

# **Standard housing temperature but not $\beta$ 3-adrenoreceptor agonism drives weight-gain and adipose tissue deposition in rats following diet induced obesity at thermoneutrality**

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## Abstract

**Background and aim:** Rodents are commonly housed below thermoneutrality and this exposure to 'cold' (i.e. 20°C) activates thermogenic brown (BAT) and beiging of white adipose tissue. Here, we examined whether a standard housing temperature (i.e. 20°C, a reduction in temperature of ~8°C) or YM-178, a highly-selective  $\beta$ 3-adrenoreceptor agonist, in obese animals raised at thermoneutrality, would impact differently on classical BAT or subcutaneous inguinal (IWAT) beige depots.

**Methods:** Eighteen weanling Sprague-Dawley rats were housed at thermoneutrality (28°C) and fed a high-fat diet. At 12 weeks, 6 animals were randomised to either standard housing temperature (20°C, n=6) or to  $\beta$ 3-AR agonist administration (28°C+ $\beta$ 3, 0.75mg/kg/d, n=6) for 4 weeks. Metabolic assessment was undertaken during the final 48h, followed by interscapular, perivascular BAT and IWAT sampling for the analysis of thermogenic genes and the proteome.

**Results:** Exposure to 20°C increased weight gain, BAT and IWAT mass. Proteomic analysis of BAT revealed novel pathways associated with cold-induced weight gain (i.e. histone deacetylation, glycosaminoglycan degradation and glycosphingolipid biosynthesis) whilst  $\beta$ 3-adrenoreceptor agonism impacted on proteins involved in skeletal muscle contraction and cell differentiation. IWAT of cold-exposed animals exhibited an enrichment of proteins involved NAD<sup>+</sup> binding, plus retinol and tyrosine metabolic pathways whilst  $\beta$ 3-AR agonism downregulated ribosomal and upregulated acute phase response proteins.

**Conclusion:** Following diet-induced obesity at thermoneutrality, exposure to 20°C promotes subcutaneous fat deposition in order to reduce heat loss and defend body temperature. In contrast, chronic administration of  $\beta$ 3-AR agonist has minimal metabolic-related effects on adipose tissue.

## Introduction

Therapeutic activation of thermogenic brown adipose tissue (BAT) may be feasible to prevent, or treat, cardiometabolic disease [1]. In rodent models of obesity, the activation of BAT and UCP1-positive beige adipocytes in white adipose tissue (WAT) by cold exposure and sympathomimetics (i.e.  $\beta$ 3-agonists) can attenuate or reverse obesity, diabetes and atherosclerosis, thus eliciting beneficial effects on metabolic health [1]. One factor which could influence these outcomes is that animals are typically housed at temperatures well below their thermoneutral zone (which for a rodent is c. 28°C) [2]. Under these conditions, not only is BAT active, but UCP1+ beige adipocytes are readily seen in the inguinal WAT (IWAT) depot which is considered 'beige'.

Housing animals at thermoneutrality modifies the thermogenic and metabolic response. For example, fasting glucose, food intake and metabolic rate are reduced at a higher ambient temperature [3]. Furthermore, markers of 'browning' and BAT activation are reduced at thermoneutrality, whilst the primary organ responsible for glucose uptake becomes skeletal muscle and not BAT [3]. Long term housing at thermoneutrality reduces sympathetic drive in BAT, as demonstrated by reduced tyrosine hydroxylase and norepinephrine turnover [4]. This results in increased adiposity at thermoneutrality, despite a lower energy intake compared to animals housed at 20°C. Thermoneutral housing also attenuates the response to common thermogenic stimuli. For instance, the thermogenic effects of thyroxine become independent of UCP1 due to the onset of pyrexia [5]. Similarly, the induction of 'browning' seen with exercise training at standard housing is attenuated in animals housed at thermoneutrality [6].

The 'cold-stressed' animal has been widely studied but much less is known about the underlying adaptations in adipose tissue in animals maintained at thermoneutrality. Usually, temperatures as low as 4°C, which represents an 'extreme cold', are used to activate BAT, with the induction of UCP1 and subsequent thermogenic response primarily seen in

subcutaneous IWAT and other 'beige' depots [7]. Conversely, when animals which have been housed at thermoneutrality and are then exposed to 20°C, the induction of UCP1 is primarily seen in BAT. These differences highlight that there are two key steps to the 'browning' process and emphasise the need to study metabolism under more translationally relevant conditions [7]. Importantly, it was recently demonstrated that BAT from thermoneutrally housed, obese animals closer resembles human BAT and, that this model of 'humanised BAT' represents the best choice for modelling BAT physiology [8]. Here, we aimed to determine the metabolic response to modest cold exposure (i.e. standard housing temperatures (20°C)), which constitutes a drop of c. 8°C and chronic  $\beta$ 3-AR agonism in animals raised from weaning at thermoneutrality. Using this model, in which animals are not 'cold-stressed' and BAT is chronically inactive, we have demonstrated UCP1 mRNA is absent in subcutaneous IWAT (the classical 'beige' depot) and not induced with exercise training, a common response at standard housing temperatures [9]. We hypothesised that exposure to a mild cold stimulus in animals raised at thermoneutrality would have modest effects on systemic metabolism but not weight per se. Instead, we demonstrate rapid weight gain in response to an 8°C reduction in ambient temperature and deposition of substantial amounts (125%) of subcutaneous adipose tissue through an, as yet, unexplained mechanism. These effects were not seen in animals treated with the highly-selective  $\beta$ 3-adrenoreceptor agonist, YM-178, suggesting a direct effect of ambient temperature which is uncoupled from sympathetic activation. Using adipose tissue proteomics, we then uncovered novel pathways associated with this cold-induced weight gain illustrating a potentially novel mechanism through which animals defend body temperature in the absence of a thermogenic response.

## Methods

### *Animals, cold exposure and YM-178 treatment*

All studies were approved by the University of Nottingham Animal Welfare and Ethical Review Board, and were carried out in accordance with the UK Animals (Scientific Procedures) Act of 1986. Eighteen male Sprague-Dawley rats aged 3 weeks were obtained from Charles River (Kent, UK) and housed immediately at thermoneutrality (c.28°C), on a high-fat diet (HFD; 45%, 824018 SDS, Kent, UK), under a 12:12-hour reverse light-dark cycle (lights off at 08:00, on at 20:00). These conditions were chosen so as to closer mimic human physiology [7], minimise animal stress and maximise data quality and translatability [10]. At 12 weeks of age, all animals were randomised to 4 weeks of standard housing temperature (20°C, n=6), YM-178 (28°C+β3, 0.75mg/kg/day, n=6) administration or HFD controls (28°C, n=6) from 12 weeks of age. In adherence to the NC3Rs, this experiment was ran alongside our work looking at the effect of exercise training on 'browning' and utilised the same cohort of control HFD animals.

### ***Metabolic cages***

All animals were placed in an open-circuit calorimeter (CLAMS: Columbus Instruments, Linton Instrumentation, UK) for 48h towards the end of the study. Assessment of whole body metabolism was carried out as previously described [11], after which all animals were weighed and fasted overnight prior to euthanasia by rising CO<sub>2</sub> gradient. BAT, perivascular BAT (PVAT) and IWAT were then rapidly dissected, weighed, snap-frozen in liquid nitrogen and stored at -80°C for subsequent analysis.

### **Gene expression analysis**

Total RNA was extracted from each fat depot using the RNeasy Plus Micro extraction kit (Qiagen, West Sussex, UK) using an adapted version of the single step acidified phenol-chloroform method. RT-qPCR was carried out as previously described [11] using rat-specific oligonucleotide primers (Sigma) or FAM-MGB Taqman probes [11]. Gene expression was determined using the GeNorm algorithm against two selected reference genes;

*RPL19:RPL13a* in BAT and IWAT (stability value  $M = 0.163$  in BAT and  $0.383$  in IWAT) and *RPL19:HPRT1* in PVAT (stability value  $M = 0.285$ ).

### ***Serum analysis***

Serum was thawed gently on ice with concentrations of glucose (GAGO-20, Sigma Aldrich, Gillingham, UK), triglycerides (LabAssay <sup>TM</sup> Triglyceride, Wako, Neuss, Germany), non-esterified fatty acids (NEFA-HR(2), Wako, Neuss, Germany), insulin (80-INSRT-E01, Alpco, Salem, NH, USA) and leptin (EZRL-83K, Merck, Darmstadt, Germany) measured following manufacturer's instructions.

### ***Mass spectrometry***

Protein extraction, clean up and trypsinisation was carried out as previously described [11]. Briefly, 50-100 mg of frozen tissue was homogenised in 500  $\mu$ L CellLytic MT cell lysis buffer (Sigma, C3228) prior to removal of lipid and other contaminants using the ReadyPrep 2D cleanup Kit (Biorad, 1632130). Samples were then subjected to reduction, alkylation and overnight trypsinisation following which they were dried down at  $60^{\circ}\text{C}$  for 4 h and stored at  $80^{\circ}\text{C}$  before resuspension in LCMS grade 5% acetonitrile in 0.1% formic acid for subsequent analysis. Analysis by mass spectrometry was carried out as previously described on a SCIEX TripleTOF 6600 instrument [12]. Briefly, samples were analysed in both SWATH (Data Independent Acquisition) and IDA (Information Dependent Acquisition) modes for quantitation and spectral library generation respectively. IDA data was searched together using ProteinPilot 5.0.2 to generate a spectral library and SWATH data was analysed using Sciex OneOmics software [13] extracted against the locally generated library as described previously [11].

### ***2.6 Statistical analysis***

Statistical analysis was performed in GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA). Data are expressed as Mean $\pm$ SEM with details of specific statistical tests in figure legends. Functional analysis of the proteome was performed using the Advaita

Bioinformatic iPathwayGuide software ([www.advaitabio.com/ipathwayguide.html](http://www.advaitabio.com/ipathwayguide.html)) (fold change  $\pm$  0.5 and confidence score cut-off of 0.75). Significantly impacted biological processes, molecular interactions and pathways were analysed in the context of pathways obtained from the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database (Release 84.0+/10-26, Oct 17) [14] and the Gene Ontology Consortium database (2017-Nov) [15]. The Elim pruning method, which removes genes mapped to a significant GO term from more general (higher level) GO terms, was used to overcome the limitation of errors introduced by considering genes multiple times [16].

## Results

### Exposure to standard housing temperature drives weight gain and adiposity

Four weeks of exposure to 20°C increased weight gain and body weight with greater IWAT and BAT mass, an effect not seen with YM-178 (Fig. 1A-E). When body mass was incorporated into an ANCOVA, there was a significant relationship between body mass and energy expenditure ( $r^2$  0.926,  $p=0.038$ , Fig.1F) and body mass and energy intake ( $r^2=0.852$ ,  $p=0.025$ , Fig. 1H) in animals exposed to standard housing temperatures, whilst there was a change in the direction of the relationship between body mass and energy intake in animals treated with YM-178 (Fig. 1H). Cold-exposed animals exhibited an increase in light-phase, and a reduction in dark-phase ambulatory activity which was also seen in animals treated with YM-178 (Fig. 1G). There was no change in serum metabolites (i.e. glucose, triglycerides and NEFA) or hormones (i.e. insulin and leptin) (Fig. 1I-N).

Next, we analysed a panel of primary thermogenic genes in BAT and PVAT as we have previously shown these two anatomically and developmentally distinct BAT depots exhibit a divergent response to both a brief period of nutrient excess and exercise training [9, 11]. Despite an increase in BAT mass with cold, there was no reduction in thermogenic genes

(i.e. UCP1) in this depot or in PVAT with either cold or YM-178 (Fig. 2A-B). Expression of the BAT marker CITED1 and beige marker TMEM26 were reduced in BAT of both cold exposed and YM-178 treated animals whilst the beige marker P2RX5 was upregulated in PVAT of both groups. Using targeted arrays to screen for key genes involved in adipose tissue metabolism we saw an increase in the expression of FASN mRNA in both BAT and PVAT but not IWAT following cold exposure (Fig. 2A-C). There was also an increase of genes involved in glycolysis (i.e. HK2 and PDK), fatty acid oxidation (i.e. ACACA and ACACB) and insulin resistance (i.e. AdipoR1 and MAPK9) in PVAT only following cold exposure (Fig. 2D-K). This suggests that the metabolic response to cold in PVAT is uncoupled from BAT activation. We then analysed the same panel of thermogenic genes in IWAT to assess the 'browning' capacity of each intervention (Fig. 2C). UCP1 mRNA was absent in IWAT of all animals and, despite a c.125% increase in IWAT mass of cold exposed animals, there was no change in the expression of other key genes associated with thermogenesis (i.e. ADR $\beta$ 3), beige adipocytes (i.e. TMEM26) or lipogenesis (i.e. FASN).

### ***Identification of differentially regulated proteins in BAT and WAT associated with cold-induced increases in fat mass***

Given the increase in BAT and IWAT mass seen in response to standard housing temperature, we carried out proteomic analysis to elucidate potential contributors to this physiological response identifying 175 differentially regulated proteins in BAT of cold-exposed animals (Table 1 for top 20 proteins, Supp. Data for full list). This method, which quantifies the 30-40% most abundant proteins across samples, did not detect UCP1 in BAT. There was, however, an increase in numerous other metabolic proteins in BAT of cold exposed animals including a pronounced increase in the mitochondrial citrate transporter proteins (SLC25a1), together with glucose-6-phosphate dehydrogenase (G6PD), phosphogluconate dehydrogenase (PGD), succinate dehydrogenase complex flavoprotein

subunit a (SDHA), fatty acid binding protein 5 (FABP5) and the muscle isoforms of phosphoglycerate mutase (PGAM2) and creatine kinase (CKm). The increase in these metabolic proteins occurred alongside a downregulation of proteins involved in the import of membrane (TIMM9) and cytoplasmic (TIMM13) proteins to the inner mitochondrial membrane, mitochondrial amino acid production (BCAT2) and redox state (PRDX4, GLRX3 and SOD2).

Conversely, 137 proteins were differentially regulated in BAT of YM-178 treated animals (Table 1 for top 20 significant proteins, Supp. Data for full list), with an upregulation in proteins involved in skeletal muscle physiology including myosin heavy chain 4 (MYH4) and light chain 12a (MYL12a), the fast-twitch skeletal muscle isoforms troponin I2 (TNNI2) and calsequestrin 1 (CASQ1) and myoglobin (MB) in addition to proteins governing endothelial adhesion and vascular growth (PECAM1 and FBLN5). These changes occurred alongside a downregulation of the aldo-keto reductase family member proteins B15 (AKR1B15) and C3 (AKR1C3) in addition to mevalonate diphosphate decarboxylase (MVD), trans-2,3-enoyl-CoA reductase (TECR), acyl-CoA dehydrogenase short/branched chain (ACADSB), phosphate cytidyltransferase 1, choline, alpha (PCYT1A) and 3-hydroxybutyrate dehydrogenase 1 (BDH1) proteins indicating that YM-178 may be driving a muscle phenotype whilst perturbing steroid, cholesterol, acyl-CoA and phosphatidylcholine biosynthesis in BAT.

In IWAT, we detected 116 proteins differentially regulated in cold-exposed animals (Table 2 for top 20 significant proteins, Supp. Data for full list) with the upregulation of numerous lipid metabolic proteins including fatty acid binding proteins 1 (FABP1) and 3 (FABP3), fatty acid synthase (FASN) and ATP citrate lyase (ACLY) as well as those involved in beta oxidation including hydroxynacyl-CoA dehydrogenase (HADH), hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit beta (HADHB) and acyl-CoA dehydrogenase long chain (ACADL). In addition, there was an upregulation of glycerol kinase (GK), pyruvate carboxylase (PC) and monocarboxylic acid transporter 1 (MCT1) and these alterations in

IWAT metabolism were accompanied by a downregulation of multiple proteins involved in mRNA processing and splicing (i.e. RNA binding motif protein 8B, RBM8B; dexe-Box helicase 39B, DDX39B and poly(u) binding splicing factor 60, PUF60).

YM-178 administration modulated 206 proteins in IWAT (Table 2 for top 20 significant proteins, Supp. Data for full list) including an upregulation of multiple proteins involved in the nervous system including synuclein Gamma (SNCG), neurolysin (NLN) and neuronal pas domain protein 4 (NPAS4). This was associated with an increase in proteins involved in lipid and cholesterol metabolism including carnitine palmitoyltransferase 1A (CPT1A), hormone-sensitive lipase (LIPE) and apolipoprotein C1 and M (APOC1 and APOM) in addition to solute carrier family 2 member 4 (GLUT4) and ATP synthase 5MG (ATP5MG). Interestingly, YM-178 also induced an increase in multiple inflammatory proteins in IWAT including orosomucoid 1 (ORM1), complement C4A (C4A), S100 calcium binding protein A8 (S100A8) and S100 calcium binding protein B (S100B). These metabolic and inflammatory alterations in IWAT were associated with a downregulation of multiple ribosomal proteins including ribosomal protein S21 (RPS21), ribosomal protein L32 (RPL32) and ribosomal protein L10a (RPL10a).

### ***Functional analysis of the BAT and WAT proteome***

We then carried out functional analysis of the BAT and IWAT proteome in order to elucidate potential biological processes, functions and pathways in adipose tissue that may be contributing to the significant increase in BAT and IWAT mass seen following cold-exposure.

The differentially regulated proteins in BAT of cold exposed animals enriched GO terms involved '*glucose import*', '*ATP-dependent helicase activity*' and '*regulation of protein phosphorylation*' whilst there was also an enrichment of nuclear related GO terms including '*histone deacetylation*', '*nucleosomal DNA binding*', '*nuclear chromatin*' and the '*nucleosome*' (Table 3, Fig. 3A-C). Conversely, differentially regulated proteins in BAT of animals treated

with YM-178 enriched GO terms including '*positive regulation of protein kinase B signalling*', '*negative regulation of cellular carbohydrate metabolic process*' and '*positive regulation of atpase activity*' whilst there was also an enrichment of GO terms involved in both brown adipocyte and muscle biology including '*brown fat cell differentiation*', '*skeletal muscle contraction*' and '*regulation of muscle contraction*' (Table 3, Fig. 3D-F). Finally, impact analysis demonstrated that the '*glycosaminoglycan degradation*', '*notch signalling*' and '*glycosphingolipid biosynthesis – ganglio series*' pathways were affected by cold-exposure whilst YM-178 impacted pathways involved in '*Galactose metabolism*' and '*Hepatitis C*' (Table 4, Fig. 5A-F).

Differentially regulated proteins in IWAT of cold-exposed animals enriched GO terms involved in the '*DNA damage response*', '*3-hydroxyacyl-coa dehydrogenase activity*' and '*NAD<sup>+</sup> binding*' whilst YM-178 enriched inflammatory terms including the '*acute phase response*' and '*structural constituent of ribosome*' suggesting an effect of sympathetic activation on both the inflammatory system and protein synthesis (Table 5, Fig. 4A-F). Impact analysis, meanwhile, demonstrated that cold-exposure was associated with an impact on both retinol and tyrosine metabolic pathways (Table 6, Fig. 6A-B). Conversely, YM-178 treatment impacted the '*ribosome*' pathway in addition to HIF-1 signalling and cytokine-cytokine receptor pathways (Table 6, Fig. 6C-D).

### ***Elucidating the 'interactome' in BAT and IWAT***

We then sought to discover putative hub proteins and signalling pathways in these tissues, through characterisation of the 'interactome' through protein-protein interactions. Differentially regulated proteins in BAT of cold-exposed animals were involved in 22 networks (i.e. the 'continent' and 21 'islands') with the 'continent' consisting of 293 interacting proteins. We identified multiple hub proteins in this network including the G proteins G protein subunit alpha I3 (GNAI3) and G protein subunit alpha Q (GNAQ), protein

tyrosine phosphatase non-receptor type 11 (PTPN11), actin gamma 1 (ACTG1) and serine/threonine kinase 24 (STK24) (Fig. 7A). Further analysis demonstrated that these hub proteins interacted with numerous other G proteins (i.e. Adenosine A1 receptor, ADORA1; Free fatty acid receptor 3, FFAR3 and neuropeptide Y receptor 2R; NPY2R), in addition to proteins involved in growth signalling pathways (i.e. phosphoinositide-3-kinase regulatory subunit 2; PIK3R2) and adipogenesis (platelet derived growth factor receptor beta; PDGFRB) to enrich 48 biological processes (Supp. Data) including 'anatomical structure involved in morphogenesis', 'developmental growth' and 'ER nucleus signalling pathway' and 26 molecular functions (Supp. Data) including exonuclease activity, steroid dehydrogenase activity, lipase activity and neuropeptide hormone activity. Conversely, differentially regulated proteins in BAT of YM-178 treated animals were involved in 17 networks, with the 'continent' consisting of 725 proteins. Hub proteins in this main network included multiple ribosomal proteins (i.e. ribosomal protein L5 (RPL5) and ribosomal protein L17 (RPL17)), proliferating cell nuclear antigen (PCNA), signal transducer and activator of transcription 3 (STAT3) and proteasome subunit alpha 4 (PSMA4) (Fig. 7B). These hub proteins were shown to interact with numerous other ribosomal (i.e. RPL10a and RPS23) and mitochondrial ribosomal proteins (i.e. MRPS9 and MRPL13) in addition to proteins involved in cytokine signalling (i.e. SOCS1, SOCS3 and STAT1/3 and 5B) and metabolism (i.e. LEP and MTOR) to enrich 30 processes including 'generation of neurons', 'cytokine biosynthetic process' and 'generation of precursor metabolites and energy' and 23 functions including 'RNA binding' and 'transcription cofactor activity' (Supp. Data).

Differentially regulated proteins in IWAT of cold-exposed animals created 12 networks with the continent consisting of 153 interacting proteins. Hub proteins in this main network included heterogeneous nuclear ribonucleoprotein k (HNRNPK), signal transducer and activator of transcription 5A (STAT5A), RNA binding motif protein 8A (RBM8A) and histone deacetylase 1 (HDAC1) (Fig. 8A). We show these hub proteins interact with other ribonucleotide and splicing factors (i.e. small nuclear ribonucleoprotein polypeptides B And

B1; SNRPB and serine and arginine rich splicing factor 1; SRSF1) in addition to proteins involved in adipogenesis (i.e. PDGFRB) and adipose tissue hypertrophy (i.e. LEP and LEPR) with the total network enriching 37 processes including 'RAS protein signal transduction' and 'activation of JUN kinase activity' and 21 functions including 'nucleotide binding' and 'DNA binding' (Supp. Data). Conversely, the main network in IWAT of YM-178 treated animals consisted of 819 interacting proteins with numerous ribosomal (i.e. RPL17, RPL18 and RPL6) and proteasomal (i.e. PSMA4, PSMA2 and PSMB10) proteins in addition to STAT5B and growth factor receptor bound protein 2 (GRB2) constituting the main hub proteins (Fig. 8B). Again, we showed these hub proteins in YM-178 treated animals interact with numerous other ribosomal (i.e. RPL10a and RPS29) and mitochondrial ribosomal proteins (i.e. MRPL1 and MRPL13), in addition to multiple tyrosine kinases (i.e. Fms related tyrosine kinase 1; FLT1 and Fms related tyrosine kinase 4; FLT4) with this network of interacting proteins enriching processes including 'Chromatin assembly or disassembly' and 'Cytokine biosynthetic process' and functions including 'RNA binding' and 'regulation of DNA dependent transcription and elongation' (Supp. Data).

## Discussion

Housing temperature, especially cold exposure, impacts on metabolic homeostasis as illustrated by the effects it has on BAT and 'browning'. However, much less is known on the effects of 'browning' interventions in animals maintained at thermoneutrality [2, 17]. Our major finding is that chronic exposure to a mild-cold stimulus (i.e. standard housing temperature) drives weight gain and the deposition of large quantities of subcutaneous adipose tissue in obese animals rather than the activation of BAT and subsequent weight-loss as would be expected. This effect is not seen in animals treated with the highly selective  $\beta$ 3-agonist YM-178, suggesting it is independent of sympathetic activation and a direct effect of ambient temperature, pointing towards a novel mechanism whereby animals increase

body weight and fat mass in the absence of prior cold-stress to potentially reduce heat loss and thus defend body temperature.

Whilst we do not detect any change in energy expenditure and energy intake in animals exposed to 20°C, it is important to note that metabolic cages are not truly representative of the entire study period and that, even with an adaptation period, it is an artificial environment where changes in energy intake are not always be detected [18]. Furthermore, at 600-700g these animals are likely to be near the size limit of the metabolic cages, such that home cage systems would be better-suited to collect physiological data in these large animals. These issues could also explain the relationship between body weight and both energy expenditure and energy intake, which suggests a reduction in both as body mass increases though the opposite is true in animals treated with the  $\beta$ 3-agonist YM-178. Nevertheless, there is accumulating evidence that, in the absence of adaptive thermogenesis, other mechanisms compensate in order to maintain body temperature. For instance, in UCP1 k/o mice, a reduction in BAT thermogenesis leads to a compensatory increase in shivering thermogenesis in skeletal muscle [19]. Similarly, when shivering thermogenesis is impaired in sarcolipin k/o mice, there is a compensatory increase in BAT activity [19]. In obese animals lacking BAT, there seems to be an entirely different homeostatic response to cold-stress where following BAT lipectomy, obese, cold-exposed rats gain weight and exhibit nearly a one fold increase in adipose tissue mass [20]. There is also evidence to suggest that intermittent cold-exposure (from 20°C to 4°C) over a period of days increases weight gain and adiposity and that this is associated with transient, intermittent increases in energy intake [21]. Although other mechanisms such as intestinal growth and increased fatty acid absorption [22] may also play a role the impact of BAT, lipectomy on the response to cold-exposure mirrors our own findings and would suggest a compensatory mechanism for heat production in obesity when adaptive thermogenesis is insufficient. Importantly, an increase of c. 0.5g in BAT and of c. 10g in IWAT cannot account for the c. 41g higher weight gain seen in the cold-exposed animals. Given that liver mass was not higher in cold-exposed

animals, and that the increase in IWAT was highest, we speculate that this weight is not due to increased organ mass but due an increase in mass of dermal adipose tissue, given the putative role of this depot in regulating insulation and this should be investigated in future studies. A useful comparison here could be the Siberian hamster where obesity, and weight loss, can be manipulated by photoperiod. In this model, reductions in fat mass initially occur independently of reductions in energy intake and subcutaneous AT mass is preserved, likely due to its insulative properties highlighting how, at least in the short term, changes in weight and fat mass can be uncoupled from energy balance [23].

Another potentially important finding of this study is that the increase in weight gain and subcutaneous AT mass seen in cold-exposed animals was not associated with impaired metabolic parameters (i.e. fasting glucose and lipids) or any discernible adipose tissue dysfunction. Here, chronic exposure to a mild cold stimulus seemingly drives a phenotypically healthy expansion of subcutaneous AT, increasing it by 125% compared to controls. By using proteomics, we were able to elucidate processes in both BAT and IWAT associated with this expansion of AT mass. An increase in genes that modulate metabolism, including those involved in glycolysis, the TCA cycle and lipogenesis, suggests that there is an increase in metabolic flux of these depots which ultimately results in net lipogenesis. In BAT, this is associated with an enrichment of GO terms associated with DNA metabolism (i.e. histone deacetylation) with CTBP1, HDAC1 and SIRT2 all previously shown to be negative regulators of lipogenesis by either binding or deacetylating PPARY [24-26]. In IWAT, we show an enrichment of proteins involved in retinol metabolism, a well-known pathway which drives both adipogenesis and lipogenesis which is dependent on an intact NAD<sup>+</sup> binding motif [27]. The enrichment of multiple proteins involved in NAD<sup>+</sup> binding suggests this pathway is intact and upregulated and may contribute to this increase in IWAT mass.

We were also able to elucidate the impact of sympathetic activation in the absence of any change in housing temperature by administering YM-178. This highly selective  $\beta$ 3-agonist

increases BAT activity, whole body EE and induces 'browning' in humans [28-31]. Administration of YM-178 to mice for 4-6 weeks at similar dosage to this study (i.e. 0.8mg/kg) reduces fat mass, increases UCP1 in IWAT and glucose uptake in BAT at standard housing temperature (SHT) whilst driving atherosclerosis, through BAT lipolysis in ApoE<sup>-/-</sup> and LdlR<sup>-/-</sup> mice [32]. Here, we show YM-178 treatment in obese animals raised at thermoneutrality has no impact on thermogenesis although it does modulate specific proteins involved in cell differentiation and skeletal muscle contraction. YM-178 also impacted on proteins involved in the acute phase response and inflammation in both BAT and IWAT with many increasing in the latter. Taken together, this suggests that YM-178, when given to obese rats, acts negatively on adipose tissue by driving inflammatory processes. Another potentially important finding is that chronic YM-178 induces the downregulation of ribosomal proteins. Adrenergic agonists have previously been shown to modify protein synthesis in skeletal muscle but the effect of YM-178 has yet to be examined with human studies focussing on overactive bladder and associated symptoms [33, 34]. The physiological relevance of a reduction in ribosomal proteins, and perturbed proteins synthesis in IWAT with YM-178 treatment and, whether this translates to human patients is as yet unclear.

Elucidating protein-protein interactions (PPI) is an important step in understanding both communication between proteins following an intervention and the identification of novel signalling pathways. We have previously shown that ribosomal proteins are important hub proteins in PPI networks following exercise training and, in the present study, show that these also respond to YM-178 [9]. Furthermore, these interact with multiple other mitochondrial ribosomal proteins and proteins governing energy metabolism such as mTOR and leptin. The role, and physiological significance, of these adaptations in putative ribosomal regulated signalling pathways remain to be determined but may assist in our understanding of the sympathetic control of adipose tissue. In BAT, the identification of GNAI3 and GNAC as hub proteins is of particular interest given the role of GPCRs and the

Gq signalling pathway, in particular, given its role in inhibiting BAT metabolism and 'browning' [35].

## Conclusion

We propose that chronic suppression of BAT from weaning, through the use of thermoneutral housing, changes the physiological response to cold in the obese state. Instead, these animals deposit adipose tissue and gain weight, an effect not seen with YM-178, suggesting a direct effect of temperature, whereby an insulative mechanism is recruited when a thermogenic response is absent.

**Availability of data and material:** The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

**Authors' contributions:** P.A., H.B. and M.E.S. conceived the study and attained the funding; P.A. and M.E.S. developed and designed the experiments; P.A, J.E.L, I.L, A.K.M and D. J. B. performed the experiments; P.A., A.K.M. and D.J.B. analysed the data; P.A. and M.E.S. wrote the paper which was revised critically by D.J.B., H.B., F.J.P.E, and J.E.L. for important intellectual content. All authors read and approved the final manuscript.

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## Figure legends

**Figure 1.** Cold exposure (20°C) but not YM-178 (28°C+β3) drove weight gain and deposition of BAT and inguinal white adipose tissue (IWAT) with no effect on serum metabolites. (A) Final body weight, (B) 4 week intervention weight gain, (C) total fat mass, (D) BAT mass, (E) IWAT mass, (F) 24h oxygen consumption, (G) 24h ambulatory activity, (H) 24h energy intake, (I-N) serum hormones and metabolites. Data expressed as mean ± SEM, n=4-5 per group. For comparison, data was analysed by either one (A-E and H-O) or two-way ANOVA (F-G) with Sidak post-hoc tests. Significance denoted as \* <0.05; \*\* <0.01 or \*\*\* <0.001.

**Figure 2.** Cold exposure (20°C) and YM-178 (28°C+β3) do not impact key thermogenic genes. (A-C) Markers of brown and beige adipose tissue in BAT, PVAT and IWAT, (D-K) select metabolic genes in BAT and PVAT. For comparison, data was analysed one-way ANOVA with Sidak post-hoc tests.

**Figure 3.** Overview of enriched gene ontology (GO) terms in BAT following cold-exposure (20°C, B and C) and YM-178 treatment (28°C+β3, D-F). (A) Venn diagram of differentially regulated protein, (B) Histone deacetylation, (C) Enhancer sequence-specific DNA binding, (D) Skeletal muscle contraction, (E) Brown fat cell differentiation, (F) Glucocorticoid receptor binding and (G-F) terms enriched in both groups, (G) Protein tyrosine kinase activity and (H) Positive regulation of autophagy in mitochondrion. Figures created with AdvaitaBio IPPathwayGuide.

**Figure 4.** Overview of enriched gene ontology (GO) terms in IWAT following cold-exposure (20°C, B and C) and YM-178 treatment (28°C+β3, D-F). (A) Venn diagram of differentially regulated protein, (B) DNA damage response, (C) 3-hydroxyacyl-CoA dehydrogenase activity, (D) NAD<sup>+</sup> binding, (E) Acute phase response and (F) Structural constituent of ribosome. Figures created with AdvaitaBio IPPathwayGuide.

**Figure 5.** Overview of significantly impacted pathways in BAT following cold-exposure (20°C, A-C) and YM-178 treatment (28°C+β3, D-F). (A) Glycosaminoglycan degradation, (B) Notch pathway, (C) Glycosphingolipid biosynthesis ganglio series, (D) Galactose metabolism, (E) Hepatitis C and (F) Amino and sugar nucleotide sugar metabolism. Figures created with AdvaitaBio IPPathwayGuide.

**Figure 6.** Overview of significantly impacted pathways in IWAT following cold-exposure (20°C, A-C), YM-178 treatment (28°C+β3, C) and in both groups (E). (A) Retinol metabolism, (B) Tyrosine metabolism, (C) Hif1a signaling, (D) Ribosome, (E) Spliceosome. Figures created with AdvaitaBio IPPathwayGuide.

**Figure 7.** Protein-protein interaction network of differentially regulated proteins in BAT following cold-exposure (20°C, A) and YM-178 treatment (28°C+β3, B) with hub proteins in bold. Figures created with NetworkAnalyst.

**Figure 8.** Protein-protein interaction network of differentially regulated proteins in IWAT following cold-exposure (20°C, A) and YM-178 treatment (28°C+β3, B) with hub proteins in bold. Figures created with NetworkAnalyst.

## Tables

**Table 1:** Differentially regulated proteins in BAT

| GeneID          | Protein name | logfc | adjpv    |
|-----------------|--------------|-------|----------|
| <b>20°C</b>     |              |       |          |
| 80754           | Rabep2       | 3.69  | 6.35E-05 |
| 114122          | Vcan         | 2.74  | 0.000269 |
| 292073          | Galns        | -2.75 | 0.00027  |
| 64012           | Rad50        | 1.50  | 0.000288 |
| 171139          | Timm9        | -1.94 | 0.00037  |
| 295088          | Gmps         | 0.67  | 0.000396 |
| 81716           | Ggcx         | 1.44  | 0.000639 |
| 25622           | Ptpn11       | -2.47 | 0.000975 |
| 289590          | Ociad1       | -1.41 | 0.000988 |
| 29384           | H2afy        | -1.15 | 0.001077 |
| 302669          | Ca5b         | 1.18  | 0.001237 |
| 499991          | Steap4       | -3.88 | 0.00228  |
| 315265          | Twf1         | -1.54 | 0.00248  |
| 25283           | Gclc         | 2.57  | 0.002571 |
| 84474           | Ddx1         | 2.23  | 0.00326  |
| 94342           | Bag6         | -1.26 | 0.0036   |
| 89827           | Ddx39a       | 1.26  | 0.00397  |
| 64679           | Tgm4         | 0.95  | 0.00616  |
| 64517           | Thop1        | 1.25  | 0.006186 |
| 29743           | Slc25a1      | 1.10  | 0.006388 |
| <b>28°C+ β3</b> |              |       |          |
| 116689          | Ptpn6        | -3.56 | 0.000154 |
| 25650           | Atp1b1       | -1.40 | 0.000303 |
| 59108           | Mb           | 2.71  | 0.000548 |
| 306262          | Btd          | 2.72  | 0.000735 |
| 501167          | Gmppa        | 1.58  | 0.000934 |
| 64528           | Golga2       | 0.98  | 0.001723 |
| 287633          | Lrrc59       | -0.62 | 0.002902 |
| 81726           | Mvd          | -3.82 | 0.004012 |
| 363425          | Cav2         | -2.41 | 0.004547 |
| 114559          | Arhgef7      | -1.54 | 0.005187 |
| 25737           | Pcna         | 1.41  | 0.005462 |
| 171516          | Akr1c3       | -3.61 | 0.006199 |
| 311328          | Rmdn3        | -2.54 | 0.006373 |
| 312398          | Smardc1      | -2.97 | 0.006491 |
| 29583           | Pecam1       | 3.43  | 0.006613 |
| 25491           | Nes          | -1.35 | 0.008957 |
| 25106           | Rgn          | 1.79  | 0.009092 |
| 117099          | Bdh1         | -1.76 | 0.009659 |

|        |              |      |          |
|--------|--------------|------|----------|
| 1E+08  | LOC100364457 | 0.98 | 0.010813 |
| 681429 | Rps27l       | 1.68 | 0.011212 |

**Table 2:** Differentially regulated proteins in WAT

| GeneID          | Protein name | logfc | adjpv    |
|-----------------|--------------|-------|----------|
| <b>20°C</b>     |              |       |          |
| 117028          | Bin1         | -2.79 | 8.97E-06 |
| 304290          | Kdelr2       | -2.68 | 4.47E-05 |
| 24667           | Ppm1b        | -2.65 | 0.000108 |
| 84114           | Agps         | -1.35 | 0.000125 |
| 84401           | Puf60        | -2.94 | 0.000753 |
| 300983          | Abhd14b      | 0.94  | 0.000791 |
| 29218           | Rcn2         | -2.40 | 0.000854 |
| 290028          | Osgep        | -0.94 | 0.002064 |
| 24230           | Tspo         | -2.34 | 0.00248  |
| 171452          | Rab3il1      | -2.15 | 0.00629  |
| 84355           | Atox1        | -0.89 | 0.007345 |
| 311428          | RGD1311739   | 1.07  | 0.007461 |
| 25246           | Bsg          | 0.84  | 0.007876 |
| 25027           | Slc16a1      | 1.02  | 0.008061 |
| 361999          | Anp32e       | -3.03 | 0.00862  |
| 24788           | Sord         | 0.81  | 0.01132  |
| 301384          | Hibch        | 1.64  | 0.013339 |
| 83576           | Sort1        | 0.96  | 0.014054 |
| 29666           | Psmb6        | 0.72  | 0.017559 |
| <b>28°C+ β3</b> |              |       |          |
| 83730           | Vamp8        | -3.75 | 6.93E-05 |
| 29521           | Scamp1       | 1.51  | 0.000111 |
| 25116           | Hsd11b1      | 0.92  | 0.000382 |
| 117045          | Eif4e        | -0.69 | 0.000942 |
| 25342           | Oxtr         | 1.79  | 0.001104 |
| 298566          | C1qa         | 0.85  | 0.001124 |
| 445268          | Ufc1         | -0.65 | 0.001133 |
| 78947           | Gcs1         | 0.61  | 0.00204  |
| 266734          | Npas4        | 0.87  | 0.004234 |
| 246303          | Serbp1       | 0.78  | 0.004516 |
| 25139           | Slc2a4       | 1.36  | 0.005653 |
| 619574          | LOC619574    | -1.74 | 0.006446 |
| 64317           | Gpx3         | 1.00  | 0.006558 |
| 84474           | Ddx1         | -0.73 | 0.007131 |
| 24471           | Hspb1        | 1.26  | 0.007327 |

|        |        |       |          |
|--------|--------|-------|----------|
| 170673 | Palm   | 2.47  | 0.007749 |
| 313035 | Dnajc8 | -2.09 | 0.008094 |
| 64045  | Glrx   | -3.30 | 0.008229 |
| 122799 | Rps25  | -1.04 | 0.009005 |
| 252928 | Timm13 | -0.62 | 0.009087 |

**Table 3.** GO terms enriched in BAT

| gold                      | goName                                                                           | countDE | countAll | pv_elim |
|---------------------------|----------------------------------------------------------------------------------|---------|----------|---------|
| <b>20°C</b>               |                                                                                  |         |          |         |
| <b>Biological Process</b> |                                                                                  |         |          |         |
| GO:0016575                | histone deacetylation                                                            | 4       | 5        | 0.0047  |
| GO:0000122                | negative regulation of transcription from RNA polymerase II promoter             | 14      | 40       | 0.008   |
| GO:0003006                | developmental process involved in reproduction                                   | 20      | 68       | 0.0146  |
| GO:0071786                | endoplasmic reticulum tubular network organization                               | 3       | 4        | 0.0209  |
| GO:0019098                | reproductive behavior                                                            | 3       | 4        | 0.0209  |
| GO:0006544                | glycine metabolic process                                                        | 3       | 4        | 0.0209  |
| GO:0016226                | iron-sulfur cluster assembly                                                     | 3       | 4        | 0.0209  |
| GO:0090068                | positive regulation of cell cycle process                                        | 7       | 17       | 0.0231  |
| GO:0046323                | glucose import                                                                   | 6       | 14       | 0.0285  |
| GO:0001932                | regulation of protein phosphorylation                                            | 32      | 129      | 0.0316  |
| <b>Molecular Function</b> |                                                                                  |         |          |         |
| GO:0000980                | RNA polymerase II distal enhancer sequence-specific DNA binding                  | 4       | 5        | 0.0046  |
| GO:0030984                | kininogen binding                                                                | 3       | 3        | 0.006   |
| GO:0031492                | nucleosomal DNA binding                                                          | 4       | 6        | 0.0119  |
| GO:0001846                | opsonin binding                                                                  | 3       | 4        | 0.0207  |
| GO:0005212                | structural constituent of eye lens                                               | 3       | 4        | 0.0207  |
| GO:0016634                | oxidoreductase activity, acting on the CH-CH group of donors, oxygen as acceptor | 3       | 4        | 0.0207  |
| GO:0016831                | carboxy-lyase activity                                                           | 5       | 10       | 0.022   |
| GO:0016746                | transferase activity, transferring acyl groups                                   | 9       | 25       | 0.0259  |
| GO:0004616                | phosphogluconate dehydrogenase (decarboxylating) activity                        | 2       | 2        | 0.0331  |
| GO:0008484                | sulfuric ester hydrolase activity                                                | 2       | 2        | 0.0331  |
| <b>Cellular Component</b> |                                                                                  |         |          |         |
| GO:0042582                | azurophil granule                                                                | 4       | 4        | 0.0011  |
| GO:0000790                | nuclear chromatin                                                                | 12      | 27       | 0.0014  |
| GO:0031616                | spindle pole centrosome                                                          | 3       | 4        | 0.0209  |
| GO:0000786                | nucleosome                                                                       | 5       | 10       | 0.0223  |
| GO:0042719                | mitochondrial intermembrane space protein transporter complex                    | 2       | 2        | 0.0333  |
| GO:0001740                | Barr body                                                                        | 2       | 2        | 0.0333  |
| GO:0072687                | meiotic spindle                                                                  | 2       | 2        | 0.0333  |
| GO:0034751                | aryl hydrocarbon receptor complex                                                | 2       | 2        | 0.0333  |

|                           |                                                                |    |    |        |
|---------------------------|----------------------------------------------------------------|----|----|--------|
| GO:0043196                | varicosity                                                     | 2  | 2  | 0.0333 |
| GO:0001931                | uropod                                                         | 3  | 5  | 0.0453 |
| <b>28°C+ β3</b>           |                                                                |    |    |        |
| <b>Biological Process</b> |                                                                |    |    |        |
| GO:0003009                | skeletal muscle contraction                                    | 5  | 7  | 0.0015 |
| GO:0051897                | positive regulation of protein kinase B signaling              | 6  | 11 | 0.0034 |
| GO:0010677                | negative regulation of cellular carbohydrate metabolic process | 3  | 3  | 0.0039 |
| GO:1901896                | positive regulation of calcium-transporting ATPase activity    | 3  | 3  | 0.0039 |
| GO:0032781                | positive regulation of ATPase activity                         | 9  | 15 | 0.0055 |
| GO:0050873                | brown fat cell differentiation                                 | 3  | 4  | 0.0138 |
| GO:0006937                | regulation of muscle contraction                               | 8  | 22 | 0.0147 |
| GO:0071560                | cellular response to transforming growth factor beta stimulus  | 8  | 22 | 0.0147 |
| GO:0060048                | cardiac muscle contraction                                     | 6  | 15 | 0.0209 |
| GO:0048193                | Golgi vesicle transport                                        | 12 | 42 | 0.024  |
| <b>Molecular Function</b> |                                                                |    |    |        |
| GO:0035259                | glucocorticoid receptor binding                                | 3  | 4  | 0.013  |
| GO:0001671                | ATPase activator activity                                      | 3  | 4  | 0.013  |
| GO:0008134                | transcription factor binding                                   | 15 | 55 | 0.016  |
| GO:0050431                | transforming growth factor beta binding                        | 2  | 2  | 0.024  |
| GO:0031730                | CCR5 chemokine receptor binding                                | 2  | 2  | 0.024  |
| GO:0031014                | troponin T binding                                             | 2  | 2  | 0.024  |
| GO:0005044                | scavenger receptor activity                                    | 3  | 5  | 0.029  |
| GO:0019905                | syntaxin binding                                               | 4  | 9  | 0.037  |
| GO:0016779                | nucleotidyltransferase activity                                | 4  | 9  | 0.037  |
| GO:0004888                | transmembrane signaling receptor activity                      | 4  | 9  | 0.037  |
| <b>Cellular Component</b> |                                                                |    |    |        |
| GO:0005887                | integral component of plasma membrane                          | 12 | 32 | 0.002  |
| GO:0030134                | COPII-coated ER to Golgi transport vesicle                     | 5  | 9  | 0.0067 |
| GO:0005861                | troponin complex                                               | 2  | 2  | 0.0245 |
| GO:0001741                | XY body                                                        | 2  | 2  | 0.0245 |
| GO:0043596                | nuclear replication fork                                       | 3  | 5  | 0.0299 |
| GO:0044295                | axonal growth cone                                             | 3  | 5  | 0.0299 |
| GO:0016459                | myosin complex                                                 | 5  | 13 | 0.0399 |

**Table 4.** Impacted pathways in BAT with SHT and YM-178

| Pathway name                                    | pv       |
|-------------------------------------------------|----------|
| <b>20°C</b>                                     |          |
| Glycosaminoglycan degradation                   | 0.004999 |
| Notch signaling pathway                         | 0.031163 |
| Glycosphingolipid biosynthesis - ganglio series | 0.034463 |
| <b>28°C+ β3</b>                                 |          |
| Galactose metabolism                            | 0.024394 |
| Hepatitis C                                     | 0.037178 |

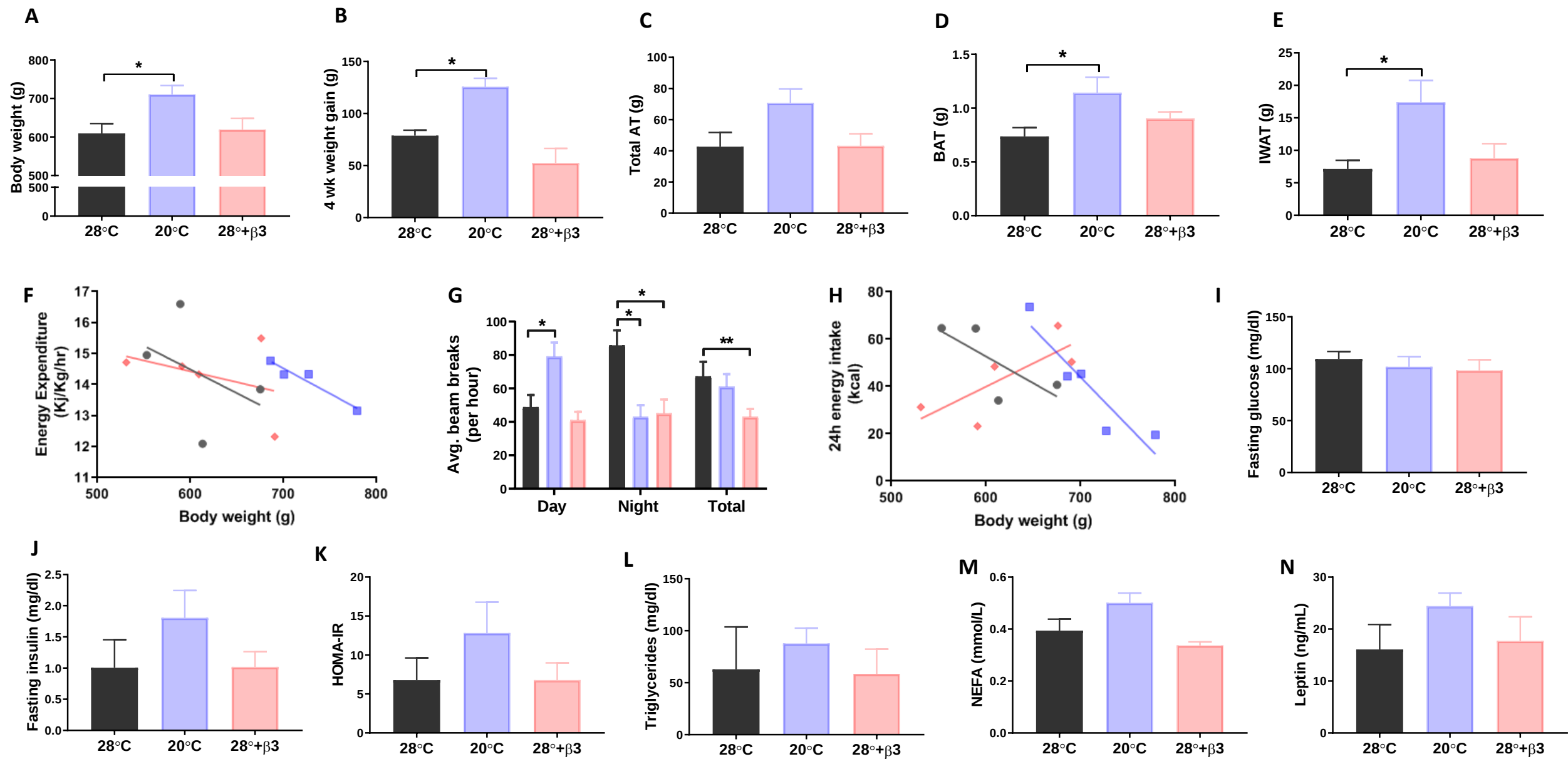
**Table 5.** GO terms enriched in WAT

| gold                      | goName                                                         | countDE | countAll | pv_elim |
|---------------------------|----------------------------------------------------------------|---------|----------|---------|
| <b>20°C</b>               |                                                                |         |          |         |
| <b>Biological Process</b> |                                                                |         |          |         |
| GO:0030330                | DNA damage response, signal transduction by p53 class mediator | 5       | 6        | 0.0022  |
| GO:0048711                | positive regulation of astrocyte differentiation               | 3       | 3        | 0.0023  |
| GO:0071498                | cellular response to fluid shear stress                        | 3       | 3        | 0.0023  |
| GO:0032780                | negative regulation of ATPase activity                         | 3       | 3        | 0.0023  |
| GO:0051607                | defense response to virus                                      | 5       | 9        | 0.003   |
| GO:0001822                | kidney development                                             | 11      | 35       | 0.0036  |
| GO:0050731                | positive regulation of peptidyl-tyrosine phosphorylation       | 7       | 17       | 0.0037  |
| <b>Molecular Function</b> |                                                                |         |          |         |
| GO:0051287                | NAD binding                                                    | 11      | 31       | 0.0013  |
| GO:0008144                | drug binding                                                   | 9       | 29       | 0.0101  |
| GO:0005001                | transmembrane receptor protein tyrosine phosphatase activity   | 2       | 2        | 0.0178  |
| GO:0005521                | lamin binding                                                  | 3       | 5        | 0.0191  |
| GO:0042393                | histone binding                                                | 5       | 13       | 0.021   |
| GO:0033613                | activating transcription factor binding                        | 3       | 6        | 0.0345  |
| GO:0003857                | 3-hydroxyacyl-CoA dehydrogenase activity                       | 3       | 6        | 0.0345  |
| GO:0045296                | cadherin binding                                               | 22      | 113      | 0.0365  |
| GO:0004028                | 3-chloroallyl aldehyde dehydrogenase activity                  | 2       | 3        | 0.0486  |
| GO:0071933                | Arp2/3 complex binding                                         | 2       | 3        | 0.0486  |
| <b>Cellular Component</b> |                                                                |         |          |         |
| GO:0005884                | actin filament                                                 | 9       | 24       | 0.0021  |
| GO:0032993                | protein-DNA complex                                            | 5       | 11       | 0.0088  |
| GO:0002102                | podosome                                                       | 7       | 14       | 0.013   |
| GO:0016607                | nuclear speck                                                  | 8       | 26       | 0.0146  |
| GO:0031209                | SCAR complex                                                   | 2       | 2        | 0.0172  |

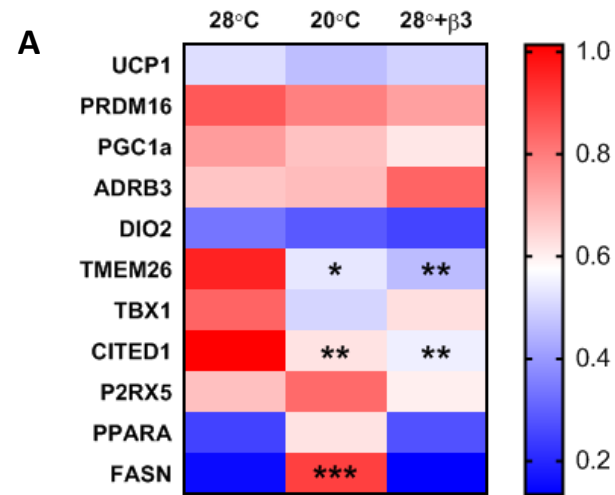
|                           |                                                          |    |     |         |
|---------------------------|----------------------------------------------------------|----|-----|---------|
| GO:0042611                | MHC protein complex                                      | 2  | 2   | 0.0172  |
| GO:0005687                | U4 snRNP                                                 | 2  | 2   | 0.0172  |
| GO:0005856                | cytoskeleton                                             | 49 | 238 | 0.0284  |
| GO:0030054                | cell junction                                            | 41 | 216 | 0.0291  |
| GO:0005681                | spliceosomal complex                                     | 9  | 22  | 0.0311  |
| <b>28°C+ β3</b>           |                                                          |    |     |         |
| <b>Biological Process</b> |                                                          |    |     |         |
| GO:0006953                | acute-phase response                                     | 7  | 12  | 0.0035  |
| GO:0000381                | regulation of alternative mRNA splicing, via spliceosome | 7  | 12  | 0.0035  |
| GO:0034113                | heterotypic cell-cell adhesion                           | 8  | 12  | 0.0061  |
| GO:0070528                | protein kinase C signaling                               | 4  | 5   | 0.0063  |
| GO:0015671                | oxygen transport                                         | 4  | 5   | 0.0063  |
|                           | positive regulation of myoblast fusion                   | 3  | 3   | 0.0077  |
|                           | CRD-mediated mRNA stabilization                          | 3  | 3   | 0.0077  |
| GO:0007566                | embryo implantation                                      | 6  | 12  | 0.0179  |
| GO:0040007                | growth                                                   | 29 | 103 | 0.0209  |
| GO:0071345                | cellular response to cytokine stimulus                   | 25 | 86  | 0.0211  |
| <b>Molecular Function</b> |                                                          |    |     |         |
| GO:0003735                | structural constituent of ribosome                       | 24 | 62  | 0.00037 |
| GO:0005344                | oxygen carrier activity                                  | 4  | 5   | 0.00657 |
| GO:0003730                | mRNA 3'-UTR binding                                      | 8  | 18  | 0.01548 |
| GO:0003682                | chromatin binding                                        | 10 | 25  | 0.01623 |
| GO:0140097                | catalytic activity, acting on DNA                        | 5  | 9   | 0.01908 |
| GO:0042162                | telomeric DNA binding                                    | 3  | 4   | 0.02688 |
| GO:0045294                | alpha-catenin binding                                    | 3  | 4   | 0.02688 |
|                           | exonuclease activity                                     | 3  | 4   | 0.02688 |
| GO:0003723                | RNA binding                                              | 73 | 281 | 0.02734 |
| GO:0019825                | oxygen binding                                           | 4  | 7   | 0.03278 |
| <b>Cellular Component</b> |                                                          |    |     |         |
| GO:0022625                | cytosolic large ribosomal subunit                        | 15 | 30  | 0.00018 |
| GO:0016323                | basolateral plasma membrane                              | 14 | 32  | 0.00164 |
|                           | hemoglobin complex                                       | 3  | 3   | 0.00782 |
| GO:0005903                | brush border                                             | 10 | 23  | 0.00812 |
| GO:0030864                | cortical actin cytoskeleton                              | 9  | 20  | 0.00918 |
| GO:0016327                | apicolateral plasma membrane                             | 3  | 4   | 0.02666 |
| GO:0044451                | nucleoplasm part                                         | 18 | 58  | 0.0269  |
| GO:0005637                | nuclear inner membrane                                   | 5  | 10  | 0.03167 |
| GO:0035770                | ribonucleoprotein granule                                | 11 | 32  | 0.03804 |
| GO:0097225                | sperm midpiece                                           | 2  | 2   | 0.03953 |

**Table 6.** Impacted pathways in WAT

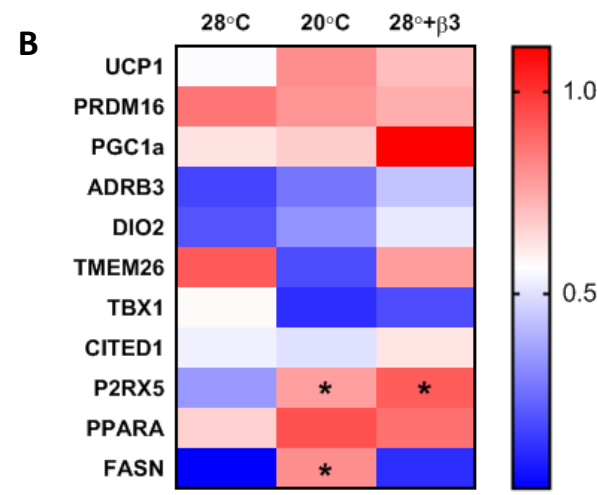
| <b>Pathway name</b>                        | <b>pv</b> |
|--------------------------------------------|-----------|
| <b>20°C</b>                                |           |
| Retinol metabolism                         | 0.02255   |
| Tyrosine metabolism                        | 0.047604  |
| <b>28°C+ <math>\beta</math>3</b>           |           |
| Ribosome                                   | 0.00024   |
| HIF-1 signaling pathway                    | 0.030833  |
| Cytokine-cytokine receptor interaction     | 0.032493  |
| Pancreatic cancer                          | 0.03371   |
| Hippo signaling pathway - multiple species | 0.036124  |
| Melanoma                                   | 0.041587  |



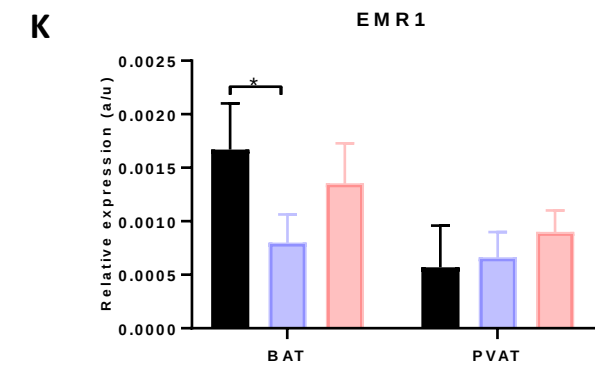
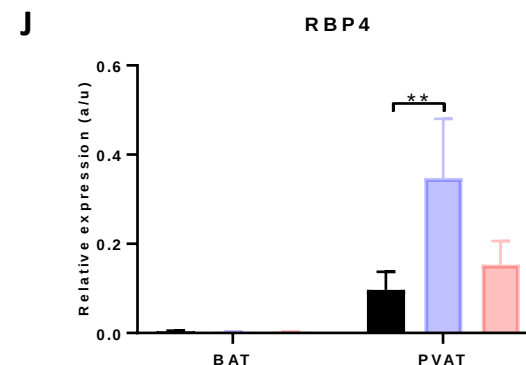
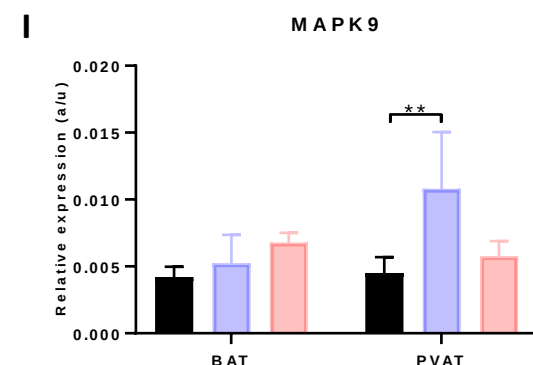
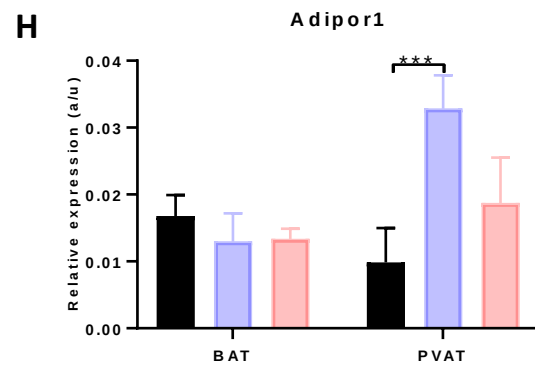
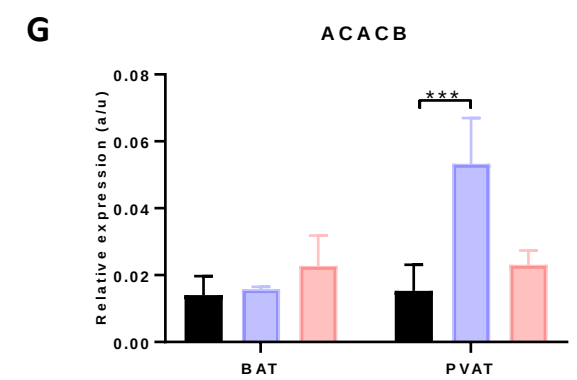
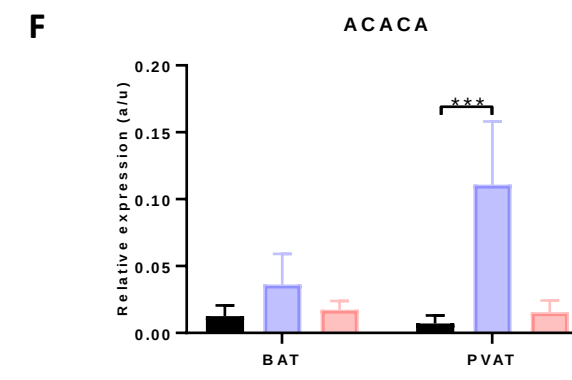
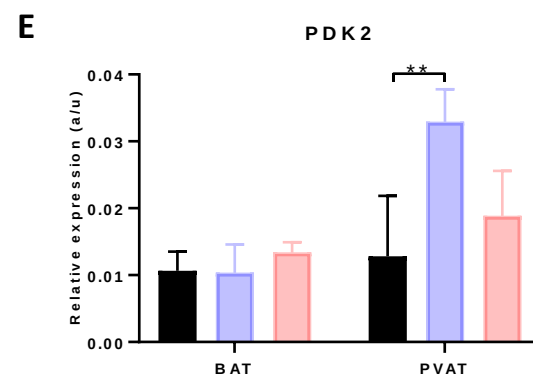
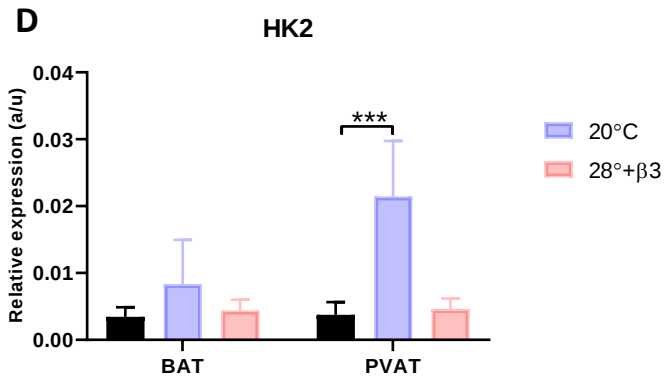
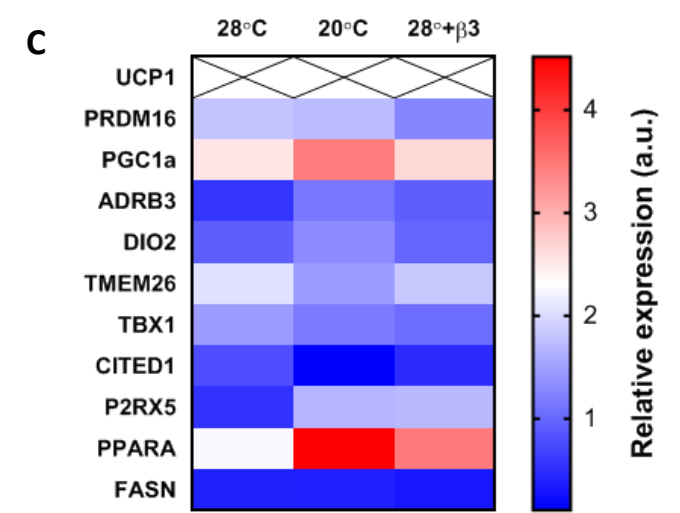
# BAT



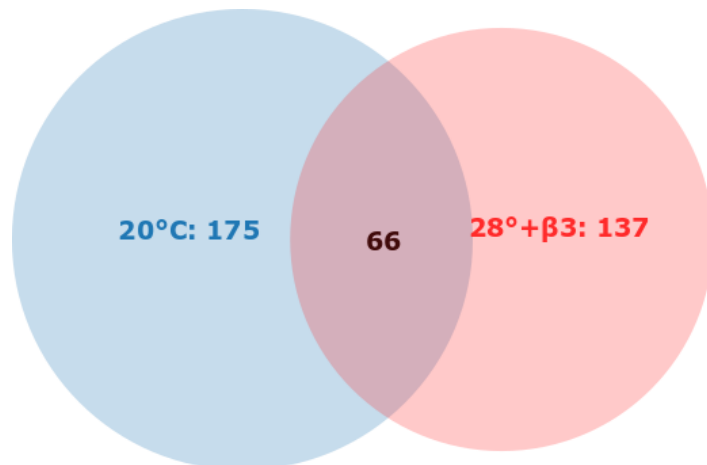
# PVAT



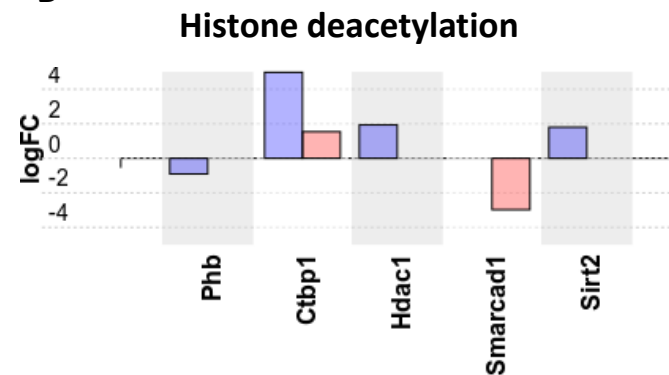
# IWAT



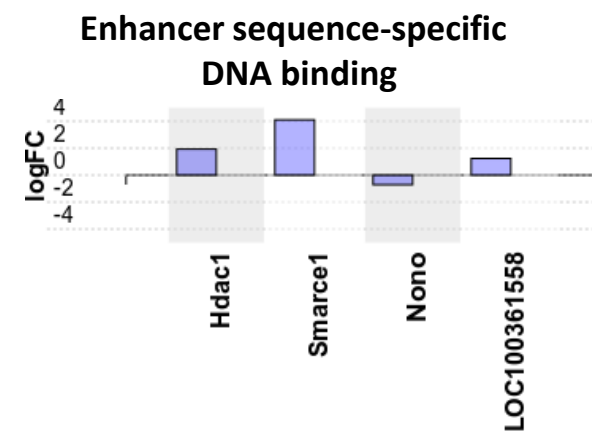
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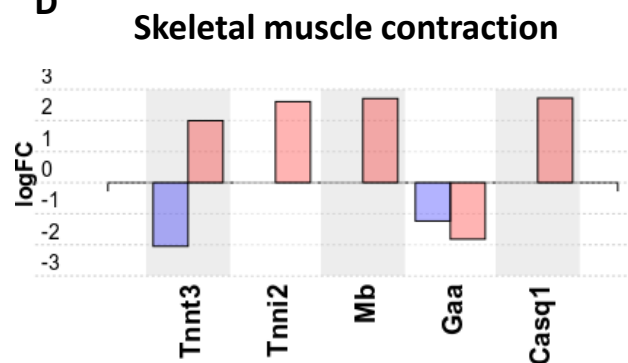
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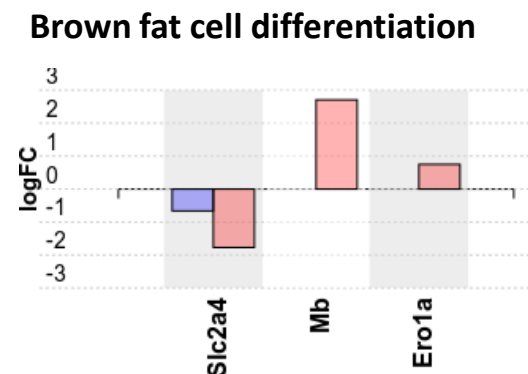
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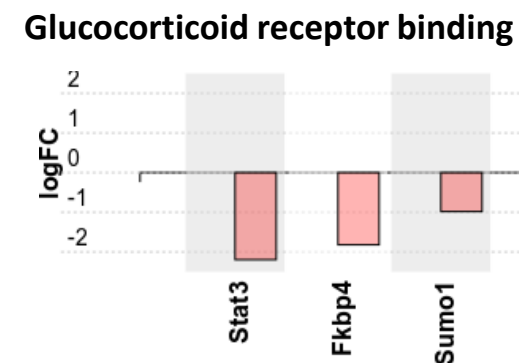
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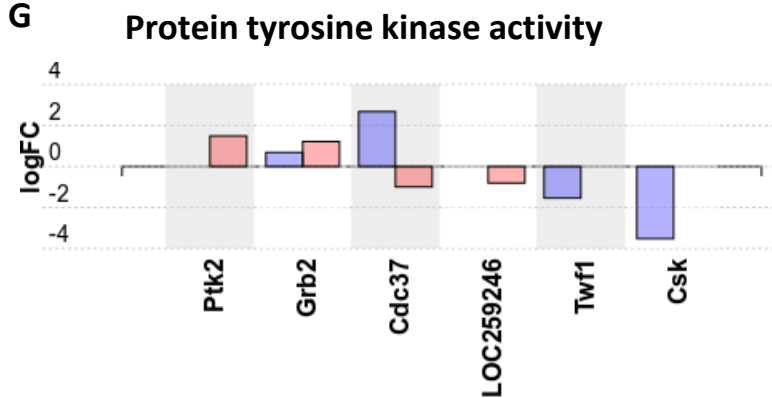
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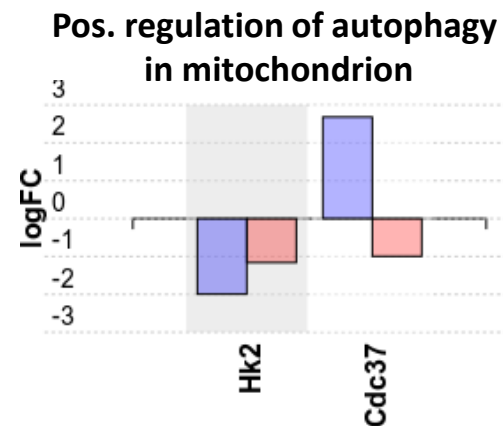
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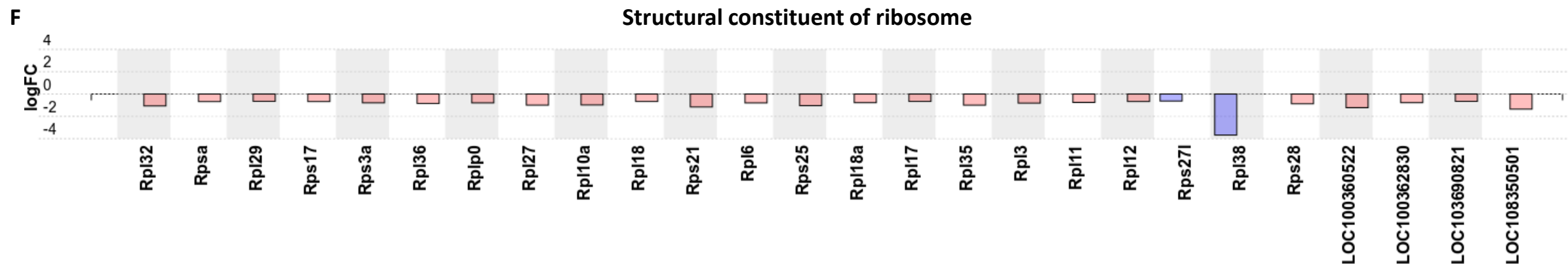
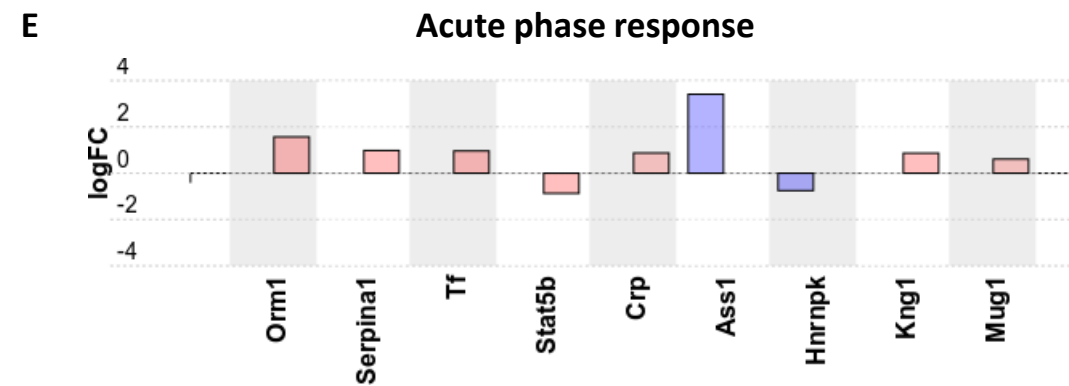
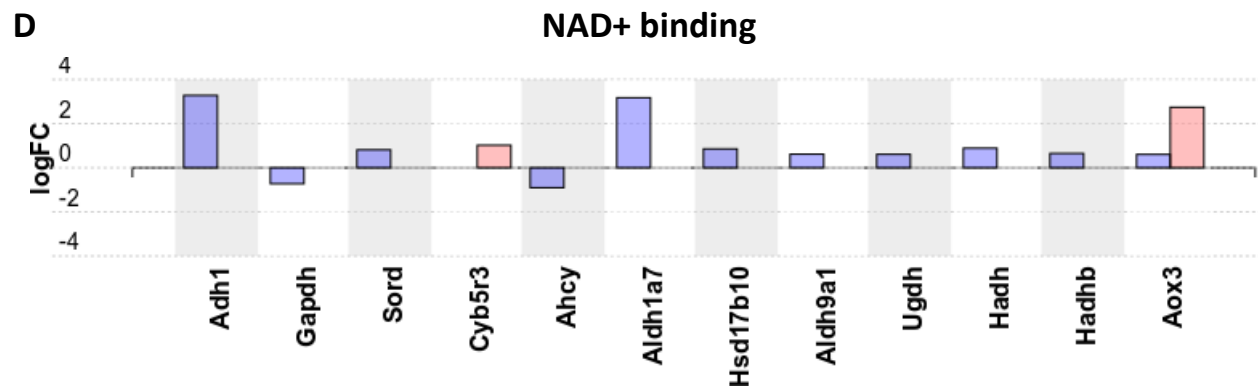
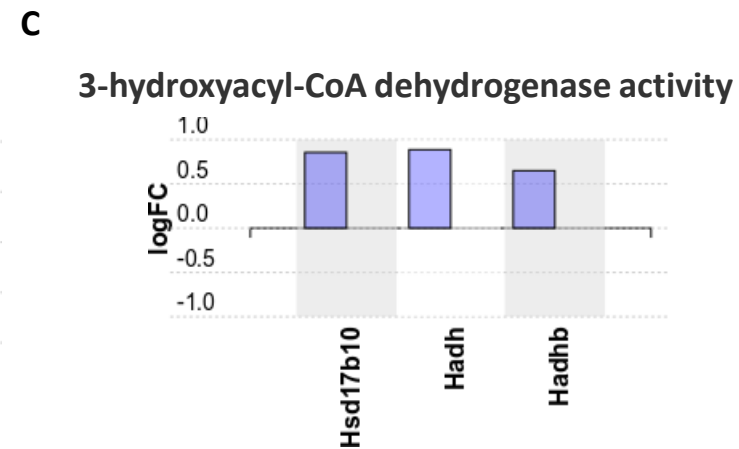
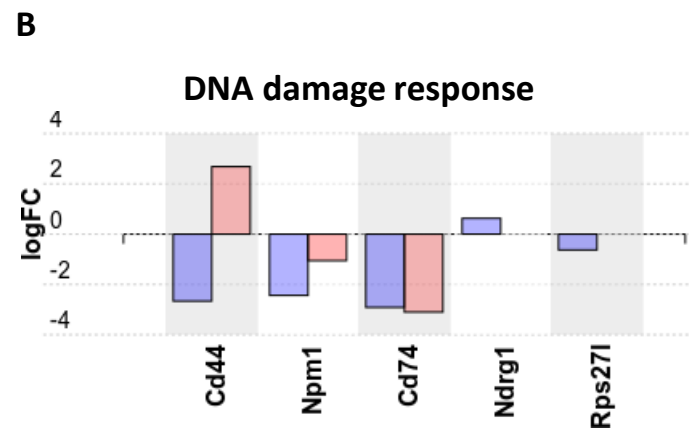
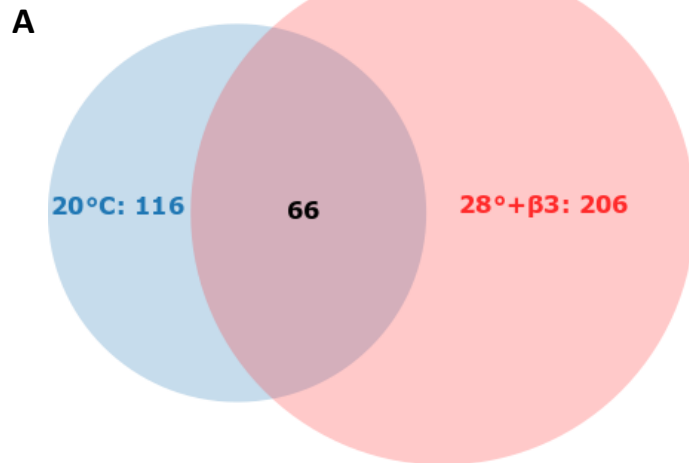


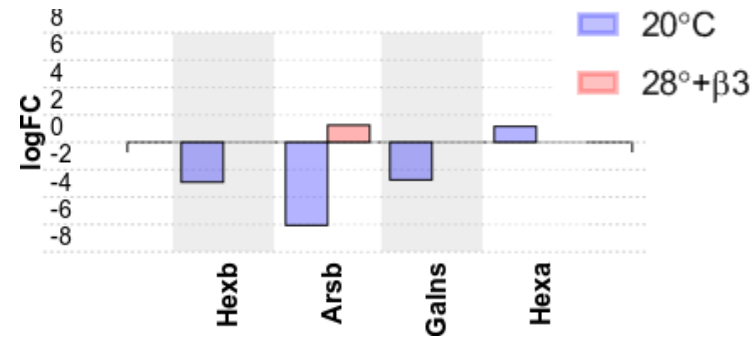
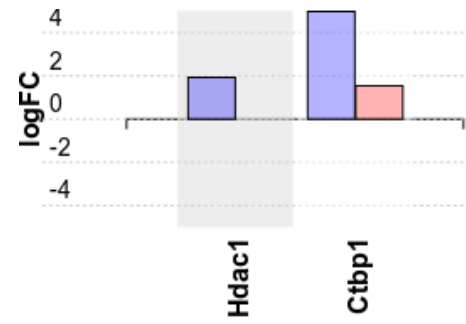
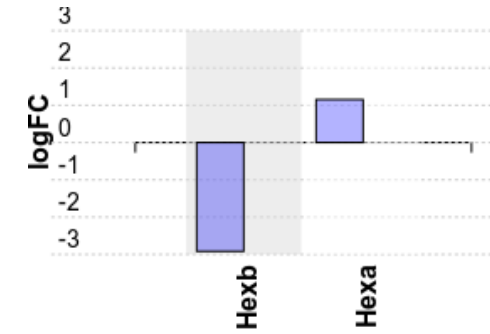
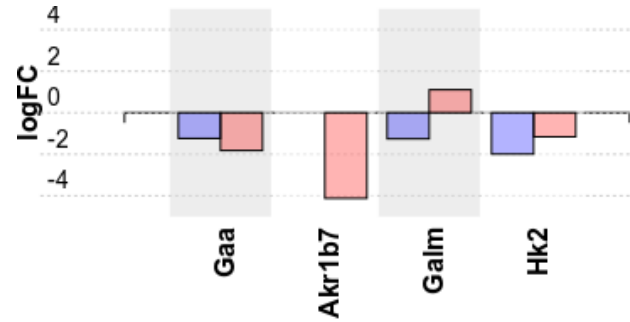
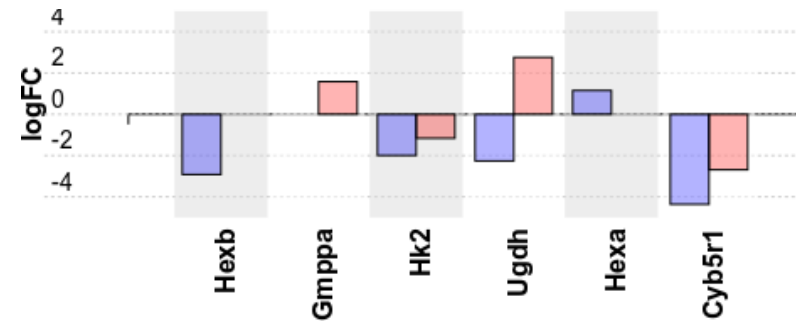
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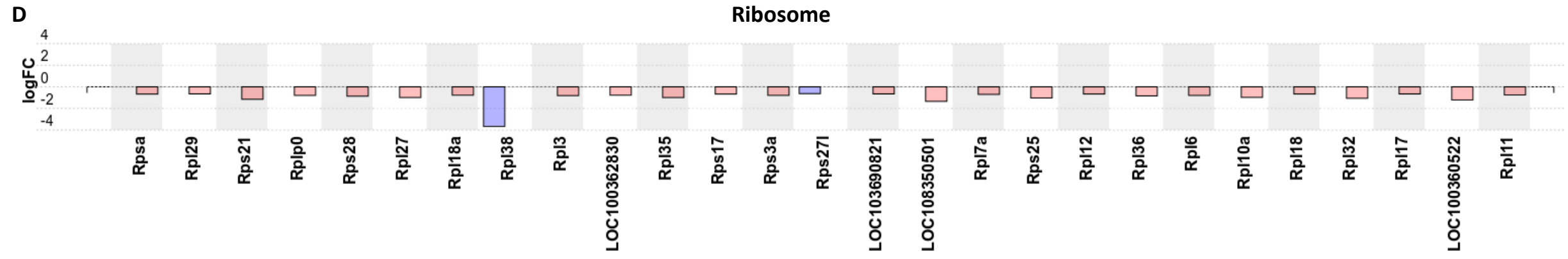
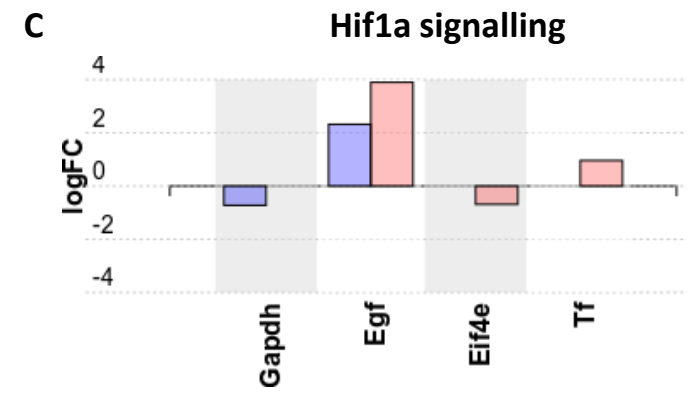
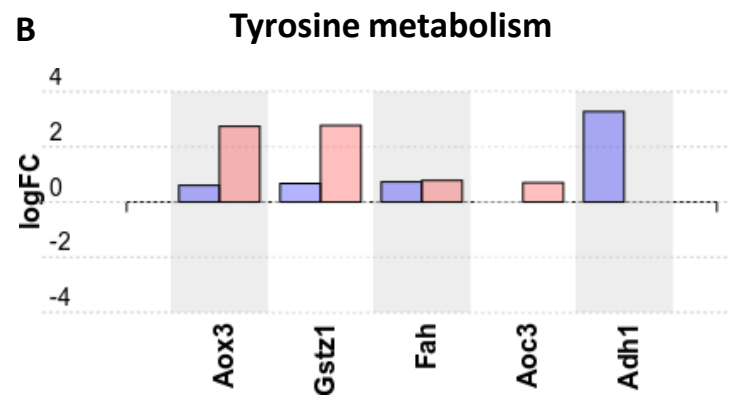
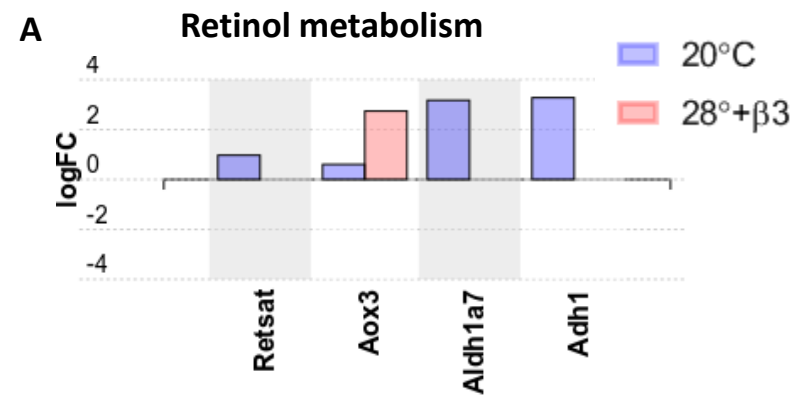


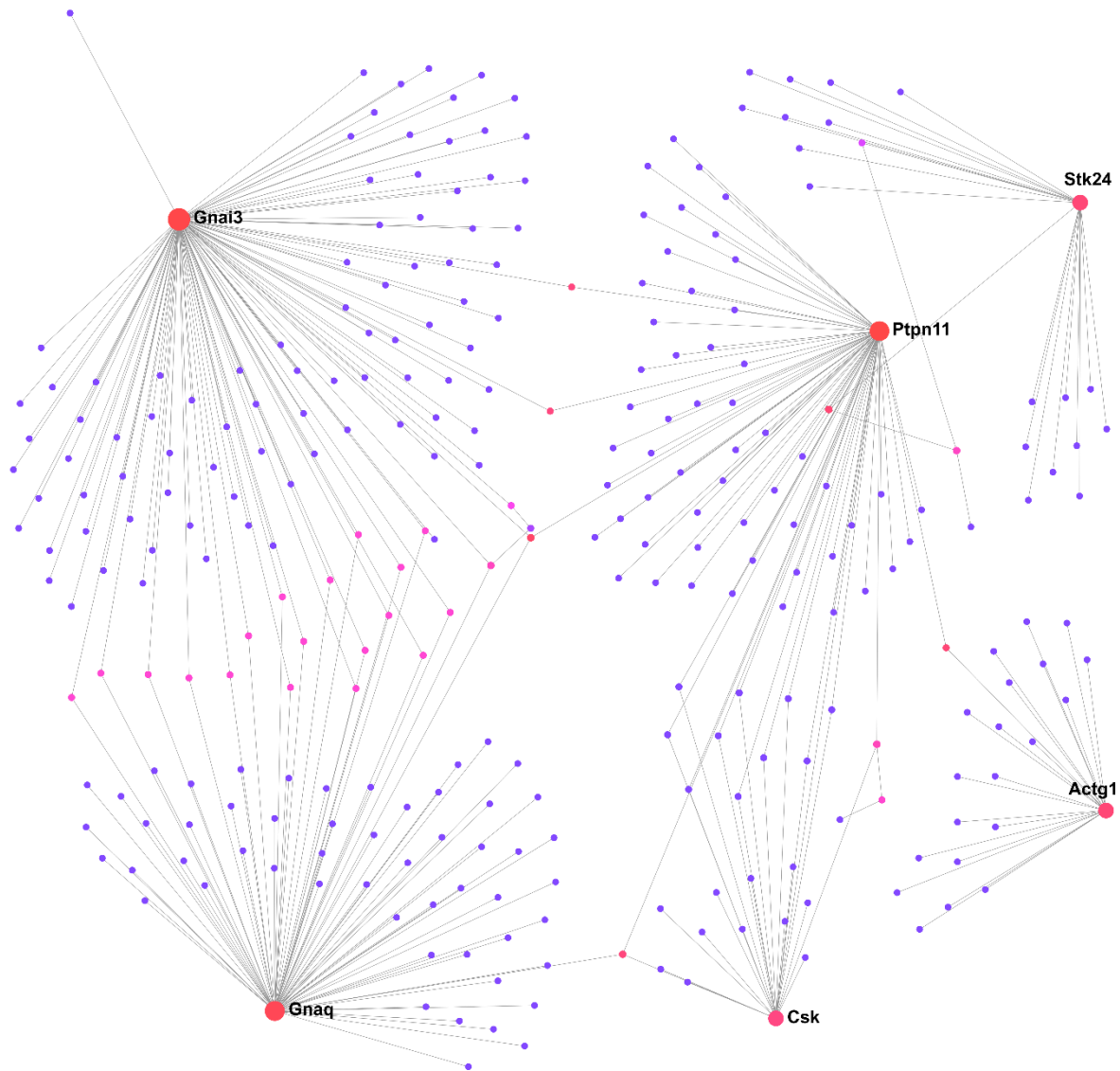
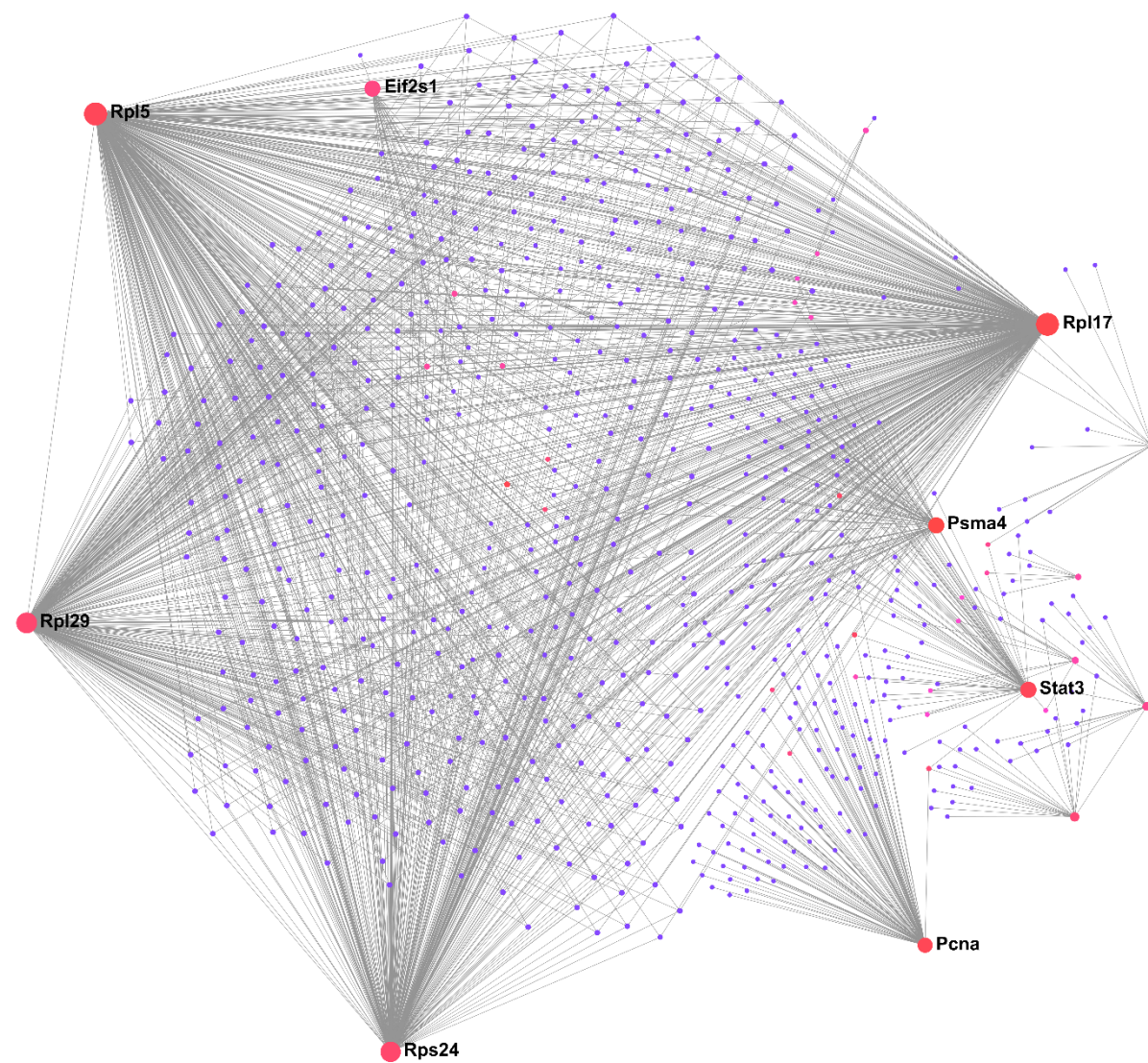
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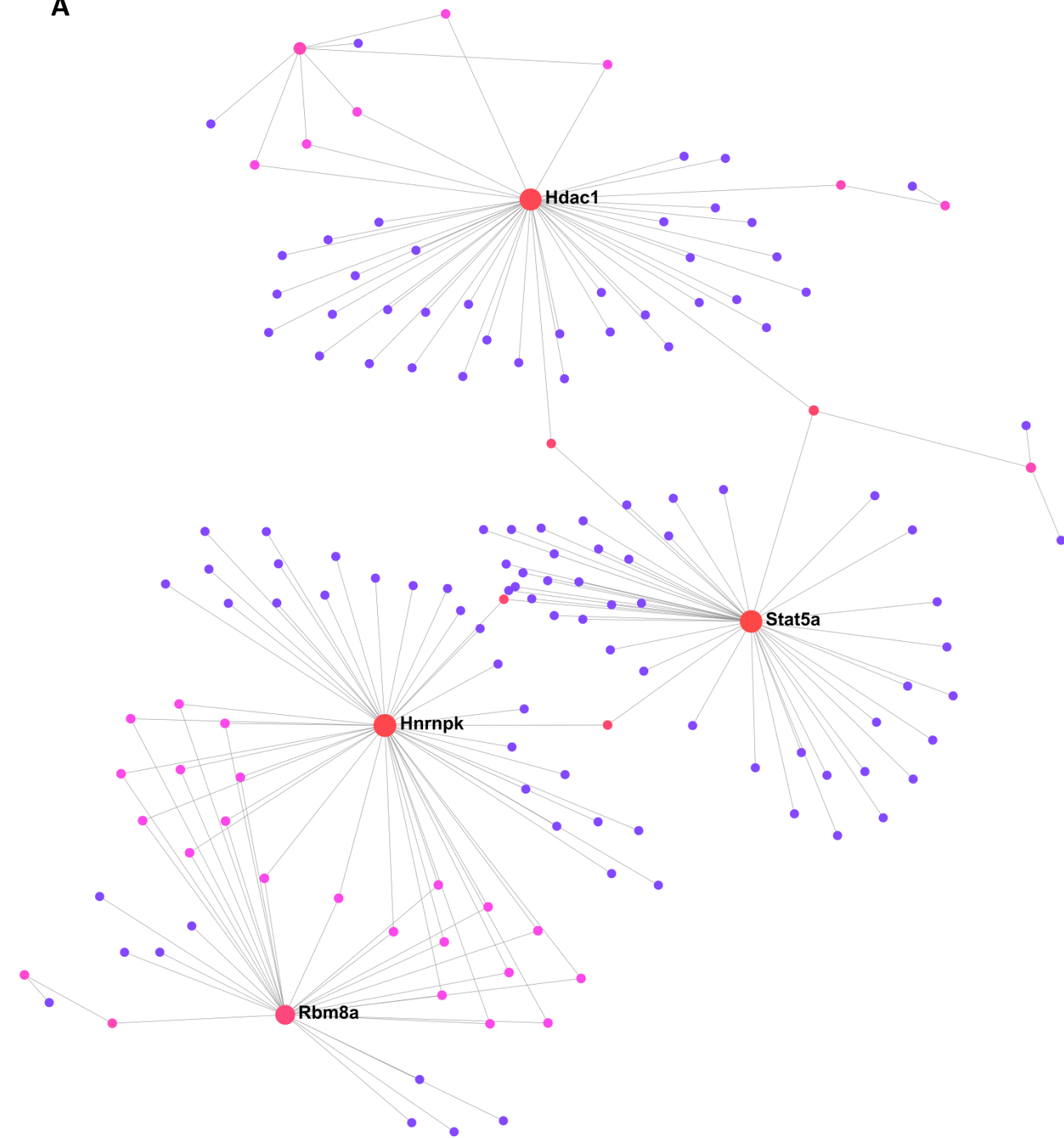


**A Glycosaminoglycan degradation****B Notch pathway****C Glycosphingolipid biosynthesis ganglio series****D Galactose metabolism****E Amino sugar and nucleotide sugar metabolism**



**A****B**

A



B

