

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.

eLife assessment

This **fundamental** study leverages natural genetic diversity in mice to discover candidate genes for insulin sensitivity, followed by experimental identification of compounds that can modulate insulin sensitivity, and finally initial mechanistic investigation of the mode of action. The generalized approach presented here – the integration of systems genetics data with drug discovery – supported by **compelling** evidence, will be a guide for others who seek to translate insights from mammalian genetics to drug discovery.

Introduction

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

Over the past decade, genetically diverse mouse panels have been used to study metabolic disease (4 [↗](#)-7 [↗](#)). This is a major step forward because these panels combine control of the environment and access to any biological tissue, with a vast phenotypic and range which can be leveraged towards understanding complex diseases. Three specific resources are the hybrid mouse diversity panel (HMDP), and the BXD (C57BL/6J x DBA) and Collaborative Cross (CC) mouse strains (7 [↗](#)-11 [↗](#)). These panels comprise large selections of inbred mice spanning vast phenotypic and genetic landscapes. Collaborative Cross mice were first generated by interbreeding five commonly used laboratory mouse strains (C57BL/6J, A/J, 129S1/SvImJ, NZO/HILtJ, NOD/ShiLtJ) and three wild-derived strains (WSB/EiJ, CAST/EiJ, PWK/PhJ) in a ‘funnel’ design. The resulting CC strains were then outbred to generate Diversity Outbred mice at Jackson Laboratories (12 [↗](#)) which have increased phenotypic diversity and resolution for genetic mapping. An independent Diversity Outbred colony was established in Western Australia using CC mice from Geniad (13 [↗](#)). This colony has since relocated to our group at The University of Sydney, termed Diversity Outbred mice from Australia (Oz) or DOz.

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

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

was also identified by us in an independent small molecule screen for effectors of insulin action in myotubes. Subsequent validation of thioestrepton uncovered profound beneficial effects on insulin resistance *in vitro* and *ex vivo*, potentially via modulation of mitochondrial function and glycolysis.

Results

DOz metabolic and proteomic variation

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

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

Role of skeletal muscle in whole-body insulin sensitivity

To leverage genetic and metabolic diversity toward uncovering new regulators of insulin action, we constructed linear models comparing insulin sensitivity (Matsuda Index) against protein abundance. Our initial analysis identified 37 mitochondrial and 40 PMF proteins that significantly associated with the Matsuda Index. Many of these appeared to be involved with adiposity rather than insulin action including adiponectin (ADIPOQ), adipisin (CFD), and the mitochondrial β -

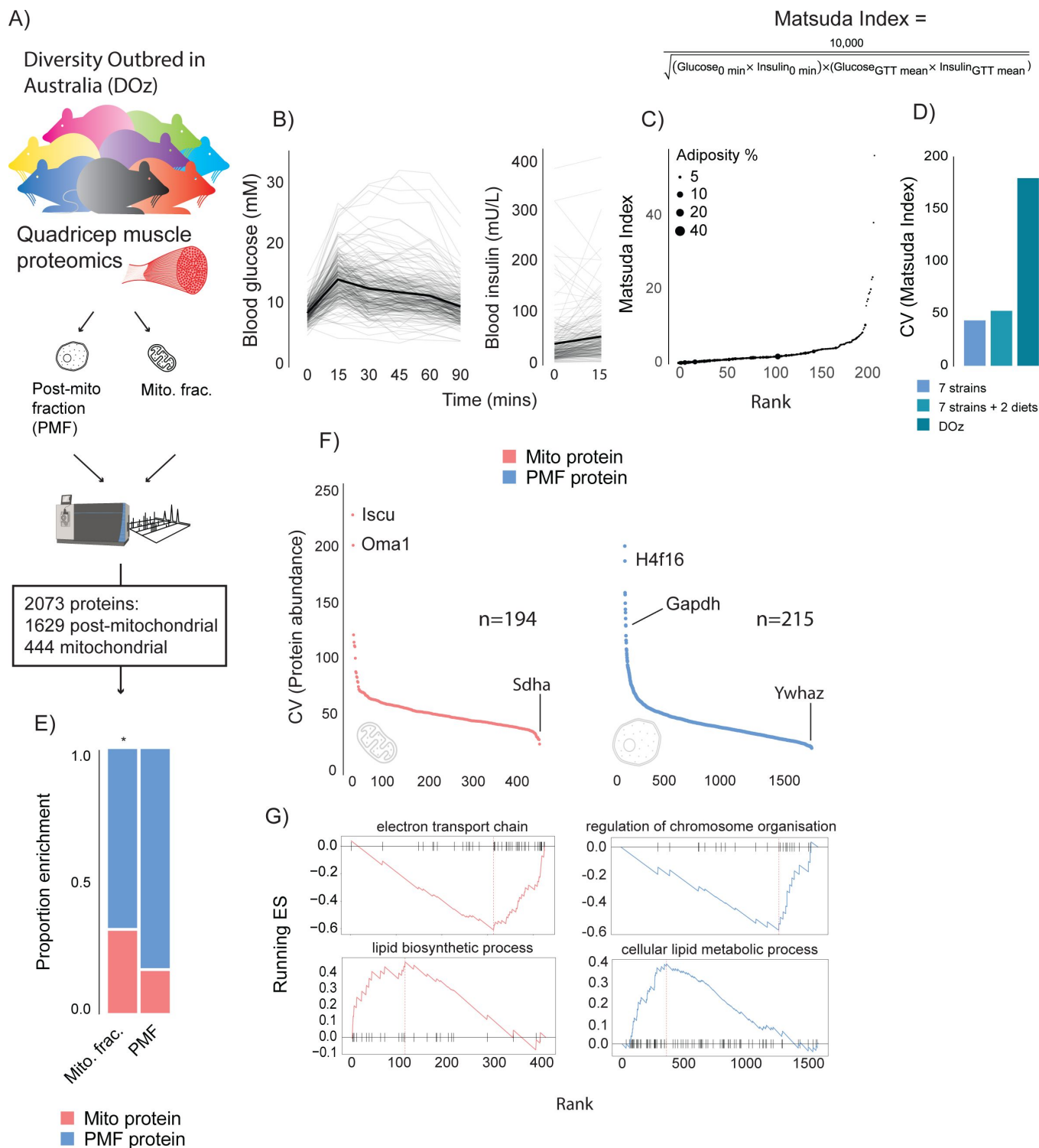


Figure 1.

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

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

To gain further insight into the biology of skeletal muscle glycaemic control, we annotated candidate proteins with PhosphoSite Plus data to test whether any protein of interest had documented roles in metabolic signalling (**Fig 2D-F**). We observed no relationship between insulin signalling substrates and regulation of whole-body insulin sensitivity (**Fig 2D**). However, there was a trend ($p = 0.08$) for AMPK substrates in the PMF to be positively associated with insulin sensitivity. Interestingly, the most positively (Canopy2; CNYP2) and most negatively (Rho GDP dissociation inhibitor alpha; ARHGDIA) associated proteins were both annotated as being insulin responsive (**Fig. 2F**). We also preformed GO gene-set enrichment analysis (GSEA) on proteins ranked by Matsuda Index effect sizes (**Fig 2G**). In the PMF, 'response to topologically incorrect protein: GO:0051788' was positively associated with insulin sensitivity while the 'proteasome complex: GO:0005839' and 'clathrin-coated pit: GO:0005905' were negatively associated. Together the unfolded protein response and proteasome are indicative of the role of proteostasis in insulin sensitivity (30, 31). Conversely, clathrin-coated pits play an important role in the internalisation of the insulin sensitive glucose transporter GLUT4 from the cell surface, a process negatively regulated by insulin (32-34). Consistent with our observation that the majority of the mitochondrial proteome is positively associated with insulin sensitivity, GSEA did not produce any negative results but did uncover a positive relationship between the mitochondrial respiratory complex I and Matsuda Index.

Integration of genetic linkage analysis and linear modelling with Connectivity Map

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

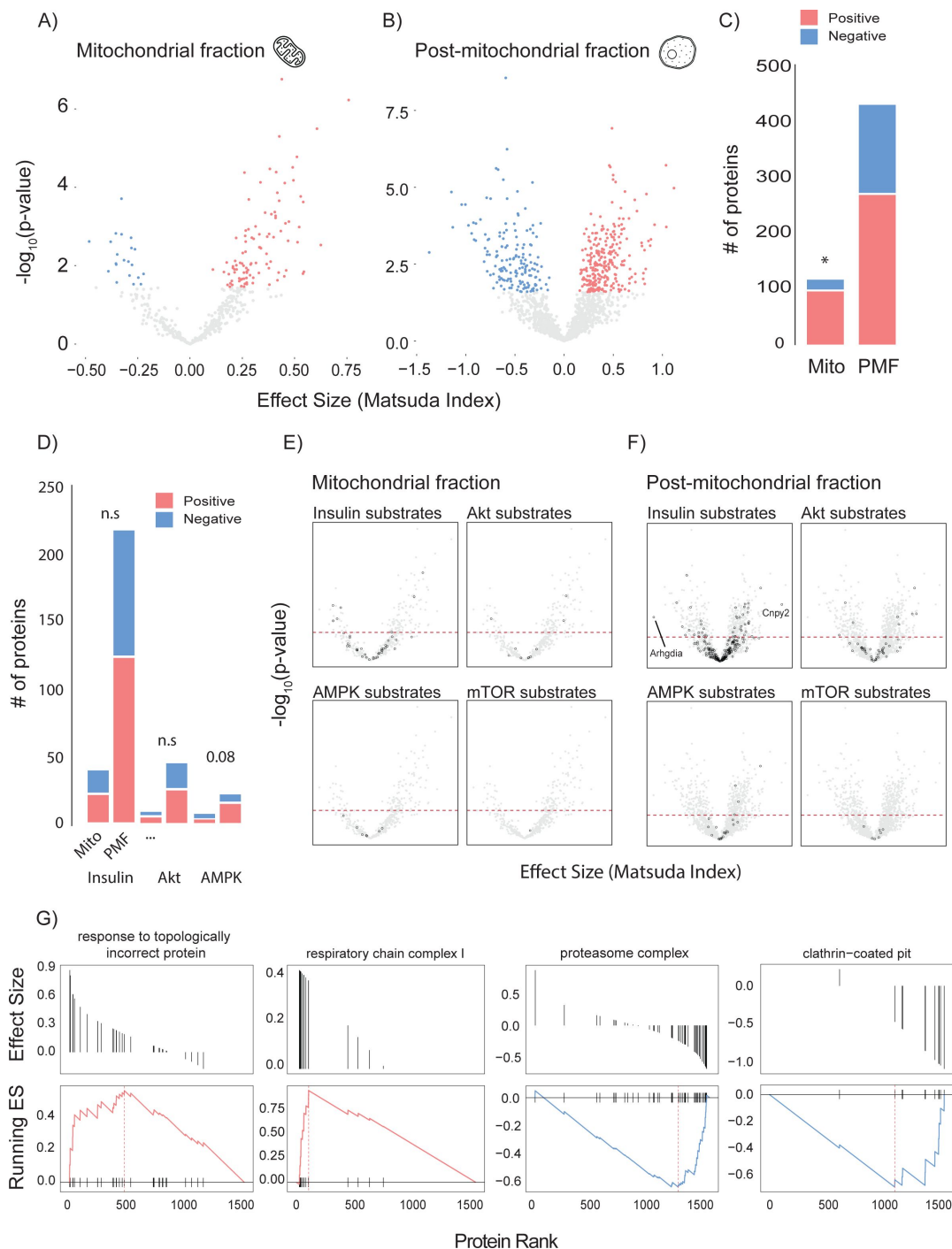


Figure 2.

Linear modelling of quadriceps proteome and whole-body insulin sensitivity. (A-B) Volcano plot with Matsuda Index effect sizes (x-axis) and significance (y-axis) for mitochondrial (A) and post-mitochondrial (B) quadriceps proteins using a linear model with adiposity as a covariate. Significant proteins with positive and negative effect sizes are indicated in red and blue, respectively. (C) Comparison of positively and negatively associated proteins between fractions. (D) Number of proteins identified in each fraction with known roles in insulin or AMPK signalling. (E-F) Volcano plot shown in A with mitochondrial (E) and post-mitochondrial fraction (F) proteins shown in black that have documented roles in indicated signalling pathways. Adjusted p-value threshold is indicated (red dotted line). (G) Pathways enriched for proteins which positively and negative associate with Matsuda Index. Effect sizes of proteins within a given pathway and running enrichment score (ES) for a that pathway on y-axis and proteins ranked by CV on x-axis. (ES) on y-axis and proteins ranked by CV on x-axis. of proteins within pathways that are enriched for proteins associated with whole-body insulin sensitivity. Proteins ranked by Matsuda Index effect size on x-axis. Linear modelling was performed using a gaussian distribution with q-value adjustment of p-values. Enrichment tests between fractions were performed by Chi-square test. * Indicates a significant difference $P < 0.01$.

proteins, and as seen (**Figure 2C**) previously, the mitochondrial proteome is enriched for positive contributors to insulin resistance. Similar approaches to identify molecular phenotypes of insulin resistance have previously been conducted using collated human transcriptomic datasets (35–45). Using a compiled list from Timmons et al (46), we searched for orthologues of proteins from our fingerprint that associate with human insulin resistance. We identified four such genes (**Fig. 3B**): MTPN (myotrophin), LGALS1 (galectin 1), PHKB (phosphorylase b kinase), and ADPRHL1 (ADP-ribosylhydrolase Like 1), which may warrant further investigation.

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

Next, we utilized Connectivity Map (CMAP) to convert our fingerprint into a list of small molecules and ligands that promote or oppose our muscle insulin resistance fingerprint. To test our assumption that pQTL filtering would improve our fingerprint we also queried CMAP with a list of the top 150 most strongly negatively associated proteins independent of pQTL presence. Intriguingly, on average CMAP scores for compounds and ligands were significantly higher when captured using a pQTL-filtered fingerprint compared to the non-pQTL filtered group, supporting the utility of this method (**Supplementary Figure 2A**). Encouragingly, the two highest scoring compounds identified using our fingerprint were Broad Institute glycogen synthase kinase (GSK3) and epidermal growth factor receptor (EGFR) inhibitors. Both of these kinases have been independently identified as drug targets that reverse insulin resistance (37, 47, 48). Many compounds listed in the Connectivity Map database are proprietary Broad Institute inhibitors which are only identified by their Broad ID and cannot be easily procured for follow-up experiments. Therefore, we excluded all Broad Institute compounds from further analysis. After this filtering, 856 small molecules and 91 ligands generated gene expression signatures matching our query. As whole-body insulin sensitivity decreased with increased fingerprint protein abundance, we focussed on molecules whose CMAP score suggested a reversal of our insulin resistance fingerprint (**Fig 3D**). Ranking these candidates based on CMAP score revealed a number of well-known potentiators of insulin sensitivity including the antioxidant resveratrol (49), the diabetes medication metformin (50) and the growth factor FGF21 (51). We also identified the antibiotic thiostrepton (52), a documented proteasome inhibitor, consistent with our enrichment analysis which identified the proteasome as negatively contributing to insulin sensitivity.

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

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

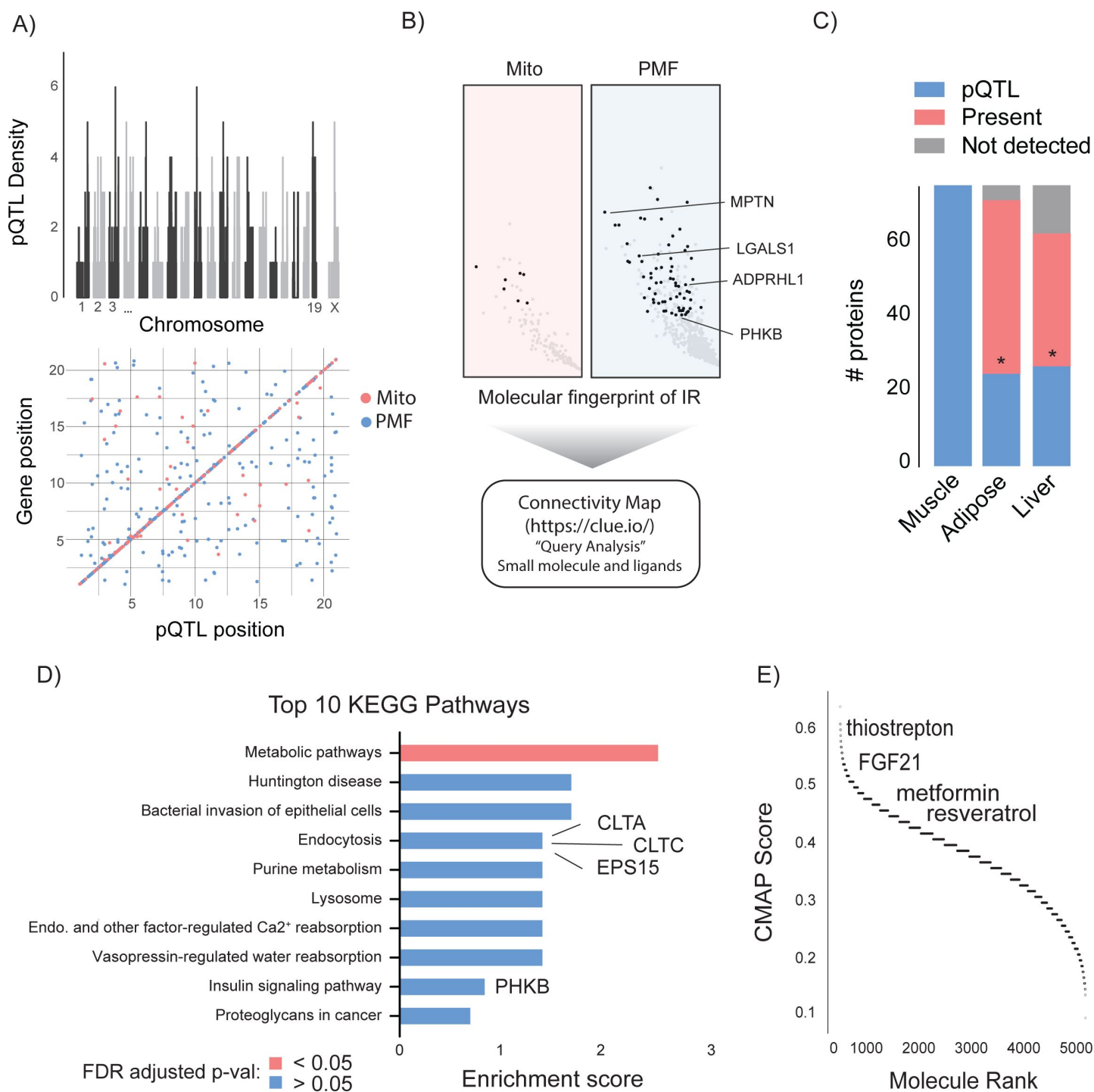


Figure 3.

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

We performed 3 separate screens (**Fig 4A**) to capture the different mechanisms by which compounds modulate glucose uptake: 1) insulin-independent activation of GLUT4 translocation to the plasma membrane (basal activators), 2) potentiation of submaximal insulin action (insulin sensitisers), and 3) rescue of palmitate-induced insulin resistance (insulin resistance reversers). We identified 22 basal agonists (**Fig 4B**), 7 insulin sensitisers (**Fig 4C**) and 16 insulin resistance reversers (**Fig 4D**). Five compounds both stimulated GLUT4 translocation and reversed insulin resistance, 4 were both basal agonists and insulin sensitisers while none met all three criteria (**Fig 4E**). Overall, we found that compounds that were identified by CMAP score (**Fig 3D**) performed better as both basal activators and as insulin resistance reversers than those that did not (**Supplementary Fig 2B**).

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

Next, we assessed the efficacy of thioestrepton to reverse insulin resistance in diet-induced obese mice. We decided to study it in isolated muscles as this circumvents potentially confounding microbiome effects due to thioestrepton's antibiotic activity and allows direct interrogation of muscle insulin action. We selected two strains of inbred mice, C57BL/6J and BXH9/TyJ, based on our previous observations that these strains are particularly amenable to developing muscle insulin resistance following high-fat, high-sugar (western diet; WD) diet feeding (4). Consistent with diet-induced perturbations in metabolic health, both C57BL/6J and BXH9 BXH9/TyJ mice fed a WD had increased body weight and adiposity, fasting hyperglycemia, fasting hyperinsulinemia, glucose intolerance and lower systemic insulin sensitivity (Matsuda Index) relative to chow-fed controls (**Supplementary Fig. 3A-E**). WD feeding also resulted in ~40% reduction in C57BL/6J soleus insulin stimulated 2-deoxyglucose uptake, a 75% reduction in BXH9 extensor digitorum longus insulin-stimulated 2-deoxyglucose uptake and 65% reduction in BXH9 soleus insulin stimulated 2-deoxyglucose uptake. Strikingly, 1 h of thioestrepton treatment prior to insulin addition was sufficient to reverse 80% of WD-induced insulin resistance in C57BL/6J EDL muscle and 50% in BXH9 EDL muscle but did not restore BXH9 soleus 2-deoxyglucose uptake (**Figure 5D**).

Thioestrepton does not affect insulin signalling

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

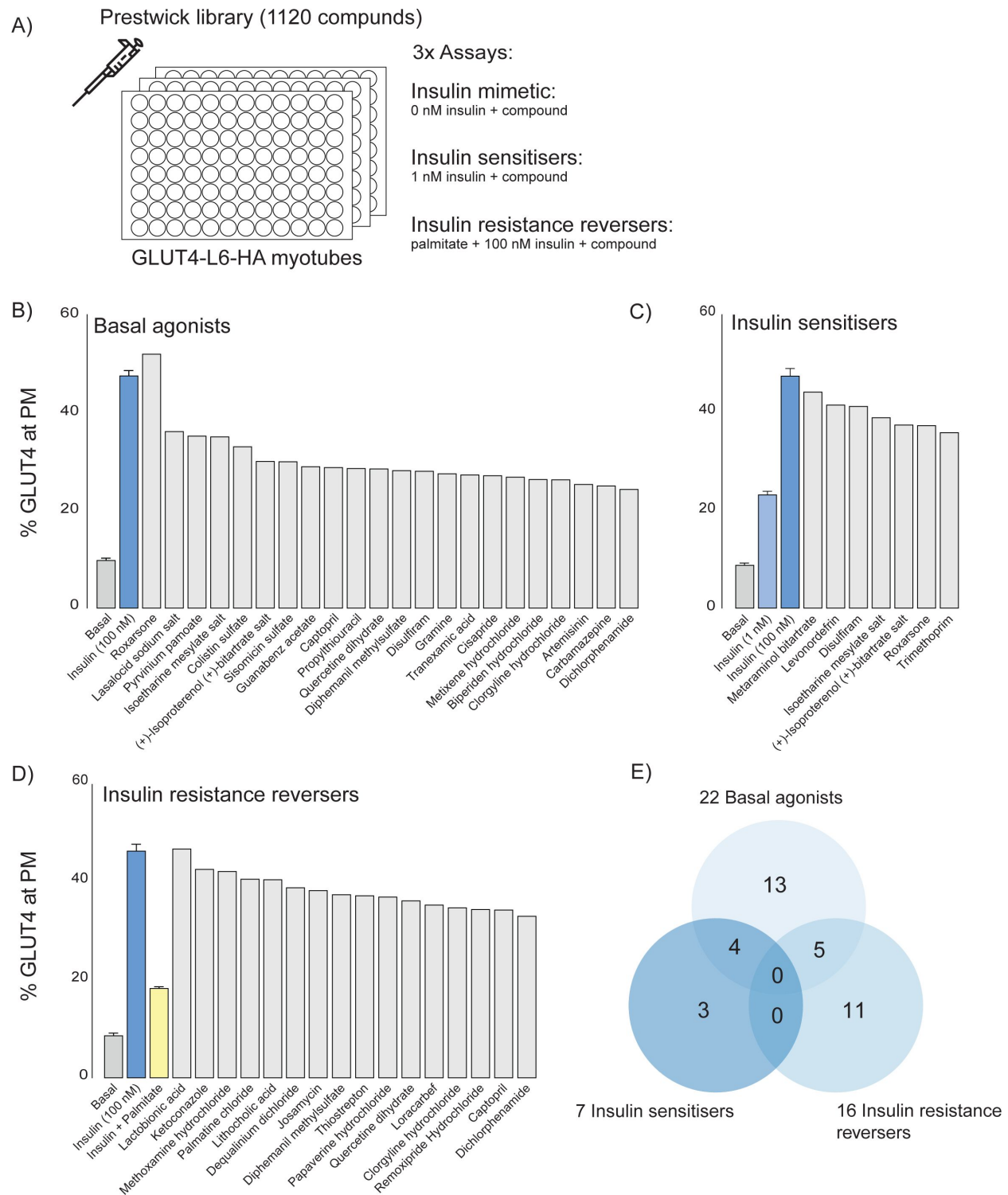


Figure 4.

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

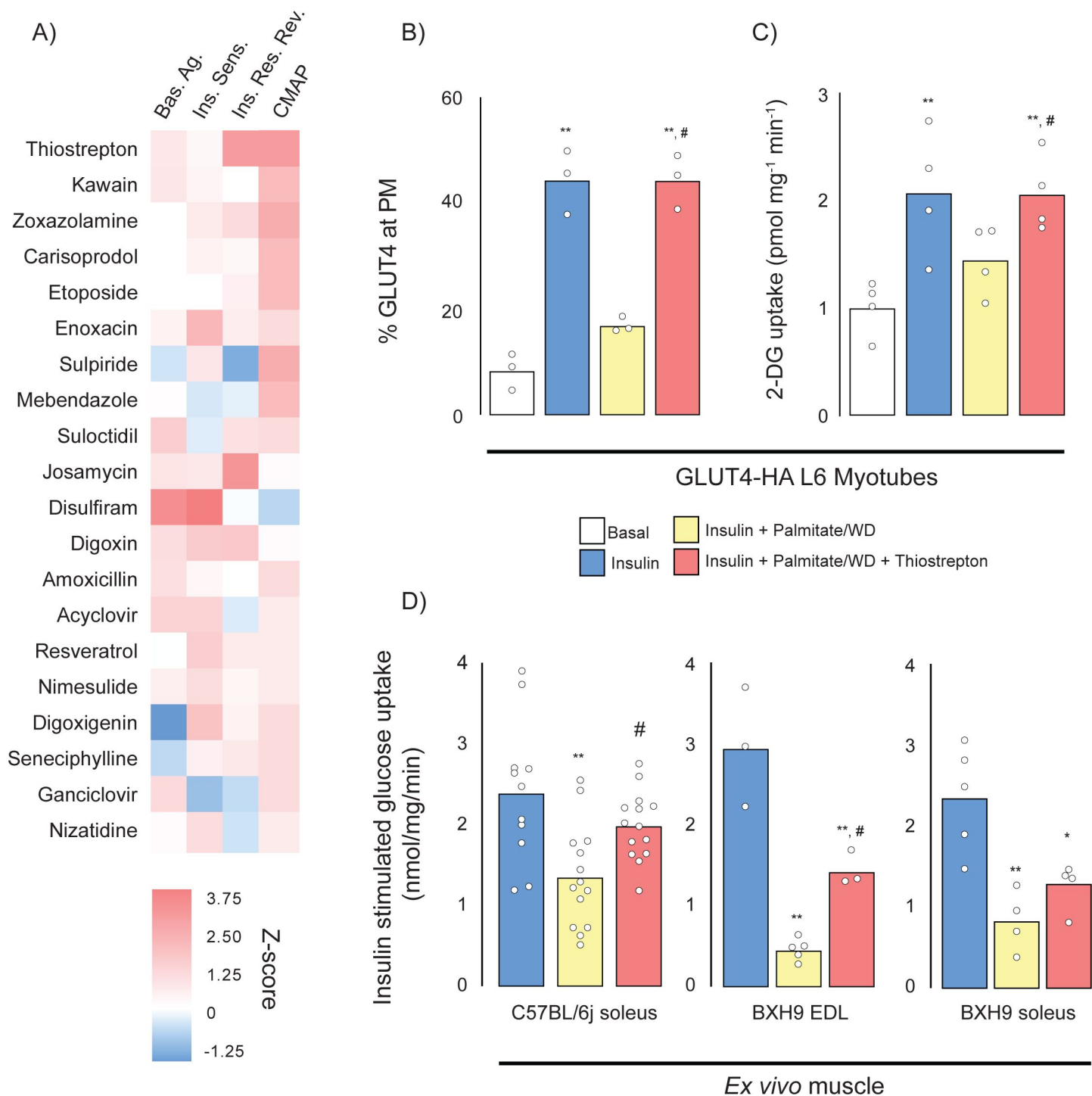


Figure 5.

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

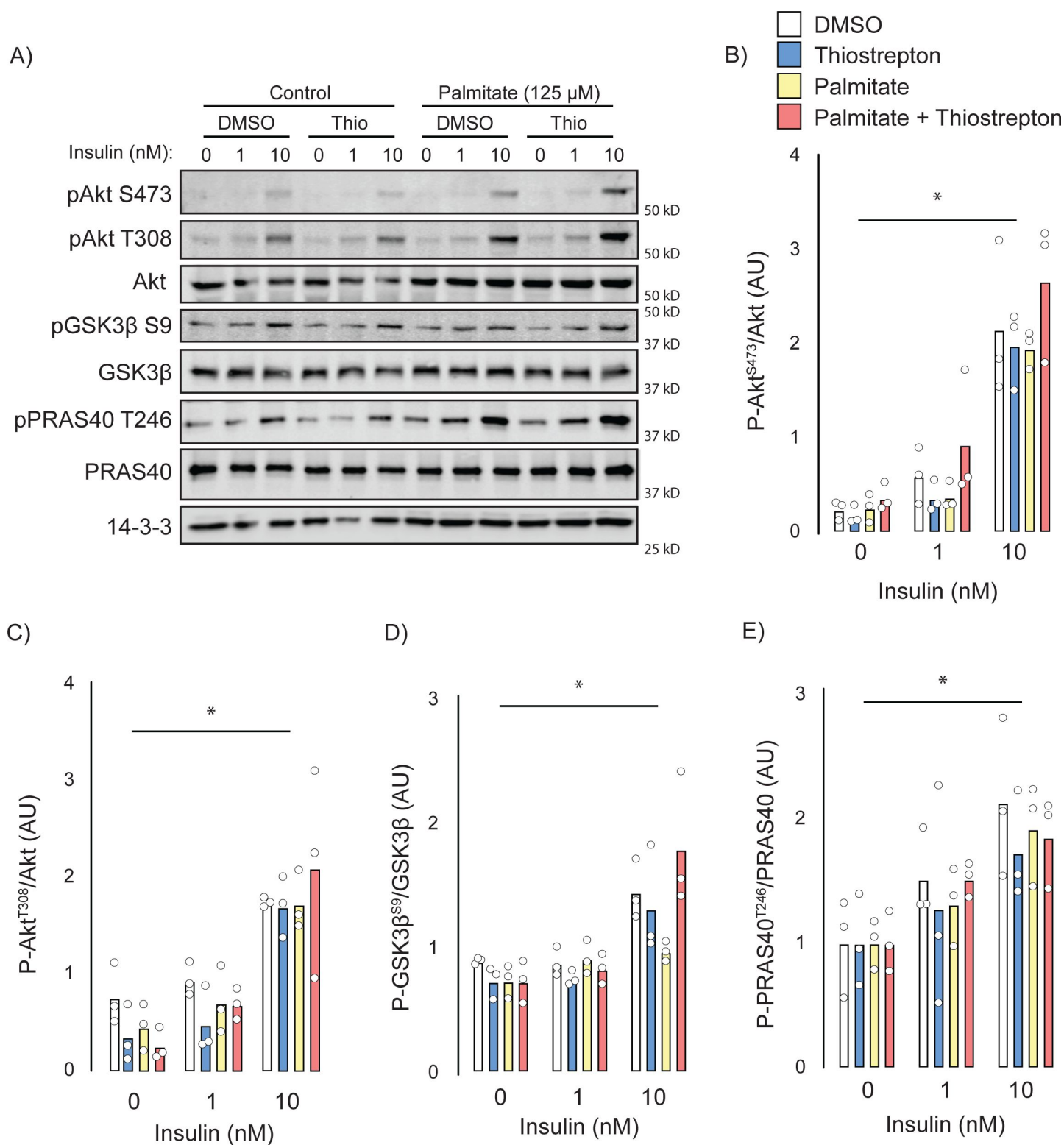


Figure 6.

Effect of thiostrepton on insulin signalling. (A-E) Immunoblotting with indicated antibodies of control and palmitate treated GLUT4-HA-L6 myotubes following treatment with thiostrepton or vehicle control and stimulation with 0, 1, or 10 nM insulin. (A) Representative immunoblot shown of 3 independent experiments. (B-E) Quantification of immunoblots in A, Akt S473 (B), Akt T308 (C), GSK3 β S9 (D) and PRAS40 T246 (E). Data are mean with individual data points, n=3. Significance was determined by two-way ANOVA with Student's post hoc test. * Indicates a significant effect of insulin $P < 0.01$.

Thiostrepton partially inhibits mitochondria and restores palmitate induced glycolysis suppression

Many insulin sensitising agents act via mitochondrial inhibition or uncoupling (58–60) and thiostrepton has been reported to inhibit mitochondrial translation (61) and respiration (62, 63). To test whether thiostrepton's ability to restore optimal insulin action occurs via mitochondria, palmitate-treated and control GLUT4-HA-L6 myotubes were incubated with thiostrepton as above. Consistent with the mitochondrial dysfunction that has been reported during insulin resistance (23, 64), we observed substantial suppression of maximal mitochondrial respiration and mitochondrial reserve capacity following palmitate treatment (Fig. 7A). Furthermore, thiostrepton alone appeared to blunt maximal respiration, albeit to a lesser extent than palmitate. When combined, thiostrepton and palmitate did not produce an additive suppression, nor did thiostrepton reverse any of the palmitate-induced defects. This suggests that this suppression of maximal respiration does not contribute to insulin resistance in this model.

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

Discussion

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

Systems-based approaches often identify networks as being drivers of disease. Empirical validation of these is difficult due to the complex interactions in biological systems. Here we took a drug-centric approach to validate our findings; this allowed targeting of entire pathways rather than singular nodes. We identified several compounds across both *in silico* and *in vitro* analyses which may restore muscle insulin action, indeed several of these have previously been investigated. Disulfiram, sold under the brand name Antabuse, is used as an alcohol-dependency medication. Two studies have described disulfiram's ability to reverse diet-induced hepatic insulin resistance and reduce adiposity (72, 73). Resveratrol, a component found in red wine, is a popular antioxidant and has been demonstrated to reverse insulin resistance via reduction of reactive oxygen species (49, 74, 75). Fibroblast growth factor 21 (FGF21) was also identified amongst the ligand dataset as reversing our insulin resistance fingerprint. FGF21 has previously been reported to promote insulin-stimulated glucose uptake in muscle fibres (76)

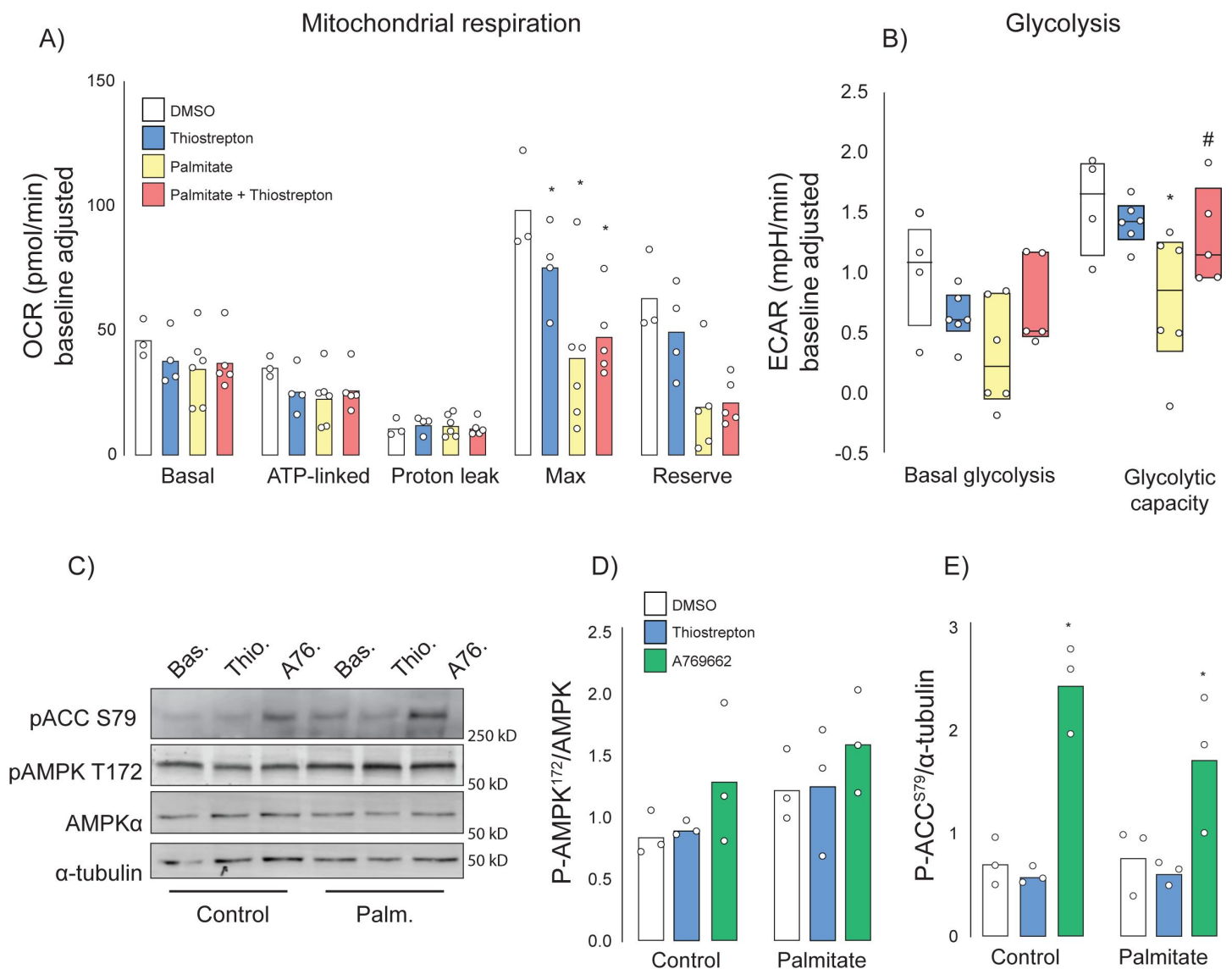


Figure 7.

Effect of thioestrepton on mitochondrial respiration and glycolysis. (A-B) Oxygen consumption rates (A) and extracellular acidification rates (B) in control and palmitate treated GLUT4-HA-L6 myotubes treated with either thioestrepton or vehicle control. (C) Immunoblotting of AMPK signalling in control and palmitate treated (Palm.) GLUT4-HA-L6 myotubes treated with either thioestrepton (Thio.), vehicle control (Bas.) or positive control A-769662 (A76.). Representative immunoblots shown of 3 independent experiments. (D-E) Quantification of immunoblots in C, AMPK T172 (D) and ACC S79 (E) phosphorylation following thioestrepton or A-769662 treatment. Data are mean with individual data points shown, $n=3$. * Indicates significant difference from control cells, # indicates significant difference from palmitate treated cells $p < 0.05$.

and can modulate mitophagy and proteostasis in muscle (77 [↗](#)). Antivirals, several classes of antibiotics, antipsychotics and cancer drugs were all identified by our analyses. Perhaps this diversity reflects the divergent and pleiotropic biology of insulin resistance.

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

A major question is what biological functions are represented by our fingerprint? One of the top pathways identified was endocytosis. This pathway featured two components of the clathrin coat and the adaptor protein EPS15. This is very exciting as endocytosis has been suggested to play a major role in stress signalling (81 [↗](#)), and in the context of insulin sensitivity this may involve internalisation of key proteins including the insulin receptor and glucose or amino acid transporters (32 [↗](#), 70 [↗](#)). The concept that variation in this process is genetically determined, and this plays a major role in governing essential processes like insulin action, adds a new dimension to the role of this pathway. A second intriguing member of the fingerprint is phosphorylase kinase which, along with glycogen synthase kinase, regulates the key glycogen storage enzymes glycogen phosphorylase and glycogen synthase (82 [↗](#)-85 [↗](#)). High levels of glycogen phosphorylase kinase may promote glycogen breakdown through activation of glycogen phosphorylase thereby altering GSK3 signalling, a process implicated in insulin resistance.

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

Integration of physiological, proteomic, genomic, and pharmaceutical data has uncovered a potent reverser of insulin resistance. By integrating proteomic diversity with the underlying genetic architecture, we believe we were able to focus on potentially causal proteins, and the use of Connectivity Map allowed us to combine these proteins into a single fingerprint to find potential modulators of insulin resistance. Our findings also build on recent reports linking glycolysis to insulin action and uncover a number of potential contributors to insulin action worthy of future study.

Methods

Mouse breeding and phenotyping

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

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

Muscle proteomic sample prep

Whole quadriceps muscle samples were prepared as previously described with modification (97 [↗](#), 98 [↗](#)). First, tissue was snap frozen with liquid nitrogen and pulverized before resuspension in 100 µL of trypsin buffer (phosphate buffered saline; PBS containing 10 mM EDTA and 0.01 ug/uL mass-spectrometry grade trypsin). Samples were incubated for 30 minutes at 37 °C before being pelleted by centrifugation (10,000 g, 5 minutes at 4 °C). Samples were then resuspended in 1.4 mL mitochondrial isolation buffer (70 mM sucrose, 220 mM Mannitol, 1 mM EGTA, 2 mM HEPES, pH at 7.4) and homogenised on ice in a glass Dounce homogeniser. Samples were then twice pelleted by centrifugation, first at 1000 g x 10 min to remove insoluble debris and second at 10,000 g x 10 min to extract the crude mitochondrial fraction, both centrifugation steps

were performed at 4 °C and the supernatant of the second step was collected as the post-mitochondrial fraction (PMF). The mitochondrial pellet was re-solubilised in 1 mL of isolation buffer by repeated pipetting on ice prior to centrifugation (10,000 g x 10 min at 4°C) and resuspension in 50 µL of isolation buffer. Protein concentration of both mitochondrial and PMF was determined by BCA assay, 10 µg of protein was then prepared as previously described ([4](#)). Reduction/alkylation (10mM TCEP, 40mM CAA) buffer was added to each sample before incubation for 20 minutes at 60 °C. Once cooled to room temperature, 0.4 mg trypsin and 0.4 mg LysC was added to each sample and incubated overnight (18h) at 37°C with gentle agitation. 30µL water and 50 µL 1% TFA in ethyl acetate was added to stop digestion and dissolve any precipitated SDC. Samples were prepared for mass spectrometry analysis by StageTip clean up using SDB-RPS solid phase extraction material ([99](#)). Briefly, 2 layers of SDB-RPS material was packed into 200 µL tips and washed by centrifugation at 1,000 x g for 2 minutes with 50 µL acetonitrile followed by 0.2% TFA in 30% methanol and then 0.2% TFA in water. 50 µL of samples were loaded to StageTips by centrifugation at 1,000 g for 3 minutes. Stage tips were washed with subsequent spins at 1,000 g for 3 minutes with 50 µL 1% TFA in ethyl acetate, then 1% TFA in isopropanol, and 0.2% TFA in 5% ACN. Samples were eluted by addition of 60µL 60% ACN with 5% NH₄OH. Samples were dried by vacuum centrifugation and reconstituted in 30 µL 0.1% TFA in 2% ACN.

Mass spectrometry analysis

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

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

Data analysis

All data analysis and visualisation was performed in either the R programming environment ([102](#)) or GraphPad Prism (GraphPad Software, San Diego, California USA). For protein-trait analysis the Matsuda Index was calculated using glucose tolerance data before being log2 transformed. Linear models were generated using the *lm()* function in R where Matsuda Index = α

+ proteinX + covariate + ϵ (α = intercept and ϵ = error) using a gaussian distribution. To correct for multiple testing, p-values were adjusted using the q-value method in the R package *qvalue* (103). Chi-square tests for distribution differences within the data and two/one-way ANOVA tests for group differences were performed in GraphPad Prism.

Gene-set enrichment

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

Genetic mapping analysis

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

Connectivity Map and scoring matrix

Insulin resistance ‘fingerprint’ proteins were queried in Connectivity Map using the CLUE software platform (16, 19). The list of 76 ‘fingerprint proteins’ were queried against the L1000 gene expression dataset in the “Query” function of CLUE and results for small molecules (trt_cp) and ligands (trt_lig) were extracted using the CLUE “Morpheus” platform. Raw connectivity score values were used to rank perturbagens. Connectivity scores were averaged across all cell lines in the L1000 dataset. The Broad Institute small molecule inhibitors denoted by the prefix were removed from our resulting dataset as they are not commercially available. Connectivity Map scores were combined with the results from our GLUT4 translocation screen to rank consensus compounds. This was done by first z-scoring each value (% GLUT4 at the plasma membrane for each assay and raw connectivity score). This produced a value which indicates how well each compound performs in a given test relative to the rest of the dataset. Then the average of all three GLUT4 assays (basal agonism, insulin sensitisation, and insulin resistance reversal) was added to the z-scored Connectivity Score to produce an overall score for each compound. Z-score adjustment for each assay and CMAP score was performed as follows:

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

Where:

- Zscore = adjusted value for a given compound
- x = observed score for given compound
- mean = average score across all compounds

- SD = standard deviation of all compounds

Overall score for each compound was calculated as follows:

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

Cell culture

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

GLUT4 translocation assays

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

2-deoxyglucose uptake

2-deoxyglucose uptake into GLUT4-HA-L6 cells was performed as previously described with modifications (108 [DOI](#), 109 [DOI](#)). Cells were incubated overnight in either α MEM overnight supplemented with either BSA-coupled 125 μ M palmitic acid or BSA vehicle control before being washed 3x with HEPES buffed saline (HBS). Cells were then incubated in HBS supplemented with 10 μ M unlabelled 2-deoxyglucose and 0.5 μ Ci/ml [3 H]-2-deoxyglucose at 37°C for 5 minutes. Cells were then washed 5x with ice-cold PBS and lysed in 1M NaOH. For non-specific background uptake, 1 well per condition was pre-treated with cytochalasin B. Counts were determined by Perkin Elmer Quantulus GCT Liquid Scintillation Counter (Perkin Elmer, Waltham, MA, USA). Glucose uptake was expressed relatively to protein concentration as determined by bicinchoninic acid (BCA) assay after subtraction of non-specific uptake.

Ex vivo glucose uptake

Ex vivo glucose uptake was performed as previously described (4 [DOI](#)). Mice were euthanized by cervical dislocation prior to rapid dissection of soleus (C57Bl/6J and BXH9/TyJ) and extensor digitorum longus (EDL) muscle (BXH9/TyJ only). Muscle selection was based on our prior observations that only soleus muscles in C57Bl/6J mice develop diet-induced insulin resistance. Both the soleus and EDL muscles were mounted and then incubated for 1 h in Krebs Henseleit

buffer (KHB; 5.5 mM glucose, 2 mM pyruvate and 0.1% BSA) that had been gassed with carbogen (95% O₂/5% CO₂) supplemented with either 10 μ M thioestrepton or a DMSO vehicle control. Glucose uptake was assessed by then switching the muscle into KHB supplemented with 0.375 mCi/ml [3H]-2-dexoyglucose (2-DG), 0.05 mCi/ml [14C]-Mannitol, 100 nM insulin and either thioestrepton (10 μ M) or DMSO vehicle control for 20 minutes at 30 °C followed by washing in ice-cold PBS and then snap frozen in liquid nitrogen. Samples were lysed in 250 mM NaOH at 70 °C. Tracer in the muscle tissue lysates was quantified by liquid scintillation counting and [3H]-2-DG was corrected for extracellular [14C]-mannitol then normalized to wet weight of the tissue.

Immunoblotting

Glut4-HA-L6 myotubes were incubated overnight in 125 μ M palmitate or BSA control prior to treatment with drugs/insulin as indicated. Cells were then washed in ice-cold PBS and lysed by scraping directly into 55 °C Laemmli sample buffer with 10 % (tris 2-carboxyethyl phosphine; TCEP). Samples were then sonicated for 24 s (3s on/3s off) and heated at 65 °C for 5 minutes. SDS-PAGE was performed. Samples were resolved by SDS-PAGE as previously described ([4](#)), transferred onto PVDF membranes and blocked in TBS-T (0.1% Tween in Tris-buffered saline) containing 5% skim milk for 1 hour. Membranes were then washed 3 x 10 minutes in TBS-T and incubated overnight in primary antibodies against phosphorylated Akt T308 (Cell Signaling Technologies #2965; diluted 1:1000), phosphorylated Akt S473 (Cell Signaling Technologies #9271; diluted 1:1000), pan-Akt (Cell Signaling Technologies #9272; diluted 1:1000), phosphorylated GSK-3 α / β S21/9 (Cell Signaling Technologies #9327; diluted 1:1000), GSK3 α / β (Cell Signaling Technologies #5676; diluted 1:1000), phosphorylated PRAS40 T246 (Cell Signaling Technologies #13175; diluted 1:1000), PRAS40 (Cell Signaling Technologies #2691; diluted 1:1000), phosphorylated AMPK (Cell Signaling Technologies, #2531; diluted 1:1000), α -tubulin (Cell Signalling Technologies #2125; diluted 1:1000), 14-3-3 (Santa Cruz #sc-1657; diluted 1:5000). The following day membranes were washed 3 x 10 minutes in TBS-T and incubated for 1 hour in species-appropriate fluorescent or HRP secondary antibodies. Imaging and densitometry were performed using LI-COR Image Studio or a Bio-Rad ChemiDoc Imaging System (Bio-Rad, Hercules, CA, USA) and Image J ([110](#)). Phosphorylated proteins were normalised against their relevant controls and data was normalised based on average band intensity.

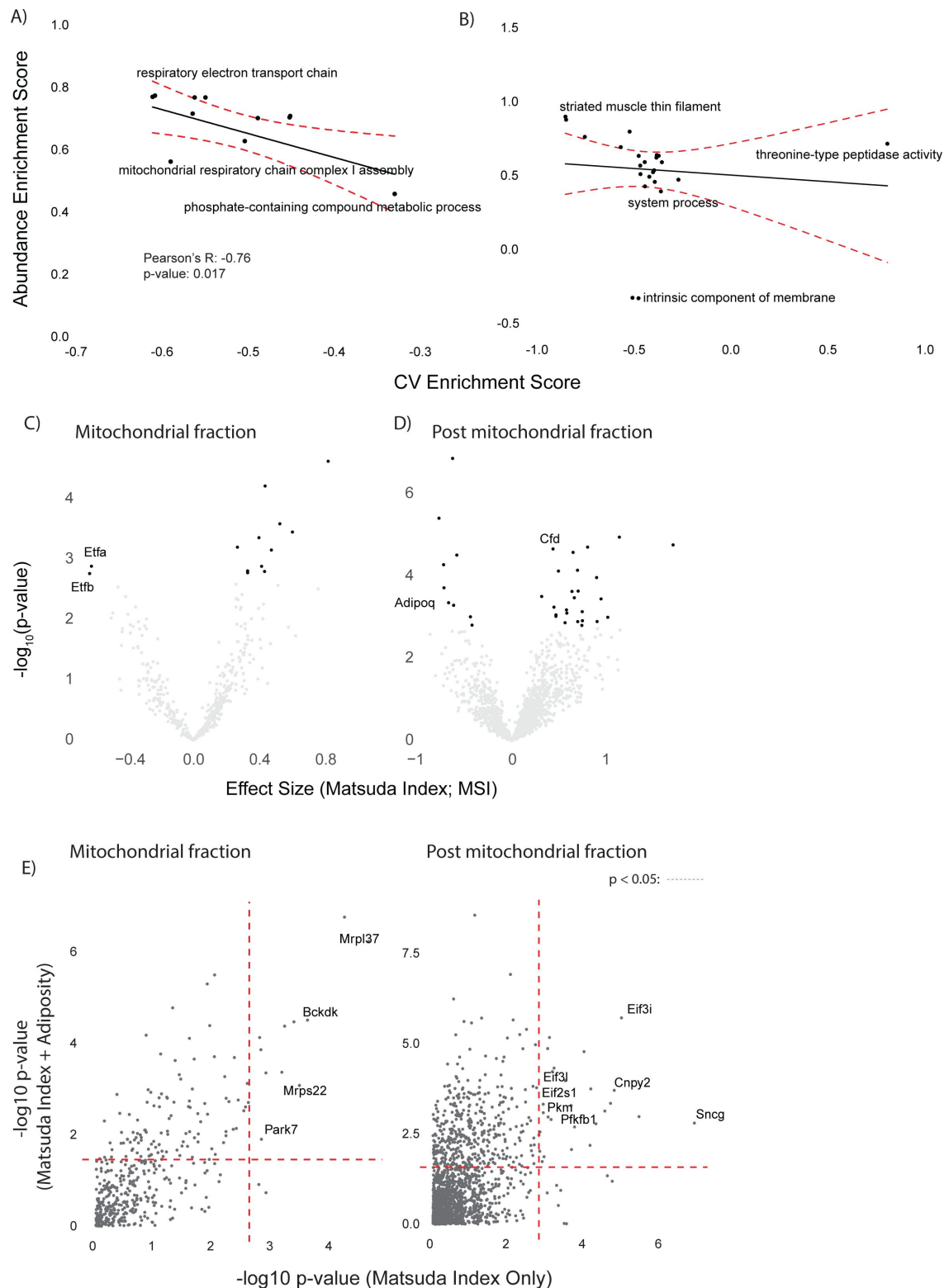
Mitochondrial stress test

Cellular respirometry (oxygen consumption rate, OCR) was performed using Seahorse XFp miniplates and a Seahorse XF HS Mini Analyzer (Seahorse Bioscience, Copenhagen, Denmark) as previously described ([111](#)). GLUT4-HA-L6 myotubes were incubated overnight in either palmitate or BSA control α MEM before treatment with either thioestrepton (10 μ M) or DMSO vehicle control. Cells were washed twice with KRBH and incubated in KRBH supplemented with 2.8 mM glucose, thioestrepton or vehicle control without BSA (150 μ l/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 (5 μ g/ml), FCCP (1 μ M) and rotenone/antimycin A (5 μ M) 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.

Acknowledgements

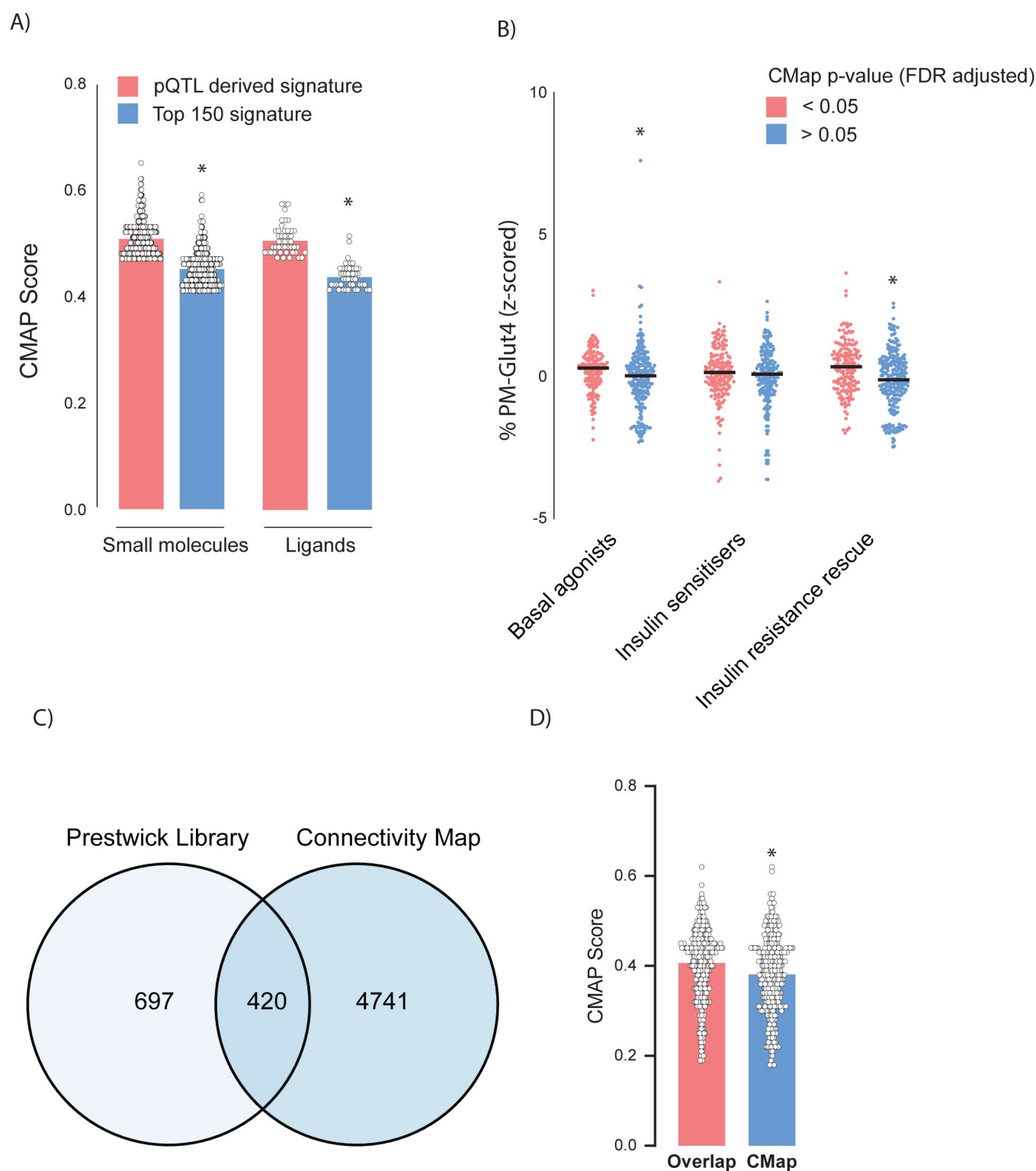
This work is dedicated to our wonderful colleague and friend Senthil Thillainadesan (1988-2023). We'd like to thank the Sydney Mass Spectrometry facility in the Charles Perkins Centre at the University of Sydney for mass spectrometry support. We'd also like to thank Large Animal Services in the Charles Perkins Centre at The University of Sydney for mouse housing support, and Cordula Hohnen-Behrens, Nicky Konstantopoulos and Briana Spolding for *in vitro* GLUT4 translocation assay assistance.

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



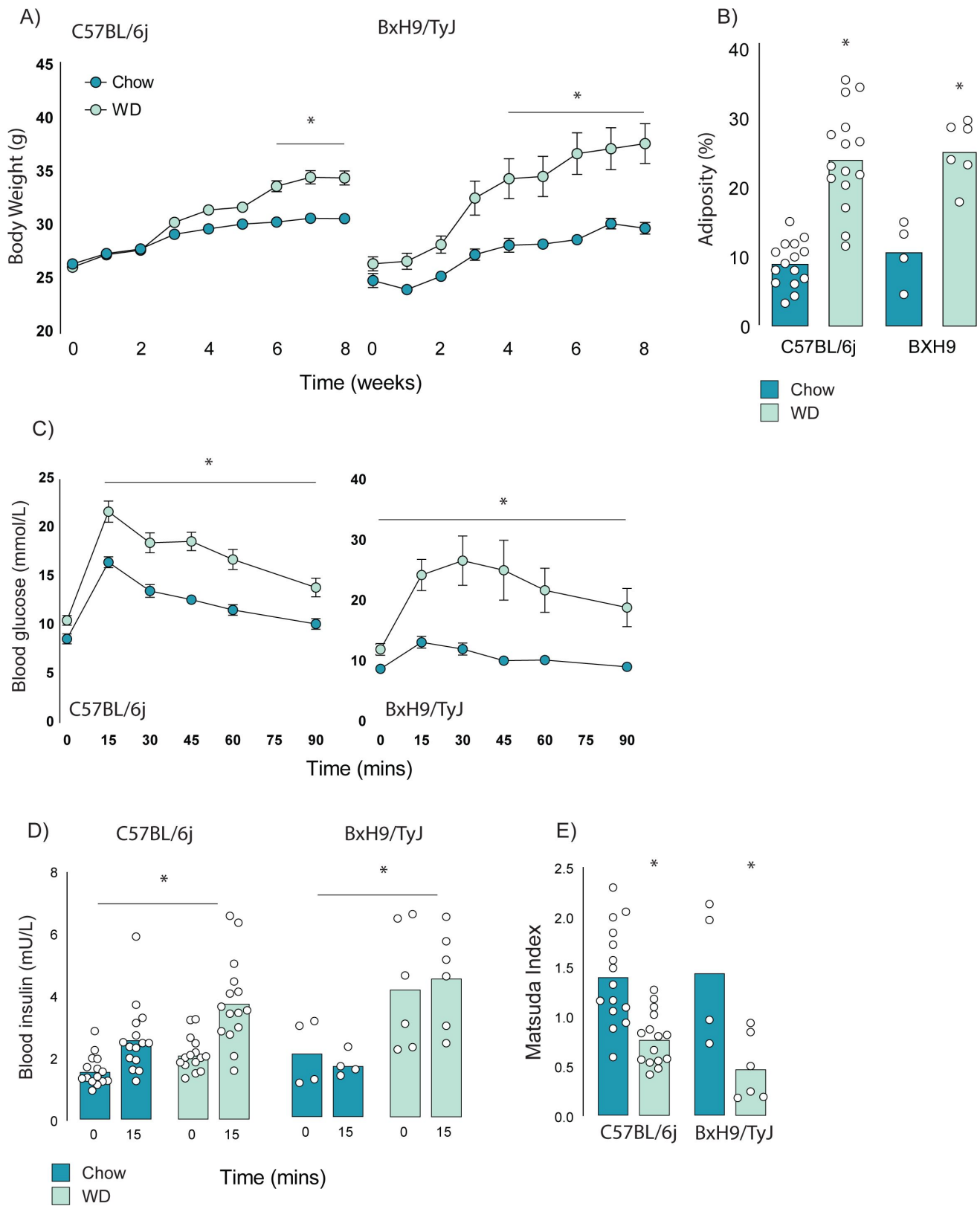
Supplementary Figure 1.

Comparison of enrichment analysis and linear modelling approaches for DOz insulin sensitivity and muscle proteomics. (A-B) Abundance (y-axis) and coefficient of variation-based (x-axis) gene-ontology enrichment scores for mitochondrial (A) and PMF (B) proteomes. (C-D) Volcano plot with Matsuda Index effect sizes (x-axis) and significance (y-axis) for mitochondrial (C) and post-mitochondrial fraction (D) proteins. (E-F) Mitochondrial (E) and post-mitochondrial fraction (F) negative $\log_{10}$ p-values for Matsuda Index effect sizes from linear models with and without adiposity as a covariate. Dashed red line indicates FDR adjusted $p < 0.05$.



Supplementary Figure 2.

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



Supplementary Figure 3.

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

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Reviewer #1 (Public Review):

Masson et al. leveraged the natural genetic diversity presented in a large cohort of the Diversity Outbred in Australia (DOz) mice (n=215) to determine skeletal muscle proteins that were associated with insulin sensitivity. The hits were further filtered by pQTL analysis to construct a proteome fingerprint for insulin resistance. These proteins were then searched against Connectivity Map (CMAP) to identify compounds that could modulate insulin sensitivity. In parallel, many of these compounds were screened experimentally alongside other compounds in the Prestwick library to independently validate some of the compound hits. These two analyses were combined to score for compounds that would potentially reverse insulin resistance. Thioestrepton was identified as the top candidate, and its ability to reverse insulin resistance was validated using assays in L6 myotubes.

Below are several comments made on the original version of this study, addressed by the authors in the current version:

- (1) Please describe the rationale of trypsinizing the tissue prior to mitochondrial isolation.
- (2) The authors mentioned that the proteomics data were Log2 transformed and median-normalized. Please provide a bit more details on this, including whether the subjects were randomized.
- (3) In Figure 1D, please give the numbers of mice the authors used for the CV comparisons in each group, whether they were of similar age and sex, and whether the differences in CV values were statistically significant

(4) The authors stated in lines 155-157 that proteins negatively associated with Matsuda index were further filtered by presence of their cis-pQTLs. Please provide more explanations to justify this filtering criterion.

(5) Please explain why the first half of the paper focused extensively on the authors' discoveries in the mitochondrial proteome, and how proteins involved in mitochondrial processes (such as complex I) were associated with Matsuda Index, but the final fingerprint list of insulin resistance, which contained 76 proteins, only had 7 mitochondrial proteins.

(6) The authors found that thiostrepton-induced insulin resistance reversal effects were not through insulin signalling. Please list the proteins in the fingerprint list that led to identification of thiostrepton on CMAP, and discuss whether you think that thiostrepton directly or indirectly acts on these protein targets.

Reviewer #2 (Public Review):

In the present study, Masson et al. provide an elegant and profound demonstration of utilization of systems genetics data to fuel discovery of actionable therapeutics. The strengths of the study are many: generation of a novel skeletal muscle genetics proteomic dataset which is paired with measures of glucose metabolism in mice, systematic utilization of these data to yield potential therapeutic molecules which target insulin resistance, cross-referencing library screens from connectivity map with an independent validation platform for muscle glucose uptake and preclinical data supporting a new mechanism for thiostrepton in alleviating muscle insulin resistance. Future studies evaluating similar integrations of omics data from genetic diversity with compound screens, as well as detailed characterization of mechanisms such as thiostrepton on muscle fibers will further inform some remaining questions. In general, the thorough nature of this study not only provides strong support for the conclusions made but additionally offers a new framework for analysis of systems-based data. I had made several comments on the prior submission, all of which have been fully addressed and incorporated.

Author Response

The following is the authors' response to the original reviews.

Reviewer #1 (Public Review):

(1) What's the rationale of trypsinizing the tissue prior to mitochondrial isolation? This is not standard for subsequent proteomics analysis. This step will inevitably cause protein loss, especially for the post mitochondrial fractions (PMF). Treating samples with 0.01ug/uL trypsin for 37oC 30 min is sufficient to partially digest a substantial portion of the proteome. If samples from different subjects were not of the same weight, then this partial digestion step may introduce artificial variability as variable proportions of proteins from different subjects would be lost during this step. In addition, the mitochondrial protein enrichment in the mito fraction, despite statistically significant, does not look striking (Figure 1E, ~30% mitochondrial proteins in the mito fraction). As a comparison, Williams et al., MCP 2018 seem to have obtained high mitochondrial protein content in the mito fraction without trypsinizing the frozen quadriceps using a similar SWATH-MS-based approach.

Trypsinisation of the tissue prior to mitochondrial isolation is based on previous work and a Nature Protocol (1, 2) which isolated mitochondria for skeletal muscle. The rationale is that it

aids in mechanical homogenisation from highly fibrous tissues such as quadriceps muscle by digesting extracellular matrix proteins. The trypsin/protein ratio used to aid in this process is at least 400 times lower than the amount of trypsin used for formal proteomic tryptic digestion. Three pieces of evidence suggest this step has negligible effect on downstream proteomic analysis. First, because the trypsinisation buffer is detergent free, trypsin will only affect extracellular or exposed membrane proteins. Filtering our PMF dataset for proteins with ‘extracellular matrix’ gene ontology identifies at least 90 unique extracellular matrix proteins indicating good retention of proteins susceptible to partial digestion. Second, the trypsin dose used is 50 times lower than the concentration used for passaging cultured cells, which retain viability after trypsinisation. Third, and contrary to the point raised by the reviewer, we observe less missingness in PMF samples compared to mitochondrial samples. We thank the reviewer for bringing the Williams et al. 2018 MCP paper to our attention. We note that mitochondrial enrichment between the two papers is comparable (~2- fold). To improve clarity line 408 now reads: “Whole quadriceps muscle samples were prepared as previously described with modification (99, 100). First, tissue was snap frozen with liquid nitrogen...” and line 95 reads: “Mitochondrial proteins were defined based on their presence in MitoCarta 3.0 (24) and consistent with previous work (25) were approximately two-fold enriched in the mitochondrial fraction relative to the PMF (Fig 1E).”

(2) The authors mentioned that the proteomics data were Log2 transformed and median-normalized. Would it be possible to provide a bit more details on this? Were the subjects randomized?

Samples were randomised prior to sample processing and mass spectrometry analysis. Because of possible variation in total protein content, it is critical to normalise protein intensities between samples. Median normalisation adjusts the samples so that they have the same median, thereby accounting for technical variation. Log2 normalisation helps to achieve normal distributions, critical for many downstream statistical tests. Line 471 now reads: “...to achieve normal distributions and account for technical variation in total protein.”

(3) In Figure 1D, what were the numbers of mice the authors used for the CV comparisons in each group? Were they of similar age and sex? Were the differences in CV values statistically significant?

The mitochondrial and PMF proteomes originated from the same quadriceps sample from the same mouse, and thus the age and sex are the same across both proteomes. After quality control, we had mitochondrial proteomes for 194 mice and PMF proteomes for 215 mice. The overall CV in the mitochondrial fraction was significantly greater than in the PMF, however whether the source of this variation is biological, or the result of mitochondrial isolation is unclear and as such we have avoided making a statement within the body of the manuscript. We have now more clearly described the nature of the samples in the revised manuscript and added sample sizes to figure 1F.

(4) The authors stated in lines 155-157 that proteins negatively associated with the Matsuda index were further filtered by presence of their cis-pQTLs. Perhaps more explanations would be needed to justify this filtering criterion? Having a cis-pQTL would mean the protein abundance variation is explained by the variation in its coding gene, this however conceptually would not be relevant to its association with the Matsuda index. With the data that the authors have in hand, would it not be natural to align the Matsuda index QTL with the pQTLs (cis and trans if available), and/or to perform mediation analysis to examine causal relationships with statistical significance?

The rationale for filtering by cis-pQTL was not to study the genetics of either Matsuda or associated proteins but rather to identify proteins that were more likely to be causally

associated with Matsuda Index as opposed to adaptively associated. To clarify this line 165 now reads: “Filtering based on cis-pQTL presence was based on the rationale that if genetic variation can explain protein abundance differences between mice, then we can be confident that phenotype (Matsuda Index) is not driving the observed differences and therefore the protein-phenotype associations are likely causal. Importantly, this assumption can only be made for cis-acting pQTLs.” Previous work by Matthew et al. (see <https://qtlviewer.jax.org/>) has demonstrated that cis-pQTL have markedly higher LOD scores than trans-pQTLs, and our own unpublished work suggests that trans-pQTLs do not reproduce well between datasets. The reviewer rightfully suggests aligning protein QTL with those for Matsuda. This is our long-term goal but to identify genome wide significant peaks associated with altered Matsuda will require many more mice than studied here.

(5) It seems a bit odd that the first half of the paper focused extensively on the authors' discoveries in the mitochondrial proteome, and how proteins involved in mitochondrial processes (such as complex I) were associated with Matsuda Index, but the final fingerprint list of insulin resistance, which contained 76 proteins, only had 7 mitochondrial proteins. Was this because many mitochondrial proteins were filtered out due to no cis-pQTL presenting?

There are three reasons our fingerprint is lacking mitochondrial proteins: 1) there are more non-mitochondrial than mitochondrial proteins in the muscle proteome; 2) we focussed on negatively associated proteins, and as demonstrated in figure 2c, the mitochondrial proteome is enriched for positively associated proteins; 3) as implied by the reviewer, we filtered for pQTL presence, further reducing the number of mitochondrial proteins in our fingerprint. To improve clarity, line 170 now reads: “Low mitochondrial representation in the fingerprint is the result of selecting negatively associating proteins, and as seen (Figure 2C) previously, the mitochondrial proteome is enriched for positive contributors to insulin resistance.”

(6) The authors found that thiostrepton-induced insulin resistance reversal effects were not through insulin signalling. It activated glycolysis but the mechanism of action was not clear. What are the proteins in the fingerprint list that led to identification of thiostrepton on CMAP?

Is thiostrepton able to bind or change the expression of these proteins? Since thiostrepton was identified by searching the insulin resistance fingerprint protein list against CMAP, it would be rational to think that it exerts the biological effects by directly or indirectly acting on these protein targets.

This is indeed the implication of our data. Because of the timescales involved it is unlikely that thiostrepton is changing fingerprint protein levels but could be binding to and inhibiting them. Searching the CMAP thiostrepton signature reveals ARHGDIB and NAGK as the fingerprint proteins with the most positive and negative fold-changes respectively perhaps suggesting they play a role in thiostrepton's mechanism of action. Experiments are underway to test this hypothesis however these are beyond the scope of the current paper.

Reviewer #2 (Public Review):

Line 105: The observation that variance in respiratory proteins is stable while lipid pathways is variable is quite interesting. Is this due to lower overall levels of lipid metabolism enzymes (ex. do these differ substantially from similar pathways ranked from high-low abundance?).

The relationship between coefficient of variation (CV) and relative abundance of proteins is important to consider. To address this, we have now also performed GSEA on proteins ranked

from high to low relative abundance. These comparisons have been added to supplementary figure 1 and line 110 now reads: “As a control experiment, we also performed enrichment analysis on proteins ranked by LFQ relative abundance. High CV pathways (enriched for high CV proteins) tended to be lower in relative abundance (enriched for low relative abundance proteins) (Supplementary Fig 1a, b). However, many high variability pathways, lipid metabolism for example, were not enriched in either direction based on relative abundance suggesting differences in relative abundance do not fully explain pathway variability differences.”

Line 154: the 664 associations are impressive and potentially informative. It would be valuable to know which of these co-map to the same locus - either to distinguish linkage in a 2mb window or identify any cis-proteins which directly exert effects in trans-

To assess this, we have analysed pQTL position relative to gene position to generate a ‘hotspot’ plot. We have also generated a histogram of this pQTL density (in a 2 Mbp window) and added these figures to figure 3. We did not detect any obvious pQTL hotspots, and the distribution of pQTLs across the genome appears fairly uniform. Line 159 now reads: “These were distributed across the genome and were predominately cis acting (Figure 3A)...”

Line 194: Cross-platform validation of the CMAP fingerprint results is an admirable set of validations. It might be good to know general parameters like how many compounds were shared/unique for each platform. Also the concordance between ranking scores for significant and shared compounds.

The Connectivity Map (CMap) query included 5163 compounds, the Prestwick library included 1120, and the overlap was 420. We have added these comparisons to supplementary figure 2. Supplementary figure 2 now also contains a comparison of CMap scores between overlapping compounds (found in CMap and the Prestwick library) against all significant compounds identified by CMap (supplementary figure 2b). Interestingly, compounds present in both platforms scored higher on average, suggesting the Prestwick library captures a significant proportion of highly scoring CMap candidates. Line 206 now reads: “In total, 420 compounds were found across both platforms, and these consensus compounds captured a significant proportion of highly scoring CMap compounds (Supplementary Figure 2A, B).”

Line 319: Another consideration in the molecular fingerprint is how unique these are for muscle. While studies evaluating gene expression have shown that many cis-eQTLs are shared across tissues, to my knowledge, this hasn't been performed systematically for pQTLs. Therefore, consider adding a point to the discussion pointing out that some of the proteins might be conserved pQTLs whereas others which would be more relevant here present unique druggable targets in muscle.

To examine tissue specificity, we determined whether our skeletal muscle fingerprint proteins were detected and contained a pQTL in two metabolically important tissues, liver and adipose. Despite detecting almost all the fingerprint proteins in both adipose and liver tissue, they were depleted for pQTL compared to skeletal muscle. These data have now been added to figure 3c. Line 172 now reads: “To assess the tissue specificity of our fingerprint we searched for the same proteins in metabolically important adipose and liver tissues. Despite detecting 94% and 82% of muscle fingerprint proteins across each tissue respectively, both adipose and liver were depleted for pQTL presence (Figure 3C) suggesting that regulation of our fingerprint protein abundance is specific to skeletal muscle.”

Line 332: These are fascinating observations. 1, that in general insulin signaling and ampk were not themselves shown as top-ranked enrichments with matsuda and that this was sufficient to alter glucose metabolism without changes in these pathways. While

further characterization of this signaling mechanism is beyond the scope of this study, it would be good to speculate as to additional signaling pathways that are relevant beyond ROS (ex. CNYP2 and others)

We have now added further discussion to the manuscript to address this point., Line 347 now reads: “Aside from glycolysis, other pathways may be involved in enhancing insulin sensitivity. For example, the negatively associated protein ARHGDIA (Figure 2F) is a potent negative regulator of insulin sensitivity, and our fingerprint of insulin resistance contained its homologue ARHGDIB. Both ARHGDIA and ARHGDIB have been reported to inhibit the insulin action regulator RAC1 thus lowering GLUT4 translocation and glucose uptake. Further investigations may uncover a role for thioestrepton in modulating the RAC1 signalling pathway via ARHGDIB.”

Line: 314: Remove the statement: "While this approach is less powerful than QTL co-localisation for identifying causal drivers," as I don't believe that this has been demonstrated. Clearly, the authors provide a sufficient framework to pinpoint causality and produce an actionable set of proteins.

We have edited line 314, which now reads: “Moreover, our approach has the major advantage that it requires far fewer mice to obtain meaningful outcomes (222 mice in this study) compared to that required for genetic mapping of complex traits like Matsuda Index.”

Line 346: I would highlight one more appeal of the approach adopted by the authors. Given that these compound libraries were prioritized from patterns of diverse genetics, these observations are inherently more-likely to operate robustly across target backgrounds.

This point is further supported by our thioestrepton results in both C57BL6/j and BXH9 mice. Line 317 now reads: “Furthermore, because we have used genetically diverse datasets (DOz mice and multiple cell lines in Connectivity Map) our findings are likely robust across diverse target backgrounds.”

Line 434: I might have missed but can't seem to find where the muscle data are available to researchers. Given the importance and novelty of these studies, it will be important to provide some way to access the proteomic data.

These data are now available via the ProteomeXchange Consortium. Line 465 now reads: “The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (104) partner repository with the dataset identifier PXD042277.”

1. Frezza C, Cipolat S, Scorrano L. Organelle isolation: functional mitochondria from mouse liver, muscle and cultured fibroblasts. *Nat Protoc.* 2007;2(2):287-95.
2. Acin-Perez R, Benador IY, Petcherski A, Veliova M, Benavides GA, Lagarrigue S, et al. A novel approach to measure mitochondrial respiration in frozen biological samples. *The EMBO Journal.* 2020;39(13):e104073.
3. Chick JM, Munger SC, Simecek P, Huttlin EL, Choi K, Gatti DM, et al. Defining the consequences of genetic variation on a proteome-wide scale. *Nature.* 2016;534(7608):500- 5.
4. Gatti DM, Svenson KL, Shabalin A, Wu L-Y, Valdar W, Simecek P, et al. Quantitative Trait Locus Mapping Methods for Diversity Outbred Mice. *G3 Genes | Genomes | Genetics.* 2014;4(9):1623-33.