Kuhl et al., 2017). Importantly, this procedure
116 was shown to efficiently exclude contaminations from other cellular compartments (Wieckowski et al.,
117 2009). Proteomes of these ultra-pure mitochondrial samples were acquired by a state-of-the-art
118 proteomics workflow (Figure 1A), allowing the robust identification and quantification (coefficient of
119 variation (CV) < 20%) of proteins that covered a dynamic range of more than five orders of magnitude
120 (Supplementary File 1).

121 In total, we identified over 7000 proteins of which 1620 were annotated as mitochondrial proteins using
122 MitoCarta3.0 (Rath et al., 2021) and the IMPI (<http://impi.mrc-mbu.cam.ac.uk/>) database. This essentially
123 covers (92%) the mitochondrial proteome by the measure of MitoCarta3.0 and even in the IMPI database,
124 which also includes predicted mitochondrial proteins, we still identified 62% (Figure 1B). For further
125 analysis, we filtered for proteins identified in more than half of the biological replicates in at least one
126 tissue, which resulted in 1548 mitochondrial proteins. This still represents over 90% of MitoCarta3.0 and
127 59% of the IMPI databases and highlights the deep and reproducible mitochondrial proteome coverage
128 of this study (Figure 1C). Interestingly, half of these mitochondrial proteins were identified across all
129 tissues while only 9% were exclusively detected in one specific tissue (Figure 1C), confirming previous

130 reports on mitochondrial proteomes by us and others (Calvo and Mootha, 2010; Forner *et al.*, 2006;
131 Johnson *et al.*, 2007; Mootha *et al.*, 2003). Of these, almost half (65 proteins) were both reproducibly
132 identified and not in the lowest 20% of ranked abundances (Supplementary File 2), making them clear
133 candidates for tissue specific mitochondrial proteins. A similar proportion of the mitochondrial proteome
134 was also exclusive to two or more tissues by the same criterion.

135 Mitochondrial enrichment efficiency can be determined by the proportion of summed signal intensity for
136 mitochondrial proteins in relation to all identified proteins in measured samples (Williams *et al.*, 2018).
137 Applying this strategy, we determined the proportions to be very high (>95%) for liver, kidney, SKM, heart
138 and BAT, but lower for brain (65%) and spleen (45%) (Figure 2A). These trends are consistent with the
139 established literature (Fecher *et al.*, 2019; McLaughlin *et al.*, 2020; Roberts *et al.*, 2021; Williams *et al.*,
140 2018) and can likely be explained by the high tissue heterogeneity of brain (Fecher *et al.*, 2019; Menacho
141 and Prigione, 2020) and spleen. Indeed, spleen tissue consists of various types of immune cells (Lewis *et*
142 *al.*, 2019), which might impede high purity enrichment of its organelles. For brain, contaminations by
143 synaptosomes, which themselves contain neuronal mitochondria, have frequently been observed
144 (Mootha *et al.*, 2003). Yet, correlation of both mitochondrial and all identified proteins between biological
145 replicates yielded Pearson correlation coefficients higher than 95% in all tissues (Figure 2B and Figure 2-
146 figure supplement 1A). Principle component analysis (PCA) further shows that biological replicates cluster
147 together and underlines functional similarities between tissues such as heart and SKM as well as tissue
148 related diversity of mitochondria proteomes (Figure 2C and Figure 2-figure supplement 1B).

149

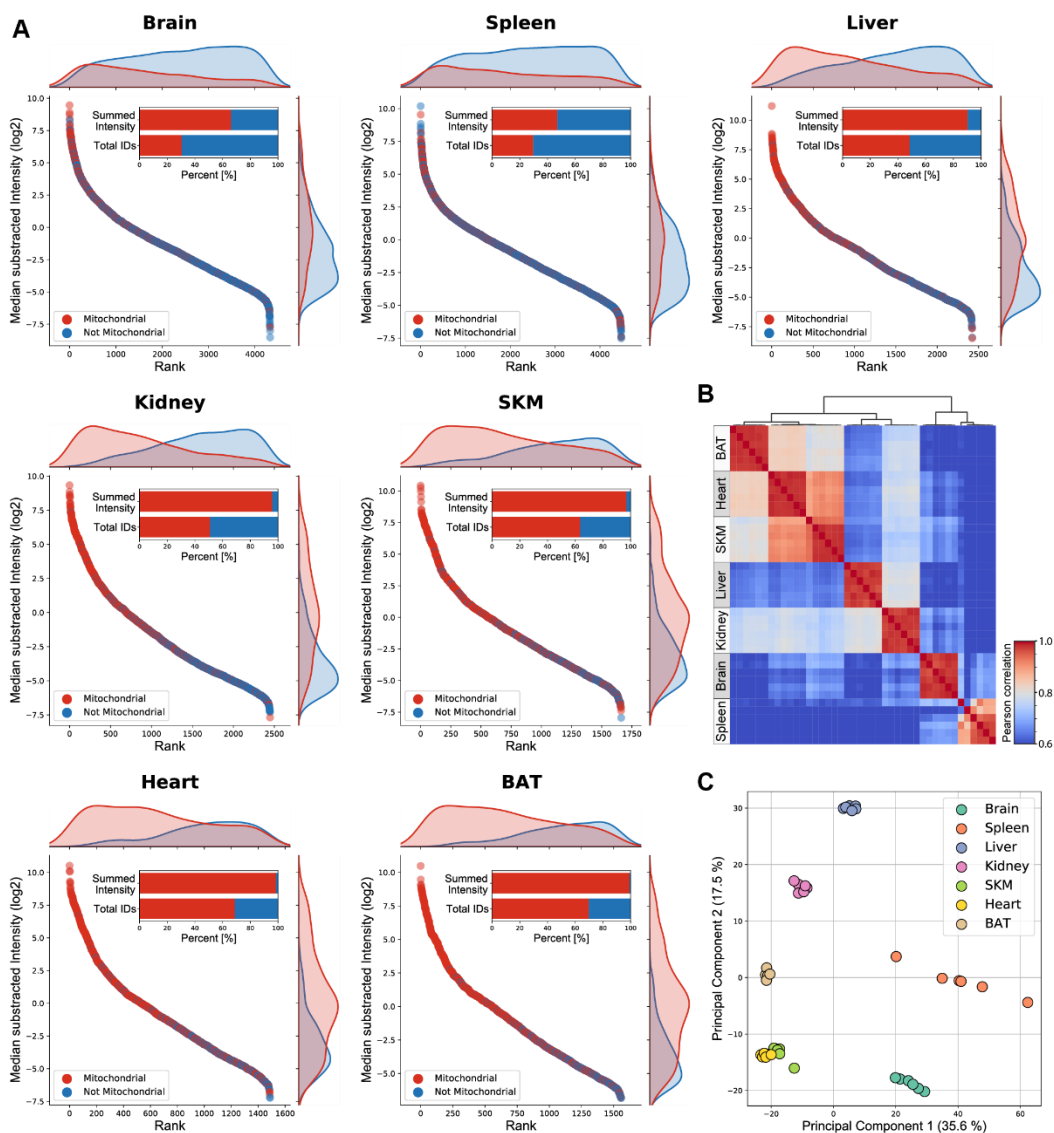


150

151 **Figure 1 Mitochondrial proteome and phosphoproteome preparation**

152 (A) Workflow of tissue preparations for mitochondrial proteome and phosphoproteome enrichment, and LC-MS/MS analysis
 153 (n=6). Tissues were first homogenized (* for skeletal muscle, see Methods), crude mitochondria were isolated and ultrapure
 154 mitochondria were obtained using a Percoll gradient. Proteins were digested and prepared for phosphoproteome analysis via
 155 TiO_2 enrichment or subjected to LC-MS/MS. (B) Distinct protein identification across biological replicates (n=6). Annotation of
 156 proteins as mitochondrial is based on MitoCarta3.0 and the IMPI database. (C) Mitochondrial protein numbers after filtering
 157 for mitochondrial proteins identified in at least 50% of biological replicates (n=6) in at least one tissue. Skeletal muscle (SKM),
 158 brown adipose tissue (BAT). See also Figure 1 – Source Data 1.

159



160

161 **Figure 2 Mitochondria enriched samples show tissue-specific clustering**

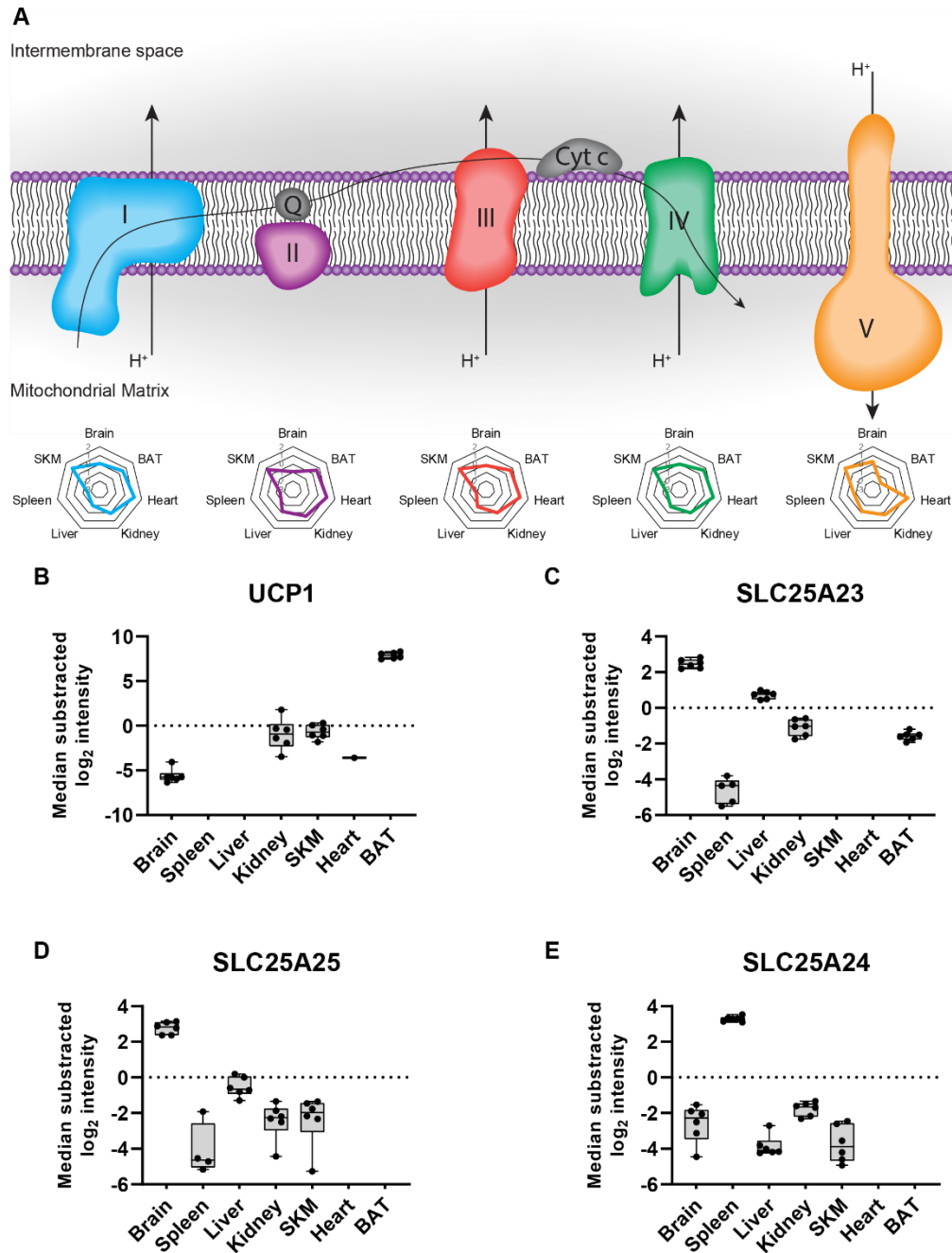
162 (A) Mitochondrial (red) and not mitochondrial (blue) proteins identified, based on the MitoCarta3.0 and IMPI database, are
 163 ranked by their intensity for each individual tissue. Histograms on the top and right display the distribution of mitochondrial
 164 (red) and not mitochondrial (blue) proteins along the rank and the Intensity axis, respectively. The percentage of all identified
 165 mitochondrial (red) and not mitochondrial (blue) proteins and their summed intensities are displayed in bar graphs. (B)
 166 Heatmap showing Pearson correlation coefficient for biological replicates (n=6) for mitochondrial proteins of all mitochondrial
 167 enriched samples. (C) Principal component analysis of mitochondrial proteins of all acquired biological replicates (n=6). Skeletal
 168 muscle (SKM), brown adipose tissue (BAT). See also Figure 2 – Source Data 1.

169 Mitochondrial proteome composition reveals tissue-specific functions

170 To gain further insights into tissue-specific functional differences in mitochondria, we investigated
171 differences in the composition of mitochondrial proteomes across tissues. First, we focused on the
172 oxidative phosphorylation (OXPHOS) system, which is essential for production of the energy-rich
173 metabolite ATP and other processes like free radical generation and apoptosis (Huttemann et al., 2007).
174 We found that proteins of the electron transport chain (Complex I – Complex IV) and ATP-synthase
175 (Complex V) displayed high abundances in heart and SKM tissues, supporting the physiological
176 requirement of high ATP levels in both muscular tissues to sustain processes like muscle contraction
177 (Ferreira et al., 2010; Ventura-Clapier et al., 2011) (Figure 3A). Conversely, levels of Complex V proteins
178 were substantially lower in mitochondria from BAT compared to all other measured tissues (Figure 3A),
179 which agrees with the specialized function of BAT in non-shivering thermogenesis (Jastroch et al., 2010;
180 Kajimura and Saito, 2014; Oelkrug et al., 2015). This was further supported by the high abundance of the
181 uncoupling protein 1 (UCP1) in our proteome measurements, the key mediator of the heat-generating
182 proton leak in the mitochondria of BAT (Figure 3B).

183 In contrast to the well described UCP1, several other members of the SLC25 family remain uncharacterized
184 (Ruprecht and Kunji, 2020). In our dataset, we identified a total of 47 members of the SLC25 family (Kunji
185 et al., 2020). We found that levels of SLC25A23 and SLC25A25, two ATP-Mg²⁺/P_i carrier paralogues, (del
186 Arco and Satrustegui, 2004; Fiermonte et al., 2004), displayed high abundance in mitochondria of brain
187 tissue (Figure 3C and Figure 3D). Interestingly, knockout of SLC25A23 was shown to increase neuronal
188 vulnerability (Rueda et al., 2015), which corroborates our observation and suggests an important role of
189 SLC25A25 in this tissue type. We also quantified a third paralogue, SLC25A24, which showed a higher
190 abundance in spleen tissue. Notably, the spleen harbors a large pool of B-cells and reduced SLC25A24
191 levels were previously linked to B-cell malignancies (Sandhu et al., 2013) (Figure 3E). Although these three
192 ATP-Mg²⁺/P_i carriers were not detected in mitochondria from heart tissue, we identified another class of

193 ATP carriers, including SLC25A4 or SLC25A31, which both showed increased abundance in heart compared
 194 to all other measured tissues. Such differences between mitochondrial proteome compositions are easily
 195 retrieved from our dataset, facilitating a better understanding of mitochondrial plasticity across tissues.



196

197 **Figure 3 Tissue-specific protein contribution of SLC25 proteins the mitochondrial proteome**

198 (A) Representation of the oxidative phosphorylation (OXPHOS) system including from left to right the electron transport chain
199 (Complex I (blue), Complex II (violet), Complex III (red), Complex IV (green)) and ATP synthase (Complex V (yellow)). Radar plots
200 show the relative contribution of the corresponding complex across the analyzed tissues to the overall mitochondrial
201 composition (see methods for detailed description). (B) Normalized intensity (median of all \log_2 transformed mitochondrial
202 proteins of a sample was subtracted from all \log_2 protein intensities of that sample) of the uncoupling protein 1 (UCP1), (C)
203 SLC25A23, (D) SLC25A25, and (E) SLC25A24 across all analyzed tissues (black dots indicate individual identifications). Protein
204 abundance differences in relation to the reference tissue (BAT in B, Brain in C and D and spleen in E) were significant (p-value
205 <0.0001) by one-way ANOVA analysis (Figure 3 - Source Data 1). Data in this figure is based on the analysis of six replicates
206 (n=6) for each tissue. Skeletal muscle (SKM), brown adipose tissue (BAT). See also Figure 3 – Source Data 1.

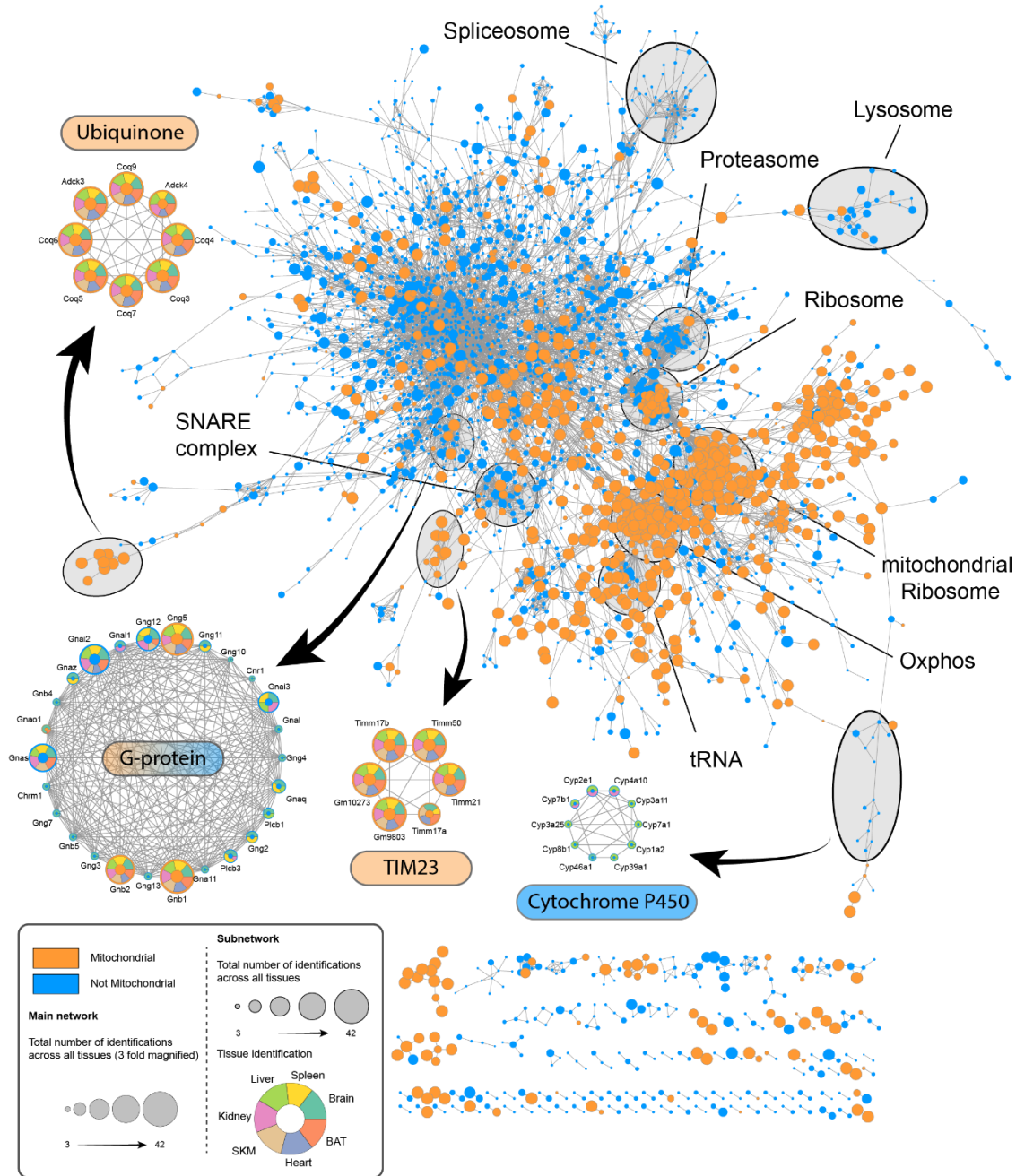
207

208 Tissue-specific function of mitochondria-associated proteins

209 The identification of key proteins mediating the crosstalk of mitochondria with their cellular environment
210 is crucial to better understand their tissue-specific regulation and this concept has already attracted
211 considerable interest in recent years (Montes de Oca Balderas, 2021). Although the characterization of
212 local proteomes of organellar contact sites usually require special centrifugation-based isolation methods,
213 we anticipated that a considerable fraction of mitochondria-associated proteins would also enrich along
214 with mitochondria in our samples. We first performed an annotation term enrichment analysis of all
215 identified proteins. While we observed significant enrichment of several mitochondria related terms in all
216 tissues as expected, the non-mitochondrial protein pool was largely enriched for terms related to the
217 tissue of origin (Figure 4 – supplement 1, Figure 4 - Source Data 1). For instance, terms like ‘epoxygenase
218 P450 pathway’ in liver, ‘positive regulation of B cell activation’ in spleen or ‘positive regulation of
219 excitatory postsynaptic potential’ in brain tissue highlight tissue-specific functions.

220 Next, we performed a network analysis to evaluate the nature and quality of co-enriched proteins, more
221 specifically whether non-mitochondrial proteins identified in these samples are associating proteins with
222 functional roles or biological contaminations that are likely tissue-specific and high abundant. This analysis

223 revealed several clusters of known mitochondrial complexes such as the Tim23 complex or processes like
224 the ubiquinone biosynthetic process, whose members were robustly identified in all tissues, and several
225 tissue-specific clusters including both mitochondrial and non-mitochondrial proteins (Figure 4). For
226 instance, in line with terms enriched for co-sedimenting proteins in liver tissue, we identified a cluster of
227 cytochrome P450 superfamily members exclusively in liver tissue (Figure 4). Intriguingly, CYP2E1 and
228 CYP1A2, two specific members of this family, were previously shown to be targeted to the mitochondria
229 (Avadhani et al., 2011; Genter et al., 2006; Robin et al., 2001), and we now find evidence of the enrichment
230 of many more family members in this tissue. Another identified cluster consisted of G proteins, some of
231 which were identified throughout all tissues and previously annotated as mitochondrial (e.g. GNB1, GNB2
232 and GNG5) or shown to localize to mitochondria (GNAI2) (Beninca et al., 2014). Interestingly, like the
233 majority of the proteins in the G protein cluster, many G protein coupled receptors (GPCR) were
234 exclusively identified in brain tissue. A prominent member of these brain specific GPCRs is CNR1, which
235 was reported to localize to mitochondria where it plays an important role in the regulation of memory
236 processes through the modulation of the mitochondrial energy metabolism (Hebert-Chatelain et al.,
237 2016). These examples highlight the value of our dataset for uncovering novel mitochondrial and
238 mitochondria-associated proteins in an unbiased way.



239

240 **Figure 4 Proteome of mitochondria enriched samples displays tissue-specific complexes**

241 Cytoscape network analysis of reproducibly (> 50% identification rate in at least one tissue, six replicates were analyzed (n=6))

242 identified proteins of all mitochondria enriched samples. The main network depicts mitochondrial (orange) and non-

243 mitochondrial (blue) proteins with at least one edge (String score >0.95). The size of individual nodes represents the number of

244 identifications ranging from 3 (small circle) to 42 (big circle). Subnetworks display mitochondrial (orange) and non-mitochondrial

245 (blue) proteins and tissues in which they were identified (dark green – brain; yellow – spleen; light green – liver; pink – kidney;
246 ochre – SKM; blue – heart; orange – BAT). Skeletal muscle (SKM), brown adipose tissue (BAT). See also Figure 4 – Source Data 1.

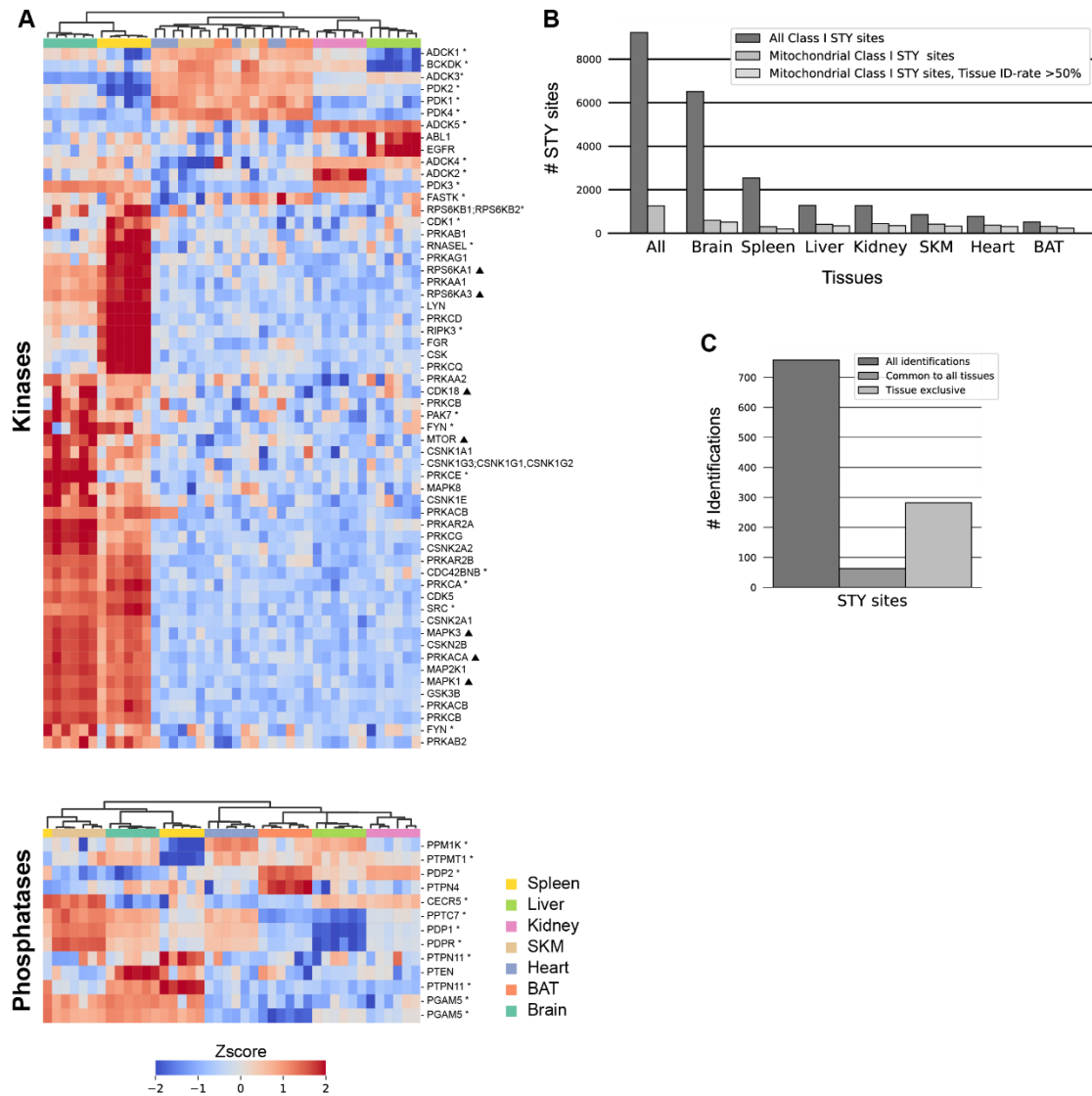
247

248 Mitochondrial kinases and phosphatases show tissue specificity

249 Post-translational modification of proteins, specifically phosphorylation, plays a crucial role in the
250 orchestration of mitochondrial protein function (Niemi and Pagliarini, 2021). However, almost no
251 mitochondrial kinases with mitochondrial targeting sequences have been consistently reported and most
252 kinases shown to associate with mitochondria have been found on or interact with the outer membrane
253 (Kotrasova et al., 2021). Given our deep mitochondrial proteomes, we investigated relative abundances
254 of kinases and phosphatases, which are annotated as or suggested to be mitochondrial, across mouse
255 tissues.

256 Firstly, we observed clear differences in abundances of identified mitochondrial kinases and
257 phosphatases, including well described matrix kinases and phosphatases, between tissues (Figure 5A). For
258 instance, PDK1, PDK2, and PDK4 contributed preferentially to the composition of heart, SKM and BAT
259 mitochondria, while the PDK3 was more abundant in brain, spleen, and kidney mitochondria. These tissue-
260 related differences are in line with earlier reports and suggest a specialized function of PDK3, which may
261 originate in its insensitivity to pyruvate inhibition (Klyuyeva et al., 2019; Sadana et al., 2018). Similarly,
262 levels of the heterodimeric pyruvate dehydrogenase phosphatase consisting of PDP1 and PDPr were
263 elevated in brain, SKM, and heart compared to the remaining tissues, whereas PDP2 contributes more to
264 the composition of liver, kidney, and BAT mitochondria. While our study confirmed previous reports on
265 the differential expressions of these proteins in a tissue-specific manner (Huang et al., 1998; Huang et al.,
266 2003), it also provided quantitative data to assess the magnitude of these differences. Together, our
267 results imply a tailored regulation of kinase and phosphatase abundances across tissues to modulate the
268 mitochondrial phosphoproteome.

269 To investigate if and how kinase and phosphatase levels translate into protein phosphorylation, we
270 analyzed the mitochondrial phosphoproteomes of the same samples collected from all seven tissues
271 (Figure 1A). This analysis resulted in the identification of 1263 phosphorylation sites on 626 mitochondrial
272 proteins (Figure 5B, Figure 5 - Source Data 1). After stringent filtering of the data for more than four
273 identifications across six biological replicates in at least one tissue and a site localization score higher than
274 75%, we obtained a dataset of 758 phosphorylation sites on 423 mitochondrial proteins (Figure 5C, Figure
275 5 - Source Data 1). Of these high confidence sites, 16% have previously not been reported in mouse
276 according to the PhosphoSitePlus (PSP) database (Hornbeck et al., 2012). Strikingly, in contrast to the
277 mitochondrial proteomes, 37% of the phosphosites identified on mitochondrial proteins were exclusive
278 to one tissue, and only 8% were identified in all the tissues measured (Figure 5C). This might indicate that
279 mitochondrial diversity is more strongly pronounced at the phosphorylation than the protein level.



280

281 **Figure 5 Tissue specificity of mitochondrial kinases and phosphatases**

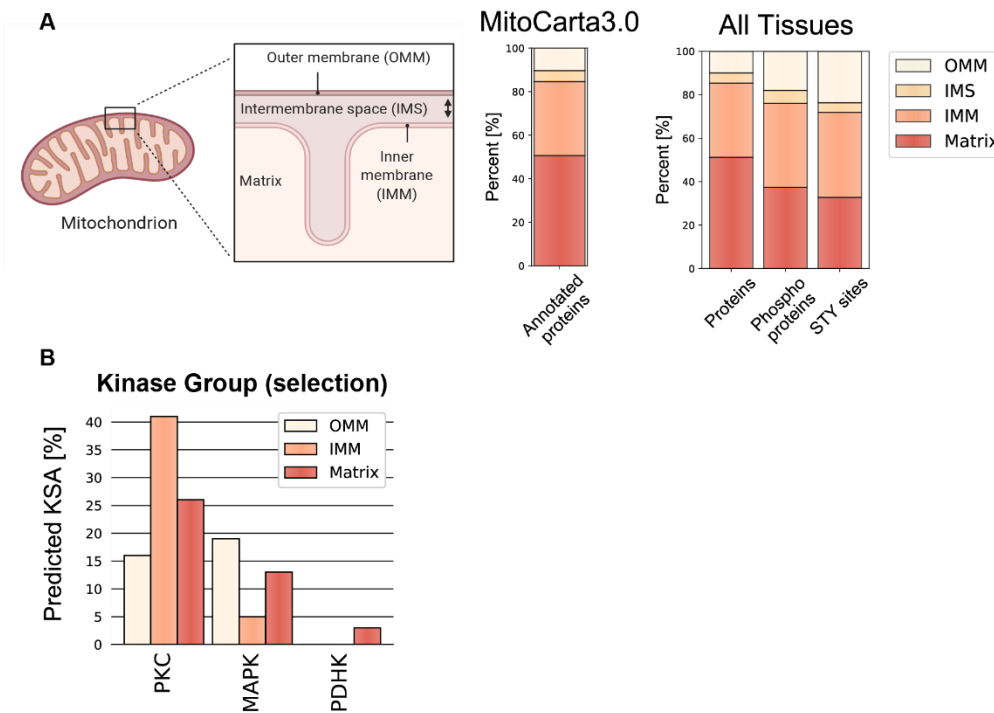
282 (A) Z-scored protein abundances for predicted (triangle), known (star) (based on MitoCarta3.0 and IMPI database), and manually
 283 curated (Figure 5 - Source Data 1) mitochondrial kinases (top) and phosphatases (bottom) across analyzed tissues. Skeletal muscle
 284 (SKM), brown adipose tissue (BAT). (B) STY site identification numbers. (C) Mitochondrial STY site numbers after filtering for
 285 mitochondrial STY sites identified in at least 5 out of 6 biological replicates in one tissue. Identification numbers for all identified
 286 STY sites (left), STY sites common to all tissues (middle), and STY sites exclusive to one tissue (right) are shown. Data in this figure
 287 is based on the analysis of six replicates (n=6) for each tissue. Skeletal muscle (SKM), brown adipose tissue (BAT). See also Figure
 288 5 – Source Data 1.

289 Mitochondrial phosphoproteomes exhibit extensive intra-mitochondrial phosphorylation

290 To understand the distribution of mitochondrial phosphoproteins across mitochondrial compartments we
291 examined the sub-mitochondrial localization based on the curated MitoCarta3.0 annotation.
292 Mitochondria are typically divided into four main compartments, i.e. mitochondrial outer membrane
293 (OMM), intermembrane space (IMS), inner mitochondrial membrane (IMM) and matrix, although the
294 complex organization of the IMM possibly may define additional compartments (Colina-Tenorio et al.,
295 2020). In line with the high proportion of shared mitochondrial proteomes across all tissues (Figure 1C),
296 the overall localization of proteins was not different between tissues and closely resembled the
297 distribution of all annotated mitochondrial proteins in the database (Figure 6A). However, when
298 performing the same analysis using the phosphorylated mitochondrial proteins, we observed a significant
299 shift towards a localization to the OMM in all tissues (adj. p-values $<6.4 \times 10^{-10}$)(Figure 6A, Figure 6 –
300 supplement 1). Surprisingly, our data also showed that depending on the tissue type, more than 60% of
301 phosphorylated mitochondrial proteins had an intra-mitochondrial annotation - IMM, IMS or matrix
302 localization - (Figure 6A, Figure 6 – supplement 1). Interestingly, 36-54% of OMM, but only 7-22% of intra-
303 mitochondrial proteins were phosphorylated. Here, especially brain (10%) and spleen (7%) tissues showed
304 low intramitochondrial phosphorylation rates.

305 This prompted us to further investigate the localization of specific kinase-substrate associations (KSA)
306 across sub-mitochondrial localizations using NetworKin3.0 (Horn et al., 2014). Prominently, more than
307 40% of the predicted KSA in the IMM were linked to the PKC kinase family (Figure 6B). Studies have already
308 reported the localization of PKC kinase family members to mitochondria, as well as an increased
309 phosphorylation of the IMM protein COX IV after PKC ϵ activation (Baines et al., 2002; Jaburek et al., 2006;
310 Majumder et al., 2000; Ogbi and Johnson, 2006; Ping et al., 2002). Moreover, the MAPK group appeared
311 to act on proteins localized to the OMM and matrix, while the PDHK family was specifically associated
312 with the matrix proteins (Figure 6B). The members of the latter kinase family are known to localize to the

313 mitochondria matrix (Hitosugi et al., 2011), further supporting the validity of identified KSA. However,
 314 molecular studies are needed to investigate such KSA, whether phosphorylation of intra-mitochondrial
 315 proteins occurs *in situ* or outside mitochondria before being imported into mitochondria, how and which
 316 kinases/phosphatases translocate to or into mitochondria and whether these phosphorylation events are
 317 functionally relevant.



318

319 **Figure 6 Localization distribution of mitochondrial phosphoproteome diverges from mitochondrial**
 320 **proteome**

321 (A) Simplified scheme of a mitochondrion with four different mitochondrial localizations – outer mitochondrial membrane
 322 (OMM), intermembrane space (IMS), inner mitochondrial membrane (IMM), matrix – and the distribution of mitochondrial
 323 proteins contained in and classified by the MitoCarta3.0 database. Bar graphs show the precentral distribution of mitochondrial
 324 proteins (left), phosphoproteins (middle) and STY sites (right) across different mitochondrial localizations. (B) Predicted Kinase
 325 substrate associations (KSA) by the NetworKin3.0 tool for selected kinase families. Data in this figure is based on the analysis of
 326 six replicates for 7 different tissues. See also Figure 6 – Source Data 1.

327

328 Mitochondrial phosphoproteome reveals tissue-specific modulation of fusion and fission 329 events

330 The tissue-specific phosphorylation of mitochondrial proteins suggested functional differences in
331 mitochondria and prompted us to investigate the influence of phosphorylation on mitochondrial
332 dynamics. We focused on proteins involved in mitochondrial fusion and fission, two important
333 counteracting events involved in organelle distribution, size balancing and maintenance of a healthy
334 mitochondrial network (Liu et al., 2020; Silva Ramos et al., 2019). Especially proteins involved in the fission
335 process are regulated by a range of protein modifications, including phosphorylation (Cribbs and Strack,
336 2007; Taguchi *et al.*, 2007; van der Blik et al., 2013).

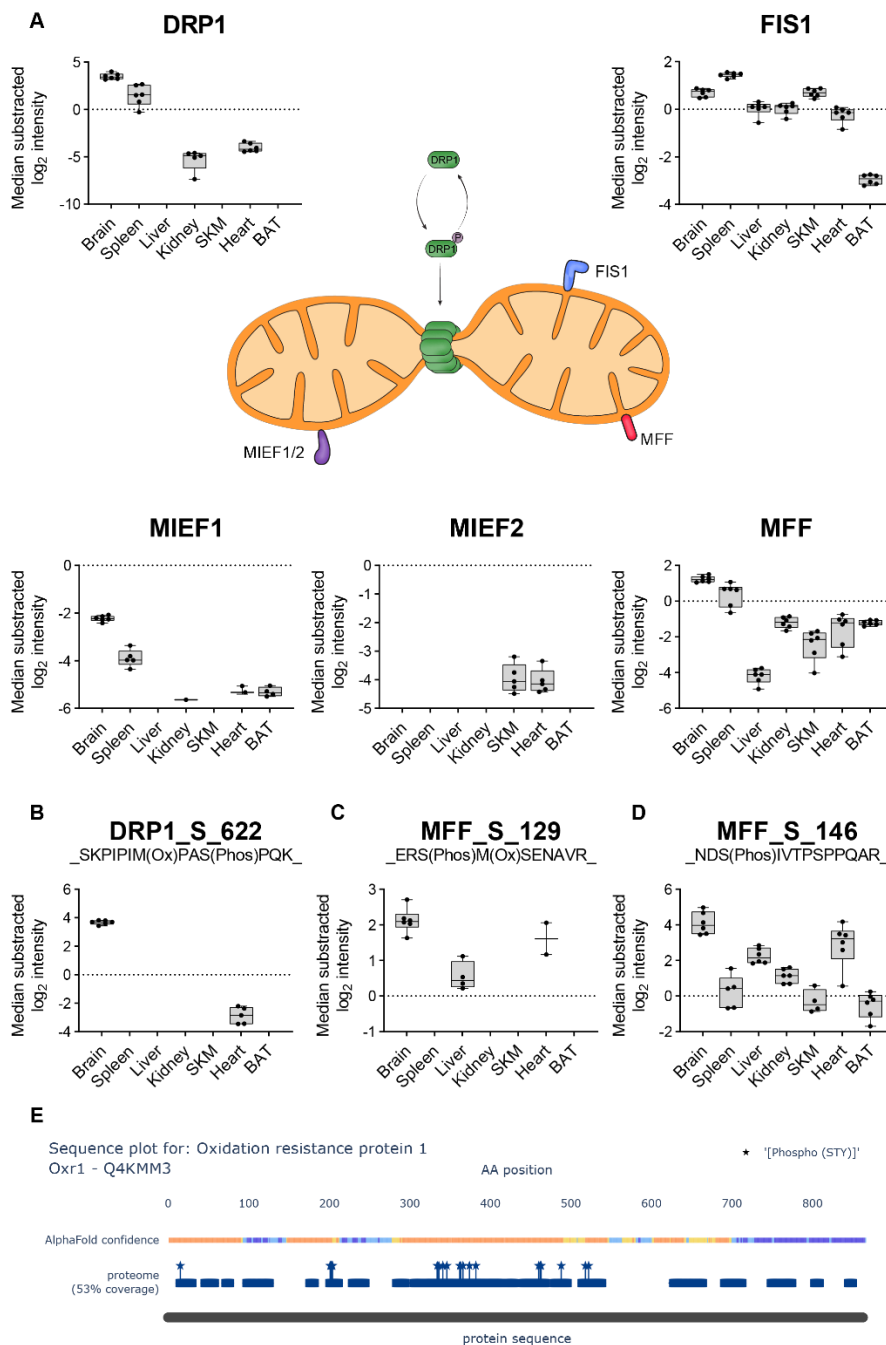
337 Throughout all tissues, MIGA1 (FAM73A) and MIGA2 (FAM73B), two homologues regulating
338 mitochondrial fusion by functioning downstream of the mitofusins, showed different abundances (Figure
339 6 – supplement 2). This is especially interesting since MIGA1 and MIGA2 can form hetero and
340 homodimers, highlighting a different regulation of fusion in different tissues (Zhang et al., 2016).
341 Moreover, we identified multiple phosphorylation site clusters on MIGA2, while none were identified on
342 MIGA1 (Figure 6 – supplement 2). Intriguingly, similar phosphorylation clusters were identified on Miga
343 in *Drosophila melanogaster* (Xu et al., 2020). Interestingly, two phosphorylation sites on Miga, S246 and
344 S249, were reported to be essential for Vap33 interaction and the establishment of endoplasmic
345 reticulum–mitochondria contact site (ERMCS), suggesting that phosphorylation on MIGA2 has similar
346 functions in mammals (Xu *et al.*, 2020).

347 Moreover, we observed that GTPase dynamin-related protein 1 (DRP1), a crucial player initiating
348 mitochondrial fission (Bleazard et al., 1999; Cereghetti et al., 2008), displayed higher abundance in brain
349 compared to other tissues (Figure 7A). This observation supports the importance of mitochondrial fission
350 in neurons, where mitochondria switch to a fragmented morphology to enter and travel through axons

351 (Lewis *et al.*, 2018). Additionally, we also found elevated levels of serine 622 phosphorylation on DRP1 in
352 brain tissue. This site has been shown to regulate DRP1 translocation to mitochondria (Cereghetti *et al.*,
353 2008; Cribbs and Strack, 2007; Taguchi *et al.*, 2007), indicating that it is actively localized to mitochondria,
354 likely to regulate constant fission events in the brain tissue (Figure 7B).

355 In mammals, four DRP1 receptor proteins, that are all integral membrane proteins of the OMM, have
356 been reported: mitochondrial fission protein 1 (FIS1), mitochondrial fission factor (MFF), and
357 mitochondrial dynamics protein MiD49 (MIEF1) and MiD51 (MIEF2) (Loson *et al.*, 2013). MFF and FIS1,
358 the fission promoting receptors, were robustly quantified in all tissues and displayed higher abundances
359 in brain and spleen compared to other tissues (Figure 7A). However, MIEF1/2, which counteract DRP1-
360 mediated fission (Dikov and Reichert, 2011; Liu *et al.*, 2013), were generally too low to be robustly
361 quantified in the measured tissues. Additionally, we detected higher levels of MFF phosphorylation at the
362 serine 129 and 146 residues in brain tissue compared to all other tissues in which they were detected
363 (Figure 7C and 7D). Both sites are essential for the recruitment of DRP1 and initiation of fission
364 (Ducommun *et al.*, 2015; Lewis *et al.*, 2018; Toyama *et al.*, 2016).

365 Elevated DRP1 levels have been shown to increase ROS levels (Watanabe *et al.*, 2014). Intriguingly, we
366 identified oxidation resistance 1 (OXR1), exclusively in the mitochondria of brain tissue, where it plays an
367 important role in the protection of neuronal cells from oxidative stress (Volkert and Crowley, 2020). This
368 likely indicates a protective function of OXR1 in brain tissue as a response to the prevalent fragmented
369 organellar morphology induced by DRP1. In addition, we found OXR1 to be hyperphosphorylated and
370 three out of 12 high confidence sites were novel (Figure 7E). Tissue specificity and the lack of functional
371 annotation of phosphorylation sites that are identified in our study make OXR1 an exciting candidate to
372 be investigated in the future.



373

374 **Figure 7 Phosphoproteome reveals tissue-specific functionality for mitochondrial fission**

375 (A) Scheme displays the reversible phosphorylation and the connected localization change to mitochondria of DRP1 (green).
 376 Normalized intensities (median of all \log_2 transformed mitochondrial proteins of a sample was subtracted from all \log_2 protein
 377 intensities of that sample) of DRP1 across all analyzed tissues (black dots indicate individual identifications) are displayed in the
 378 upper left. Mitochondrial DRP1 receptors – FIS1 (blue), MIEF1/2 (violet), MFF (red) – and corresponding box plots are shown next

379 to their receptors. (B) Normalized intensities (median of all \log_2 transformed mitochondrial phosphopeptide of a sample was
380 subtracted from all \log_2 peptide intensities of that sample) of the phosphopeptide showing S622 phosphorylation on DRP1 (black
381 dots indicate individual identifications). (C) Same as (B) showing S129 phosphorylation on MFF. (D) Same as (B) showing S146
382 phosphorylation on MFF. Significance of protein abundance differences in relation to the reference tissue (brain for DRP1 panel,
383 brain and spleen in FIS1 and MFF panel) were estimated (p-values <0.001, except for SKM in FIS1 panel) by one-way ANOVA
384 analysis (Figure 7 - Source Data 1). Significance of phosphosites abundance differences in (B) and (C, only brain and liver) were
385 estimated by a two-side t-test (Figure 7 - Source Data 1). Significance of MFF_S_146 abundance differences in relation to the
386 reference tissue (brain) were estimated (p-value<0.05) by one-way ANOVA analysis (Figure 7 - Source Data 1). Data in this figure
387 is based on the analysis of six replicates (n=6). Skeletal muscle (SKM), brown adipose tissue (BAT). See also Figure 7 – Source
388 Data 1.

389

390 Web application makes Mouse Mitochondria Atlas data readily accessible

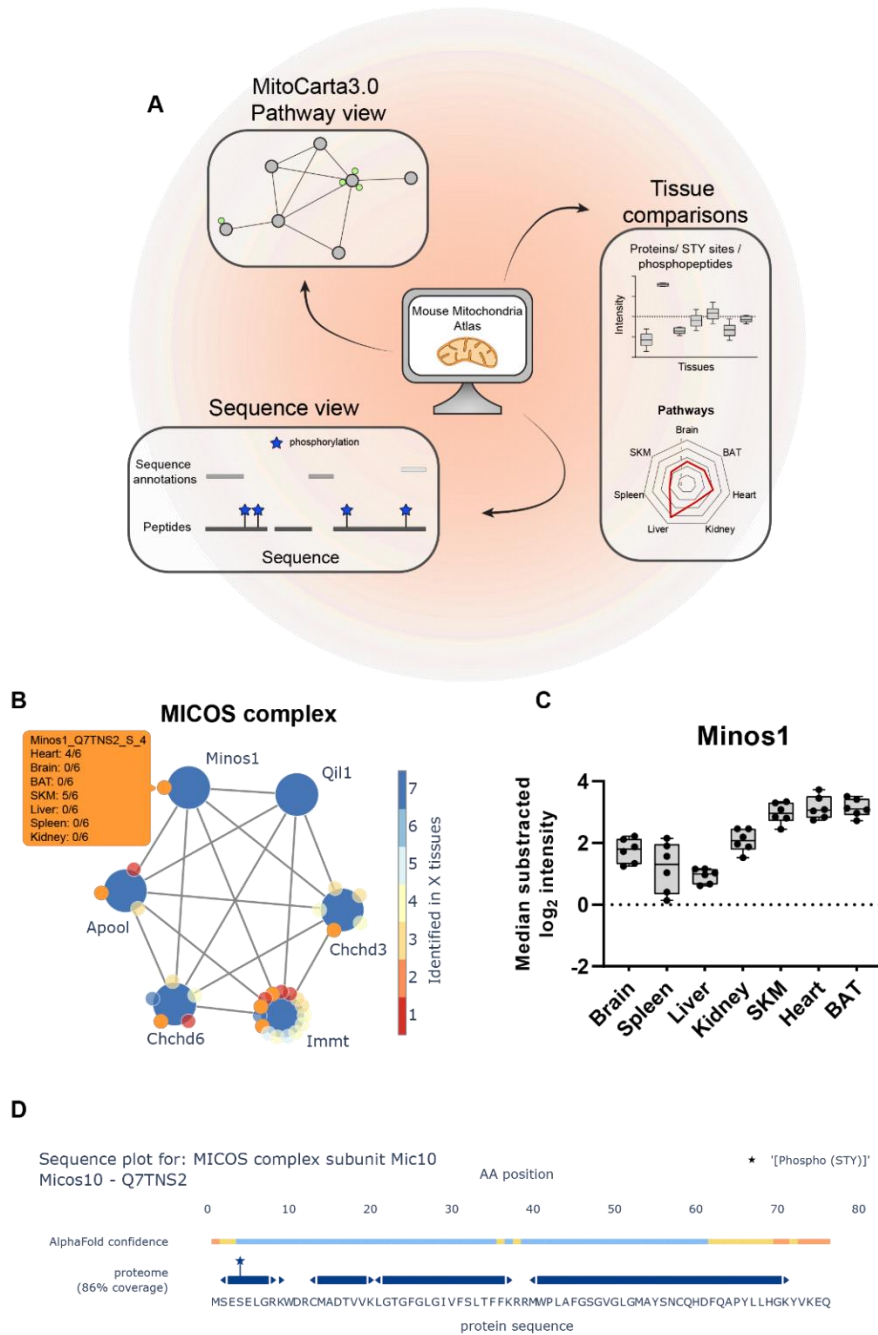
391 As indicated by the above examples, this study presents a rich resource to explore the mitochondrial
392 proteomes and phosphoproteomes across mouse tissues. Preceding examples show the potential of this
393 resource for investigation of tissue-specific mitochondrial regulations on the proteome and
394 phosphoproteome level, ultimately permitting the generation and analysis of new hypotheses. However,
395 utilization of such resources largely depends on the ease of data access for exploration.

396 To this end, we created a web application mitophos.de offering the end user an interface to easily explore
397 datasets, including MitoCarta3.0 networks, abundance comparisons across tissues and sequence analysis
398 (Figure 8A). As an example, Figure 8 illustrates the MICOS complex, which has a central role in
399 mitochondria (Khosravi and Harner, 2020). In the network view one can see (I) members of the complex
400 as well as the phosphorylation sites identified on these proteins and (II) in which tissues and how
401 reproducibly they are identified in our dataset (Figure 8B). Moreover, the user can inspect individual
402 abundance distributions of all identified proteins/STY sites across all measured tissues. For instance,
403 MINOS1, a core component of the MICOS complex (Bohnert et al., 2015), displayed high abundance in

404 mitochondria isolated from heart, SKM and BAT tissues (Figure 8C). Moreover, in the sequence view the
405 AlphaMap tool (Voytik et al., 2021) is integrated to map all identified peptides, including phosphorylated
406 peptides, onto their respective protein sequence along with structural information such as topological
407 domains and transmembrane regions (Figure 8D) and to visualize phosphorylation sites in their 3D
408 structures (unpublished data) as predicted by AlphaFold (Jumper et al., 2021).

409 Together, this data-rich and comprehensive tool is an entry point to investigate the herein presented
410 resource and will assist in future efforts to functionally characterize mitochondrial proteins and their
411 respective phosphorylation sites.

412



413

414 **Figure 8 Web application readily enables easy data access**

415 (A) Scheme of Mouse Mitochondria Atlas application features (mitophos.de). (B) MICOS complex view based on MitoCarta3.0
 416 annotation. Large and small nodes represent proteins and class I STY sites, respectively. Edges represent on String interaction
 417 scores >0.4 and color of nodes indicate the number of tissues in which proteins/STY sites were identified. (C) Normalized
 418 intensities (median of all \log_2 transformed mitochondrial proteins of a sample was subtracted from all \log_2 protein intensities of

419 that sample) for Micos1 across all analyzed tissues (black dots indicate individual identifications, box and error bar). (D) Sequence
420 plot of Micos1 shows structural information (based on UniProt annotations) and protein coverage based on identified peptides.
421 All identified STY sites are marked with a star. Data in this figure is based on the analysis of six replicates (n=6). Skeletal muscle
422 (SKM), brown adipose tissue (BAT).

423 Discussion

424 Here we present a tissue-specific atlas of mouse mitochondrial proteomes and phosphoproteomes, an in-
425 depth resource towards a better understanding of the composition and function of this vital organelle in
426 a tissue-specific manner. Previous MS-based studies combining mitochondrial phosphoproteome and
427 proteome measurements typically focused on a single or few tissues and were generally shallower than
428 our study. In addition, differences in study designs such as utilization of various organisms or
429 mitochondria/phosphopeptide enrichment protocols, analysis pipelines, and mitochondrial protein
430 annotation databases, complicate the integration of such datasets to understand tissue specificity. We
431 now globally and precisely quantified different protein expression and phosphorylation patterns at
432 subcellular level across seven mouse tissues, providing a detailed view on mitochondrial diversity. The
433 breadth and depth of coverage achieved by the integration of tissue-specific mitochondrial proteomes
434 and phosphoproteomes provide unbiased insights into the mitochondrial composition and function. This
435 allows the generation and assessment of novel hypotheses related to mitochondrial biology, which cannot
436 be generated with focused studies alone. Integrated mitochondrial proteomes and phosphoproteomes
437 that are diverse between tissues can readily be explored at mitophos.de.

438 Our data revealed that the functional diversity of mitochondria is defined by protein abundance rather
439 than compositional differences since more than half of the mitochondrial proteome was shared by all
440 analyzed tissues and 90% by at least two tissues. For instance, the electron transport chain is an integral
441 part of the mitochondrial composition and its components are found across all tissues, however, their
442 abundance shows substantial differences to meet tissue-specific energy demands. Thus, dysregulation of
443 individual proteins can strongly affect mitochondria in one tissue, leading to severe diseases, while
444 mitochondria in a different tissue remain largely unaffected. Moreover, we found that 9% of the proteome
445 displays tissue specificity, further contributing to our understanding of tissue-specific effects of

446 mitochondrial protein dysregulation (Russell et al., 2020). This is an important concept, which can now be
447 studied in an unbiased manner using our resource data, providing opportunities for development of
448 targeted treatments for mitochondrial diseases. For instance, members of the SLC25 family are linked to
449 metabolic diseases in distinct tissues (Palmieri and Monne, 2016) and various cancers (Rochette et al.,
450 2020). However, biological functions of a large repertoire of mitochondrial SLCs are still unknown. For
451 example, inactivation of SLC25A25 was assessed in mouse skeletal muscle tissue where it caused a
452 reduced metabolic efficiency (Anunciado-Koza et al., 2011). Given its high abundance in mitochondria of
453 brain tissue and in glioma cells (Traba et al., 2012), it will also be interesting to investigate its role in this
454 tissue, particularly whether SLC25A25 deficiency influences neuronal fitness.

455 Mitochondria are essential cellular entities that are involved in a wide variety of cellular processes
456 (McBride et al., 2006) through dynamic interaction and constant communication with other organelles
457 such as the (ER), nucleus and peroxisomes via membrane contact sites (Desai et al., 2020; Perrone et al.,
458 2020; Shai et al., 2018) or protein complexes, such as the ribosome (Lashkevich and Dmitriev, 2021). We
459 suggest that non-mitochondrial proteins identified in the samples might present signatures that could
460 convey important biological information regarding mitochondria-associated structures. Given the high
461 level of mitochondrial enrichment combined with highly reproducible LC-MS/MS measurements, such
462 protein signatures are unlikely to be solely based on unspecific enrichment of abundant proteins. For
463 example, the proteasome is robustly identified in most of the mitochondria enriched samples, which is in
464 line with its involvement in the degradation of misfolded mitochondrial proteins (Basch et al., 2020;
465 Kodron et al., 2021). We also prominently observed that several members of the cytochrome P450
466 superfamily are enriched in liver mitochondria, demonstrating the versatile interaction of mitochondria
467 with their cellular environment. Furthermore, our data identified non-mitochondrial ribosomal proteins
468 in all tissues, which could be explained by the local translation of nuclear-encoded mitochondrial mRNAs
469 (Lashkevich and Dmitriev, 2021). It was recently shown that RNA-bearing late endosomes associate with

470 mitochondria and ribosomes forming hotspots of local protein synthesis in axons (Cioni et al., 2019) and
471 that mitochondria fuel such local translation in neurons, enabling synaptic plasticity (Rangaraju et al.,
472 2019).

473 There is mounting evidence that phosphorylation of mitochondrial proteins fulfills important functions to
474 maintain cellular health as exemplified by the fission process in this study. Deregulation of mitochondrial
475 protein phosphorylation can lead to diseases such as cancer, diabetes, heart and neurological disorders.
476 It was recently reported that 91% of mitochondrial proteins on MitoCarta3.0 have at least one
477 phosphorylation site reported on the PSP database (Niemi and Pagliarini, 2021). However, this analysis
478 also includes proteins that do not always localize to mitochondria (Ben-Menachem et al., 2011) and
479 phosphorylation sites that can specifically be captured upon perturbations and stimuli. Our study revealed
480 that around half of the mitochondria-localized proteins were phosphorylated in tissues at steady state -
481 39% in brain, 15% in spleen, 29% in liver, 28% in kidney, 32% in SKM, 31% in heart and 22% in BAT. This
482 suggests that the mitochondrial proteome and phosphoproteome compositions are dynamically
483 modulated in response to environmental changes. Furthermore, we identified over 60 kinases and 10
484 phosphatases that either are localized to mitochondria or associate with mitochondria, providing a global
485 view on important modulators of mitochondrial protein phosphorylation. Mapping tissue-specific
486 mitochondrial kinases and phosphatases is an important step towards understanding their role in the
487 regulation of the mitochondrial phosphoproteome in different tissues and hence developing therapeutics
488 for mitochondrial diseases. For example, a mitochondrial phosphatase, phosphoglycerate mutase family
489 member 5 (PGAM5), has recently emerged as an important regulator of mitochondrial homeostasis.
490 Deletion of PGAM5 has been shown to result in Parkinson's-like movement disorder in mice (Lu et al.,
491 2014) and T cell dysfunction in primary cells (Panda et al., 2016). While its diverse roles largely remain to
492 be uncovered (Liang et al., 2021), a novel PGAM5 inhibitor was recently suggested as a potential
493 therapeutic for brain ischemic stroke (Gao et al., 2021). We observed that PGAM5 displays elevated levels

494 in the mitochondria isolated from brain, SKM and spleen, possibly explaining the tissue-specific
495 phenotypes induced by its absence and where in the body molecules targeting its phosphatase activity
496 would exert their effects.

497 The data in this study will contribute to our understanding of the tissue-specific composition and function
498 of mitochondria and serve as a gateway for investigation of specific questions related to mitochondrial
499 biology. Future biochemical and more focused investigations are needed to validate our findings and test
500 the hypotheses arising from our study. For instance, it is pivotal to experimentally validate kinase-
501 substrate associations of previously unknown phosphorylations on mitochondrial proteins and their
502 functional implications in the cell. Additionally, the impact of those phosphorylations on the localization
503 of target proteins and, more specifically, the question of whether mitochondrial proteins are
504 phosphorylated before or after entering the mitochondria remain to be investigated. Accessibility, for
505 instance, of previously undescribed phosphorylation sites on mitochondrial proteins can be assessed using
506 advanced structural tools (Jumper *et al.*, 2021) to determine if they are likely targeted by a mitochondrial
507 kinase or a cytoplasmic kinase before being imported (Schober *et al.*, 2021). Moreover, future
508 developments towards better enrichment strategies for the isolation of mitochondria from different
509 tissues and advances in the MS technology will aid to further improve the depth and quality of the
510 mitochondrial proteomes and phosphoproteome.

511 **Materials and Methods**

512 **Experimental model and subject details**

513 Six C57BL/6N mice (3male, 3 female) were housed in a 12-hours light/dark cycle in standard ventilated
514 cages under specific-pathogen-free conditions with constant temperature (21°C) and humidity (50 - 60%)
515 and fed ad libitum with a standard mouse diet. At the age of 18-21 weeks mice were sacrificed by cervical
516 dislocation. The study was approved by the by the Landesamt für Natur, Umwelt und Verbraucherschutz
517 Nordrhein–Westfalen, Germany, and performed in accordance with European law.

518 **Tissue preparation and isolation of ultra-pure mitochondria**

519 Mice were sacrificed by cervical dislocation and the 7 tissues - heart, skeletal muscle (SKM), brown adipose
520 tissue (BAT), spleen, kidney, liver, and brain – were rapidly removed. Heart, spleen, and kidney, tissues
521 were homogenized in mitochondrial isolation buffer containing 320 mM sucrose, 1 mM EDTA, and 10 mM
522 Tris-HCl, pH 7.4, supplemented with EDTA-free complete protease inhibitor cocktail and PhosSTOP tablets
523 (Roche). For isolation of mitochondria from BAT, liver, and brain the mitochondrial isolation buffer was
524 additionally supplemented with 0.2% bovine serum albumin (Sigma-Aldrich). Subsequently, crude
525 mitochondria were isolated from the homogenates by two rounds of differential centrifugation (see
526 Figure 1a). Isolation of crude mitochondria from SKM was performed as previously described (Frezza et
527 al., 2007)). Crude mitochondrial pellets from all tissues were further purified on a Percoll density gradient
528 as described recently (Kuhl *et al.*, 2017). Briefly, mitochondrial pellets were washed once in 1xM buffer
529 (220 mM mannitol, 70mM sucrose, 5mM HEPES pH 7.4, 1 mM EGTA pH 7.4; pH was adjusted with
530 potassium hydroxide; supplemented with EDTA-free complete protease inhibitor cocktail and PhosSTOP
531 tablets (Roche)) and subsequently purified on a Percoll (GE healthcare) density gradient of 12%:19%:40%
532 via centrifugation in a SW41 rotor at 42, 000 g at 4°C for 30 min in a Beckman Coulter Optima L- 100 XP
533 ultracentrifuge using 14 mm × 89 mm Ultra-Clear Centrifuge Tubes (Beckman Instruments Inc.). Ultra-

534 pure mitochondria were harvested at the interphase between 19% and 40% and washed three times with
535 1xM buffer. Dry mitochondrial pellets were snap-frozen in liquid nitrogen and stored at -80°C until further
536 use.

537 Mitochondrial (phospho)proteome sample preparation

538 Frozen ultra-pure mitochondria pellets were resuspended in lysis buffer (4%SDC, 100mM Tris/HCl, pH8.5),
539 boiled for 5 min at 95°C and sonicated in 30 s intervals for 15 min (Bioruptor). Protein concentration was
540 estimated via Tryptophan assay (Kulak et al., 2014) and was adjusted with lysis buffer to a total volume of
541 270 µl containing 400 µg of protein for brain, SKM, liver, heart, kidney, and 140 µg of protein for BAT
542 samples. Proteins were reduced and alkylated by adding 30 µl of 10x reduction/alkylation solution (100
543 mM Tris (2-carboxyethyl)phosphine hydrochloride (TCEP) and 400 mM 2-chloroacetamide (CAA), followed
544 by 5min incubation at 45°C. Subsequently, 1:100 Trypsin and LysC were added for overnight protein
545 digestion at 37°C. For proteome analysis 10 µl (brain, SKM, liver, heart, kidney) and 20 µl (BAT, spleen)
546 aliquots were taken and loaded on SDB-RPS StageTips. Peptides were washed with 200µl wash buffer
547 (0.2% TFA/2% ACN (vol/vol)) and then eluted with SDB-RPS elution buffer (1.25% NH₄OH, 80% ACN
548 (vol/vol)) and dried in a SpeedVac. Dried peptides were resuspended in A* buffer (2% ACN/0.1% TFA).

549 The remaining samples were processed following the EasyPhos protocol for phosphopeptide enrichment
550 (Humphrey *et al.*, 2018). In brief, samples were first mixed with isopropanol and EP buffer (48% TFA, 8
551 mM KH₂PO₄), followed by phosphopeptide enrichment with 5mg TiO₂ beads per sample (GL Sciences). For
552 this, samples were mixed with TiO₂ beads in loading buffer (6% TFA/80% ACN (vol/vol)) at a concentration
553 of 1 mg/µl and incubated for 5 min at 40°C by shaking at 1200rpm. Subsequently beads were washed four
554 times with 1 ml of wash buffer (5% TFA,60% isopropanol (vol/vol)) and phosphopeptides were eluted from
555 beads using 60 µl of elution buffer (40% ACN, 5% NH₄OH) and concentrated in a SpeedVac for 30 min at
556 45°C. Samples were immediately diluted with 100 µl of SDBRPS loading buffer (99% isopropanol, 1% TFA

557 (vol/vol)) and loaded on SDB-RPS StageTips. Thereafter, phosphopeptides were washed and eluted as
558 described above and resuspended in 6 μ l A*.

559 LC-MS/MS

560 For all measurements peptides were loaded onto a 50cm, in-house packed, reversed-phase column (75 μ m
561 inner diameter, 1. diameter, ReproSil-Pur C18-AQ 1.9 μ m resin [Dr. Maisch GmbH]) and separated with
562 and binary buffer system consisting of buffer A (0.1% formic acid (FA)) and buffer B (0.1% FA in 80% ACN).
563 The column temperature was controlled by a homemade column oven and maintained at 60°C. For
564 nanoflow liquid chromatography an EASY-nLC 1200 system (Thermo Fisher Scientific), directly coupled
565 online with a Q Exactive HF-X (Thermo Fisher Scientific) via a nano-electrospray source, was operated at
566 a flow rate of 300 nl/min and 350 nl/min for mitochondrial proteome and phosphoproteome
567 measurements, respectively.

568 For mitochondrial proteome measurements 500 μ g of peptides were loaded and separated using a
569 gradient starting at 5% buffer B, increasing to 30% buffer B in 80 min, 60% buffer B in 4 min and 95%
570 buffer B in 4 min. The MS was operated in DDA mode (Top12) with a full scan range of 300-1650 m/z and
571 a MS1 and MS2 resolution of 60,000 and 15,000, respectively. The automatic gain control (AGC) was set
572 to 3e6 and 1e5 for MS1 and MS2, while the maximum injection time was set to 20 ms and 60 ms,
573 respectively. Precursor ion selection width was kept at 1.4 m/z and fragmentation was achieved by higher-
574 energy collisional dissociation (HCD) (NCE 27%). Dynamic exclusion was enabled and set to 20 s.

575 For mitochondrial phosphoproteome measurements, 5 μ l as loaded and separated using a gradient
576 starting at 3% buffer B, increasing to 19% buffer B in 40 min, 41% buffer B in 20 min and 90% buffer B in
577 5 min. The MS was operated in DDA mode (Top10) with a full scan range of 300-1600 and a MS1 and MS2
578 resolution of 60,000 and 15,000, respectively. The automatic gain control (AGC) was set to 3e6 and 1e5
579 for MS1 and MS2, while the maximum injection time was set to 120 ms and 60 ms, respectively. Precursor

580 ion selection width was kept at 1.6 m/z and fragmentation was achieved by higher-energy collisional
581 dissociation (HCD) (NCE 27%). Dynamic exclusion was enabled and set to 30 s.

582 Raw data analysis

583 DDA raw data were analyzed with MaxQuant (1.6.14.0) against the mouse fasta file (downloaded 19.
584 October 2020) using default settings. PSM and protein dales discovery rate were controlled at 1% FDR.
585 The match between runs (MBR) functionality was enabled and set in a way that only biological replicates
586 belonging to the same tissue type were allowed to match each other. This eliminates the possibility of
587 potentially false MBR identification transfer between tissues. Carbamidomethyl (C) was selected as fixed
588 modification and Acetyl (Protein N-term) and oxidation (M) were defined as variable modifications. For
589 mitochondrial phosphoproteome analysis, STY site phosphorylation was additionally selected as variable
590 modification.

591 Bioinformatics analysis

592 Data analysis was performed using the python programing language using python (3.8.12) and the
593 following packages: alphamap, matplotlib (3.5.0), mygene (3.2.2), numpy (1.19.2), pandas (1.1.3),
594 pyteomics (4.3.3), requests (2.26.0), scipy (1.7.2), seaborn (0.11.2), sklearn (0.0), upsetplot (0.6.0). All
595 notebooks used for data analysis are available at GitHub (<https://github.com/MannLabs>). Identified
596 proteins were filtered for at least 3 valid values in at least one tissue. Similarly, phosphorylation sites were
597 filtered for at least 5 valid values in at least one tissue and a localization probability >75%. The UniProt
598 API was used to map 'ACC+ID' protein group identifiers provided by the MQ analysis to 'ENSEMBL_ID',
599 'P_ENTREZGENEID' and 'STRING_ID' for further analysis. 'ENSEMBL_ID' and 'P_ENTREZGENEID' identifier
600 were used for mitochondrial protein annotation based on the IMPI (IMPI_2020_Q3, downloaded 27.
601 October 2020) and MitoCarta3.0 (downloaded 1. January 2021) databases, respectively. Network analysis
602 and visualization were performed with the StringApp (1.6.0) in Cytoscape (3.8.2). Kinase annotations are

603 based on manual annotations (Figure 6 - Source Data 1) and 'pkinfam' (downloaded 9. April 2021).
604 Networkin3.0 was used for kinase substrate association (KSA) predictions, while the Networkin score was
605 set to 1. Gene Ontology (GO) annotations for the GOBP term enrichment analysis were retrieved from
606 UniProt (accessed 8. March 2021). Enrichment analysis was performed in Perseus (1.6.7.0) against the set
607 of identified proteins in the corresponding tissue and the results were filtered for an intersection size >10.
608 Missing values were only imputed for PCA and heatmap analysis. For this, a Gaussian normal distribution
609 with a width of 0.3 relative to the standard deviation of measured values and a downshift of 1.8 standard
610 deviations were used. For data normalization, intensity values were \log_2 transformed and then filtered for
611 known and predicted mitochondrial proteins. The median value of these mitochondrial proteins was
612 subtracted from all \log_2 transformed values. Significance testing for individual proteins and
613 phosphopeptides as shown in Figure 3 and Figure 7 was performed with the ordinary one-way ANOVA
614 method or by two-sided t-tests in GraphPad Prism (9.3.1) (Figure 3 - Source Data 1, Figure 7 - Source Data
615 1). Significance testing for differences in mitochondrial protein, phosphoprotein and phosphosite
616 localization was performed in RStudio (1.3.1093) (Figure 6 - Source Data 1). OMM proportions were used
617 for fitting a beta-regression model using the betareg R package with default settings (Cribari-Neto and
618 Zeileis, 2010; Grun et al., 2012). P-values were estimated with the lrtest function of the lrttest package
619 (Zeileis and Hothorn, 2015) and p-values were adjusted with the "fdr" method of the p.adjust function of
620 the stats base package (Benjamini and Hochberg, 1995)

621 Website tool

622 The website tool is structured into four sections. The first three 'Pathway view', 'Sequence view', and
623 'Tissue comparison' are for displaying data, while the fourth section provides explanations for each
624 individual section. In the 'Pathway view' and 'Tissue comparison', proteome and phosphoproteome data
625 filtered for at least three and 5 identifications in at least one tissue, respectively. Intensity values were
626 normalized as described above and used for data representation in the 'Tissue comparison' tab or z-scored

627 across tissues and used for the 'Pathway view tab'. Here, median z-score values of the six biological
628 replicates per tissue are displayed in the data table. The polar plot represents the median z-score of all
629 pathway/complex members of a given tissue. Network/complex annotations were retrieved from
630 MitoCarta3.0 and protein interactions are based on STRING interaction scores. These STRING interaction
631 scores were retrieved from STRING (17. November 2021) using the 'STRING_ID' and the STRING API. For
632 the 'Sequence view', the 'evidence.txt' of the MaxQuant output files was directly used as input to
633 annotated sequences.

634 The python programming language was used for data processing and visualization for the Dashboard. The
635 following libraries were used for data processing: numpy (1.19.2), pandas (1.19.2), re, sys, os, and
636 pyteomics (4.3.3). Several libraries from the HoloViz family of tools were used for data visualization and
637 creation of the dashboard, including panel (1.14.6), holoviews (1.14.6), bokeh (2.2.2), plotly (4.12.0), and
638 param (1.10.0). Network visualization was achieved with the NetworkX package (Hagberg et al., 2008).
639 The Alphamap tool (Voytik *et al.*, 2021) was integrated to display linear protein sequence annotations as
640 well as to visualize 3D protein structures.

641

642 Resource availability

643 Data and code availability

644 Datasets generated in this study have been deposited at ProteomeXchange and are publicly available as
645 of the date of publication. The accession number is listed in the key resource table. (Identifier:
646 PXD030062).

647 All original code has been deposited on GitHub (<https://github.com/MannLabs>) and is available as of the
648 date of publication.

649 Any additional information required to reanalyze the data reported in this paper is available from the lead
650 contact upon request.

651

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664 with BioRender.com.

665 Author contributions

666 F.M.H., L.S.K. designed experiments. Mouse work was performed by L.S.K. and I.K.. Proteomic
667 experiments were conducted by F.M.H. and O.K.. Website was constructed by F.M.H. and I.B.. Data were
668 analyzed by F.M.H.. All authors contributed to writing and editing of the manuscript.

669 Competing interests

670 The authors declare no competing interests.

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extensive phosphorylation of inner membrane protein complexes and enzymes. *Mol Cell Proteomics* 10, M110 000299. 10.1074/mcp.M110.000299.

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Supplementary Figures

Mitochondrial phosphoproteomes are functionally specialized across tissues

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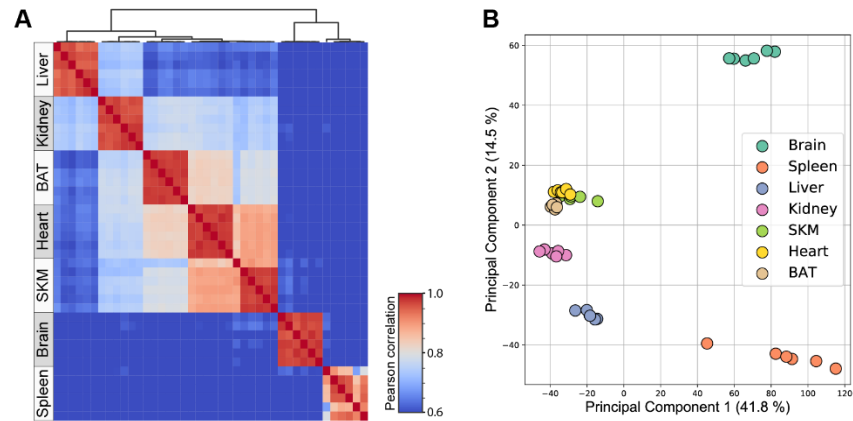


Figure 2 – figure supplement 1 Mitochondria enriched samples show tissue-specific clustering

(A) Heatmap showing Pearson correlations for biological replicates (n=6) for proteins of all mitochondrial enriched samples. (B) Principal component analysis of all proteins of all acquired biological replicates (n=6) (Figure 2 – Source Data 1). Skeletal muscle (SKM), brown adipose tissue (BAT).

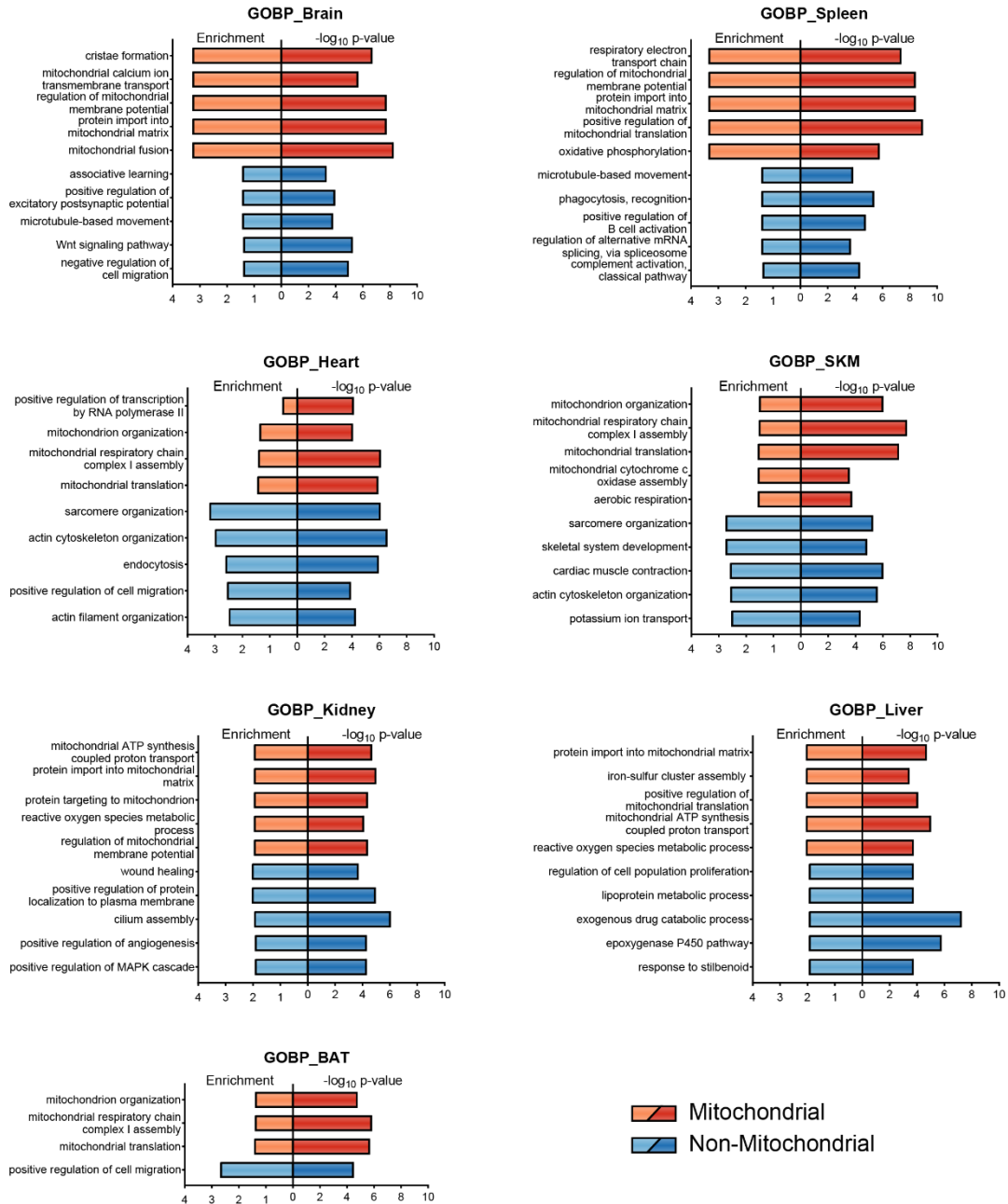


Figure 4 – figure supplement 1 Gene Ontology enrichment displays tissue specificity

Gene Ontology biological process (GOBP) enrichment for mitochondrial (orange) and non-mitochondrial (blue) proteins. Enrichment analysis was performed in Perseus (1.6.7.0) against the set of identified proteins in the corresponding tissue and the results were filtered for an intersection size >10 and the top 5 enriched terms of each tissue are displayed (Figure 4 – Source Data 1). Skeletal muscle (SKM), brown adipose tissue (BAT).

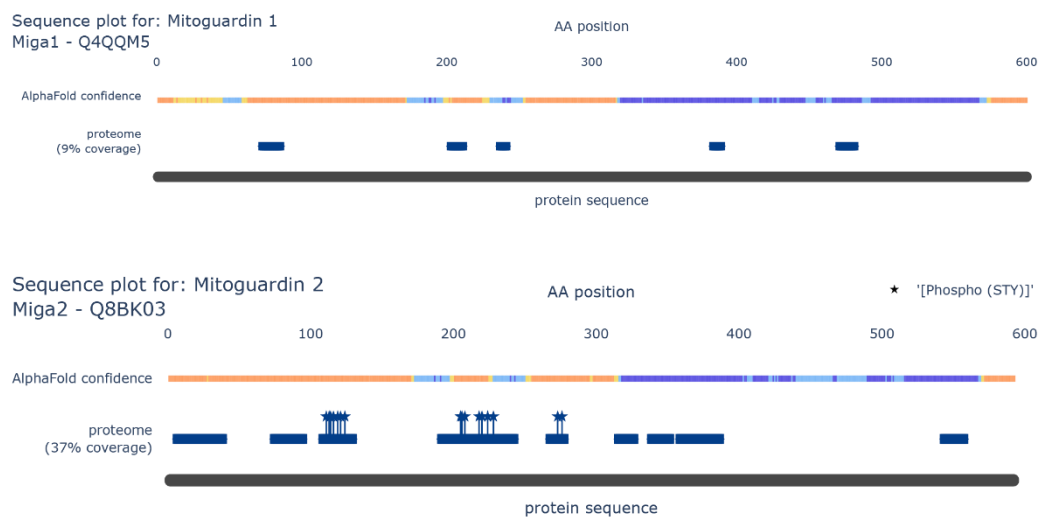


Figure 6 – figure supplement 1 Sequence annotation highlights phosphorylation clusters on MIGA2

Sequence plot of MIGA1 (top panel) and MIGA2 (bottom panel) show structural information (AlphaFold confidence scores) and protein coverage based on identified peptides. All identified STY sites are marked with a star.

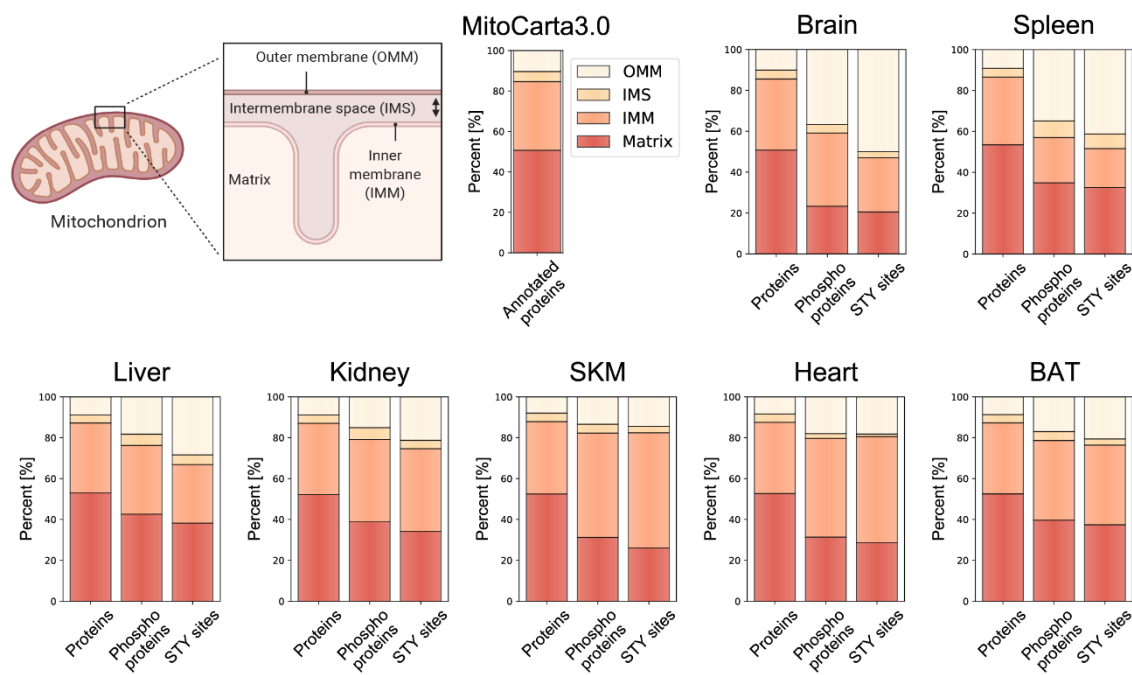


Figure 6 – figure supplement 2 Localization distribution of mitochondrial phosphoproteome diverges from mitochondrial proteome

(A) Scheme of a mitochondrion shows four different mitochondrial localizations – outer mitochondrial membrane (OMM), intermembrane space (IMS), inner mitochondrial membrane (IMM), Matrix – and the distribution of mitochondrial proteins contained in and classified by the MitoCarta3.0 database. Bar graphs for each individual tissue show the precentral distribution of mitochondrial proteins (left), phosphoproteins (middle) and STY sites (right) across different mitochondrial localizations. Fitting of OMM proportions to a beta-regression model shows significant differences between protein-phosphoprotein and protein-STY sites for all tissues (adjusted p-value <0.0001) (see Methods and Figure 6 – Source Data 1). Skeletal muscle (SKM), brown adipose tissue (BAT).

Supplemental information

Mitochondrial phosphoproteomes are functionally specialized across tissues

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Supplementary Table 1 Overview of mitochondrial phosphoproteome studies in mammalian tissue

The table was adapted and extended from Kruse et al. (Kruse and Hojlund, 2017).

Strategy	Species	Tissue	Phosphoproteins and phosphorylation sites identified	Reference
2-DE-ProQ staining-MS	Bovine	Heart	13 phosphoproteins	(Schulenberg et al., 2003)
2-DE-ProQ staining-MS or P32 labelling-2-DE-MS	Pig	Heart	45 phosphoproteins	(Hopper et al., 2006)
IMAC-MS/MS/MS	Mouse	Liver	84 phosphorylation sites in 62 distinct proteins	(Lee et al., 2007)
2D-BN-PAGE-phos—anti-Ser/Thr/Tyr Ab-MS SCX/IMAC-LC-MS/MS	Rat	Heart	2-DE: 45 phosphoproteins LC-MS/MS: 26 phosphorylation sites in 19 distinct proteins	(Feng et al., 2008)
Anti-Tyr Ab-MS	Rat	Brain	7 tyrosine-phosphorylation sites in 7 distinct proteins	(Lewandrowski et al., 2008)
32-P labelling/phos-tag 540 gel staining-2-DE-MS	Pig	Liver, Heart	68 phosphoproteins	(Aponte et al., 2009)
iTRAQ labelling- SCX/HILIC/TiO2-LC- MS/MS-HCD	Pig	Heart	56 phosphorylation sites in 38 distinct proteins	(Boja et al., 2009)
IMAC/TiO2-2D-nano LC- MS/MS	Rat	INS-1 β cells	84 distinct phosphoproteins	(Cui et al., 2010)
SAX/SCX-LC-MS/MS	Rat	Liver	447 phosphorylation sites in 228 distinct proteins	(Deng et al., 2010)
SDS-PAGE-TiO2-LC- MS/MS	Mouse	Heart	236 phosphorylation sites in 181 distinct proteins	(Deng et al., 2011)
SDS-PAGE- TiO2/PPP/HILIC-LC- MS/MS	Human	Muscle	155 phosphorylation sites in 77 distinct proteins	(Zhao et al., 2011)
IMAC-SCX-nano-LC- MS/MS	Mouse	Liver	811 phosphosites in 295 distinct proteins	(Grimsrud et al., 2012)
TiO2-HILIC-LC-MS/MS	Rat	Liver, Heart, Skeletal muscle	899 phosphorylation sites in 354 distinct proteins	(Bak et al., 2013)
TiO2-HILIC-LC-MS/MS	Human	Skeletal muscle	207 phosphorylation sites in 95 distinct proteins	(Zhao et al., 2014)
iTRAQ labelling-TiO2-LC- MS/MS	Human	Ovary	124 phosphorylation sites in 67 distinct proteins	(Li et al., 2019)
Anti-Tyr Ab-MS	Mouse	Liver	79 tyrosine-phosphorylated mitochondrial proteins	(Guedouari et al., 2020)
TMT labeling - IMAC-LC- MS/MS	Human	Skeletal muscle	480 phosphopeptides that correspond to 196 proteins	(Sathe et al., 2021)