Title: Leveraging genetic diversity to identify small molecules that reverse mouse skeletal muscle insulin resistance

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Abstract

Systems genetics has begun to tackle the complexity of insulin resistance by capitalising on computational advances to study high-diversity populations. "Diversity Outbred in Australia (DOz)" is a population of genetically unique mice with profound metabolic heterogeneity. We leveraged this variance to explore skeletal muscle's contribution to whole-body insulin action through metabolic phenotyping and skeletal muscle proteomics of 215 DOz mice. Linear modelling identified 553 proteins that associated with whole-body insulin sensitivity (Matsuda Index) including regulators of endocytosis and muscle proteostasis. To enrich for causality, we refined this network by focussing on negatively associated, genetically regulated proteins, resulting in a 76-protein fingerprint of insulin resistance. We sought to perturb this network and restore insulin action with small molecules by integrating the Broad Institute Connectivity Map platform and *in vitro* assays of insulin action using the Prestwick chemical library. These complimentary approaches identified the antibiotic thiostrepton as an insulin resistance reversal agent. Subsequent validation in *ex vivo* insulin resistant mouse muscle, and palmitate induced insulin resistant myotubes demonstrated potent insulin action restoration, potentially via up-regulation of glycolysis. This work demonstrates the value of a drug-centric framework to validate systems level analysis by identifying potential therapeutics for insulin resistance.

23 **Introduction**

24 Skeletal muscle is a key determinant of whole-body glycaemic control. Under optimal conditions,
25 insulin secreted from the pancreas initiates a signalling program in muscle, and other tissues,
26 culminating in translocation of the insulin-responsive glucose transporter (GLUT4) to the plasma
27 membrane (1). Increased plasma membrane GLUT4 lowers circulating glucose by increasing cellular
28 influx for either storage as glycogen, or subsequent metabolism via the glycolytic pathway. Insulin
29 resistance is the progressive failure of these processes, and often precedes a number of metabolic
30 disorders including type 2 diabetes (2). Advances in genomics and computational biology have begun
31 to shed new light on molecular drivers of insulin resistance. A recent study in humans undertook a
32 GWAS of 188,577 individuals unveiling 53 genetic loci associated with a surrogate insulin resistance
33 signature (3). Such studies are important as they point toward genetic lesions in metabolic tissues like
34 skeletal muscle and adipose tissue as playing a key causal role in the development of insulin resistance.
35 This emphasises the importance of focusing on genetics and peripheral tissues for new therapeutic
36 targets and strategies to overcome insulin resistance. Recent developments in systems biology
37 provides unique opportunities for discovering ways of reversing and or preventing insulin resistance.
38 This will have enormous clinical benefit since insulin resistance is a gateway to an expanding family of
39 diseases.

40

41 Over the past decade, genetically diverse mouse panels have been used to study metabolic disease
42 (4-7). This is a major step forward because these panels combine control of the environment and
43 access to any biological tissue, with a vast phenotypic and range which can be leveraged towards
44 understanding complex diseases. Three specific resources are the hybrid mouse diversity panel
45 (HMDP), and the BXD (C57BL/6J x DBA) and Collaborative Cross (CC) mouse strains (7-11). These
46 panels comprise large selections of inbred mice spanning vast phenotypic and genetic landscapes.
47 Collaborative Cross mice were first generated by interbreeding five commonly used laboratory mouse

48 strains (C57BL/6J, A/J, 129S1/SvlmJ, NZO/HILtJ, NOD/ShiLtJ) and three wild-derived strains (WSB/EiJ,
49 CAST/EiJ, PWK/PhJ) in a 'funnel' design. The resulting CC strains were then outbred to generate
50 Diversity Outbred mice at Jackson Laboratories (12) which have increased phenotypic diversity and
51 resolution for genetic mapping. An independent Diversity Outbred colony was established in Western
52 Australia using CC mice from Geniad (13). This colony has since relocated to our group at The
53 University of Sydney, termed Diversity Outbred mice from Australia (Oz) or DOz.

54

55 The use of such rodent models for studying complex traits has given rise to the field of Systems
56 Genetics. System genetics uses global quantification of 'intermediate phenotypes' i.e., gene
57 transcripts, proteins, metabolites to provide mechanistic links between genetic variation and complex
58 traits/disease (14, 15). Unlike traditional genetic studies that identify single or multiple loci of interest,
59 systems genetics often identifies entire biological pathways or networks that are inherently more
60 difficult to empirically test. In an attempt to streamline interrogation of molecular pathways, several
61 large-scale perturbation screens/projects have been undertaken. One such example is the Broad
62 Institute's Connectivity Map that integrated mRNA expression levels from 1.5 million combinations of
63 different cell lines and perturbations (small molecule inhibitors, receptor ligands, genetic
64 manipulations) into a searchable database (16-19). These kinds of tools provide invaluable resources
65 for testing hypotheses generated from systems genetics experiments, and for broadly linking
66 molecular networks to phenotypic outcomes.

67

68 Here we have utilised DOz mice to interrogate insulin resistance. By combining metabolic phenotyping
69 and skeletal muscle proteomics we have identified an insulin resistance fingerprint of 76 proteins. We
70 then used Connectivity Map to identify small molecules that give rise to an overlapping transcriptional
71 signature across a number of cell lines, and therefore have the potential to affect insulin action.
72 Strikingly, one of these compounds, the antibiotic thiostrepton, was also identified by us in an

73 independent small molecule screen for effectors of insulin action in myotubes. Subsequent validation
74 of thiostrepton uncovered profound beneficial effects on insulin resistance *in vitro* and *ex vivo,*
75 potentially via modulation of mitochondrial function and glycolysis.

76

77 **Results**

78 *DOz metabolic and proteomic variation*

79 Diversity Outbred from Oz (DOz) mice were metabolically phenotyped by oral glucose tolerance test
80 (GTT) and echoMRI to determine body composition (Fig 1A-C). We integrated glucose and insulin
81 levels during the GTT into a surrogate measure of whole-body insulin sensitivity referred to as the
82 Matsuda Index (20). Similar to HOMA-IR, the Matsuda Index uses blood glucose and insulin values to
83 predict whole-body insulin sensitivity. However, an advantage of the Matsuda Index over HOMA-IR is
84 that it includes values over a range of GTT time-points, this better incorporates the dynamics of
85 glycaemic control. Furthermore, the Matsuda Index is better correlated to the euglycemic-
86 hyperinsulinaemic clamp, the gold standard measurement for insulin sensitivity, in humans (20).
87 Consistent with studies in other DO mouse populations (12, 21), we observed profound phenotypic
88 diversity in DOz mice with 20-to-400-fold differences in insulin sensitivity, adiposity, and fasting insulin
89 levels across all DOz animals (Fig 1C). Notably, the metabolic variation we observed in DOz mice is
90 markedly greater than the variation typically observed in similar studies using inbred mouse strains
91 (Fig 1D). Since skeletal muscle, and mitochondrial function are major contributor to whole body insulin
92 sensitivity in mammals (4, 22, 23), we performed proteomic analysis on quadriceps muscles that were
93 fractionated into mitochondrial and post-mitochondrial fractions (PMF, Fig 1A). We identified 2073
94 total proteins (444 mitochondrial and 1629 PMF) present in at least 50% of mice. Mitochondrial
95 proteins were defined based on their presence in MitoCarta 3.0 (24) and consistent with previous
96 work (25) were approximately two-fold enriched in the mitochondrial fraction relative to the PMF (Fig
97 1E).

98

99 As with glycaemic control, muscle proteomes exhibited profound variation, with approximately 200-
100 fold differences in coefficient of variation (CV) across both fractions (Fig 1F). Interestingly,
101 glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the often-reported western blot loading
102 control was the 11th most variable protein in our dataset. Amongst the other highly variable proteins
103 were the histone subunit H4F16, the mitochondrial iron-sulfur cluster assembly regulator ISCU, and
104 the metalloendopeptidase OMA1. Among proteins with low variability between mice was the
105 mitochondrial respiratory complex II subunit SDHA, and the 14-3-3 zeta isoform, YWHAZ. To uncover
106 how variation differed across biological processes, gene ontology (GO) enrichment analysis was
107 performed on proteins ranked by CV. Among the low variance processes were the electron transport
108 chain (mitochondrial fraction) and regulation of chromosome organisation (PMF), while lipid
109 metabolic pathways were highly variable across both mitochondrial and post-mitochondrial fractions
110 (Fig 1G). As a control experiment, we also performed enrichment analysis on proteins ranked by LFQ
111 relative abundance. High CV pathways (enriched for high CV proteins) tended to be lower in relative
112 abundance (enriched for low relative abundance proteins) (Supplementary Fig 1a, b). However, many
113 high variability pathways, lipid metabolism for example, were not enriched in either direction based
114 on relative abundance suggesting differences in relative abundance do not fully explain pathway
115 variability differences.

116 *Role of skeletal muscle in whole-body insulin sensitivity*

117 To leverage genetic and metabolic diversity toward uncovering new regulators of insulin action, we
118 constructed linear models comparing insulin sensitivity (Matsuda Index) against protein abundance.
119 Our initial analysis identified 37 mitochondrial and 40 PMF proteins that significantly associated with
120 the Matsuda Index. Many of these appeared to be involved with adiposity rather than insulin action
121 including adiponectin (ADIPOQ), adipsin (CFD), and the mitochondrial β-oxidation proteins ETFA and
122 ETFB (Supplementary Fig 1C, D). Because we were mainly interested in identifying muscle-specific

123 factors that regulate insulin sensitivity, we next constructed a model that included adiposity (the
124 percentage of body mass which is adipose tissue) as a covariate. Using this approach, we identified
125 120 mitochondrial (Fig. 2A-B) and 433 PMF proteins that significantly associated with whole-body
126 insulin sensitivity. Comparison of the results from each model (with and without adiposity as a
127 covariate), revealed consensus proteins that associated with insulin sensitivity independently of
128 model design (Supplementary Fig 1E). These included the glycolytic enzymes PFKFB1 and PKM, which
129 have previously been identified as regulating muscle insulin action (4). Using adiposity as a covariate
130 not only increased the number of proteins identified but also uncovered relationships between
131 Matsuda Index and the GLUT4 trafficking regulators RAB10 (26, 27), SNAP23 (28) and IQGAP1 (29)
132 which were not seen in the original model. Comparison of the mitochondrial and PMF proteomes
133 revealed a mitochondrial enrichment for proteins that positively associate with insulin sensitivity,
134 highlighting the broadly positive role mitochondria play in muscle's contribution to metabolic health
135 (Fig 2C).

136

137 To gain further insight into the biology of skeletal muscle glycaemic control, we annotated candidate
138 proteins with PhosphoSite Plus data to test whether any protein of interest had documented roles in
139 metabolic signalling (Fig 2D-F). We observed no relationship between insulin signalling substrates and
140 regulation of whole-body insulin sensitivity (Fig 2D). However, there was a trend ($p = 0.08$) for AMPK
141 substrates in the PMF to be positively associated with insulin sensitivity. Interestingly, the most
142 positively (Canopy2; CNYP2) and most negatively (Rho GDP dissociation inhibitor alpha; ARHGDIA)
143 associated proteins were both annotated as being insulin responsive (Fig. 2F). We also preformed GO
144 gene-set enrichment analysis (GSEA) on proteins ranked by Matsuda Index effect sizes (Fig 2G). In the
145 PMF, 'response to topologically incorrect protein: GO:0051788' was positively associated with insulin
146 sensitivity while the 'proteasome complex: GO:0005839' and 'clathrin-coated pit: GO:0005905' were
147 negatively associated. Together the unfolded protein response and proteasome are indicative of the

148 role of proteostasis in insulin sensitivity (30, 31). Conversely, clathrin-coated pits play an important
149 role in the internalisation of the insulin sensitive glucose transporter GLUT4 from the cell surface, a
150 process negatively regulated by insulin (32-34). Consistent with our observation that the majority of
151 the mitochondrial proteome is positively associated with insulin sensitivity, GSEA did not produce any
152 negative results but did uncover a positive relationship between the mitochondrial respiratory
153 complex I and Matsuda Index.

154

155 *Integration of genetic linkage analysis and linear modelling with Connectivity Map*

156 Changes in protein levels may be either cause or consequence of changes in insulin sensitivity. In an
157 attempt to select for proteins with a potentially causal relationship, we performed genetic mapping
158 analysis of both the mitochondrial and PMF proteomes (Fig 3A). Across both proteomes we identified
159 624 protein quantitative trait loci (pQTL). These were distributed across the genome and were
160 predominately cis acting (Figure 3A), indicating that a significant proportion of variation in these
161 proteins can be explained by their local genetic architecture. Next, we filtered proteins that were
162 negatively associated with Matsuda Index by cis-pQTL presence to generate a molecular fingerprint of
163 insulin resistance (Fig 3B). We focussed on negatively associated proteins based on the assumption
164 that inhibiting deleterious proteins is easier than promoting the activity of beneficial ones. Filtering
165 based on cis-pQTL presence was based on the rationale that if genetic variation can explain protein
166 abundance differences between mice, then we can be confident that phenotype (Matsuda Index) is
167 not driving the observed differences and therefore the protein-to-phenotype associations are likely
168 causal. Importantly, this assumption can only be made for cis-acting pQTLs. Our analysis yielded a list
169 of 76 (69 PMF and 7 mitochondrial) proteins that encompassed a wide range of biological processes
170 (Supplementary Table 1). Low mitochondrial representation in the fingerprint is the result of selecting
171 negatively associating proteins, and as seen (Figure 2C) previously, the mitochondrial proteome is
172 enriched for positive contributors to insulin resistance. Similar approaches to identify molecular

173 phenotypes of insulin resistance have previously been conducted using collated human transcriptomic
174 datasets (35-45). Using a compiled list from Timmons et al (46), we searched for orthologues of
175 proteins from our fingerprint that associate with human insulin resistance. We identified four such
176 genes (Fig. 3B): MTPN (myotrophin), LGALS1 (galectin 1), PHKB (phosphorylase b kinase), and
177 ADPRHL1 (ADP-ribosylhydrolase Like 1), which may warrant further investigation.

178 To assess the tissue specificity of our fingerprint we searched for the same proteins in metabolically
179 important adipose and liver tissues. Despite detecting 94% and 82% of muscle fingerprint proteins
180 across each tissue respectively, both adipose and liver were depleted for pQTL presence (Figure 3C)
181 suggesting that regulation of our candidate protein abundance is somewhat specific to skeletal
182 muscle. Finally, we queried our fingerprint for any biological pathways that could represent novel
183 drivers of insulin resistance by performing KEGG pathway enrichment (Fig 3C). Both 'endocytosis:
184 mmu04144' (clathrin light chain A, clathrin light chain C, epidermal growth factor receptor pathway
185 substrate 15) and 'insulin signalling pathway; mmu04910' (phosphorylase kinase regulatory subunit
186 beta) featured in the top 10 providing further supportive evidence for the biological relevance of our
187 fingerprint in the context of insulin sensitivity.

188

189 Next, we utilized Connectivity Map (CMAP) to convert our fingerprint into a list of small molecules and
190 ligands that promote or oppose our muscle insulin resistance fingerprint. To test our assumption that
191 pQTL filtering would improve our fingerprint we also queried CMAP with a list of the top 150 most
192 strongly negatively associated proteins independent of pQTL presence. Intriguingly, on average CMAP
193 scores for compounds and ligands were significantly higher when captured using a pQTL-filtered
194 fingerprint compared to the non-pQTL filtered group, supporting the utility of this method
195 (Supplementary Figure 2A). Encouragingly, the two highest scoring compounds identified using our
196 fingerprint were Broad Institute glycogen synthase kinase (GSK3) and epidermal growth factor
197 receptor (EGFR) inhibitors. Both of these kinases have been independently identified as drug targets

198 that reverse insulin resistance (37, 47, 48). Many compounds listed in the Connectivity Map database
199 are proprietary Broad Institute inhibitors which are only identified by their Broad ID and cannot be
200 easily procured for follow-up experiments. Therefore, we excluded all Broad Institute compounds
201 from further analysis. After this filtering, 856 small molecules and 91 ligands generated gene
202 expression signatures matching our query. As whole-body insulin sensitivity decreased with increased
203 fingerprint protein abundance, we focussed on molecules whose CMAP score suggested a reversal of
204 our insulin resistance fingerprint (Fig 3D). Ranking these candidates based on CMAP score revealed a
205 number of well-known potentiators of insulin sensitivity including the antioxidant resveratrol (49), the
206 diabetes medication metformin (50) and the growth factor FGF21 (51). We also identified the
207 antibiotic thiostrepton (52), a documented proteasome inhibitor, consistent with our enrichment
208 analysis which identified the proteasome as negatively contributing to insulin sensitivity.

209

210 *Cross-validation of thiostrepton by Prestwick library screen of GLUT4 translocation*

211 To obtain independent validation of some of the candidates revealed from Connectivity Map we
212 performed a screen for compounds that affect GLUT4 translocation to the cell surface in L6 myotubes
213 expressing HA-tagged GLUT4 (GLUT4-HA-L6), a readout of insulin action that is defective in insulin
214 resistance. For this we used our established high sensitivity, high-throughput 96-well plate format
215 screen that is amenable to physiological models of insulin resistance (53, 54), combined with the
216 Prestwick library of FDA-approved drugs. In total, 420 compounds were found across both platforms,
217 and these consensus compounds captured a significant proportion of highly scoring CMap compounds
218 (Supplementary Figure 2C, D).

219 We performed 3 separate screens (Fig 4A) to capture the different mechanisms by which compounds
220 modulate glucose uptake: 1) insulin-independent activation of GLUT4 translocation to the plasma
221 membrane (basal activators), 2) potentiation of submaximal insulin action (insulin sensitisers), and 3)
222 rescue of palmitate-induced insulin resistance (insulin resistance reversers). We identified 22 basal

223 agonists (Fig 4B), 7 insulin sensitisers (Fig 4C) and 16 insulin resistance reversers (Fig 4D). Five
224 compounds both stimulated GLUT4 translocation and reversed insulin resistance, 4 were both basal
225 agonists and insulin sensitisers while none met all three criteria (Fig 4E). Overall, we found that
226 compounds that were identified by CMAP score (Fig 3D) performed better as both basal activators
227 and as insulin resistance reversers than those that did not (Supplementary Fig 2B).

228

229 To cross-reference our CMAP data with the Prestwick screen in an unbiased way, we constructed a
230 scoring matrix to rank compounds found by both our CMAP query and in the Prestwick library. First,
231 we z-scored the values for each category (basal agonists, insulin sensitisers, insulin resistance reversal,
232 CMAP score). Next, we averaged the three *in vitro* assays z-scores and added it to the CMAP score.
233 This overall score represents how each compound modulates GLUT4 translocation, and potentially
234 reverses our insulin resistance fingerprint, relative to the rest of the compound library. Using this final
235 value, we ranked each compound and displayed the top 20 in a heat map (Fig 5A). Based on this
236 metric, thiostrepton was identified as the highest-ranking compound and was selected for subsequent
237 validation by further GLUT4 translocation (Fig. 5B) and 2-deoxyglucose uptake (Fig. 5C) experiments
238 in GLUT4-HA-L6 myotubes. We observed a consistent reversal of insulin resistance across both assays.

239

240 Next, we assessed the efficacy of thiostrepton to reverse insulin resistance in diet-induced obese mice.
241 We decided to study it in isolated muscles as this circumvents potentially confounding microbiome
242 effects due to thiostrepton's antibiotic activity and allows direct interrogation of muscle insulin action.
243 We selected two strains of inbred mice, C57BL/6J and BXH9/TyJ, based on our previous observations
244 that these strains are particularly amenable to developing muscle insulin resistance following high-fat,
245 high-sugar (western diet; WD) diet feeding (4). Consistent with diet-induced perturbations in
246 metabolic health, both C57BL/6J and BXH9 BXH9/TyJ mice fed a WD had increased body weight and
247 adiposity, fasting hyperglycemia, fasting hyperinsulinemia, glucose intolerance and lower systemic

248 insulin sensitivity (Matsuda Index) relative to chow-fed controls (Supplementary Fig. 3A-E). WD
249 feeding also resulted in ~40% reduction in C57BL/6J soleus insulin stimulated 2-deoxyglucose uptake,
250 a 75% reduction in BXH9 extensor digitorum longus insulin-stimulated 2-deoxyglucose uptake and
251 65% reduction in BXH9 soleus insulin stimulated 2-deoxyglucose uptake. Strikingly, 1 h of thiostrepton
252 treatment prior to insulin addition was sufficient to reverse 80% of WD-induced insulin resistance in
253 C57Bl/6J EDL muscle and 50% in BXH9 EDL muscle but did not restore BXH9 soleus 2-deoxyglucose
254 uptake (Figure 5D).

255

256 *Thiostrepton does not affect insulin signalling*

257 Next, we attempted to identify the potential mechanisms by which thiostrepton relieved insulin
258 resistance. Canonically, insulin stimulated GLUT4 translocation is facilitated by a signalling cascade
259 comprising PI3K/Akt and dysfunction in this pathway has been implicated in insulin resistance (55),
260 although this is controversial (2). To assess insulin signalling we treated control and palmitate-treated
261 GLUT4-HA-L6 myotubes with either thiostrepton or vehicle for 1 h prior to insulin stimulation. Unlike
262 GLUT4 translocation or 2-deoxyglucose uptake, palmitate did not perturb proximal insulin signalling.
263 We detected no effect of palmitate treatment or thiostrepton on the phosphorylation of Akt-T308,
264 Akt-S473, or the Akt-substrates GSK3-S21/9 and PRAS40-T246 (Fig. 6A-E). These findings are
265 consistent with the view that insulin resistance occurs independently of canonical insulin signalling
266 (56, 57) and suggests that thiostrepton is acting independently of signalling to reverse insulin
267 resistance.

268

269 *Thiostrepton partially inhibits mitochondria and restores palmitate induced glycolysis suppression*

270 Many insulin sensitising agents act via mitochondrial inhibition or uncoupling (58-60) and thiostrepton
271 has been reported to inhibit mitochondrial translation (61) and respiration (62, 63). To test whether

272 thiostrepton's ability to restore optimal insulin action occurs via mitochondria, palmitate-treated and
273 control GLUT4-HA-L6 myotubes were incubated with thiostrepton as above. Consistent with the
274 mitochondrial dysfunction that has been reported during insulin resistance (23, 64), we observed
275 substantial suppression of maximal mitochondrial respiration and mitochondrial reserve capacity
276 following palmitate treatment (Fig. 7A). Furthermore, thiostrepton alone appeared to blunt maximal
277 respiration, albeit to a lesser extent than palmitate. When combined, thiostrepton and palmitate did
278 not produce an additive suppression, nor did thiostrepton reverse any of the palmitate-induced
279 defects. This suggests that this suppression of maximal respiration does not contribute to insulin
280 resistance in this model.

281

282 We also assessed glycolysis by way of extra-cellular acidification rate (ECAR). Like mitochondrial
283 respiration, palmitate suppressed maximal glycolytic capacity, however unlike respiration this was
284 potently reversed by co-treatment with thiostrepton (Fig. 7B). To test whether this increase in
285 glycolytic flux could be explained by changes in cellular energy status due to mitochondrial inhibition,
286 we investigated the energy sensor AMP-dependent kinase (AMPK). AMPK can promote glycolysis (65),
287 GLUT4 translocation (66, 67), and glucose uptake in skeletal muscle independently of insulin.
288 However, unlike the AMPK activator A-769662, we observed no effect of thiostrepton on the
289 phosphorylation of AMPK or its substrate acetyl-CoA carboxylase in either control cells or cells treated
290 overnight with palmitate (Fig. 7C-E). Although A-769662 potently increases AMPK substrate
291 phosphorylation in muscle cells, AMPK phosphorylation itself is not observed, consistent with a
292 previous study (68). These data suggest that if thiostrepton activates glycolysis via mitochondrial
293 inhibition, it occurs independently of AMPK.

294

295 **Discussion**

296 By leveraging genetic and phenotypic diversity of DOz mice we have explored skeletal muscle's
297 contribution to whole-body insulin action at the molecular level. Our approach was validated by the
298 identification of various 'positive controls' at each level of analysis. Firstly, utilising adiposity as a
299 covariate during linear modelling uncovered relationships between whole-body insulin sensitivity and
300 muscle GLUT4 trafficking proteins; secondly, pathway enrichment revealed proteostasis (30, 69) and
301 endocytosis (32, 70) as key contributors to whole-body insulin sensitivity; and thirdly, querying
302 Connectivity Map with our fingerprint of insulin resistance returned metformin, GSK3 (37, 71) and
303 EGFR (47) inhibitors as potential insulin resistance therapeutics (50). The identification of these
304 proteins, pathways and drugs by our strategy gives us confidence in our approach and the novel
305 players identified.

306

307 Systems-based approaches often identify networks as being drivers of disease. Empirical validation of
308 these is difficult due to the complex interactions in biological systems. Here we took a drug-centric
309 approach to validate our findings; this allowed targeting of entire pathways rather than singular
310 nodes. We identified several compounds across both *in silico* and *in vitro* analyses which may restore
311 muscle insulin action, indeed several of these have previously been investigated. Disulfiram, sold
312 under the brand name Antabuse, is used as an alcohol-dependency medication. Two studies have
313 described disulfiram's ability to reverse diet-induced hepatic insulin resistance and reduce adiposity
314 (72, 73). Resveratrol, a component found in red wine, is a popular antioxidant and has been
315 demonstrated to reverse insulin resistance via reduction of reactive oxygen species (49, 74, 75).
316 Fibroblast growth factor 21 (FGF21) was also identified amongst the ligand dataset as reversing our
317 insulin resistance fingerprint. FGF21 has previously been reported to promote insulin-stimulated
318 glucose uptake in muscle fibres (76) and can modulate mitophagy and proteostasis in muscle (77).
319 Antivirals, several classes of antibiotics, antipsychotics and cancer drugs were all identified by our
320 analyses. Perhaps this diversity reflects the divergent and pleiotropic biology of insulin resistance.

321

322 Key to our approach is the insulin resistance muscle proteomic fingerprint. This was generated by
323 filtering proteins that associated with whole-body insulin sensitivity and that possessed significant cis-
324 pQTLs. The latter was particularly important as we postulated that this would select for proteins that
325 were likely to be causal drivers of insulin resistance. We hypothesed that a protein whose expression
326 is post-translationally regulated in response to insulin resistance would not show a genetic signal and
327 therefore be excluded from our fingerprint. Filtering on this basis improved the overall Connectivity
328 Map score, and ultimately identified thiostrepton. One reason for this could be the discordance
329 between mRNA and protein (78, 79). Connectivity Map uses mRNA expression data whereas our
330 fingerprint uses protein. By restricting our fingerprint to proteins with significant cis-pQTLs we may
331 have inadvertently selected for genes whose mRNA expression closely matches their protein thereby
332 increasing the overlap between fingerprint and perturbagen signatures. Moreover, our approach has
333 the major advantage that it requires far fewer mice to obtain meaningful outcomes (222 mice in this
334 study) compared to that required for genetic mapping of complex traits like Matsuda Index (80).,
335 Furthermore, because we have used genetically diverse datasets (DOz mice and multiple cell lines in
336 Connectivity Map) our findings are likely robust across diverse target backgrounds.

337

338 A major question is what biological functions are represented by our fingerprint? One of the top
339 pathways identified was endocytosis. This pathway featured two components of the clathrin coat and
340 the adaptor protein EPS15. This is very exciting as endocytosis has been suggested to play a major role
341 in stress signalling (81), and in the context of insulin sensitivity this may involve internalisation of key
342 proteins including the insulin receptor and glucose or amino acid transporters (32, 70). The concept
343 that variation in this process is genetically determined, and this plays a major role in governing
344 essential processes like insulin action, adds a new dimension to the role of this pathway. A second
345 intriguing member of the fingerprint is phosphorylase kinase which, along with glycogen synthase

346 kinase, regulates the key glycogen storage enzymes glycogen phosphorylase and glycogen synthase
347 (82-85). High levels of glycogen phosphorylase kinase may promote glycogen breakdown through
348 activation of glycogen phosphorylase thereby altering GSK3 signalling, a process implicated in insulin
349 resistance.

350

351 Neither AMPK nor Akt signalling account for the profound effect of thiostrepton on insulin action. This
352 is exciting as it suggests both a novel mechanism of action and a novel insulin resistance defect. So
353 far, the most enticing potential mechanism is restoration of glycolysis. Thiostrepton restores normal
354 glycolytic function in palmitate treated cells and we have previously reported links between glycolysis
355 and insulin action in skeletal muscle (4, 86). Mechanistically, thiostrepton could promote glycolysis via
356 attenuation of mitochondrial oxidative phosphorylation, and this has previously been demonstrated
357 in acute myeloid leukemia and malignant mesothelioma cell lines (62, 87). Our data supports this work
358 and identifies a similar, albeit mild, effect on myotube respiration. Thiostrepton can also increase
359 cellular exposure to mitochondrial reactive oxygen species (ROS) as it inhibits peroxiredoxin-3 (63, 87),
360 a key antioxidant enzyme. Perhaps, as seen during the Warburg effect (88-90), increased
361 mitochondrial ROS can act as a signal to promote glycolysis and relieve mitochondrial energetic
362 demands. Aside from glycolysis, other pathways may be involved in enhancing insulin sensitivity. For
363 example, the negatively associated protein ARHGDIA (Figure 2F) is a potent negative regulator of
364 insulin sensitivity, and our fingerprint of insulin resistance contained its homologue ARHGDIB. Both
365 ARHGDIA and ARHGDIB have been reported to inhibit the insulin-action regulator RAC1 (91-93), and
366 thus may lower GLUT4 translocation and glucose uptake. Further investigations may uncover a role
367 for thiostrepton in modulating the RAC1 signalling pathway via ARHGDIB.

368 Integration of physiological, proteomic, genomic, and pharmaceutical data has uncovered a potent
369 reverser of insulin resistance. By integrating proteomic diversity with the underlying genetic
370 architecture, we believe we were able to focus on potentially causal proteins, and the use of

371 Connectivity Map allowed us to combine these proteins into a single fingerprint to find potential
372 modulators of insulin resistance. Our findings also build on recent reports linking glycolysis to insulin
373 action and uncover a number of potential contributors to insulin action worthy of future study.

374

375 **Methods**

376 Mouse breeding and phenotyping

377 Male 'Diversity Outbred from Oz' (DOz) mice were bred and housed at the Charles Perkins Centre,
378 University of Sydney, NSW, Australia. They were originally established at Geniad, Western Australia,
379 Australia and then relocated to The University of Sydney. The DOz population comprises 46 breeding
380 pairs and the breeding strategy avoids mating's between siblings or first cousins. Breeders are selected
381 based on the genotype of the *R2d2* locus to limit the meiotic drive favouring the WSB allele on
382 chromosome 2 (94). The DOz mice used in the current study were outbred for 27 to 33 generations
383 and comprised a total of 250 male DOz mice that were studied as 5 separate cohorts. Genomic DNA
384 was isolated from each mouse and subjected to SNP genotyping (95), followed by genotyping
385 diagnostics and cleaning as described (96). Experiments were performed in accordance with NHMRC
386 guidelines and under approval of The University of Sydney Animal Ethics Committee. To delineate
387 genetic from cage-effects, mice were randomised into cages of 3-5 at weaning. All mice were
388 maintained at 23°C on a 12-hour light/dark cycle (0600-1800) and given *ad libitum* access to a standard
389 laboratory chow diet containing 16% calories from fat, 61% calories from carbohydrates, and 23 %
390 calories from protein or a high-fat high-sugar diet (western diet; WD) containing 45% calories from
391 fat, 36% calories from carbohydrate and 19% calories from protein (3.5%g cellulose, 4.5%g bran, 13%g
392 cornstarch, 21%g sucrose, 16.5%g casein, 3.4%g gelatine, 2.6%g safflower oil, 18.6%g lard, 1.2%g AIN-
393 93 vitamin mix (MP Biomedicals), 4.95%g AIN-93 mineral mix (MP Biomedicals), 0.36%g choline and
394 0.3%g L-cysteine). Fat and lean mass measures were acquired via EchoMRI-900 (EchoMRI Corporation
395 Pte Ltd, Singapore) at 14 weeks of age. Glucose tolerance was determined by oral glucose tolerance
396 test (GTT) at 14-weeks of age by fasting mice for 6-hours (0700-1300 hrs) before oral gavage of 20%
397 glucose solution in water at 2 mg/kg lean mass. Blood glucose concentrations was measured directly
398 by handheld glucometer (Accu-Chek, Roche Diabetes Care, NSW, Australia) from tail blood 0, 15, 30,
399 45, 60, 90 minutes after oral gavage of glucose. Blood insulin levels at the 0- and 15-minute time points
400 were measured by mouse insulin ELISA Crystal Chem USA (Elk Grove Village, IL, USA) according to

401 manufacturer instructions. Blood glucose and insulin levels were integrated into a surrogate measure
402 of whole-body insulin sensitivity using the Matsuda Index:

403

404 $$Matsuda\ Index = \frac{10{,}000}{\sqrt{(Glucose_0 \times\ Insulin_0) \times (Glucose_{\mathrm{GTT}\ mean} \times\ Insulin_{GTT\ mean})}}$$

405

406 Muscle proteomic sample prep

407 Whole quadriceps muscle samples were prepared as previously described with modification (97, 98).
408 First, tissue was snap frozen with liquid nitrogen and pulverized before resuspension in 100 µL of
409 trypsin buffer (phosphate buffered saline; PBS containing 10 mM EDTA and 0.01 ug/uL mass-
410 spectrometry grade trypsin). Samples were incubated for 30 minutes at 37 °C before being pelleted
411 by centrifugation (10,000 g, 5 minutes at 4 °C). Samples were then resuspended in 1.4 mL
412 mitochondrial isolation buffer (70 mM sucrose, 220 mM Mannitol, 1 mM EGTA, 2 mM HEPES. pH at
413 7.4) and homogenised on ice in a glass Dounce homogeniser. Samples were then twice pelleted by
414 centrifugation, first at 1000 g x 10 min to remove insoluble debris and second at 10,000 g x 10 min to
415 extract the crude mitochondrial fraction, both centrifugation steps were performed at 4 °C and the
416 supernatant of the second step was collected as the post-mitochondrial fraction (PMF). The
417 mitochondrial pellet was re-solubilised in 1 mL of isolation buffer by repeated pipetting on ice prior to
418 centrifugation (10.000 g x 10 min at 4*C) and resuspension in 50 µL of isolation buffer. Protein
419 concentration of both mitochondrial and PMF was determined by BCA assay, 10 µg of protein was
420 then prepared as previously described (4). Reduction/alkylation (10mM TCEP, 40mM CAA) buffer was
421 added to each sample before incubation for 20 minutes at 60 °C. Once cooled to room temperature,
422 0.4 mg trypsin and 0.4 mg LysC was added to each sample and incubated overnight (18h) at 37C with
423 gentle agitation. 30µL water and 50 µL 1% TFA in ethyl acetate was added to stop digestion and
424 dissolve any precipitated SDC. Samples were prepared for mass spectrometry analysis by StageTip

425 clean up using SDB-RPS solid phase extraction material (99). Briefly, 2 layers of SDB-RPS material was
426 packed into 200 µL tips and washed by centrifugation at 1,000 x g for 2 minutes with 50 µL acetonitrile
427 followed by 0.2% TFA in 30% methanol and then 0.2% TFA in water. 50 µL of samples were loaded to
428 StageTips by centrifugation at 1,000 g for 3 minutes. Stage tips were washed with subsequent spins at
429 1,000 g for 3 minutes with 50 µL 1% TFA in ethyl acetate, then 1% TFA in isopropanol, and 0.2% TFA
430 in 5% ACN. Samples were eluted by addition of 60µL 60% ACN with 5% NH_4OH_4. Samples were dried
431 by vacuum centrifugation and reconstituted in 30 µL 0.1% TFA in 2% ACN.

432

433 Mass spectrometry analysis

434 Proteomic sample analysis was conducted using a Dionex UltiMate 3000 RSLCnano LC coupled to a
435 Exploris Orbitrap mass spectrometer. 2 µL of sample was injected on to an in-house packed 150 µm x
436 15 cm column (1.9 mm particle size, ReproSilPurC18-AQ) and separated using a gradient elution and
437 with column temperature of 60 °C, with Buffer A consisting of 0.1% formic acid in water and Buffer B
438 consisting of 0.1% formic acid in 80% ACN. Samples were loaded to the column at a flow rate 3 µL min^{-}
439 ^{1} at 3% B for 3 minutes, before dropping to 1.2 µL $min-^{1}$ over 1 minute for the gradient elution. The
440 gradient was increased to 32% B over 50 min, then to 60% B over 0.5 min and 98% B over 0.5 min and
441 held for 1.5 min, before returning to a flow rate of 3 µL min^{-1} at 3% B. Eluting peptides were ionized
442 by electrospray with a spray voltage of 2.3kV and a transfer capillary temperature of 300 °C. Mass
443 spectra were collected using a DIA method with varying isolation width windows (widths of *m/z* 22-
444 589) between 350 - 1650 according to Table S1. MS1 spectra were collected between *m/z* 350-1650
445 at a resolution of 60,000 and an AGC target of 4e5 with a 50 ms maximum injection time. Ions were
446 fragmented with stepped HCD collision energy at 27.5% and MS2 spectra collected between m/z 300-
447 2000 at resolution of 30,000, with an AGC target of 3e5 and the maximum injection time of 54 ms.

448

449 Proteomics raw data files were searched using DIA-NN using a library free FASTA search against the
450 reviewed UniProt mouse proteome (downloaded May 2020) with deep learning enabled (99, 100).
451 The protease was set to Trypsin/P with 1 missed cleavage, N-term M excision, carbamidomethylation
452 and M oxidation options on. Peptide length was set to 7-30, precursor range 350-1650, and fragment
453 range 300-2000, and FDR set to 1%. Both the PMF and mitochondrial fractions were filtered for
454 mitochondrial proteins using based on MitoCarta 3.0 and presence in 50% in mice. Across both
455 fractions we quantified 2073 proteins (1629 proteins in the PMF and 444 in the mitochondrial
456 fraction). Proteomic intensities were log2 transformed and median normalised prior to analysis to
457 achieve normal distributions and account for technical variation in total protein. The mass
458 spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the
459 PRIDE (101) partner repository with the dataset identifier PXD042277.

460

461 Data analysis

462 All data analysis and visualisation was performed in either the R programming environment (102) or
463 GraphPad Prism (GraphPad Software, San Diego, California USA). For protein-trait analysis the
464 Matsuda Index was calculated using glucose tolerance data before being log2 transformed. Linear
465 models were generated using the *lm()* function in R where Matsuda Index = α + proteinX + covariate
466 + ε (α = intercept and ε = error) using a gaussian distribution. To correct for multiple testing, p-values
467 were adjusted using the q-value method in the R package *qvalue* (103). Chi-square tests for
468 distribution differences within the data and two/one-way ANOVA tests for group differences were
469 performed in GraphPad Prism.

470

471 Gene-set enrichment

472 Gene-set enrichment analysis for each mitochondrial and post-mitochondrial fraction was conducted
473 using Matsuda Index effect sizes for each protein and performed in R using the *gseGO()* function within
474 the clusterprofiler package (104). Over-representation analysis of protein-protein interaction clusters
475 and KEGG pathway analysis of insulin resistance fingerprint proteins were performed in WebGestalt
476 (105). All enrichment tests were performed using all quantified proteins within a given fraction as a
477 background gene-set. P-value correction was performed using false-discovery rate correction.

478

479 Genetic mapping analysis

480 Genetic mapping analysis was performed in R using the QTL2 package (106). The GIGA-MUGA single
481 nucleotide polymorphism array was used as genomic inputs for mapping (95). pQTL analysis was
482 performed by linear mixed modelling on z-scored protein abundance data with probabilistic
483 estimation of expression residuals (PEER) factor adjustment, a covariate, and a kinship matrix to
484 account for genetic relatedness amongst the DOz animals. PEER factor adjustment was performed
485 using the top 10 calculated PEER factors, as described (107). Significance thresholds were established
486 by performing 1000 permutations and set at $P < 0.1$ for cis-acting pQTL and $P < 0.05$ for trans acting
487 pQTL. The cis-pQTL window was set as ± 2 Mbp.

488

489 Connectivity Map and scoring matrix

490 Insulin resistance 'fingerprint' proteins were queried in Connectivity Map using the CLUE software
491 platform (16, 19). The list of 76 'fingerprint proteins' were queried against the L1000 gene expression
492 dataset in the "Query" function of CLUE and results for small molecules (trt_cp) and ligands (trt_lig)
493 were extracted using the CLUE "Morpheus" platform. Raw connectivity score values were used to rank
494 perturbagens. Connectivity scores were averaged across all cell lines in the L1000 dataset. The Broad
495 Institute small molecule inhibitors denoted by the prefix were removed from our resulting dataset as

496 they are not commercially available. Connectivity Map scores were combined with the results from
497 our GLUT4 translocation screen to rank consensus compounds. This was done by first z-scoring each
498 value (% GLUT4 at the plasma membrane for each assay and raw connectivity score). This produced a
499 value which indicates how well each compound performs in a given test relative to the rest of the
500 dataset. Then the average of all three GLUT4 assays (basal agonism, insulin sensitisation, and insulin
501 resistance reversal) was added to the z-scored Connectivity Score to produce an overall score for each
502 compound. Z-score adjustment for each assay and CMAP score was performed as follows:

503 $$Zscore = \frac{x - mean}{SD}$$

504 Where:

505 - Zscore = adjusted value for a given compound
506 - x = observed score for given compound
507 - mean = average score across all compounds
508 - SD = standard deviation of all compounds

509 Overall score for each compound was calculated as follows:

510 $$\frac{\text{z-scored }(bas) + \text{ z-scored }(ins.sens) + \text{z-scored }(ins.res.rev)}{3} + \text{ z-scored }(CMAP)$$

511 $$= Overall\ score$$

512 Cell culture

513 GLUT4-HA-L6 myoblasts (108) were grown in α-MEM supplemented with 10% fetal bovine serum.
514 Differentiation was induced by changing media to α-MEM supplemented with 2% horse serum for 5
515 days.

516

517 GLUT4 translocation assays

518 GLUT4 exocytosis was determined as previously reported (53). Briefly, GLUT4-HA-L6 myotubes were
519 serum starved overnight in α-MEM containing 0.2% BSA before being washed 3x with Krebs Ringer
520 phosphate buffer supplemented with 0.2% BSA. Cells were stimulated with either 1 nM or 100 nM
521 insulin for 20 minutes before being washed with ice-cold PBS and placed on ice. Cells were then fixed
522 for 30 minutes in 3% paraformaldehyde, washed with phosphate buffered saline. Remaining
523 paraformaldehyde was quenched with 50 mM glycine. Cells were then blocked for 20 minutes in either
524 5% normal swine serum (NSS) or 5% NSS with 0.1% saponin (for measurement of total GLUT4 levels).
525 After blocking cells were washed and incubated with anti-HA antibody (Convance, 1:200 in 5% NSS)
526 for 45 minutes before incubation with secondary antibody for 20 minutes. Total and plasma
527 membrane GLUT4-HA was determined by fluorescence plate reader at 485/520 nm. GLUT4
528 translocation was calculated as % of total GLUT4 at the plasma membrane. For palmitate induced
529 insulin resistance assays myotubes were incubated in αMEM overnight supplemented with either 125
530 µM palmitic acid conjugated to BSA or equivalent BSA as a vehicle control before performing assay as
531 above. For assessment of the Prestwick library of compounds, each compound was dissolved in DMSO
532 and added for 1 hour at a final concentration of 10 µM (0.2% DMSO) prior to experimentation.
533 Biological significance for each assay was defined as 50% of control. For basal agonists this was > 50%
534 of 100 nM insulin, for insulin sensitisers this was > 50% of the difference between 1 and 100 nM insulin,
535 and for insulin resistance reversers this was > 50% of the difference between 100 nM insulin and 100
536 nM insulin + palmitate.

537

538 2-deoxyglucose uptake

539 2-deoxyglucose uptake into GLUT4-HA-L6 cells was performed as previously described with
540 modifications (108, 109). Cells were incubated overnight in either αMEM overnight supplemented
541 with either BSA-coupled 125 µM palmitic acid or BSA vehicle control before being washed 3x with
542 HEPES buffed saline (HBS). Cells were then incubated in HBS supplemented with 10 µM unlabelled 2-

543 deoxyglucose and 0.5 µCi/ml [^{3}H]-2-deoxyglucose at 37°C for 5 minutes. Cells were then washed 5x
544 with ice-cold PBS and lysed in 1M NaOH. For non-specific background uptake, 1 well per condition was
545 pre-treated with cytochalasin B. Counts were determined by Perkin Elmer Quantulus GCT Liquid
546 Scintillation Counter (Perkin Elmer, Waltham, MA, USA). Glucose uptake was expressed relatively to
547 protein concentration as determined by bicinchoninic acid (BCA) assay after subtraction of non-
548 specific uptake.

549

550 Ex vivo glucose uptake

551 Ex vivo glucose uptake was performed as previously described (4). Mice were euthanized by cervical
552 dislocation prior to rapid dissection of soleus (C57Bl/6J and BXH9/TyJ) and extensor digitorum longus
553 (EDL) muscle (BXH9/TyJ only). Muscle selection was based on our prior observations that only soleus
554 muscles in C57Bl/6J mice develop diet-induced insulin resistance. Both the soleus and EDL muscles
555 were mounted and then incubated for 1 h in Krebs Henseleit buffer (KHB; 5.5 mM glucose, 2 mM
556 pyruvate and 0.1% BSA) that had been gassed with carbogen (95% O2/5% CO2) supplemented with
557 either 10 µM thiostrepton or a DMSO vehicle control. Glucose uptake was assessed by then switching
558 the muscle into KHB supplemented with 0.375 mCi/ml [3H]-2-dexoyglucose (2-DG), 0.05 mCi/ml [14C]-
559 Mannitol, 100 nM insulin and either thiostrepton (10 µM) or DMSO vehicle control for 20 minutes at
560 30 °C followed by washing in ice-cold PBS and then snap frozen in liquid nitrogen. Samples were lysed
561 in 250 mM NaOH at 70 °C. Tracer in the muscle tissue lysates was quantified by liquid scintillation
562 counting and [3H]-2-DG was corrected for extracellular [14C]-mannitol then normalized to wet weight
563 of the tissue.

564

565 Immunoblotting

566 Glut4-HA-L6 myotubes were incubated overnight in 125 µM palmitate or BSA control prior to
567 treatment with drugs/insulin as indicated. Cells were then washed in ice-cold PBS and lysed by
568 scraping directly into 55 °C Laemmli sample buffer with 10 % (tris 2-carboxyethyl phosphine; TCEP).
569 Samples were then sonicated for 24 s (3s on/3s off) and heated at 65 °C for 5 minutes. SDS-PAGE was
570 performed. Samples were resolved by SDS-PAGE as previously described (4), transferred onto PVDF
571 membranes and blocked in TBS-T (0.1% Tween in Tris-buffered saline) containing 5% skim milk for 1
572 hour. Membranes were then washed 3 x 10 minutes in TBS-T and incubated overnight in primary
573 antibodies against phosphorylated Akt T308 (Cell Signaling Technologies #2965; diluted 1:1000),
574 phosphorylated Akt S473 (Cell Signaling Technologies #9271; diluted 1:1000), pan-Akt (Cell Signaling
575 Technologies #9272; diluted 1:1000), phosphorylated GSK-3α/β S21/9 (Cell Signaling Technologies
576 #9327; diluted 1:1000), GSK3α/β (Cell Signaling Technologies #5676; diluted 1:1000), phosphorylated
577 PRAS40 T246 (Cell Signaling Technologies #13175; diluted 1:1000), PRAS40 (Cell Signaling
578 Technologies #2691; diluted 1:1000), phosphorylated AMPK (Cell Signaling Technologies, #2531;
579 diluated 1:1000), α-tubulin (Cell Signalling Technologies #2125; diluted 1:1000), 14-3-3 (Santa Cruz
580 #sc-1657; diluted 1:5000) . The following day membranes were washed 3 x 10 minutes in TBS-T and
581 incubated for 1 hour in species-appropriate fluorescent or HRP secondary antibodies. Imaging and
582 densitometry were performed using LI-COR Image Studio or a Bio-Rad ChemiDoc Imaging System (Bio-
583 Rad, Hercules, CA, USA) and Image J (110). Phosphorylated proteins were normalised against their
584 relevant controls and data was normalised based on average band intensity.

585

586 Mitochondrial stress test

587 Cellular respirometry (oxygen consumption rate, OCR) was performed using Seahorse XFp miniplates
588 and a Seahorse XF HS Mini Analyzer (Seahorse Bioscience, Copenhagen, Denmark) as previously
589 described (111). GLUT4-HA-L6 myotubes were incubated overnight in either palmitate or BSA control
590 αMEM before treatment with either thiostrepton (10 µM) or DMSO vehicle control. Cells were washed

twice with KRBH and incubated in KRBH supplemented with 2.8 mM glucose, thiostrepton or vehicle control without BSA (150 µl/well) at 37°C for 1h in non-CO2 incubator. Cells were then assayed in XFp Analyzer. The OCR was measured after a 12-min equilibration period followed by 3/0/3 min of mix/wait/read cycles. Following stabilization of baseline rates, compounds were injected sequentially to reach a final concentration of: 20 mM glucose, oligomycin (5µg/ml), FCCP (1 µM) and rotenone/antimycin A (5 µM) to assess glucose-dependent respiration (calculated by baseline – glucose OCR), ATP-linked respiration (determined by glucose – oligomycin OCR), maximal respiration (calculated by FCCP – AntA/Rot OCR) and non-mitochondrial respiration respectively (equal to AntA/Rot OCR). Data were normalised against protein concentration and presented as baseline adjusted.

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Figure 1:

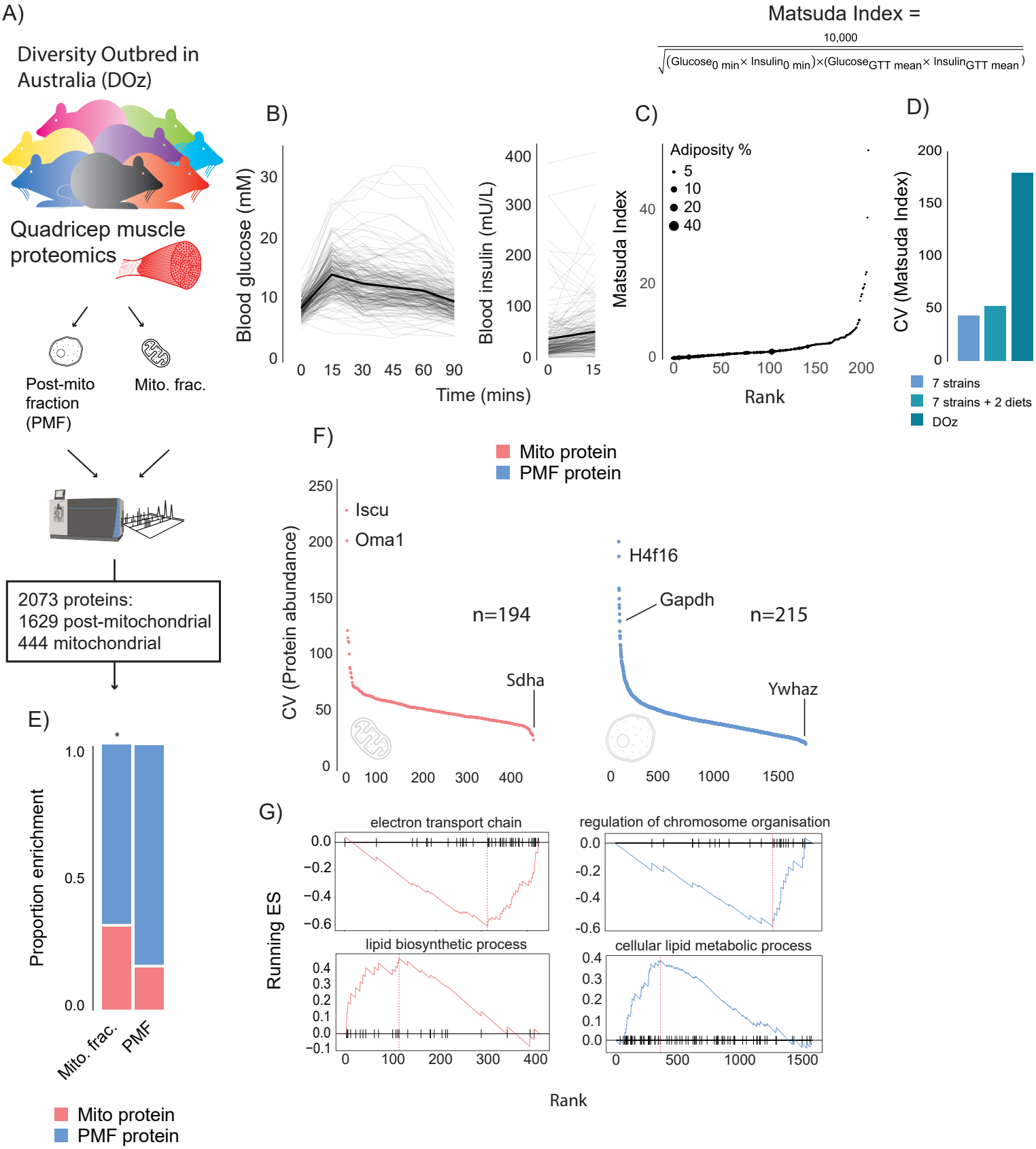

A)
Diversity Outbred in Australia (DOz)
Quadricep muscle proteomics
Post-mito fraction (PMF)
Mito. frac.
2073 proteins:
1629 post-mitochondrial
444 mitochondrial
Matsuda Index =
10,000
√((Glucose0 min × Insulin0 min)×(GlucoseGTT mean × InsulinGTT mean))
B)
Blood glucose (mM)
Time (mins)
Blood insulin (mU/L)
C)
Adiposity %
5
10
20
40
Matsuda Index
Rank
D)
CV (Matsuda Index)
7 strains
7 strains + 2 diets
DOz
E)
Proportion enrichment
Mito. frac.
PMF
Mito protein
PMF protein
F)
Mito protein
PMF protein
CV (Protein abundance)
Iscu
Oma1
n=194
Sdha
H4f16
Gapdh
n=215
Ywhaz
G)
electron transport chain
regulation of chromosome organisation
lipid biosynthetic process
cellular lipid metabolic process
Running ES
Rank

Figure 2

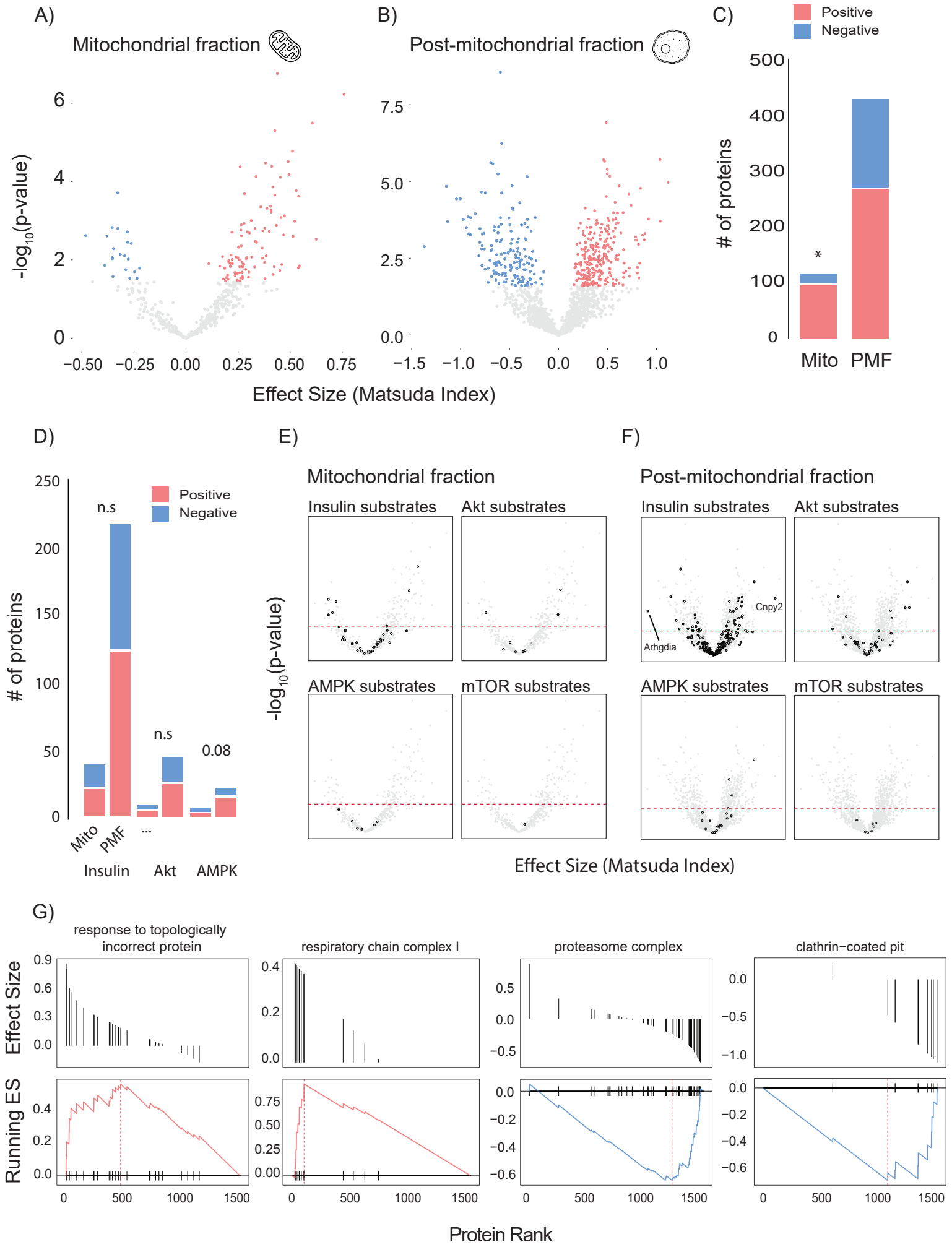

A)
Mitochondrial fraction
B)
Post-mitochondrial fraction
C)
Positive
Negative
-log10(p-value)
Effect Size (Matsuda Index)
# of proteins
Mito
PMF
D)
n.s
n.s
0.08
Insulin
Akt
AMPK
E)
Mitochondrial fraction
F)
Post-mitochondrial fraction
Insulin substrates
Akt substrates
AMPK substrates
mTOR substrates
Cnpy2
Arhgdia
G)
response to topologically incorrect protein
respiratory chain complex I
proteasome complex
clathrin−coated pit
Effect Size
Running ES
Protein Rank

Figure 3:

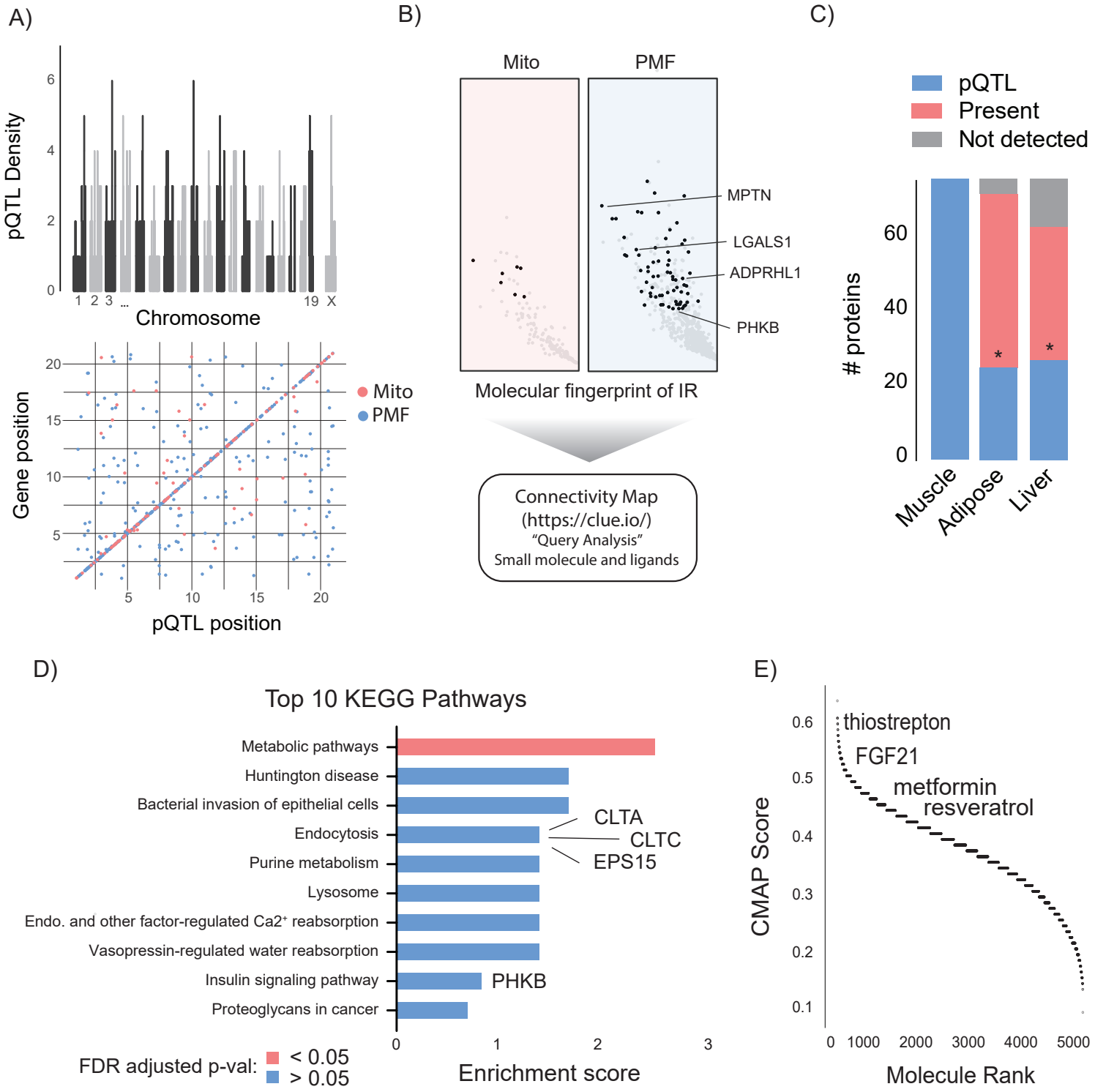

A)
pQTL Density
Chromosome
Gene position
pQTL position
Mito
PMF
B)
Mito
PMF
MPTN
LGALS1
ADPRHL1
PHKB
Molecular fingerprint of IR
Connectivity Map
(https://clue.io/)
"Query Analysis"
Small molecule and ligands
C)
pQTL
Present
Not detected
proteins
Muscle
Adipose
Liver
D)
Top 10 KEGG Pathways
Metabolic pathways
Huntington disease
Bacterial invasion of epithelial cells
Endocytosis
Purine metabolism
Lysosome
Endo. and other factor-regulated Ca2+ reabsorption
Vasopressin-regulated water reabsorption
Insulin signaling pathway
Proteoglycans in cancer
CLTA
CLTC
EPS15
PHKB
FDR adjusted p-val:
< 0.05
> 0.05
Enrichment score
E)
CMAP Score
thiostrepton
FGF21
metformin
resveratrol
Molecule Rank

Figure 4:

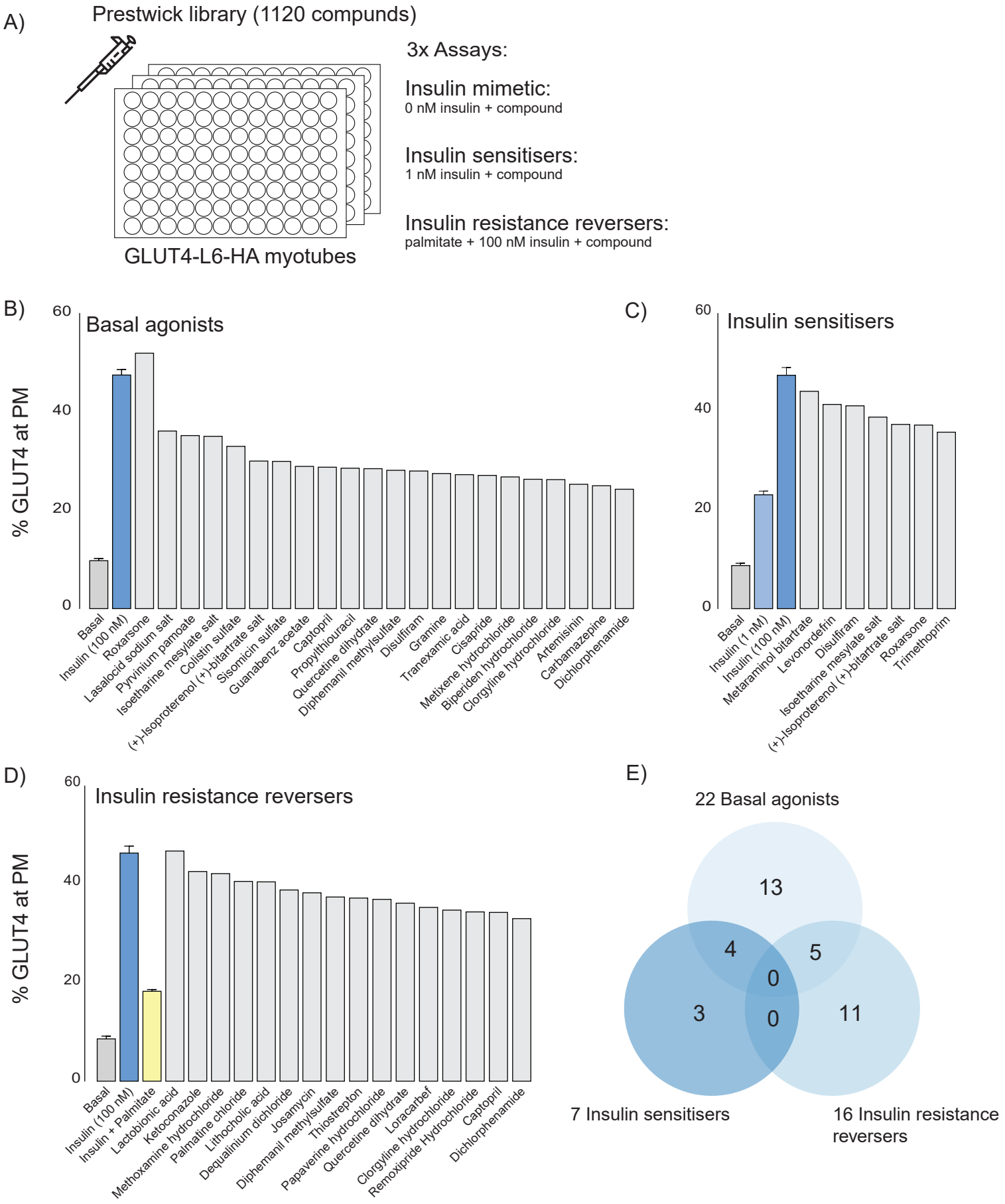

A)
Prestwick library (1120 compunds)
3x Assays:
Insulin mimetic:
0 nM insulin + compound
Insulin sensitisers:
1 nM insulin + compound
Insulin resistance reversers:
palmitate + 100 nM insulin + compound
GLUT4-L6-HA myotubes
B)
Basal agonists
% GLUT4 at PM
60
40
20
0
Basal
Insulin (100 nM)
Roxarsone
Lasalocid sodium salt
Pyrvinium pamoate
Isoetharine mesylate salt
Colistin sulfate
(+)-Isoproterenol (+)-bitartrate salt
Sisomicin sulfate
Guanabenz acetate
Captopril
Propylthiouracil
Quercetine dihydrate
Diphemanil methylsulfate
Disulfiram
Gramine
Tranexamic acid
Cisapride
Metixene hydrochloride
Biperiden hydrochloride
Clorgyline hydrochloride
Artemisinin
Carbamazepine
Dichlorphenamide
C)
Insulin sensitisers
60
40
20
0
Basal
Insulin (1 nM)
Insulin (100 nM)
Metaraminol bitartrate
Levonordefrin
Disulfiram
Isoetharine mesylate salt
(+)-Isoproterenol (+)-bitartrate salt
Roxarsone
Trimethoprim
D)
Insulin resistance reversers
% GLUT4 at PM
60
40
20
0
Basal
Insulin (100 nM)
Insulin + Palmitate
Lactobionic acid
Ketoconazole
Methoxamine hydrochloride
Palmatine chloride
Lithocholic acid
Dequalinium dichloride
Josamycin
Diphemanil methylsulfate
Thiostrepton
Papaverine hydrochloride
Quercetine dihydrate
Loracarbef
Clorgyline hydrochloride
Remoxipride Hydrochloride
Captopril
Dichlorphenamide
E)
22 Basal agonists
13
4
5
0
3
0
11
7 Insulin sensitisers
16 Insulin resistance reversers

Figure 5:

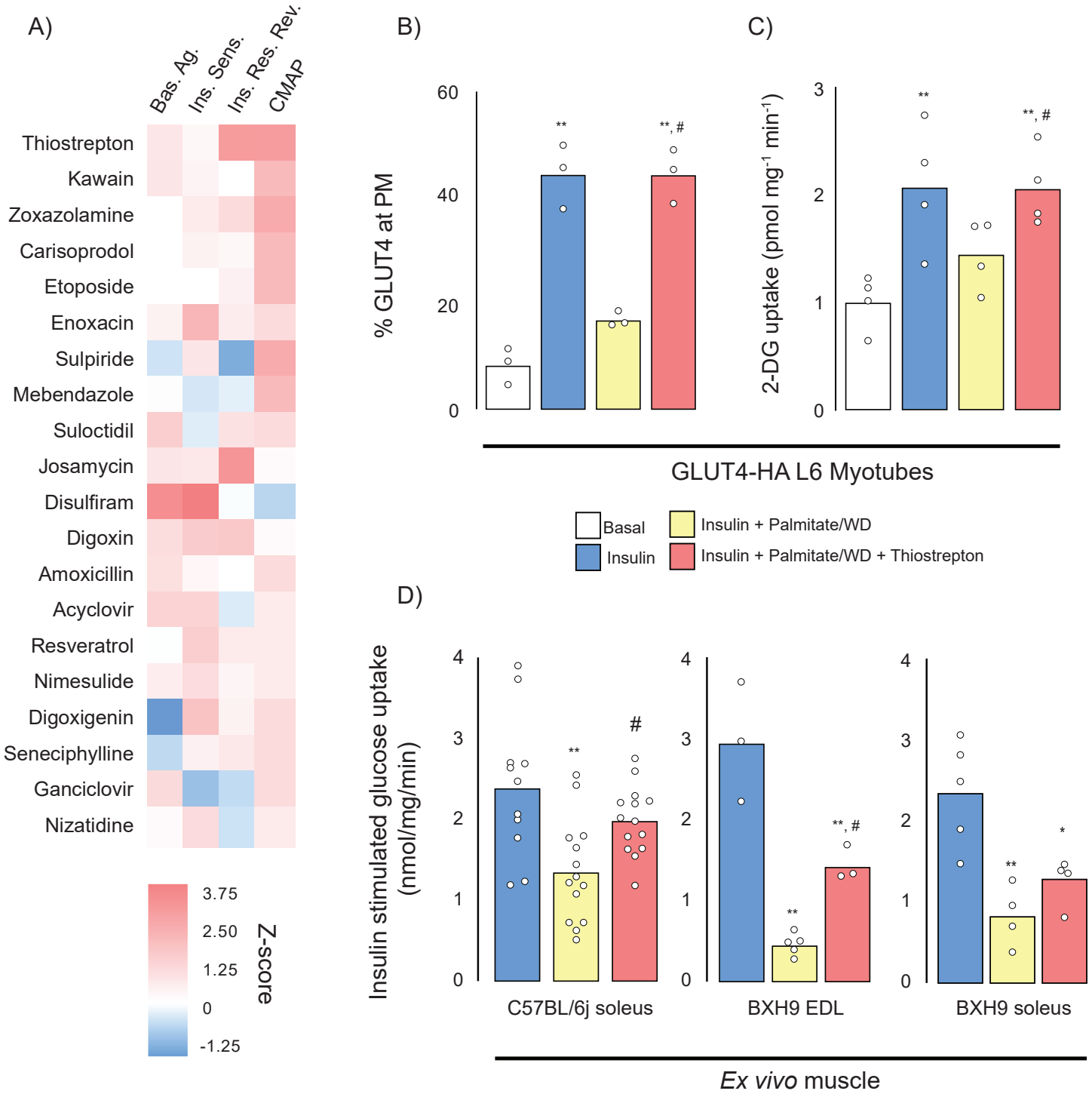

A)
Bas. Ag.
Ins. Sens.
Ins. Res.
Rev. CMAP
Thiostrepton
Kawain
Zoxazolamine
Carisoprodol
Etoposide
Enoxacin
Sulpiride
Mebendazole
Suloctidil
Josamycin
Disulfiram
Digoxin
Amoxicillin
Acyclovir
Resveratrol
Nimesulide
Digoxigenin
Seneciphylline
Ganciclovir
Nizatidine
3.75
2.50
1.25
0
-1.25
Z-score
B)
% GLUT4 at PM
60
40
20
0
**
**, #
C)
2-DG uptake (pmol mg-1 min-1)
3
2
1
0
**
**, #
GLUT4-HA L6 Myotubes
Basal
Insulin
Insulin + Palmitate/WD
Insulin + Palmitate/WD + Thiostrepton
D)
Insulin stimulated glucose uptake (nmol/mg/min)
4
3
2
1
0
**
#
**
**, #
**
*
C57BL/6j soleus
BXH9 EDL
BXH9 soleus
Ex vivo muscle

Figure 6

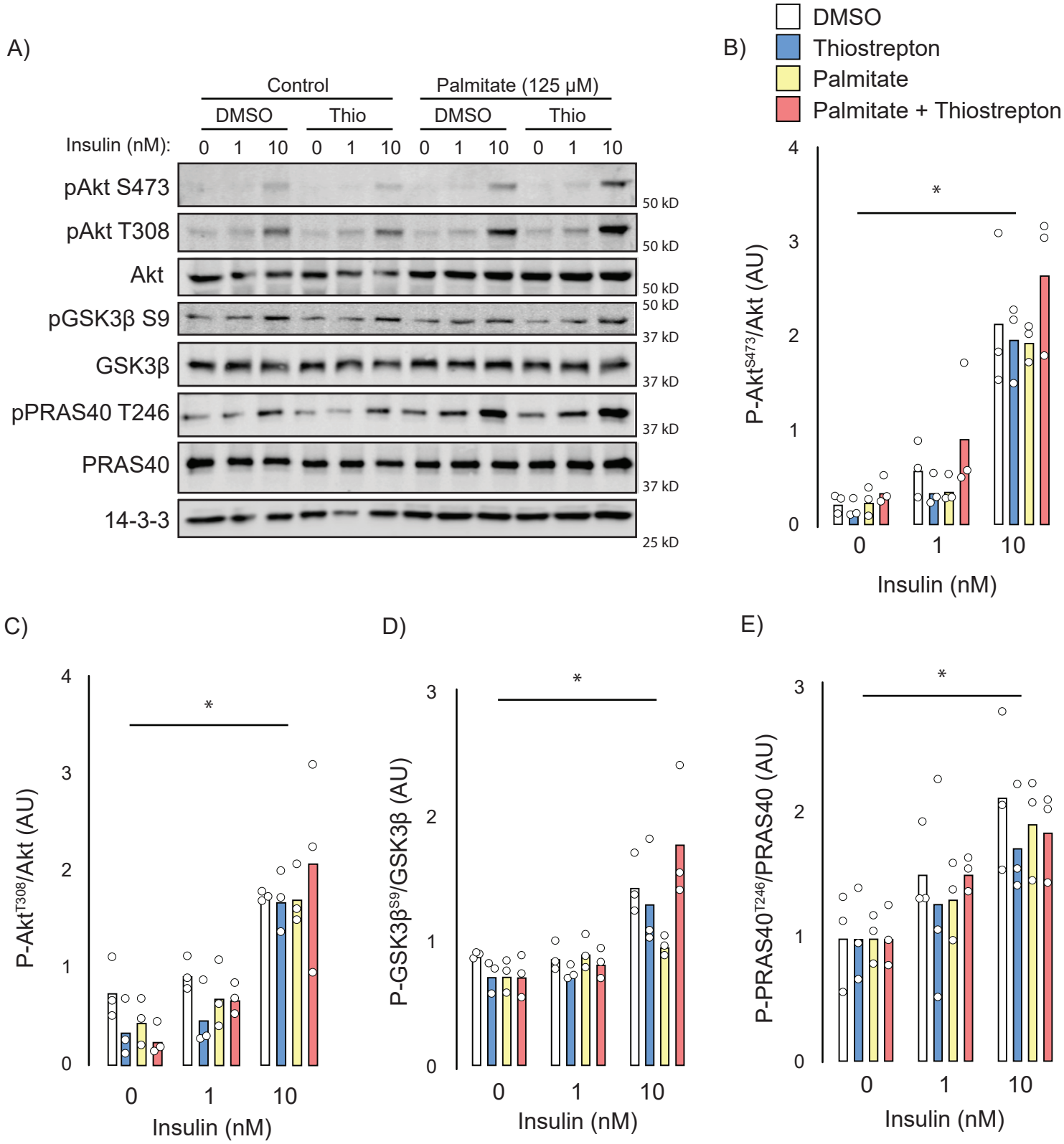
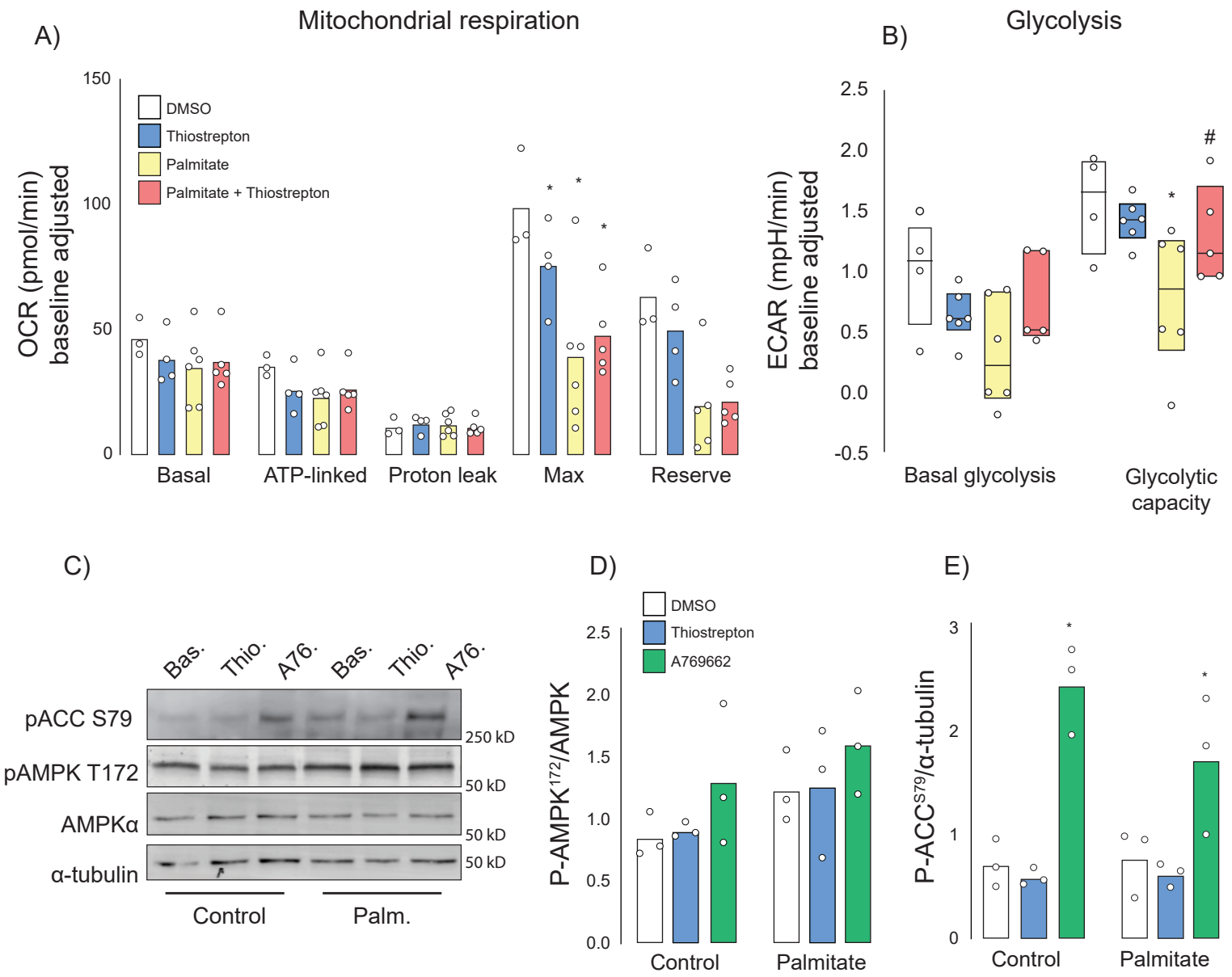
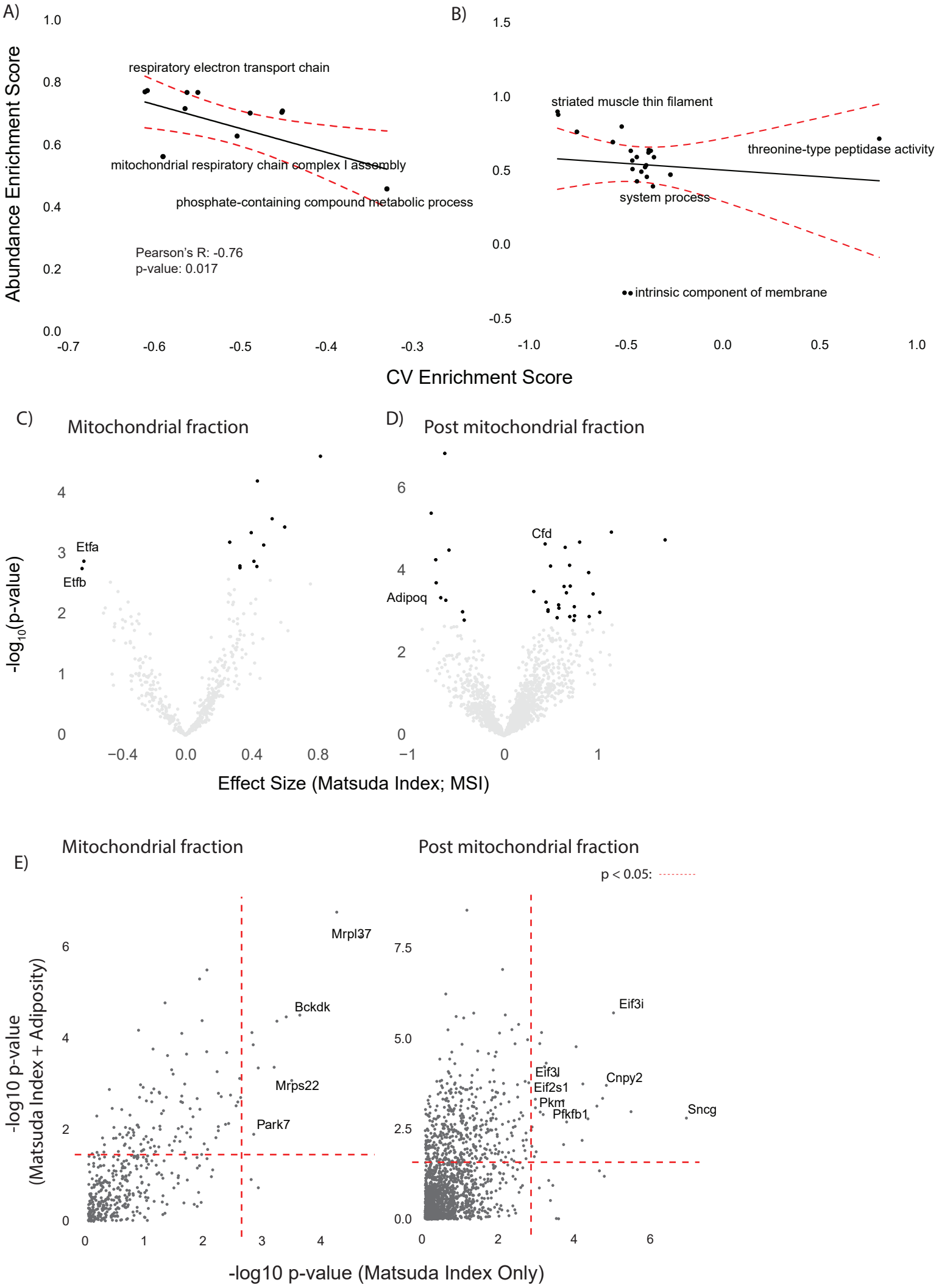

A)
Control
Palmitate (125 μM)
DMSO
Thio
DMSO
Thio
Insulin (nM): 0 1 10 0 1 10 0 1 10 0 1 10
pAkt S473
pAkt T308
Akt
pGSK3β S9
GSK3β
pPRAS40 T246
PRAS40
14-3-3
50 kD
50 kD
50 kD
50 kD
37 kD
37 kD
37 kD
37 kD
25 kD
B)
DMSO
Thiostrepton
Palmitate
Palmitate + Thiostrepton
P-Akt^{S473}/Akt (AU)
Insulin (nM)
C)
P-Akt^{T308}/Akt (AU)
Insulin (nM)
D)
P-GSK3β^{S9}/GSK3β (AU)
Insulin (nM)
E)
P-PRAS40^{T246}/PRAS40 (AU)
Insulin (nM)

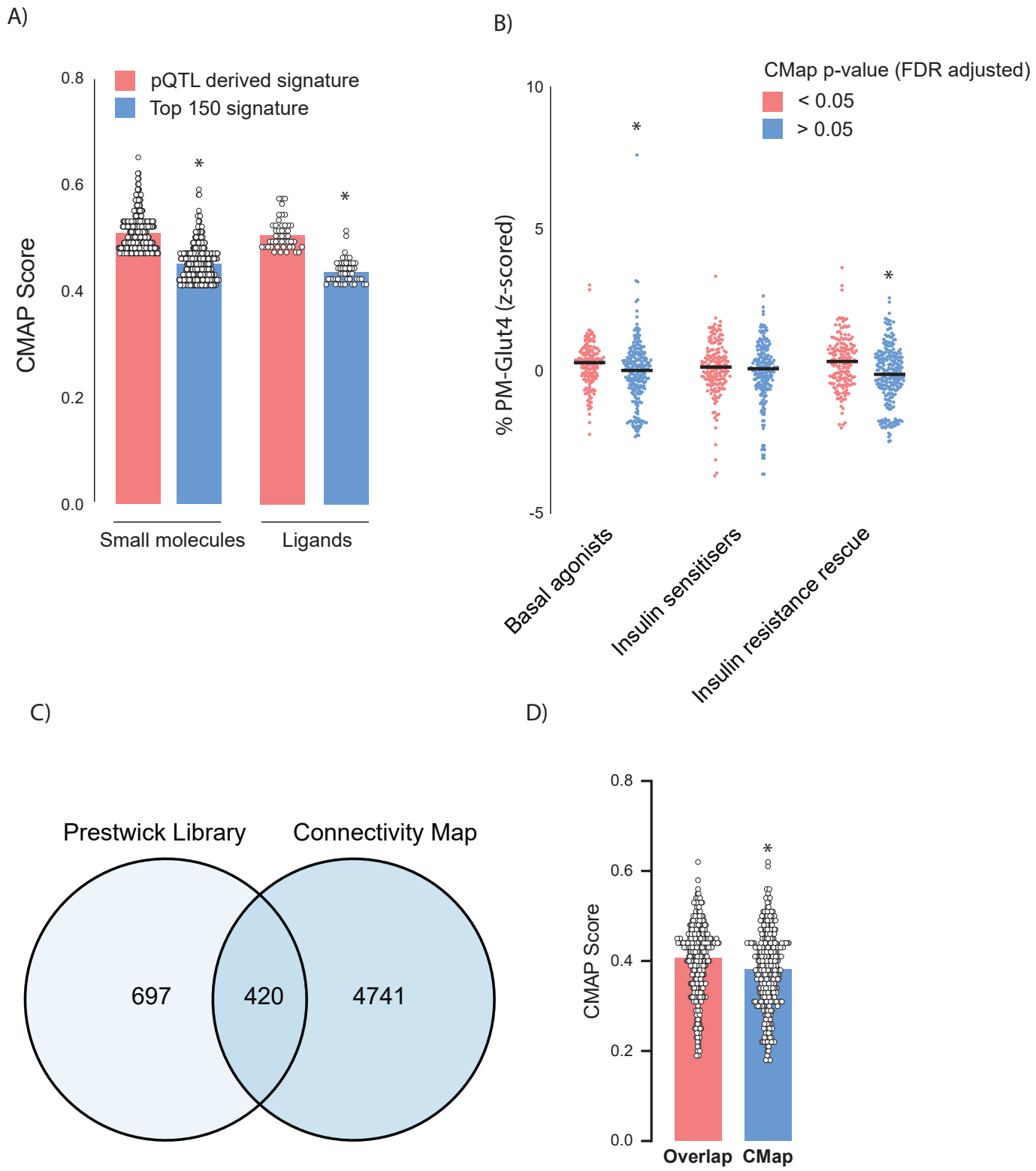
Figure 7


A)
Mitochondrial respiration
OCR (pmol/min) baseline adjusted
150
100
50
0
DMSO
Thiostrepton
Palmitate
Palmitate + Thiostrepton
Basal
ATP-linked
Proton leak
Max
Reserve
B)
Glycolysis
ECAR (mpH/min) baseline adjusted
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
#
Basal glycolysis
Glycolytic capacity
C)
Bas.
Thio.
A76.
Bas.
Thio.
A76.
pACC S79
pAMPK T172
AMPKα
α-tubulin
250 kD
50 kD
50 kD
50 kD
Control
Palm.
D)
DMSO
Thiostrepton
A769662
P-AMPK172/AMPK
2.5
2.0
1.5
1.0
0.5
0.0
Control
Palmitate
E)
P-ACCS79/α-tubulin
3
2
1
0
Control
Palmitate

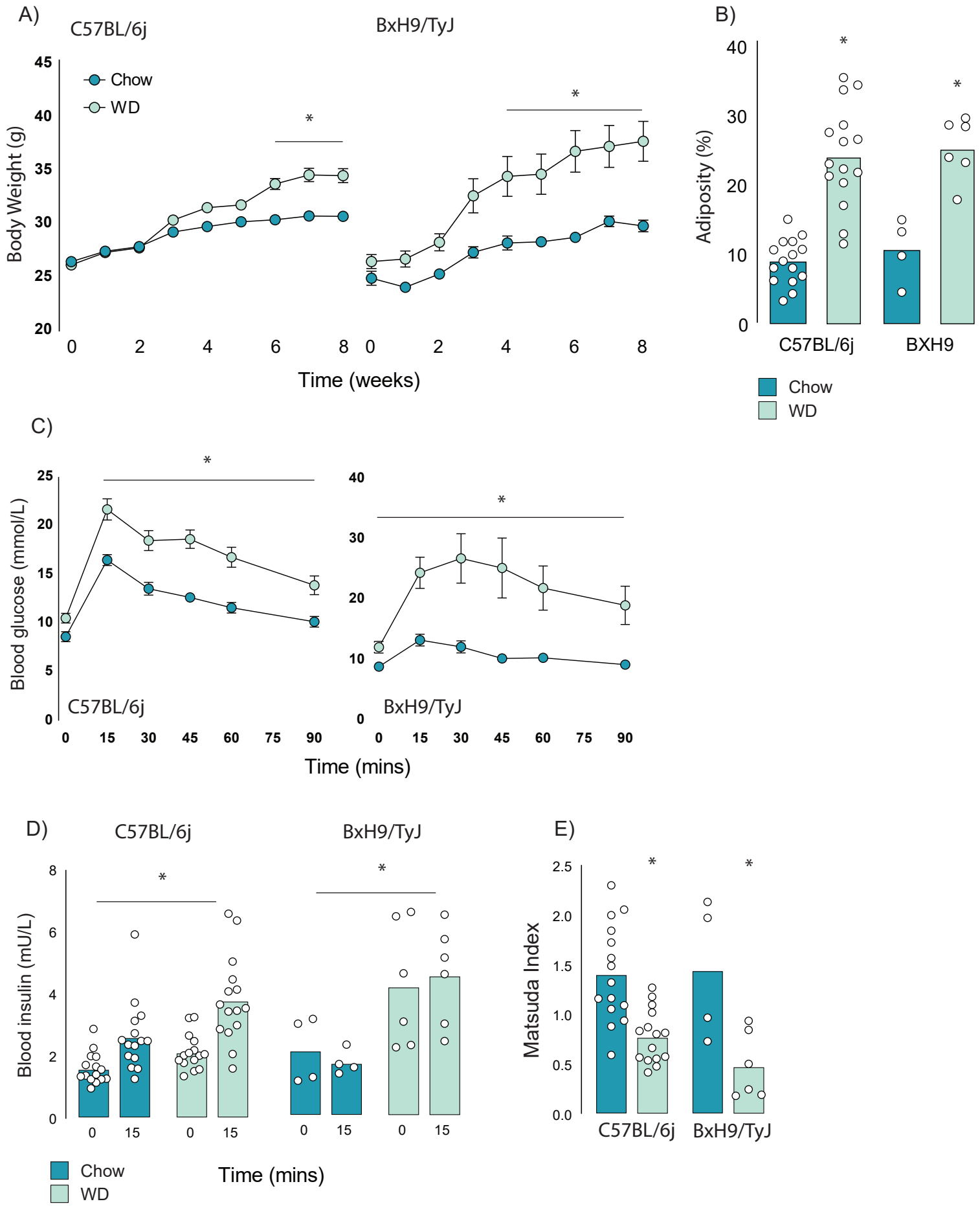
Supplementary Figure 1:



Supplementary Figure 2:



Supplementary Figure 3:



890 Figure Legends

891 Figure 1. Metabolic and proteomic diversity of Diversity Outbred in Oz (DOz) mice. (A) Schematic of
892 metabolic phenotyping and quadricep proteomics in chow fed DOz mice. (B) Blood glucose and insulin
893 levels during a GTT. (C) Whole-body insulin sensitivity (Matsuda Index, formula shown above) and
894 adiposity of DOz mice (n= 215). (D) Comparison of coefficient of variation (CV) of insulin of Matsuda
895 Index across inbred strains and diets versus chow fed DOz mice. (E) Relative enrichment of
896 mitochondrial (Mito) proteins in mitochondrial fraction and post-mitochondrial fraction (PMF) of
897 quadricep proteomes. (F) Relative protein CV across mitochondrial and post-mitochondrial quadricep
898 fractions. (G) Biological pathways enriched in mitochondrial and post-mitochondrial quadricep
899 fractions, running enrichment score for a given pathway (ES) on y-axis and proteins ranked by CV on
900 x-axis. Significance testing was performed by Chi-square test. * Indicates a significant difference $P <$
901 0.01.

902

903 Figure 2. Linear modelling of quadricep proteome and whole-body insulin sensitivity. (A-B) Volcano
904 plot with Matsuda Index effect sizes (x-axis) and significance (y-axis) for mitochondrial (A) and post-
905 mitochondrial (B) quadricep proteins using a linear model with adiposity as a covariate. Significant
906 proteins with positive and negative effect sizes are indicated in red and blue, respectively. (C)
907 Comparison of positively and negatively associated proteins between fractions. (D) Number of
908 proteins identified in each fraction with known roles in insulin or AMPK signalling. (E-F) Volcano plot
909 shown in A with mitochondrial (E) and post-mitochondrial fraction (F) proteins shown in black that
910 have documented roles in indicated signalling pathways. Adjusted p-value threshold is indicated (red
911 dotted line). (G) Pathways enriched for proteins which positively and negative associate with Matsuda
912 Index. Effect sizes of proteins within a given pathway and running enrichment score (ES) for a that
913 pathway on y-axis and proteins ranked by CV on x-axis. (ES) on y-axis and proteins ranked by CV on x-
914 axis. of proteins within pathways that are enriched for proteins associated with whole-body insulin

915 sensitivity. Proteins ranked by Matsuda Index effect size on x-axis. Linear modelling was performed
916 using a gaussian distribution with q-value adjustment of p-values. Enrichment tests between fractions
917 were performed by Chi-square test. * Indicates a significant difference P < 0.01.

918

919 Figure 3. Integration of proteomic data via Connectivity Map. (A-B) Workflow includes filtering for
920 proteins with cis-pQTL (A) and negative association with Matsuda Index (B) prior to Connectivity Map
921 query. (A) Distribution of cis and trans-pQTL across mitochondrial and PMF proteome. (B) Left side of
922 volcano plots from Figure 2A-B (proteins negatively associated with Matsuda Index) is shown with
923 proteins comprised in Molecular Fingerprint of insulin resistance (IR) indicated in black. Proteins with
924 human homologues are highlighted. (C) Comparison of Molecular Fingerprint of insulin resistance
925 across muscle adipose and liver proteomes. (D) Top 10 KEGG pathways enriched in the molecular
926 fingerprint of insulin resistance with proteins of interest highlighted. (E) Distribution of Connectivity
927 Map (CMAP) scores for identified small molecules and ligands with compounds of interest indicated.
928 Significance testing was performed by Chi-square test. * Indicates a significant difference P < 0.001.

929

930 Figure 4. Prestwick library of FDA-approved drugs that modulate GLUT4 translocation in L6 myotubes.
931 (A) Schematic representation of the three assays performed. (B) Small molecules that promote GLUT4
932 exocytosis to the plasma membrane independently of insulin with controls (Basal, Insulin) on the left.
933 (C) Small molecules that potentiate a submaximal dose of insulin (1 nM) with controls (Basal, 1 nM
934 and 100 nM Insulin) on the left. Compounds were added in combination with 1 nM insulin. (D) Small
935 molecules that reverse palmitate induced insulin resistance with controls (Basal, Insulin, Insulin +
936 Palmitate) on the left. Compounds were added in combination with 100 nM insulin following palmitate
937 treatment. (E) Venn diagram of compound overlap between assays. Biological significance for each
938 assay was defined as 50% of corresponding control, see methods for details. Plasma-membrane; PM.

939

940 Figure 5. Cross-validation of Connectivity Map and Prestwick library. (A) Scoring matrix of top 20
941 scoring compounds present in both Connectivity Map (CMAP) and the Prestwick library screens (Basal
942 Agonists, Bas.Ag.; Insulin Sensitizer, Ins. Sens; Insulin Resistance Reversers, Ins. Res. Rev). (B-C) Insulin
943 stimulated GLUT4 translocation to the plasma membrane (PM, B) and 2-deoxyglucose uptake (C) in
944 control and insulin resistant L6 myotubes (Palmitate) treated with thiostrepton or vehicle control. (D)
945 Insulin stimulated 2-deoxyglucose uptake in soleus and extensor digitorum longus (EDL) muscles from
946 chow and WD fed C57BL/6J and BXH9/TyJ following ex vivo treatment with thiostrepton or vehicle
947 control. Data are mean with individual data points shown, n=3-4 (B-C), n=3-5 (BXH9) n=11-14
948 (C57BL/6J). Significance was determined by one-way ANOVA with Student's post hoc test. ** Indicates
949 significant difference from control (Basal or chow fed C57BL6/J) group ($p < 0.01$), * Indicates significant
950 difference from control ($p < 0.05$). # Indicates significant difference from palmitate-treated or WD-fed
951 fed control group ($p < 0.05$).

952

953 Figure 6. Effect of thiostrepton on insulin signalling. (A-E) Immunoblotting with indicated antibodies
954 of control and palmitate treated GLUT4-HA-L6 myotubes following treatment with thiostrepton or
955 vehicle control and stimulation with 0, 1, or 10 nM insulin. (A) Representative immunoblot shown of
956 3 independent experiments. (B-E) Quantification of immunoblots in A, Akt S473 (B), Akt T308 (C),
957 GSK3β S9 (D) and PRAS40 T246 (E). Data are mean with individual data points, n=3. Significance was
958 determined by two-way ANOVA with Student's post hoc test. * Indicates a significant effect of insulin
959 $P < 0.01$.

960

961 Figure 7. Effect of thiostrepton on mitochondrial respiration and glycolysis. (A-B) Oxygen consumption
962 rates (A) and extracellular acidification rates (B) in control and palmitate treated GLUT4-HA-L6

963 myotubes treated with either thiostrepton or vehicle control. (C) Immunoblotting of AMPK signalling
964 in control and palmitate treated (Palm.) GLUT4-HA-L6 myotubes treated with either thiostrepton
965 (Thio.), vehicle control (Bas.) or positive control A-769662 (A76.). Representative immunoblots shown
966 of 3 independent experiments. (D-E) Quantification of immunoblots in C, AMPK T172 (D) and ACC S79
967 (E) phosphorylation following thiostrepton or A-769662 treatment. Data are mean with individual data
968 points shown, n=3. * Indicates significant difference from control cells, # indicates significant
969 difference from palmitate treated cells $p < 0.05$.

970

971 Supplementary Figure 1. Comparison of enrichment analysis and linear modelling approaches for DOz
972 insulin sensitivity and muscle proteomics. (A-B) Abundance (y-axis) and coefficient of variation-based
973 (x-axis) gene-ontology enrichment scores for mitochondrial (A) and PMF (B) proteomes. (C-D) Volcano
974 plot with Matsuda Index effect sizes (x-axis) and significance (y-axis) for mitochondrial (C) and post-
975 mitochondrial fraction (D) proteins. (E-F) Mitochondrial (E) and post-mitochondrial fraction (F)
976 negative log10 p-values for Matsuda Index effect sizes from linear models with and without adiposity
977 as a covariate. Dashed red line indicates FDR adjusted $p < 0.05$.

978

979 Supplementary Figure 2. Evaluation of Connectivity Map analysis. (A) Raw Connectivity Map (CMAP)
980 scores for compounds and ligands identified by Connectivity map using either a pQTL filtered signature
981 (Fingerprint) or a signature of the 150 proteins with the largest negative effect sizes. (B) Comparison
982 of z scored GLUT4 at plasma membrane (PM) after treatment with Prestwick library compounds based
983 on CMAP significance. (C) Overlap of compounds found in both the Prestwick library and Connectivity
984 Map. (D) Comparison of Connectivity Map scores between overlapping compounds and all of
985 Connectivity Map. Data are mean with individual data points. Significance was determined by two-
986 way ANOVA with Student's post hoc test or Student's t-test. * Indicates a significant difference
987 between groups $P < 0.01$.

988

989 Supplementary Figure 3. Effect of western diet feeding on C57BL/6J and BxH9/TyJ mice body
990 composition and insulin sensitivity. (A-E) Body weights (A), adiposity (B), blood glucose (C) and insulin
991 (D) during a glucose tolerance test, and Matsuda Index (E) of C57BL/6J and BxH9/TyJ mice fed a chow
992 of high-fat/high-sugar (western diet; WD) for 8 weeks. Data are mean with individual data points, n=
993 4-6 (BxH9), n=15 (C57BL/6J).

994

995 Supplementary Table 1. List of proteins and their Matsuda Index effect sizes which comprise our pQTL-
996 filtered molecular fingerprint of insulin resistance.

Figure 1:

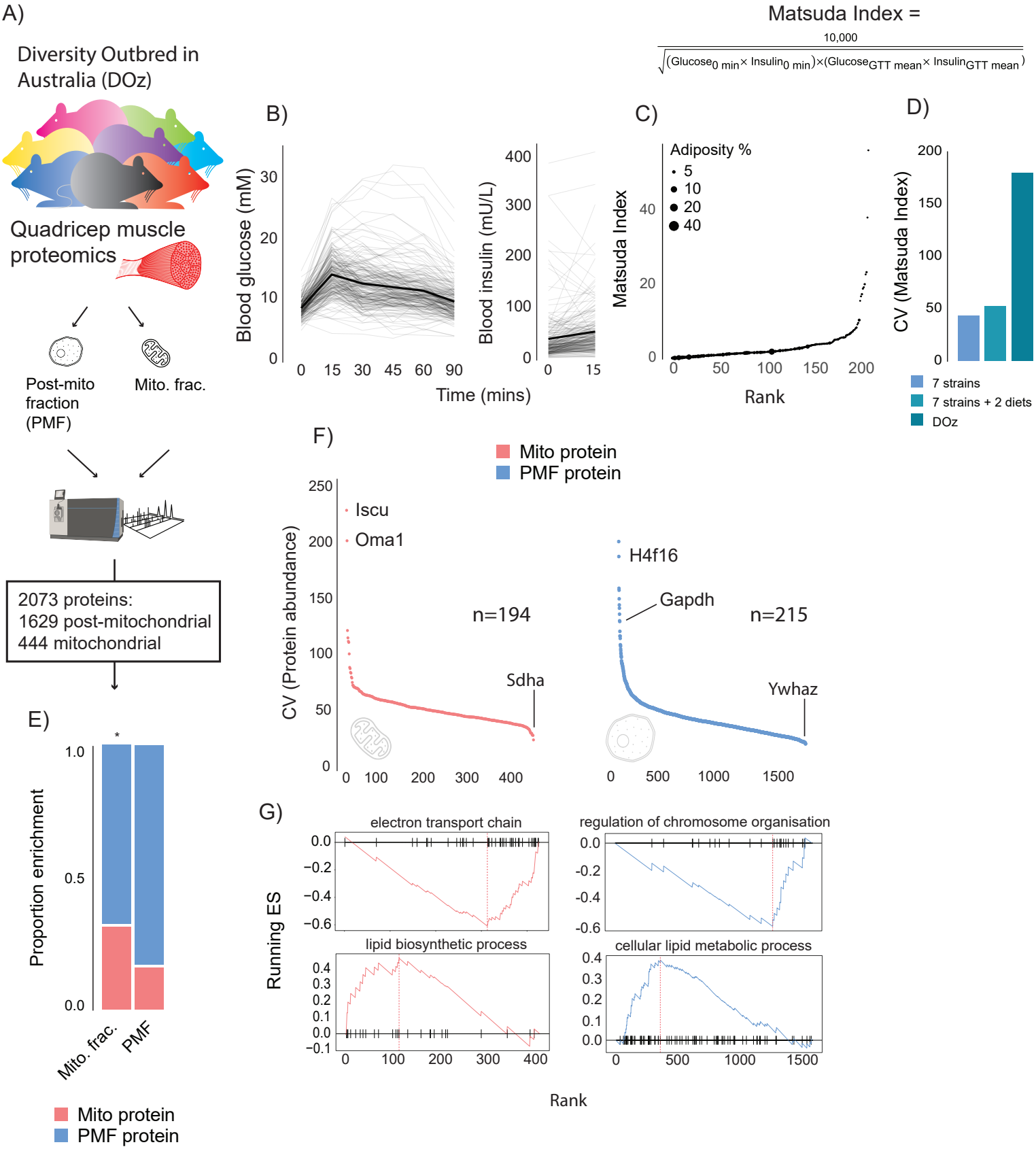

A)
Diversity Outbred in Australia (DOz)
Quadricep muscle proteomics
Post-mito fraction (PMF)
Mito. frac.
2073 proteins:
1629 post-mitochondrial
444 mitochondrial
Matsuda Index =
10,000
√((Glucose0 min × Insulin0 min)×(GlucoseGTT mean × InsulinGTT mean))
B)
Blood glucose (mM)
Time (mins)
Blood insulin (mU/L)
C)
Adiposity %
5
10
20
40
Matsuda Index
Rank
D)
CV (Matsuda Index)
7 strains
7 strains + 2 diets
DOz
E)
Proportion enrichment
Mito. frac.
PMF
Mito protein
PMF protein
F)
Mito protein
PMF protein
CV (Protein abundance)
Iscu
Oma1
n=194
Sdha
H4f16
Gapdh
n=215
Ywhaz
G)
electron transport chain
regulation of chromosome organisation
lipid biosynthetic process
cellular lipid metabolic process
Running ES
Rank

Figure 2

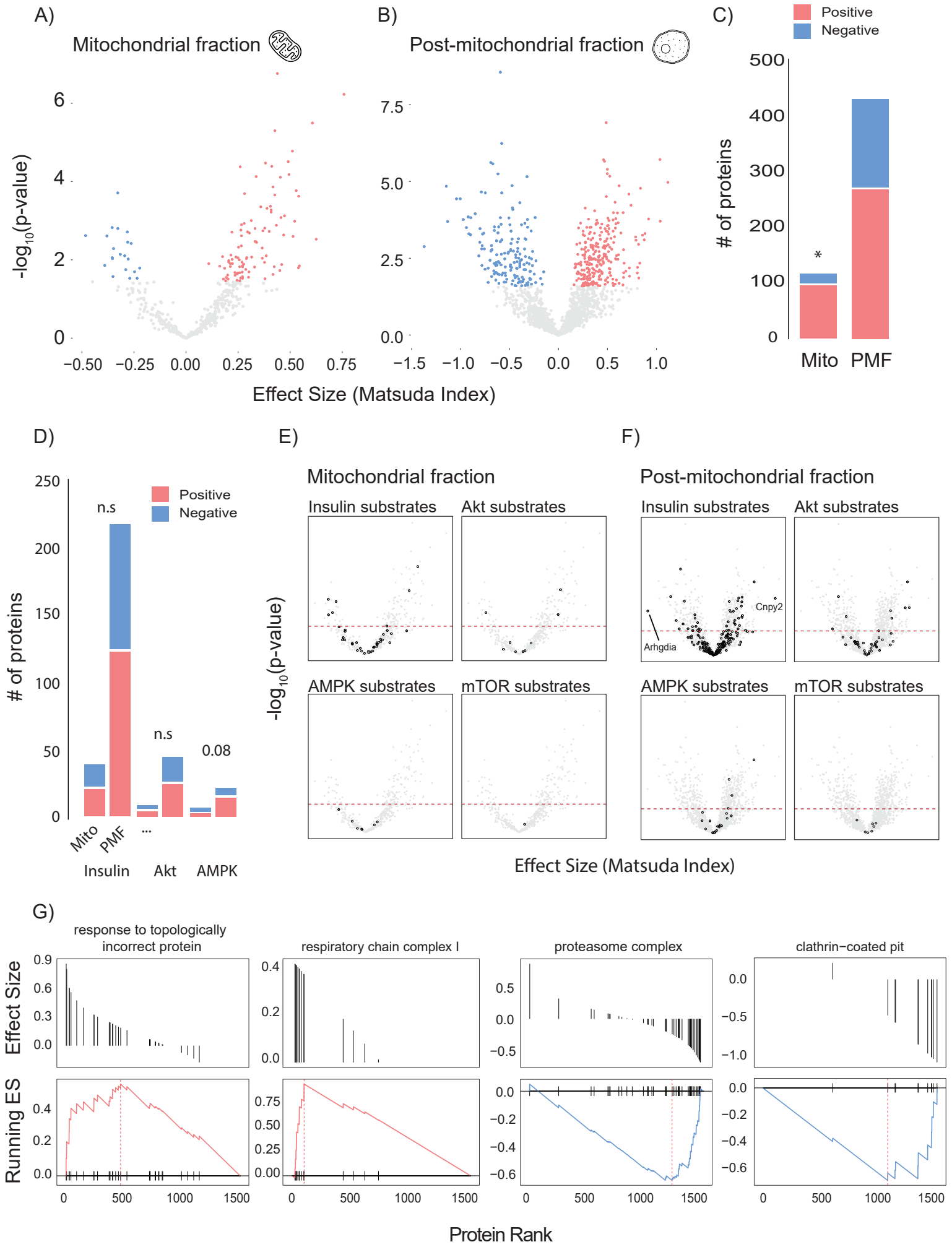

A)
Mitochondrial fraction
B)
Post-mitochondrial fraction
C)
Positive
Negative
-log10(p-value)
Effect Size (Matsuda Index)
# of proteins
Mito
PMF
D)
n.s
n.s
0.08
Insulin
Akt
AMPK
E)
Mitochondrial fraction
F)
Post-mitochondrial fraction
Insulin substrates
Akt substrates
AMPK substrates
mTOR substrates
Cnpy2
Arhgdia
G)
response to topologically incorrect protein
respiratory chain complex I
proteasome complex
clathrin−coated pit
Effect Size
Running ES
Protein Rank

Figure 3:

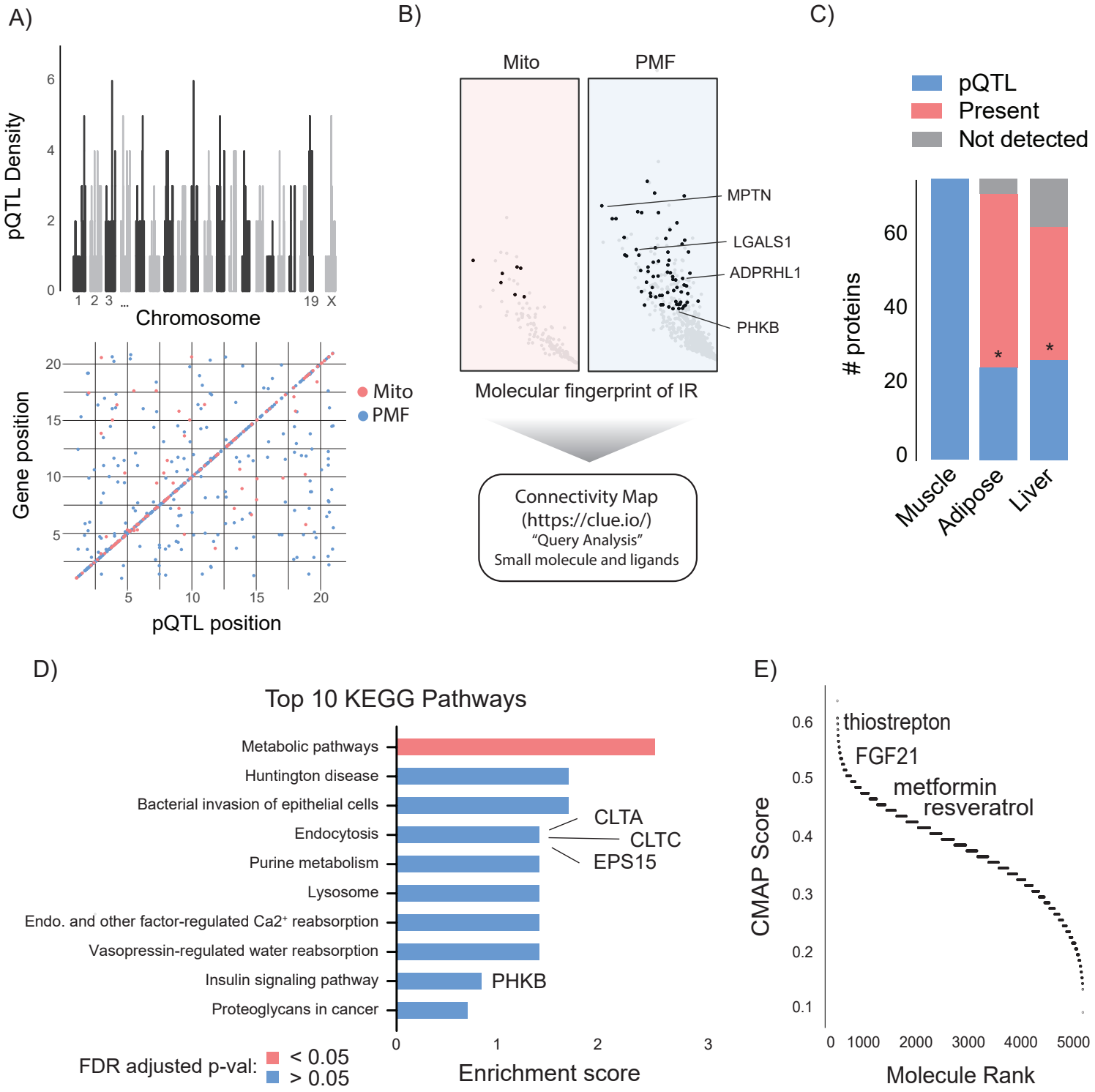

A)
pQTL Density
Chromosome
Gene position
pQTL position
Mito
PMF
B)
Mito
PMF
MPTN
LGALS1
ADPRHL1
PHKB
Molecular fingerprint of IR
Connectivity Map
(https://clue.io/)
"Query Analysis"
Small molecule and ligands
C)
pQTL
Present
Not detected
proteins
Muscle
Adipose
Liver
D)
Top 10 KEGG Pathways
Metabolic pathways
Huntington disease
Bacterial invasion of epithelial cells
Endocytosis
Purine metabolism
Lysosome
Endo. and other factor-regulated Ca2+ reabsorption
Vasopressin-regulated water reabsorption
Insulin signaling pathway
Proteoglycans in cancer
CLTA
CLTC
EPS15
PHKB
FDR adjusted p-val:
< 0.05
> 0.05
Enrichment score
E)
CMAP Score
thiostrepton
FGF21
metformin
resveratrol
Molecule Rank

Figure 4:

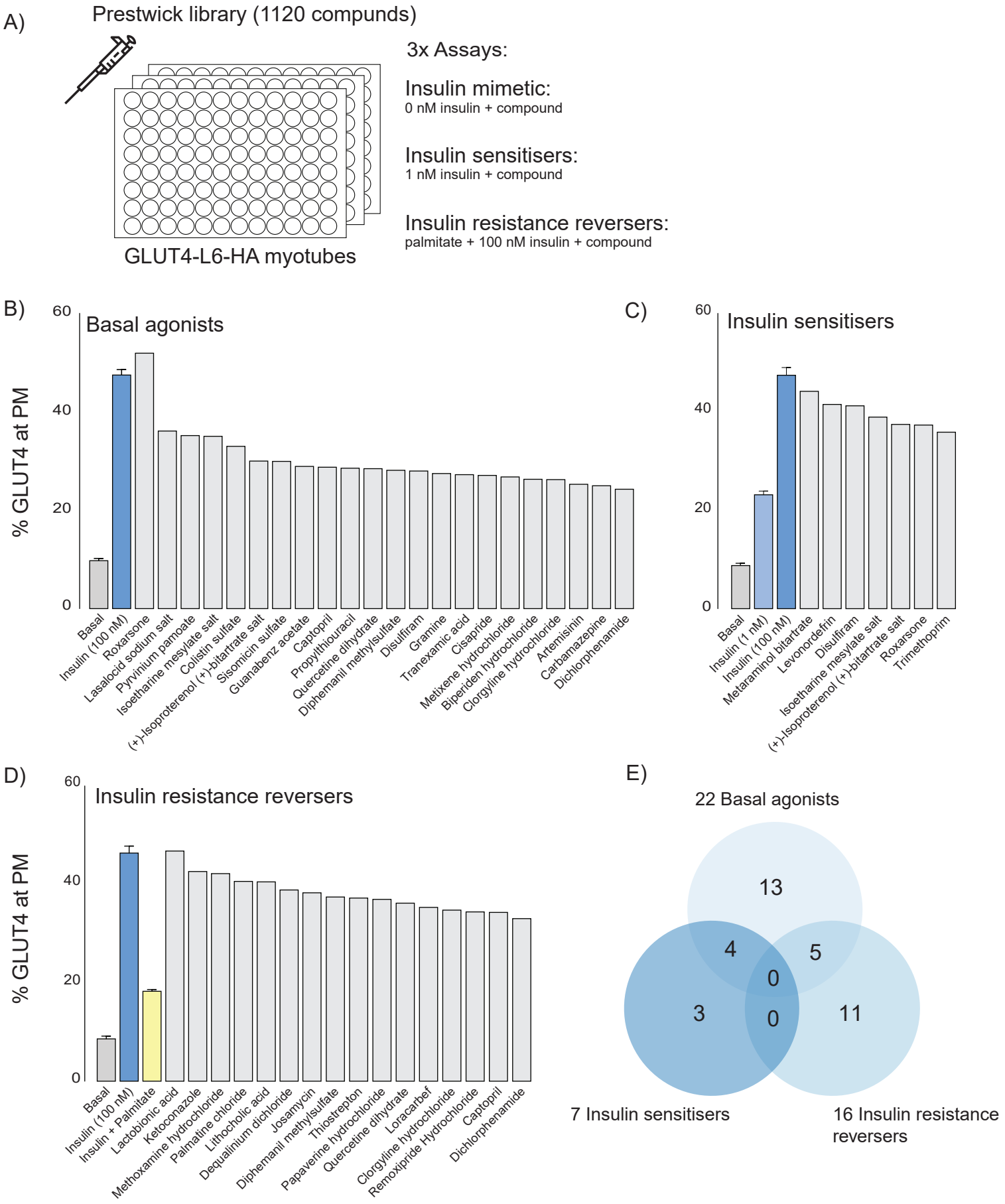

A)
Prestwick library (1120 compunds)
3x Assays:
Insulin mimetic:
0 nM insulin + compound
Insulin sensitisers:
1 nM insulin + compound
Insulin resistance reversers:
palmitate + 100 nM insulin + compound
GLUT4-L6-HA myotubes
B)
Basal agonists
% GLUT4 at PM
60
40
20
0
Basal
Insulin (100 nM)
Roxarsone
Lasalocid sodium salt
Pyrvinium pamoate
Isoetharine mesylate salt
Colistin sulfate
(+)-Isoproterenol (+)-bitartrate salt
Sisomicin sulfate
Guanabenz acetate
Captopril
Propylthiouracil
Quercetine dihydrate
Diphemanil methylsulfate
Disulfiram
Gramine
Tranexamic acid
Cisapride
Metixene hydrochloride
Biperiden hydrochloride
Clorgyline hydrochloride
Artemisinin
Carbamazepine
Dichlorphenamide
C)
Insulin sensitisers
60
40
20
0
Basal
Insulin (1 nM)
Insulin (100 nM)
Metaraminol bitartrate
Levonordefrin
Disulfiram
Isoetharine mesylate salt
(+)-Isoproterenol (+)-bitartrate salt
Roxarsone
Trimethoprim
D)
Insulin resistance reversers
% GLUT4 at PM
60
40
20
0
Basal
Insulin (100 nM)
Insulin + Palmitate
Lactobionic acid
Ketoconazole
Methoxamine hydrochloride
Palmatine chloride
Lithocholic acid
Dequalinium dichloride
Josamycin
Diphemanil methylsulfate
Thiostrepton
Papaverine hydrochloride
Quercetine dihydrate
Loracarbef
Clorgyline hydrochloride
Remoxipride Hydrochloride
Captopril
Dichlorphenamide
E)
22 Basal agonists
13
4
5
0
3
0
11
7 Insulin sensitisers
16 Insulin resistance reversers

Figure 5:

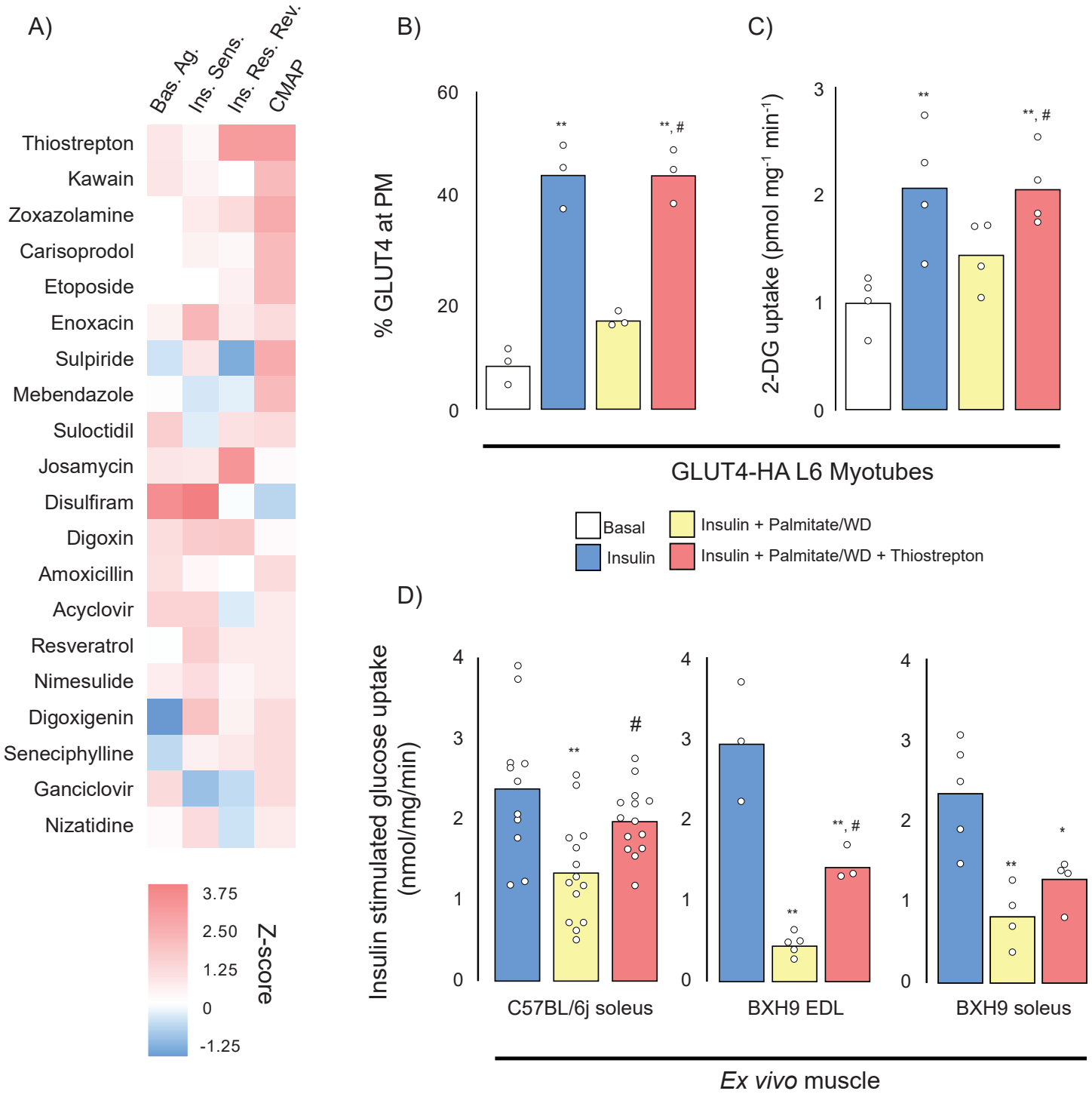

A)
Bas. Ag.
Ins. Sens.
Ins. Res.
Rev. CMAP
Thiostrepton
Kawain
Zoxazolamine
Carisoprodol
Etoposide
Enoxacin
Sulpiride
Mebendazole
Suloctidil
Josamycin
Disulfiram
Digoxin
Amoxicillin
Acyclovir
Resveratrol
Nimesulide
Digoxigenin
Seneciphylline
Ganciclovir
Nizatidine
3.75
2.50
1.25
0
-1.25
Z-score
B)
% GLUT4 at PM
60
40
20
0
**
**, #
C)
2-DG uptake (pmol mg-1 min-1)
3
2
1
0
**
**, #
GLUT4-HA L6 Myotubes
Basal
Insulin
Insulin + Palmitate/WD
Insulin + Palmitate/WD + Thiostrepton
D)
Insulin stimulated glucose uptake (nmol/mg/min)
4
3
2
1
0
**
#
**
**, #
**
*
C57BL/6j soleus
BXH9 EDL
BXH9 soleus
Ex vivo muscle

Figure 6

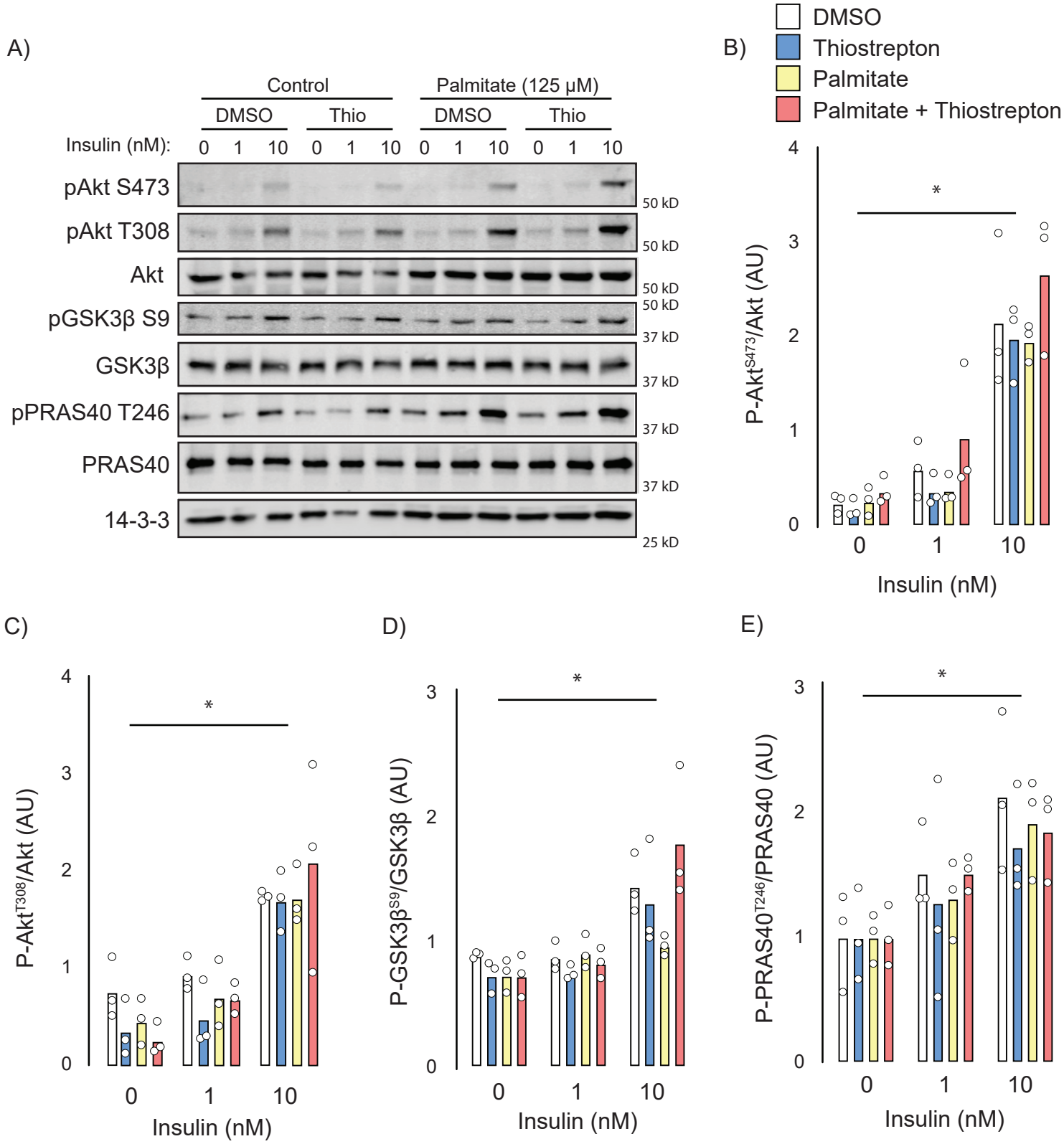
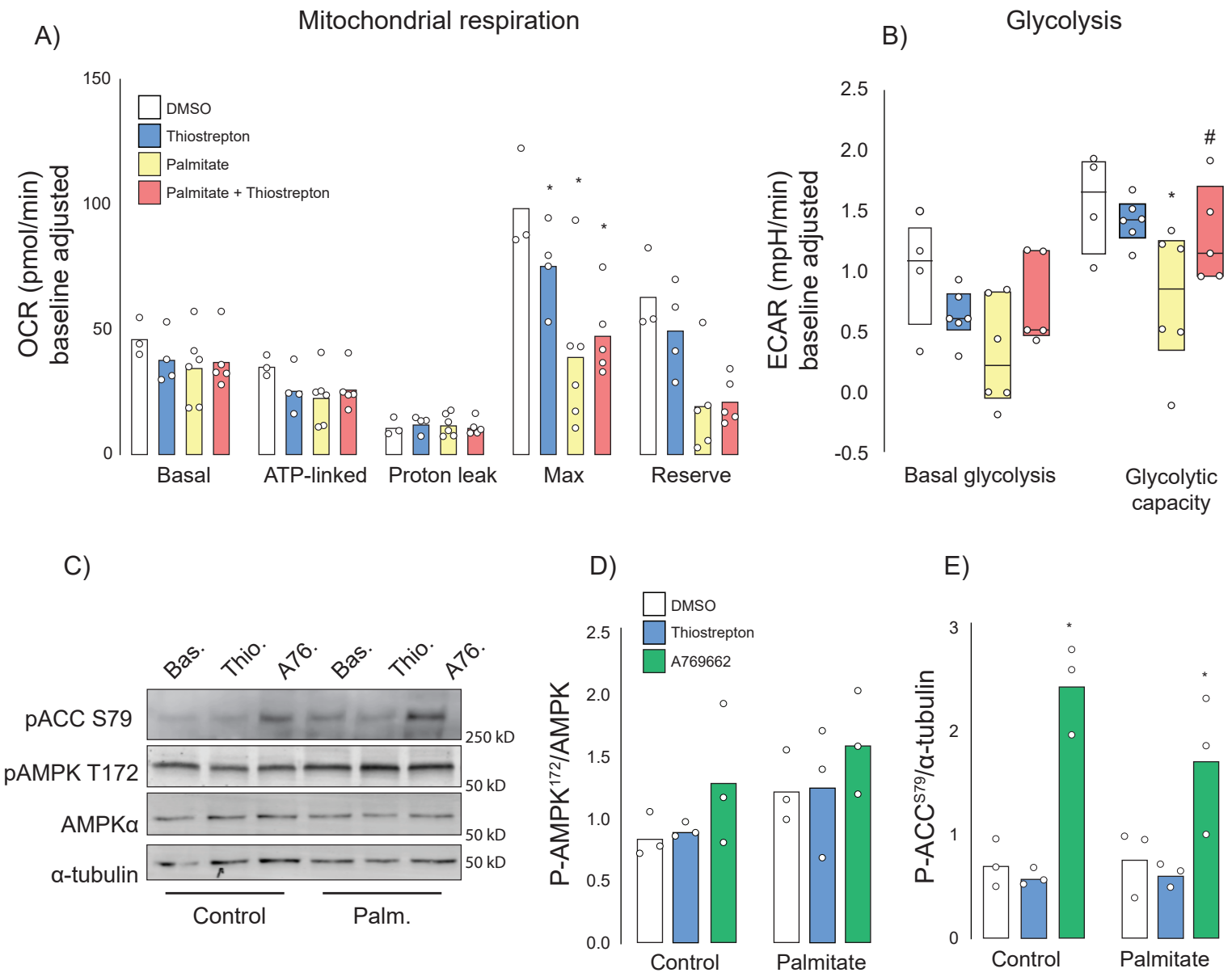
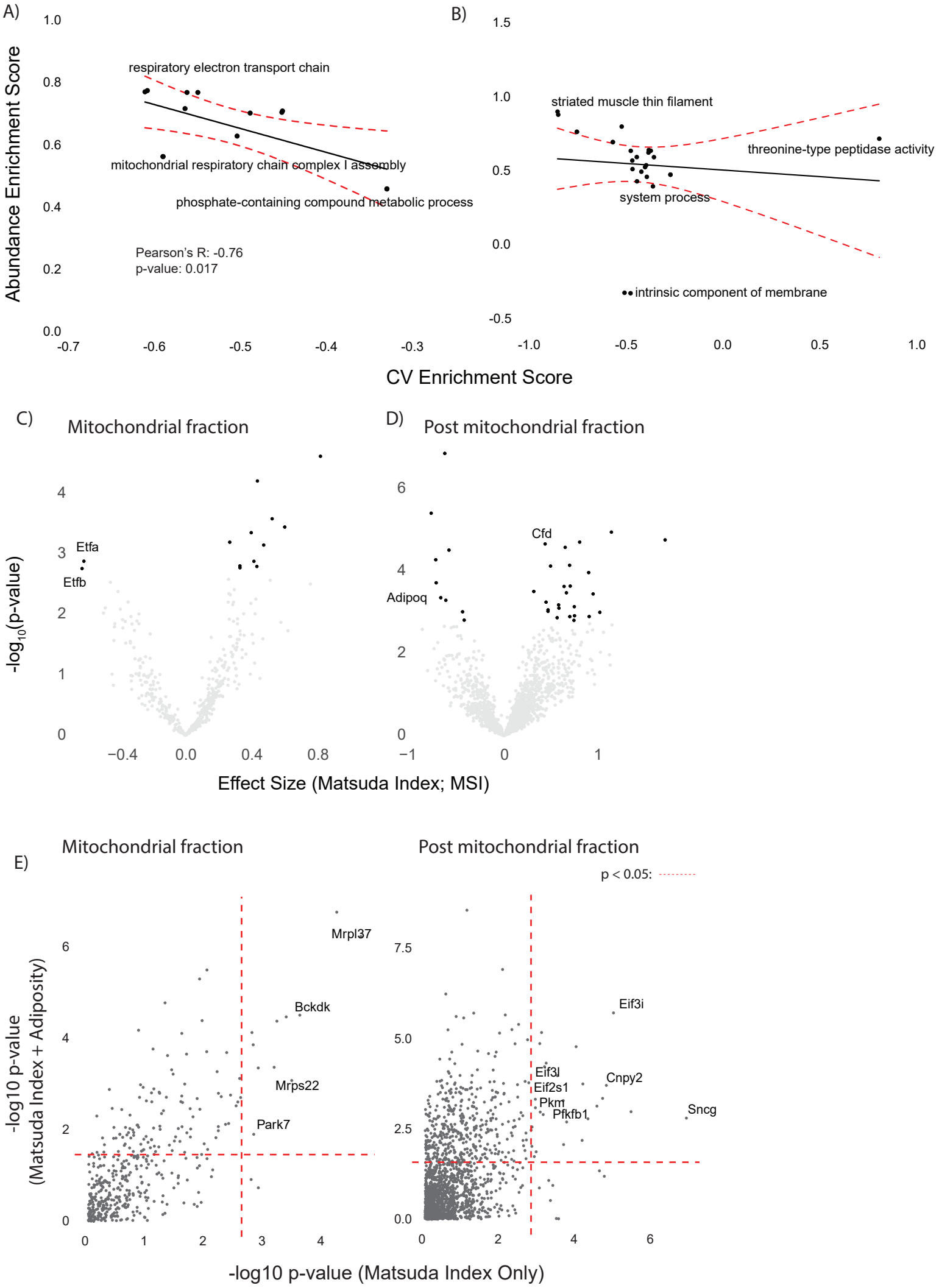

A)
Control
Palmitate (125 μM)
DMSO
Thio
DMSO
Thio
Insulin (nM): 0 1 10 0 1 10 0 1 10 0 1 10
pAkt S473
pAkt T308
Akt
pGSK3β S9
GSK3β
pPRAS40 T246
PRAS40
14-3-3
50 kD
50 kD
50 kD
50 kD
37 kD
37 kD
37 kD
37 kD
25 kD
B)
DMSO
Thiostrepton
Palmitate
Palmitate + Thiostrepton
P-Akt^{S473}/Akt (AU)
Insulin (nM)
C)
P-Akt^{T308}/Akt (AU)
Insulin (nM)
D)
P-GSK3β^{S9}/GSK3β (AU)
Insulin (nM)
E)
P-PRAS40^{T246}/PRAS40 (AU)
Insulin (nM)

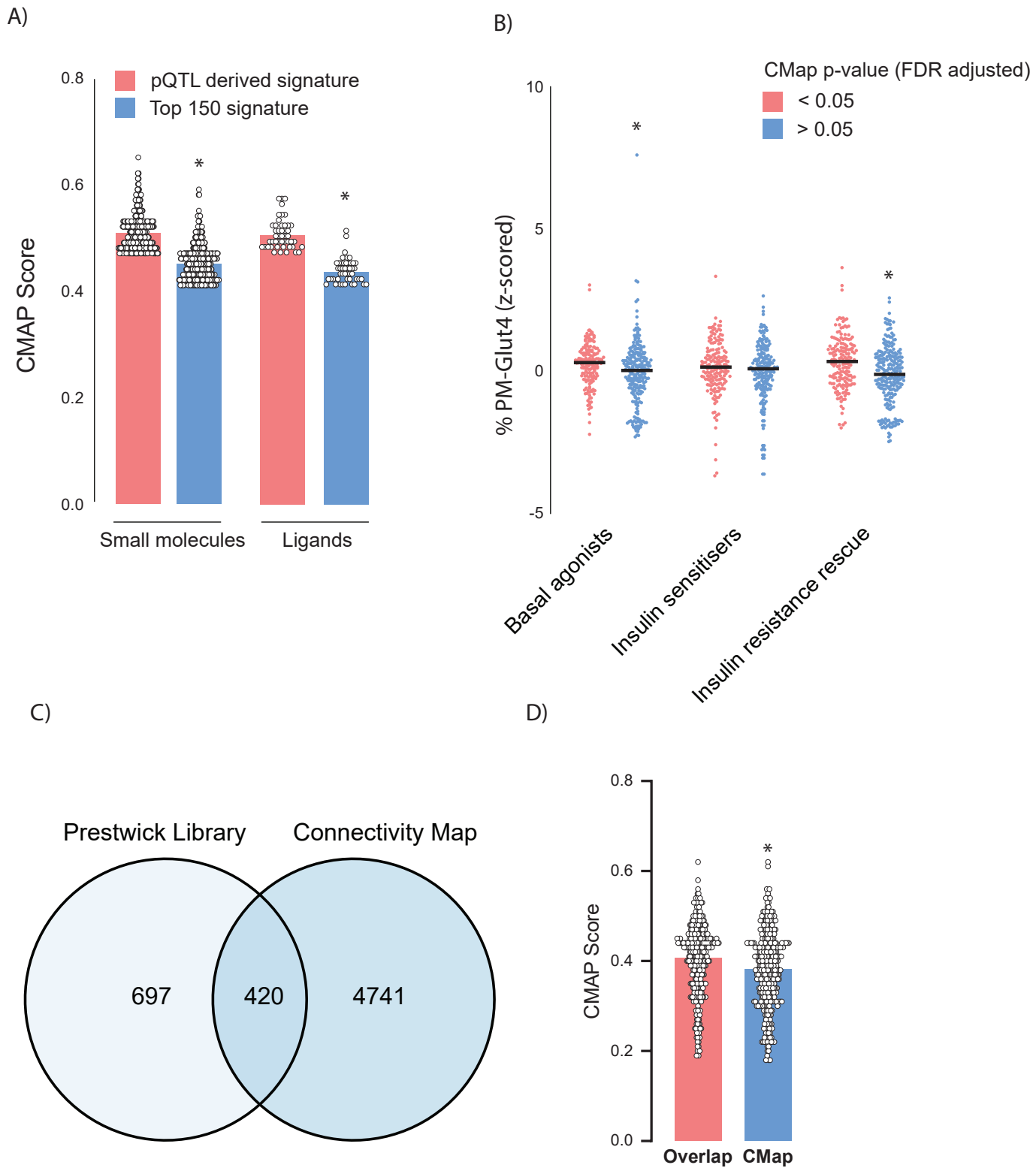
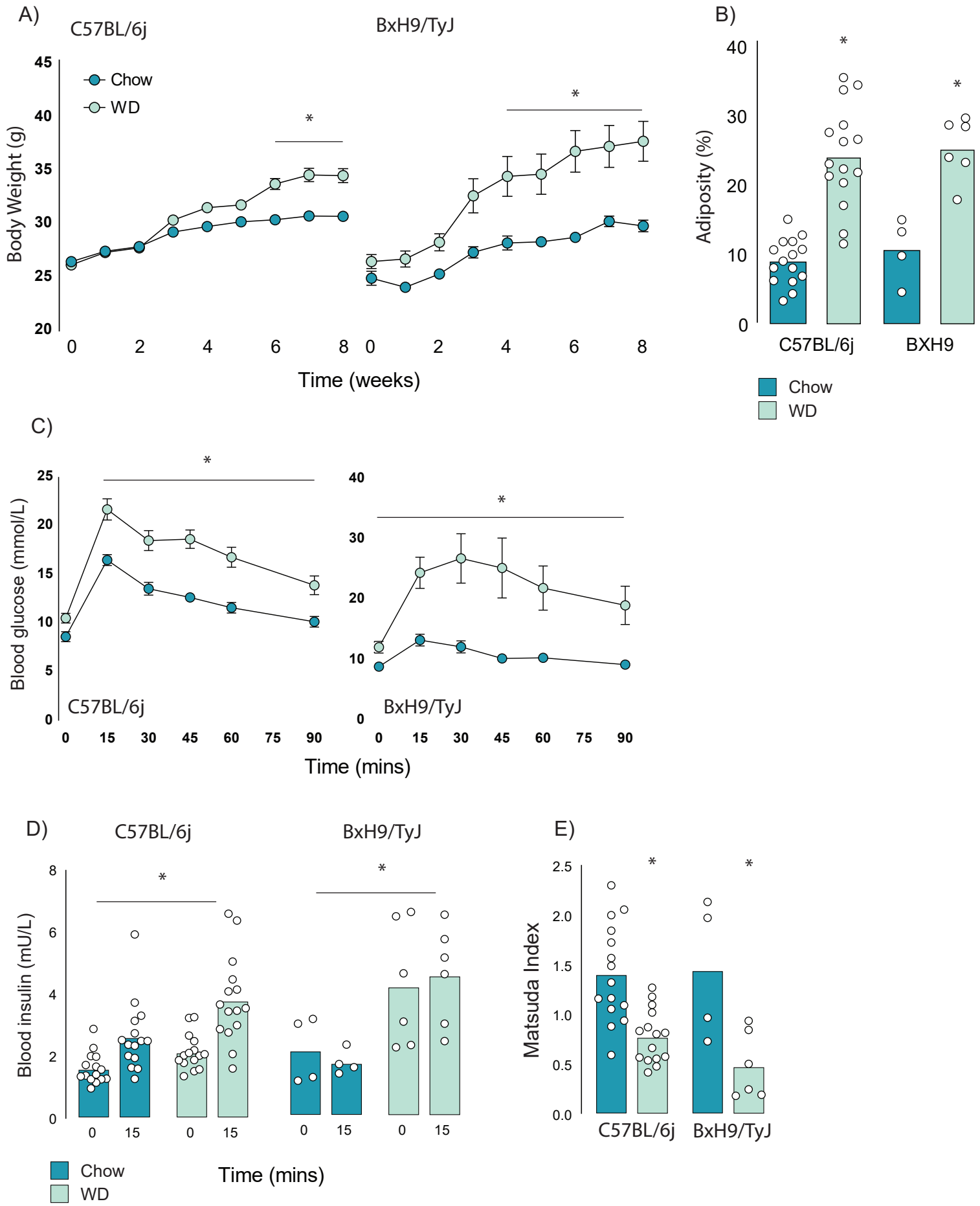
Figure 7


A)
Mitochondrial respiration
OCR (pmol/min) baseline adjusted
150
100
50
0
DMSO
Thiostrepton
Palmitate
Palmitate + Thiostrepton
Basal
ATP-linked
Proton leak
Max
Reserve
B)
Glycolysis
ECAR (mpH/min) baseline adjusted
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
#
Basal glycolysis
Glycolytic capacity
C)
Bas.
Thio.
A76.
Bas.
Thio.
A76.
pACC S79
pAMPK T172
AMPKα
α-tubulin
250 kD
50 kD
50 kD
50 kD
Control
Palm.
D)
DMSO
Thiostrepton
A769662
P-AMPK172/AMPK
2.5
2.0
1.5
1.0
0.5
0.0
Control
Palmitate
E)
P-ACCS79/α-tubulin
3
2
1
0
Control
Palmitate

Supplementary Figure 1:



Supplementary Figure 2:



Supplementary Figure 3: