Single-nucleus RNA sequencing reveals cellular and molecular dynamics of white and brown adipose tissue in a mouse model of type-2 diabetes

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Summary

Excessive adipose tissue expansion is often linked with type-2 diabetes. Despite recent efforts mapping adipose tissue changes in obesity using single-cell omics, an understanding of cellular and gene expression changes in a model of type 2 diabetes, and the transcriptional circuitry controlling it, is still lacking. Here, we use single-nucleus RNA sequencing to analyse the remodelling of gonadal white and interscapular brown adipose tissue from female and male mice with or without diabetes. Analysis of 51,877 nuclei revealed altered phenotypes in every cell population in type 2 diabetes. This included an immunoregulatory response, and changes in extracellular matrix organisation, energy generation, and hormone stimuli. Key transcription factors were inferred as cell-specific and non-specific nodes controlling diabetes-linked phenotypes. Finally, female-to-male population heterogeneity and gene expression differences were observed. Here we provide a resource detailing how adipose tissue remodelling, and the molecular mechanisms governing it, may contribute to cardiometabolic disease.

Keywords – Adipose, adipocytes, single-cell, single-nucleus, transcriptomics, diabetes, single-nucleus RNA sequencing, flow cytometry, microscopy

Introduction

Cardiometabolic diseases, such as type-2 diabetes mellitus (T2D)[1], have been linked with excessive expansion of adipose tissue. Adipose depots can be broadly classified as white and brown adipose tissue (WAT and BAT, respectively), with WAT being recognised as an endocrine organ having a primary function in lipid storage while BAT drives thermogenesis. The anatomical location of each adipose depot can dictate the tissue's function systemically[2]. Specifically, the expansion of visceral WAT can exert pathogenic effects through the release of pro-inflammatory adipokines[3]. Conversely, subcutaneous WAT and interscapular BAT can be protective[4,5] with research actively focussing on inducing 'browning' in WAT to improve energy expenditure and metabolic outcomes[6].

Many single cell-omic studies have provided detailed characterisations of adipose depot cellular heterogeneity in health and how this is altered in diseases such as obesity[7–14]. Moreover, disease-specific populations in obese WAT from individuals with and without T2D have been identified[14,15]. Yet, despite these advancements, a comprehensive analysis of both WAT and BAT in an animal model of T2D is lacking. Further, with biological sex being more widely recognised as a factor influencing disease processes including cardiovascular disease[16], the role of sex in adipose tissue remodelling remains unclear.

To study the remodelling of WAT and BAT in T2D, we used single-nucleus transcriptomics to profile the entire adipose cellular landscape of female and male mice with or without T2D (*db/db* and *db/h,* respectively). Single-nucleus RNA-sequencing (snRNA-seq) analyses of these depots revealed complex cellular landscapes, identifying important cell-specific gene expression patterns and regulatory circuits that govern cell identity and phenotype. We found that T2D significantly alters the relative abundance of adipose cell types and gene expression of every cell type, resulting in inflammation and tissue remodelling. Further, we identified sex differences in gene expression and cellular abundance, providing a possible basis for varying adipose accretion. Together, these findings provide a valuable resource for understanding WAT and BAT cellular landscapes, their changes in T2D and their potential contribution to metabolic disorders.

Results

Leptin-receptor deficient *db/db* mice display hyperglycaemia and increased fat mass

Both female and male *db/h* and leptin-receptor deficient *db/db* mice were used in this study. To validate hyperglycaemia in *db/db* animals, blood glucose levels over an extended time were measured using an HbA1c test (Supplementary Table S1). Higher blood glucose levels were seen in *db/db* mice when compared to *db/h* controls across both females and males ($P<0.0001$ for both) with no sex differences being observed across the genotypes. Next, EchoMRI measurements of lean and fat mass relative to total body weight were taken to assess body composition. EchoMRI showed a greater lean mass in males across both *db/h* and *db/db* genotypes ($P=0.0036$ and $P=0.0042$, respectively). Both sexes had lower lean mass and greater fat mass in *db/db* counterparts ($P<0.0001$ for all). Finally, *db/db* females had greater fat mass than male counterparts ($P=0.0017$). These findings highlight T2D-related hyperglycaemia and increased fat mass in *db/db* mice.

Novel high throughput nuclei isolation technique to characterise the adipose cellulome

Single-cell transcriptomics has been instrumental in shedding new light on tissue cellularity and disease responses in multiple tissues, including adipose[7,9–12,14,15,17–20]. Due to the lipid-filled nature of mature adipocytes, a key approach for single-cell transcriptomic profiling of adipose involves crushing tissue to extract nuclei using a dounce homogeniser[12,21,22]. While this technique has proven useful for nuclei extraction, a limitation of this approach is the number of samples that can be processed promptly. This is important for limiting spurious gene expression signatures during subsequent sequencing. To address this issue, we developed a nuclei isolation technique to isolate both mature adipocyte and stromal vascular fraction (SVF) nuclei from samples in a high-throughput manner. The automated technique involves the mechanical digestion of snap-frozen adipose tissue, and subsequent removal of cellular debris using fluorescence-activated cell sorting. This technique yields high-quality, intact nuclei for snRNA-seq and allows the isolation of nuclei from most adipose cell populations (Figure 1A; Supplementary Figure 1A and 1B).

snRNA-seq profiling and flow cytometry reveal cellular heterogeneity of adipose depots

Using the aforementioned protocol to isolate nuclei for subsequent snRNA-seq, we were able to generate a dataset containing 51,877 nuclei comprising 22,526 nuclei from gonadal WAT and 29,351 nuclei from interscapular BAT after quality control filtering (Figure 1B). WAT and BAT demonstrated distinct cellular compositions in both *db/h* and *db/db* conditions (Figures 1B and 1C). Cell populations were identified using their top enriched genes and established marker genes uncovering the cellular heterogeneity of both tissues (Figures 1D and 1E; Supplementary Table S2). Populations identified included macrophages (*Adgre1*, *Fcgr1*, *Csf1r, Cd68*, *Mrc1*), including a proliferating subpopulation (*Top2a*, *Aurka*, *Mki67*), lymphocytes (*Thy1*, *Cd19*, *Cd5*, *Cd3e*), endothelial cells (ECs; *Pecam1*, *Ly6c1*), lymphatic endothelial cells (LECs; *Cldn5, Prox1*), mesothelial cells (*Msln*), smooth muscle cells (SMCs; *Acta2*, *Myh11*), Schwann cells (*Plp1*, *Kcna1*), pericytes (*Pdgfrb*, *Vtn*), multipotent progenitors (*Cd34*, *Dpp4*, *Pi16*, *Creb5*), white adipocytes (*Slc7a10*), and brown adipocytes (*Ucp1*, *Cidea*). Brown adipocytes identified in WAT likely correspond to beige or brite adipocyte subpopulations but are referred to collectively as brown adipocytes throughout analysis. Finally, as fibroblasts and adipocyte progenitors/pre-adipocytes share many of the same marker genes, cells expressing *Pdgfra* and *Col1a1* were collectively referred to as fibroblasts. Epididymal cells (*Adam7*, *Pemt*, *Abcb5*, *Ces5a*), ovarian epithelial cells (*Pax8*, *Epcam*, *Krt7*, *Cdh1*) and skeletal muscle cells (*Ttn*, *Myl1*) were identified and subsequently removed from analysis alongside clusters with high mitochondrial RNA content and low RNA counts.

Adipose cell populations show tissue-dependent compositional and transcriptional individuality

To next compare and validate the cellular compositions of *db/h* WAT and BAT, flow cytometric analysis was conducted on SVF cell populations with mature adipocytes being excluded due to their fragile and buoyant nature (Figure 2A; Supplementary Figure 1C). Populations were labelled according to their expression, and thus fluorescent staining, of cell surface markers (Supplementary Table S1). As mentioned above, due to adipocyte progenitors, preadipocytes, and fibroblasts being challenging to distinguish from one another, in addition to the lack of clear cell surface markers[23,24], these populations were collectively labelled as fibroblasts based on their negative expression of mural cell markers. Distinct compositional differences were observed between WAT and BAT. This included variations in most immune and mural cell populations with higher contributions to the viable cell count being observed in WAT when compared to BAT. Of note, vascular EC (VEC) contribution was highest in BAT when compared to WAT, while LECs showed the opposite trend (Figure 2A; $P<0.0001$ and $P=0.0061$, respectively). Histological validation and quantification of ECs using isolectin B4 (IB4) showed a greater percentage of IB4 positive (or vascularised) tissue area in BAT corroborating the flow cytometry findings ($P<0.0001$), however, vascularisation area per nucleus (stained with DAPI) was not different between depots (Figure 2B).

Assessing transcriptomic data, differential gene expression (DGE) analysis showed variations between depots across all cell populations. White and brown adipocytes, fibroblasts and ECs showed the most marked differences across *db/h* depots (Figure 2C; Supplementary Table S3). In BAT, the upregulation of up to 3275 genes in white adipocytes, 3172 genes in brown adipocytes, 2507 genes in fibroblasts, and 3124 genes in ECs was observed and in WAT the upregulation of up to 2880 genes in white adipocytes, 2116 genes in brown adipocytes, 1914 genes in fibroblasts, and 2164 genes in ECs was seen (Supplementary Table S4). Gene ontology (GO) term analysis of these differentially expressed genes (DEGs) uncovered upregulation of biological pathways in BAT cell populations including g*eneration of precursor metabolites and energy*, *oxidative phosphorylation*, and *ribose phosphate metabolic process* emphasising the heat-producing role of this tissue (Supplementary Table S5). GO terms in WAT included *small GTPase mediated signal transduction*, *actin filament organization*, and c*ell substrate adhesion.* GTP-mediated RHO signalling has been implicated in insulin-mediated glucose uptake and homeostasis in adipocytes, highlighting the endocrine role of this tissue[25,26] (Supplementary Table S6). These findings highlight the distinct gene expression programs of individual cell populations across disparate adipose depots.

Adipocyte subpopulation profiles differ between disparate adipose depots

With white and brown adipocytes being present in both WAT and BAT, we subset these nuclei from the dataset and conducted DGE analysis between tissues to determine possible depot-dependent roles (Figure 2D; Supplementary Table S7). White adipocytes in BAT showed greater expression of genes including *Fabp3,* of which deficient mice have extreme cold sensitivity[27]. They also showed higher expression of *Ntrk3*, a key receptor regulating innervation and sympathetic neuron growth in adipose tissue[28] suggesting possible implications for white adipocytes in sympathetically activated BAT thermogenic processes. Contrarily, in WAT, white adipocytes were enriched for *Lcp1*, a gene involved in leptin and glucose homeostasis, and in the inhibition of WAT 'browning'[29]. Expectedly for brown adipocytes in BAT, greater expression of thermogenesis-related genes including *Ucp1*, *Cidea*, *Pparg, Ppara,* and *Ppargc1b* was observed while brown-like adipocytes in WAT were enriched for *Nnat, Aldh1a1*, *Cers6* and *Nrip1,* genes implicated in suppressing thermogenic activity[30–33]. GO term analysis of DEGs uncovered pathways such as *generation of precursor metabolites and energy*, *adaptive thermogenesis* and *oxidative phosphorylation*, among others, as upregulated in adipocytes (irrespective of subtype) from BAT reflecting its heat-producing capacity (Supplementary Table S8). While in WAT, both subpopulations showed expression of genes related to hormonal responses (Supplementary Table S9). In addition, terms relating to transforming growth factor beta (TGFβ) and WNT signalling were seen with the latter involving the expression of *Tle3*. TLE3 is a transcription factor co-factor that is selective for white adipocytes by repressing PRDM16 and PPARγ to drive white adipose-related genes and impair thermogenesis[34]. These data underscore the importance of TGFβ and WNT in driving progenitor proliferation and suppressing adipogenesis[35–41]. Together these findings highlight the cellular landscapes of WAT and BAT, the roles of adipocytes within these depots, and how tissue function determines gene expression in cell subsets.

Distinct gene regulatory circuitry governs brown and white adipose tissue phenotypes and cellularity

Next, we looked to determine the key gene regulatory elements that underlie differences between WAT and BAT and the cells within them. To achieve this, we applied the single-cell regulatory network inference and clustering (SCENIC) pipeline[42]. In brief, SCENIC links single-cell sequencing and cis-regulatory data to infer transcriptional modules—termed 'regulons'—comprising transcription factors (TFs) and their target genes (Supplementary Table S10). Cells were first clustered based on their expression of regulons and corresponding gene regulatory networks (GRNs; denoted as TF gene(+)) showing clear clustering patterns that aligned with the previous RNA annotations (Figure 3A; Figure 1B). Moreover, examination of the top 5 regulon markers for each cell population in WAT and BAT demonstrated entirely distinct top GRNs governing cell population phenotypes for brown adipocytes and mesothelial cells, while ECs had shared marker GRNs between tissues with varying levels of similarity observed for other cell populations (Figure 3B; Supplementary Tables S11 and S12, respectively). Noteworthy regulons supporting this inference-based analysis included the high expression of Erg(+) and Tal1(+) both important for EC homeostasis and angiogenesis[43,44], Tfec(+) and Irf5(+) both implicated in macrophage functions and polarisation[45,46], Meox1(+) and Smad3(+) which govern fibroblast activation[47], and TGFβ-directed fibrosis[48] in a cardiac context, respectively. Finally, Esrrg(+), Esrra(+), and Ppara(+) are all implicated in thermogenesis and were observed as markers for brown adipocytes. Altogether these data show an agreement between RNA-seq-based GRN inferences with established transcriptional regulators in various cell populations. This analysis highlights how GRN activity differs between adipose depots to yield tissue-specific cellular phenotypes.

Inflammation and macrophage accumulation in type-2 diabetes adipose depots

To next identify the impact of T2D on WAT and BAT we compared the cellular abundance of *db/h* and *db/db* samples for each depot. Analysis revealed an increase in macrophages across both WAT and BAT from *db/db* depots (Figure 4A) with statistical analysis of snRNA-seq counts and flow cytometry data further supporting this ($P<0.01$ for WAT and $P<0.05$ for BAT snRNA-seq data, Supplementary Figure 2A and 2B, respectively; $P<0.0001$ for both WAT and BAT flow cytometry data, Supplementary Figure 2C and 2D, respectively). These observed cellular changes were possibly attributed to lipid droplet expansion (measured by Ferets diameter) in adipocytes across both depots thus reducing the abundance and diversity of cells captured in an equal tissue weight across conditions (Figure 4B). DGE analysis of WAT and BAT between *db/h* and *db/db* tissues (Supplementary Tables S13 and S14) was followed by subsequent GO terms analysis of genes up and downregulated (Supplementary Tables S15 to S17, and Supplementary Tables S18 to S20, respectively). Vast changes in macrophages were observed across both depots. Linked GO terms that were upregulated included *phagocytosis* and *leukocyte migration* (Figure 4C and 4D). A possible consequence of lipotoxicity and adipocyte hypertrophy is the formation of macrophage crown-like structures (CLSs). CLSs can accumulate in dysfunctional adipose depots through macrophages clustering around dead or dying adipocytes to clear lipid and cell debris[49,50]. Recent work has highlighted the importance of macrophage proliferation in the formation of these structures and subsequent inflammation[51–54]. Indeed, histological validation using phosphohistone H3 validated the presence of proliferative cells in the CLSs of WAT corroborating the snRNA-seq findings and affirming that macrophage crowns exist in adipose tissue from T2D mice (Figure 4E; Supplementary Figure 2). Moreover, subpopulation-specific expression of proliferative markers *Top2a*, *Mki67*, and *Aurka* was observed (Figure 4F). Finally, as it is challenging to attribute macrophage-related pathogenesis solely to a T2D-derived phenotype, exclusive of obesity, we looked to assess the similarity of gene expression patterns between our dataset and a previously published human obesity dataset[14]. Approximately 62% of DEGs across all cell populations in *db/db* mice were unique to our dataset and not differentially expressed in human obesity. Assessing macrophage-specific changes, 88% of *db/db* genes were uniquely altered in our dataset. While these findings indicate that a high proportion of gene expression changes in T2D may not be attributable to obesity, differences relating to the model system, experimental setup, and the number of identified DEGs, should be considered carefully.

Type-2 diabetes influences energy generation in BAT and endocrine functions in WAT

Other cell populations that displayed altered gene expression patterns in T2D included white and brown adipocyte subpopulations and fibroblasts. Notable GO terms upregulated in BAT included *extracellular matrix*

organisation and *extracellular structure organisation* suggesting tissue remodelling. Downregulated terms included *generation of precursor metabolites and energy*, and *oxidative phosphorylation* suggesting decreased energy utilisation and possible whitening of this classically thermogenic tissue. Genes that were associated encoded complexes that are essential for oxidative phosphorylation such as ATP synthase or cytochrome bc 1 complex. In WAT, downregulated terms in WAT included *cellular response to hormone stimulus*, and *hormone-mediated signaling pathway* indicating a possible dampened hormone response in the tissue (Figure 4C). Analysis of the top 50 upregulated (UR) and downregulated (DR) genes for each depot showed a notable upregulation of genes in macrophages (including proliferating subtypes) (Supplementary Figures 3A to 3C; Supplementary Tables S21 to S24). High expression of lysosomal proteases (cathepsins; Cts genes) and leukocyte immunoglobulin-like receptor, Lilr, encoding genes was observed including *Lilr4b*, *Lilrb4a* and *Pirb* (*Lilrb3*), with the latter being the only Lilr gene differentially expressed in *db/db* BAT. Moreover, in *db/db* BAT, the downregulation of heat shock proteins (Hsp genes) was observed suggesting decreased thermogenesis. Collectively, these findings support the possibility of a concerted inflammatory response existing across both depots, with a concomitant dampening of the cellular response to hormone in WAT and downregulation of energy generation in BAT in T2D.

snRNA-seq derived gene regulatory network inferences in type-2 diabetes

We next sought to determine the GRNs within each depot that were driving the changes in adipose tissue cell subsets in disease, focusing on adipocytes, fibroblasts, and macrophages (Figure 5A). We found that the top 5 GRNs of these cell types in *db/h* mice were almost always unique across depots. These were altered in *db/db* counterparts and exhibited signatures consistent with the activation of known gene expression programs. For example, Cebpd(+), which is involved in cell apoptosis, proliferation and adipocyte differentiation, appeared important across adipocytes, fibroblasts and macrophage populations in T2D. Additionally, Irf4(+), a critical regulator of lipid handling and of which deficiency induces macrophage-related inflammation[55], appeared important for BAT *db/h* samples and less so for *db/db* counterparts. Immunohistochemical analyses revealed IRF4 staining around nuclei (indicating its presence in the nucleus for transcriptional regulation) - including those near blood vessels. IRF4 staining was less notable in *db/db* tissue (Figure 5B) suggesting possible transcriptional regulation driving impaired lipid handling in diabetic BAT.

Next, differentially expressed regulons were assessed (Figure 5C; Supplementary Tables S25 and S26). Regulons that showed common changes across both depots included Tfeb(+), Klf13(+), Hmga2(+), and Atf3(+) - all being upregulated in a disease context. Regulons including Tfec(+) and Tfeb(+), among others, showed marked upregulation in macrophages specifically. TFEB is a transcription factor that plays an essential role in the biogenesis of lysosomal machinery aligning with the upregulated lysosomal protease (Cts gene) expression observed above[56,57] (Supplementary Figure 3; Supplementary Table S21). To further dissect the influence of transcriptional regulation on the concerted immune response observed in Figure 4, gene targets linked to the GO term *leukocyte migration* were mapped to transcription factors (Figure 5C; Supplementary Table S27). Fli1(+), which plays an important role in cell proliferation, activation, differentiation, survival and the regulation of chemokines[58], showed the greatest link to this *leukocyte migration* with 92 target genes being associated with this regulon. Other notable regulons included Ikzf1(+) and Spi1(+) which showed 74 and 68 leukocyte migration-related genes, respectively. Together, these findings highlight the importance of transcriptional regulation for processes, such as inflammation, in T2D adipose depots and highlight possible avenues of targeting TF candidates for therapeutic purposes.

Biological sex differences in classical adipose depots relating to adipocyte subpopulations

Given the considerable sex differences that exist in obesity and diabetes-associated disorders, we explored sex-dependent mechanisms regulating adipose tissue homeostasis and diabetes-induced remodelling. Female and male samples were barcoded using a previously described cholesterol-moiety attached-oligonucleotide approach[59] (Supplementary Table S1). Samples were separated downstream based on barcodes, with cells having undetectable barcodes being further delineated using sex-specific gene expression (*Xist*, females; *Ddx3y*, *Eif2s3y*, *Gm29650*, *Kdm5d*, *Uty*, males)[16]. Assessment of DEGs between females and males for each depot (Supplementary Tables S28 and S29) uncovered differing gene expression

patterns for mesothelial cells, fibroblasts, and white adipocytes in WAT (Figure 6A; Supplementary Tables S30 and S31). Interestingly, macrophages showed relatively little variation in gene expression between sexes across both *db/h* depots. For BAT, high levels of variation across adipocyte subpopulation gene expression were seen. When the cellular composition of these depots was considered, it became evident that females and males had differing contributions of adipocyte subpopulations to their overall cellular composition (Figure 6B). Specifically, *db/h* female BAT had high proportions of BA1 and BA2 cells, while *db/h* male BAT had higher contributions of BA3 and BA4. Assessment of key genes involved in thermogenesis showed higher expression levels in female *db/h* BAT while these dimorphisms were not as evident in a diabetic context (Figure 6C). Analysis of the top 5 GO terms for brown adipocytes in *db/h* BAT provided some insight into biological pathway differences showing female upregulation of *adaptive thermogenesis*, *cellular response to hormone stimulus* and *fatty acid metabolic process* in females, and upregulation of *protein folding*, *oxidative phosphorylation*, *ATP metabolic process,* and *proton motive force-driven ATP synthesis* pathways in males (Figure 6D; Supplementary Tables S32 to S33, respectively). Genes linked to these pathways encoded ATPase subunits, and heat shock proteins in males while in females they encoded thermogenesis-related genes, including *Ppargc1a* and *Ucp1*, alongside insulin receptor substrate 1 (*Irs1*). These findings align with the already recognised increased thermogenic capacity of female BAT driven by mitochondrial size, abundance and UCP1 content[60–66].

Female BAT is more densely packed and vascularised than male counterparts

Finally, as many endothelial-related GO terms were noted in the analysis for female BAT we looked to assess vascular differences between sexes (Figure 6D; Supplementary Table S34). Efficient blood flow to the tissue is essential for the supply of free fatty acids for energy metabolism and generation. Differences between biological sexes have previously been observed in gonadal WAT[67,68] yet, to date, no sex differences in BAT vascular networks have been characterised. IB4 staining was used to mark endothelial cells in female and male *db/h* BAT. Female tissue appeared to have more cells and vascularity than males (P=0.0075 and P=0.0206, respectively; Figure 6F). However, normalisation of IB4 staining to the number of nuclei revealed that the ratios were similar (Figure 6F). These data indicate that, while female tissue may be more densely packed, vascularisation-to-cell ratios remain comparable. Strikingly, cell density and vascularisation density sex differences were lost in T2D alongside decreased gene expression dimorphisms across adipocyte populations in *db/db* BAT (Figure 6F; P=0.002 and P=0.0399, respectively; Supplementary Tables S34 and S35). Together, these data highlight BAT sex differences that exist in a healthy state but appear to be dampened or lost in disease.

Discussion

Pathogenic adipose tissue expansion is a well-established risk factor for cardiometabolic disease. Recent research has been directed into understanding how the deterioration of adipose tissue can contribute to disease in both an obese and high-fat diet context at a single-cell level[7–14]. Limited T2D-focussed human work has characterised insulin-regulating adipocyte subpopulations and molecularly distinct preadipocyte populations associated with diabetes[14,15,22]. However, despite this, we are still lacking a precise understanding of the cellular and molecular underpinnings of how these tissues change in the context of T2D. To address this, we conducted snRNA-seq on classical adipose depots including gonadal WAT and interscapular BAT from both *db/h* and *db/db* female and male mice.

Characterisation of these depots using snRNA-seq showed that while similarity in cellular heterogeneity existed between depots, the contribution of various cell subsets to the overall tissue cellularity differed. Moreover, gene expression patterns differed across WAT and BAT highlighting the distinct functions of these tissues. WAT is classically recognised for its lipid-storing functions, while BAT is known for its heat-producing and energy-expending role. Analysis of adipocyte subtypes highlighted that cell populations have unique functions across depots with BAT populations upregulating metabolic processes and WAT populations upregulating WNT-related pathways. WNT signalling has important adipose-specific functions including regulating lipogenesis, inhibiting adipogenesis[37], and the modulation of structural WNT-regulated adipose tissue-resident cells, termed SWAT cells, which can move toward both adipogenic or progenitor-like states[69]. Finally, the assessment of gene regulatory network inferences validated the observed depot-specific gene expression patterns.

Our analysis of non-diabetic and T2D mice also revealed several features of pathogenic remodelling of adipose. These include an increase in macrophage abundance across both WAT and BAT, consistent with observations by others[7,9–14]. Our findings align with the more recent recognition of proliferative macrophage importance in CLS formation[51–54] and highlight their presence in a model of T2D. Assessment of gene regulatory network changes in T2D also showed notable Tfeb(+) upregulation in macrophage subsets. TFEB is an important transcription factor in the biogenesis of lysosomal machinery[56,57]. Lysosomal degradation of hypertrophic adipocytes is an important function of macrophage CLSs where these phagocytic cells secrete lysosomal enzymes to create an environment wherein dying adipocytes are degraded through a process known as exophagy. Lysosomal proteases are vital for this process with CTSB being specifically identified as key for perilipin 1 (PLIN1; a lipid-droplet coating protein) degradation[70–72]. Indeed, these lysosomal proteases were upregulated in *db/db* samples (most predominantly in macrophages) which was further corroborated through Tfeb(+) expression –TFEB being a known regulator of Cts genes[56]. These data identify targets at both a single gene and broader regulatory level that could be investigated for therapeutic development.

In line with the noted adipocyte lipid droplet expansion, BAT showed downregulation of energy generation in T2D. BAT whitening has gathered much attention in the literature due to the hijacking of BAT-driven energy expenditure to improve metabolic health. A wealth of research has been directed into exploring therapeutic options to increase this expenditure including the use of cold exposure, exercise, and pharmacological and dietary interventions[6]. Assessment of genes showing decreased expression in T2D included heat shock proteins. Moreover, downregulated GO terms showed decreased gene expression related to vital oxidative phosphorylation complexes – again supporting the notion of BAT moving into a lipid-storing state. Interestingly, insulin receptor substrate 1 and 2 (*Irs1* and *Irs2*) expression was also decreased with these GO terms. IRSs are important for adipocyte differentiation and insulin sensitivity[73], suggesting that brown adipocyte differentiation in BAT may be downregulated.

Finally, with previous research highlighting clear biological sex differences in adipose depot localisation, moreover functionality differences in BAT, the inclusion of both female and male mice in this study was paramount. Female BAT has greater mitochondrial abundance and size, in addition to higher mRNA and protein levels of UCP1 and other thermogenic genes[60–66] of which our data corroborates. Further, single-cell datasets have shown brown adipocyte populations with differing heat-producing capacities[19,74–76], yet sex differences were not characterised. This dataset has, for the first time, shown that differences exist in

adipocyte subpopulation abundance across sexes. Moreover, histological analysis showed greater cellular and vascular density in female BAT – with differences only being identified in gonadal WAT in the past[67,68]. VEGFA is long linked to BAT activity, with *Vegfa* deletion driving BAT whitening resulting in decreased expression of thermogenic gene *Ucp1* and *Ppargc1a*[77]. Female sex hormone estrogen receptor 1 is known to regulate VEGFA in adipose tissue possibly driving the prior dimorphism[78]. T2D appeared to override these sex differences with vascular and gene expression dimorphisms being less striking or absent.

While this paper profiles disparate adipose depots in unprecedented detail through the inclusion of a large sample size of both mature adipocyte and SVF nuclei, some limitations to the study should be acknowledged. First, the model of T2D used in this study (*db/db*) is a leptin receptor mutant meaning changes in depots may be driven by the loss of this signalling rather than the diabetic phenotype. While we analysed our data alongside that from humans with obesity, data from this study should be further compared across other models of T2D to ensure confidence in our findings. Second, it is important to note that *db/db* mice are characterised by obesity, thus findings in the study may not be strictly attributed to T2D alone. It has been shown, however, that *db/db* mice appear more 'diabetic' than *ob/ob* mice and have less liver failure[81]. Third, mouse adipose depots, particularly BAT, do not always perfectly recapitulate that of a human context. Thermoneutral BAT is closer to human BAT and could be used in future experiments – particularly those relating to biological sex differences[79,80]. Finally, as this dataset was produced using nuclei, cytosolic mRNA is missing and therefore important transcriptomic information could be absent from our dataset. Despite this, previous work characterising snRNA-seq and scRNA-seq simultaneously has indicated that both techniques yield a similar level of cellular heterogeneity[82].

Overall, this dataset provides a resource detailing the cellular heterogeneity of two disparate adipose depots and how they are altered in a murine model of T2D. Changes identified included dampened hormone-responsiveness and reduced energy generation in WAT and BAT, respectively. Alongside this, apparent adipocyte hypertrophy was observed and the appearance of macrophage crown-like structures for which the importance of lysosomal machinery was highlighted. Key sex differences were observed between female and male depots with the uncovering of vascular dimorphisms of which T2D appeared to override. Finally, the availability of this dataset through an online open-access web portal enables other research groups to explore candidate genes (including transcription factors and their corresponding regulons). This provides ample opportunity for the possible identification of novel therapeutic targets to ameliorate pathogenic adipose-derived effects on cardiometabolic health.

Research design and methods

Animals, physiological assessment, and tissue isolation

All animal-related experiments were approved by the Alfred Research Alliance (ARA) Animal Ethics Committee (Ethics number: E/1990/2020/B) and were performed in accordance with the National Health and Medical Research Council of Australia. 20-week-old female and male B6.BKS(D)-Lepr$^{db/J}$ (*db/db;* referred to as T2D) (strain #:000697, The Jackson Laboratory) or heterozygous littermates (*db/h;* referred to as ND) in the C57BL/6J background[83–85] were housed under 12h light/dark cycles at 22°C with access to both food and water *ad libitum*. Homozygous *db/db* mice are a well-established model of T2D, having a spontaneous autosomal recessive global knockout for the leptin receptor. At 19-weeks of age, whole-body composition analysis was performed on mice using Echo-MRI™ 4-in-1 700 Analyser (EchoMRI, Houston, TX, USA). Long-term blood glucose levels were measured via HbA_{1c} at endpoint (Cobas b 101 POC system, Roche Diagnostics). At 20 weeks of age, mice were administered an intraperitoneal injection of heparin (100 units per mouse) 20-30 minutes prior to euthanasia using CO_2 inhalation, which was followed by subsequent cardiac exsanguination. As described previously[86], the thoracic cavity was exposed, and the right atrium was cut. The pericardium was removed, and the inferior vena cava was cut. The apex of the left ventricle was pierced using a 30-gauge hypodermic needle for perfusion with buffers specified below.

Stromal Vascular Fraction Flow Cytometry Processing

Mice were perfused with cold ethylenediaminetetraacetic acid (EDTA) at a rate of 2 mL/min/mouse before gonadal white adipose and interscapular white and brown adipose depots were excised and snap frozen. Tissues were washed briefly in clean PBS with 0.9 mM $CaCl_2$ (C4901, Sigma) before being minced using curved scissors into ~2 mm pieces in a petri dish and transferred into 3 mL of enzymatic solution (2 mg/mL collagenase IV (LS004188, Worthington Biochem) and 1 mg/mL dispase II (04942078001, Roche) in PBS with 0.9 mM $CaCl_2$). Samples were triturated three times in 15-minute intervals using a P1000 pipette for a total of 45 minutes then centrifuged at 400xg for 4 minutes at 4°C. The floating fraction was then carefully removed using a P1000 pipette and discarded. Using the remaining supernatant, the pellet was resuspended before being filtered through a 70 µm filter (43-50070-50, pluriSelect) into a tube containing PBS with 0.9 mM $CaCl_2$. The sample was pulled through the filter twice, followed by the addition of count beads (C36950, Invitrogen) before centrifugation at 200xg for 15 minutes at 4°C - no breaks - for debris clearance. The supernatant was removed carefully, and the pellet was resuspended in Fx buffer (1XHBSS (14185-052, Gibco), 2% FCS in ddH2O (10977015, Invitrogen)) before centrifugation at 400xg for 4 minutes at 4°C. The supernatant was removed, and the pellet was resuspended in 50 µL of Fx buffer. A volume of 43µL for each sample was transferred to 1.5 mL tubes containing Fc block (101320, BioLegend) on ice for 5 minutes and the remaining samples were pooled and used for single colour controls. Samples were then incubated in tubes containing antibody mix (Supplementary Table S1) on ice for 20 minutes covered from light. Calcein Blue (1:500; 65-0855-39, Invitrogen) was added to each sample and incubated at 37°C for 10 minutes. Samples were washed using Fx buffer before centrifugation at 400xg for 4 minutes at 4°C. Supernatant was removed and samples were resuspended in Sytox Green (1:1000; S34860, Invitrogen) in Fx buffer before being filtered into FACS tubes (38030, StemCell) and analysed on a BD Fortessa II flow cytometer.

Statistical Analysis

FlowJo™ V10.8 Software (for Windows BD Life Sciences) was utilised to compensate and analyse flow cytometry data. A detailed gating strategy is outlined in Supplementary Figure 1C. For sex-independent analyses within each depot, raw data were normalised to the average value for all *db/h* samples for that batch. For sex-dependent analyses, the prior was conducted with only female samples for each batch. GraphPad Prism v9.2.0 (GraphPad Software San Diego, California USA, www.graphpad.com) was subsequently used to analyse data and conduct statistical analyses. Outliers were excluded using GraphPad Prism's inbuilt ROUT outlier identification method (Q=1%) on data within each cell population. For between depot analysis, data were treated as non-parametric and analysed using a Kruskal-Wallis test. For within depot analysis of T2D changes and sex differences, data were treated as non-parametric and analysed using a Mann-Whitney test. *P* values less than 0.05 were considered statistically significant.

Nuclei extraction

Mice were perfused with cold ethylenediaminetetraacetic acid (EDTA) at a rate of 2 mL/min/mouse before gonadal white adipose and interscapular white and brown adipose depots were excised and snap frozen. Frozen adipose tissue samples (ranging from ~25 to 34.7 mg for WAT and ~60.9 to 65.5 mg for BAT) were mechanically homogenised in a previously published[87] nuclei purification buffer (NPB) (containing 5% bovine serum albumin, 0.2% Nonidet™ P 40 Substitute (74385, Sigma-Aldrich), 1 mM dithiothreitol (D0632, Sigma-Aldrich), 1X cOmplete™ EDTA-free Protease Inhibitor Cocktail (11873580001, Roche), 0.2 U/µL of Protector RNase Inhibitor (3335399001, Roche) in Dulbecco's PBS (14190-250, Gibco)) with a 5 mm diameter stainless steel ball (Qiagen, 69989) in Eppendorf Safe-Lock LoBind tubes (0030120.094, Eppendorf) using a Qiagen TissueLyser II. BAT and WAT were homogenised at 30 Hz for 30 and 50 seconds, respectively, in 10-second intervals. Samples were placed on ice post-homogenisation and incubated for 15 minutes. Homogenised samples were then pulled through 20 µM pluriSelect Cell Strainers (43-50020-03, pluriSelect) to remove debris and centrifuged at 1000 xg for 5 minutes at 4°C. Pellets were then resuspended in cold nuclear Fx Buffer (containing 1X HBSS, 2% fetal calf serum, 0.2 U/µL of Protector RNase Inhibitor). Nuclei were barcoded using cholesterol modified barcodes and anchors as previously described[59]. Briefly, nuclei were first incubated with the anchor-barcode mix at a final concentration of 200nM for 5 minutes on ice before being further incubated with a co-anchor at a final concentration of 200 nM for 20 minutes (Barcodes in Supplementary Table S1). Samples were twice washed in cold nuclear Fx buffer before samples within each group were pooled and stained with DAPI (1 µg/mL; D1306, Invitrogen) in cold nuclear Fx buffer. Nuclei were filtered through a 35 µM filter (38030, StemCell) before being sorted on the BD FacsARIA Fusion to remove debris (Alfred Research Alliance Flow Core).

Single-nucleus transcriptomic library preparation and sequencing

FACS isolated nuclei were counted using a haemocytometer and pooled as detailed in Supplementary Table S1 for single nucleus transcriptomic library preparation using the Chromium controller (10X Genomics). Approximately 40,000 nuclei were loaded into each channel and processed using the Chromium Single Cell 3' v3.1 reagent kit (10X Genomics). After capture and lysis, cDNA was synthesised and amplified for 12 cycles following the manufacturer's protocol. Once amplified, the cDNA was used for downstream sequencing on four lanes of an Illumina NovaSeq 6000 to an approximate depth of 50,000 reads and 3000 genes per nucleus and a sequencing saturation of around 50-61.5% (see Supplementary Table S1).

Analysis of single-nucleus RNA sequencing data

Raw sequencing data in FASTQ format was processed using Cell Ranger v6.1.1 (10X Genomics) with the inclusion of intronic reads before downstream analysis. Reads that were confidently mapped to the genome were aligned to the Mus musculus mm10 transcriptome before the expression of each transcript was quantified for each nucleus. Analysis was carried out using R v4.1 and v4.2 and Seurat v4.1.1 (satijalab.org/seurat). Nuclei with <200 and >6500 and >7000 unique genes expressed for GEX_A (BAT_dbh) and GEX_B (BAT_dbdb), respectively, and nuclei having <200 and >7500 unique genes expressed were excluded for GEX_C (WAT_dbh) and GEX_D (WAT_dbdb), respectively. Nuclei with a percentage of reads mapping to mitochondrial genes greater than 2, 1, 1, and 0.5 standard deviations were removed from GEX_A, GEX_B, GEX_C and GEX_D, respectively, to keep the top 80% of nuclei for analysis. SCTransform[88] was used to normalise the data and regress out ribosomal protein genes (genes starting with Rps and Rpl) and mitochondrial genes to reduce technical artifacts. Four objects were created for each sequencing run and nuclei were clustered. Low quality clusters were removed based on nFeature_RNA, nCount_RNA and percent.mt. Remaining cells were re-clustered, and doublets were removed using DoubletFinder[89] prior to sample integration. Standard Seurat integration approach was performed with 4000 integration features used and canonical correlation analysis (CCA) was utilised to find the integration anchors. Sex-based separation was primarily conducted using the barcoding approach described above. Sex barcodes were demultiplexed using MULTIseqDeemux() function in Seurat. Nuclei that were predicted as doublets were either further separated based on our previously published approach[16] that considers the expression of sex-specific genes (*Xist*, females; *Ddx3y*, *Eif2s3y*, *Gm29650*, *Kdm5d*, *Uty*, males) or discarded from analysis if they showed co-expression. Sex information then was embedded into the integrated Seurat object. Dimensionality reduction

was conducted using 40 principal components and a resolution of 0.8 to explain variability not expected by chance to cluster the nuclei into cell populations. Uniform Maximum Approximation and Projection (UMAP) was used to visualise the data in two dimensions and 'FindAllMarkers' was used to identify cell clusters. As observed in previous adipose single cell datasets, contaminating epididymal cells, ovarian epithelial cells and skeletal muscle cells were removed from analysis. The final dataset included 51,877 nuclei across 28 samples (7 samples in each of the 4 groups) comprising 22,526 nuclei in WAT and 29,351 nuclei in BAT from db/h and db/db samples.

Differential expression analysis

To assess differentially expressed (DE) genes between conditions and sexes, 'MASTcpmDetRate' was used. MASTcpmDetRate is a modified version of 'MAST'[90] which considers cellular detection rate and has been successfully used in the past for benchmarking experiments that deemed this approach superior to others[91]. First, ribosomal RNA was removed from the dataset and DE analysis was conducted. Genes with an uncorrected *P* value less than 0.01 were considered statistically significant and, thus, differentially expressed between groups. Data was primarily visualised using the ggplot2 package.

Gene ontology analysis

Gene ontology (GO) enrichment analysis of the above DE genes was performed using the 'enrichGO' function from the clusterProfiler package (v4.4.4)[92]. DE genes were mapped to genomes from geneontology.org. Enrichment analysis of GO Biological Process terms (GO terms) was calculated using all genes identified in the dataset as the background gene set (with minimum and maximum gene set sizes as 10 and 500, respectively). The 'simplify' function was used to calculate similarities between GO terms. GO terms with a similarity higher than the 0.7 cut-off were discarded from analysis. Terms with a *P* value less than 0.05 were considered statistically significant GO terms. Data was visualised using the ggplot2 package.

Gene expression overlap analysis

To determine the similarity of differentially expressed genes between mouse *db/db* and human obesity datasets, a publicly available scRNA-seq dataset[14] of visceral WAT and present data on gonadal (visceral) WAT samples from *db/db* mice were compared. For the human obesity dataset, samples with a BMI greater than 30 were classed as 'obese' while those less than 25 were classed as 'lean'. DE analysis was conducted between control and treatment groups (lean vs. obese for humans and *db/h* vs. d*b/db* for mice), as described above. Human gene orthologs were converted to mouse orthologs using the AnnotationDbi package (v1.58.0). DE genes with a *P* value less than 0.01 were isolated from each dataset and the 'overlap' function within RVenn (v1.1.0) was used to determine both common and unique DE genes across datasets. The number of common and unique genes were visualised using RVenn (v1.1.0), Euller (v6.1.1), VennDiagram (v1.7.3) and Vennueler (v1.1.3).

Tissue preparation for histological validation

Age and sex-matched animals were euthanised using CO2 inhalation cardiac exsanguination. Animals were then perfused with PBS with 200mM KCl (P9541, Sigma-Aldrich) at a rate of 2 mL/min/mouse until the liver cleared of blood. Mice were then perfused with 4% PFA (P6148-500G, Sigma) in PBS with 200mM KCl at a rate of 1 mL/min/mouse. Tissue was kept in 4% PFA in PBS overnight before being stored in PBS with PBS with 0.09% NaN_3 (S2002-25G, Sigma) at 4°C short-term. Tissue was embedded in paraffin by the Monash Histology Platform (Monash University, Clayton, VIC, Australia).

Tissue section staining

FFPE blocks were cut on a microtome (RM 2235, Leica Biosystems) at 10 µm on SuperFrost plus slides then dried overnight at 37°C. Paraffin was melted at 80°C for 1 minute before slides were washed three times with fresh xylene for 10 minutes each (534056-4L, Honeywell). Slides were rehydrated in 100% (v/v) EtOH, 100% (v/v) EtOH, 90% (v/v) EtOH, 70% (v/v) EtOH and 50% (v/v) EtOH for 5 minutes each before being submerged in ddH_2O for 1 minute. Slides were washed twice in Tris-EDTA, pH 9.0 (10 mM Tris (BIO3094T-1KG, Astral Scientific), 1 mM EDTA (EDS-500G, Sigma) in ddH2O) before being microwaved for 15 minutes in 1 L of

Tris-EDTA, pH 9.0. Slides were washed twice with PBS-T (PBS, 0.05% Tween-20) before being permeabilised for 10 mins in 0.5% Triton X-100 (T9284, Sigma) in PBS. Slides were washed twice with PBS for 5 minutes each before blocking with goat block (2% goat serum (G9023-10ML, Sigma), 1% BSA (A3608-100g, Sigma), 0.1% cold fish skin (G7765-250ML, Sigma), 0.1% Triton X-100 (T9284, Sigma) for 1 hour at room temp. Slides were then incubated with primary antibody in goat block (PLIN1, 1:200, AB3526, Abcam; Isolectin B4/IB4, 1:50, L2140, Sigma-Aldrich; Interferon Regulatory Factor 4/IRF4, 1:200, PA5-115603, Invitrogen) overnight at 4°C in a humidified chamber. Sections were washed three times in PBS-T for 5 minutes each. Slides were incubated with secondary antibody in PBS-T for 1 hour at room temp. Sections were then washed three times with PBS-T with AF488 conjugated Wheat Germ Agglutinin (WGA) (29022, Jomar Life Research) for 5 minutes before a final wash with DAPI to stain nuclei in PBS-T for 10 minutes. Sections were mounted under a coverslip before imaging using a 20X multi-immersion objective and glycerol on a Nikon A1R confocal laser scanning microscope.

Statistical analysis of quantified data

For immunofluorescence-based validation, micrographs were analysed in Fiji image analysis software[95]. Macros were utilised for IB4, nuclei and adipocyte lipid size quantification. Briefly, for tissue sections stained with IB4 and DAPI, the total tissue area and IB4 stained tissue area (determined using thresholding) were measured within a field of view. The percentage of IB4-positive tissue space was calculated. Nuclei (DAPI-positive) were selected with thresholding, quantified, and divided by the total area to give the number of nuclei per μm^2. Finally, thresholding of PLIN1-positive lipid droplets facilitated the subsequent measurement of Feret's diameter (µm) for each PLIN1-positive oval lipid droplet. Calculations using raw data from Fiji were conducted in Microsoft Excel before processed data were transposed into GraphPad Prism (v9.2.0). GraphPad Prism (v9.2.0) was used to conduct unpaired t-tests on comparisons between two groups and ordinary one-way ANOVAs on comparisons between three groups. Statistical significance was determined where $P<0.05$.

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Author contributions

A.R.P. supervised the study. A.R.P., G.E.F., C.K., T.L.G., and C.D.C. conceived and designed the experiments and collected the data. I.H., M.S.I.D., T.L.G., C.K., C.D.C., and A.R.P. performed the data analysis with I.H. and M.S.I.D. leading the computational analysis. A.R.P. and T.L.G. wrote the manuscript. A.R.P., B.G.D., T.L.G., G.E.F., C.K., C.D.C., I.H., B.G.D and M.S.I.D. reviewed, edited, and approved the manuscript.

Competing interests

The authors declare no competing interests.

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Ethics approval

All animal-related experiments were approved by the Alfred Research Alliance (ARA) Animal Ethics Committee (Ethics number: E/1990/2020/B) and were in accordance with NHMRC guidelines.

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Figures

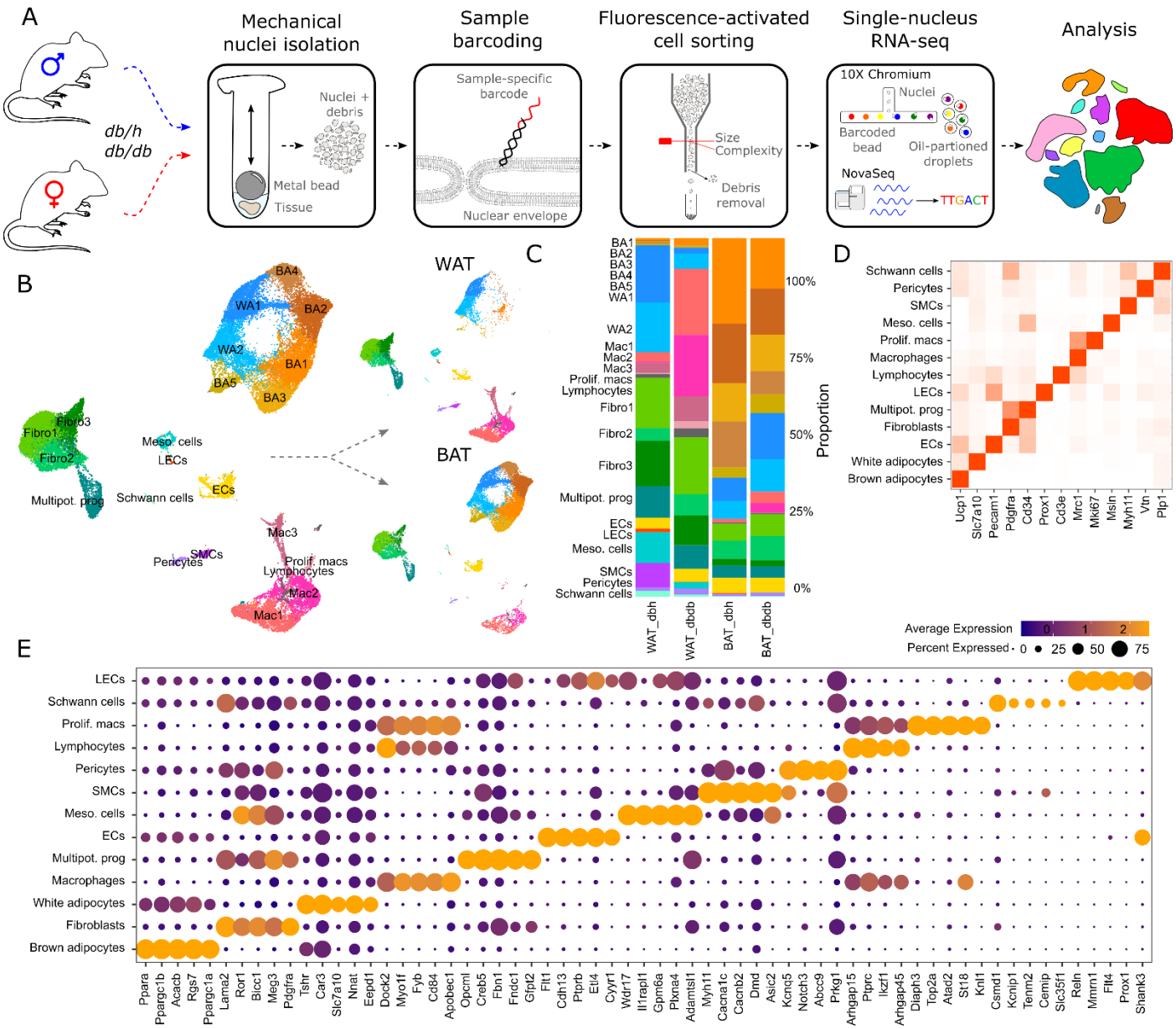


Figure 1. snRNA-seq characterisation of adipose cellulome. A, Experimental outline for novel isolation of mature adipocyte and SVF nuclei from snap-frozen adipose tissue. B, Uniform Manifold Approximation and Projection (UMAP) of nuclei representing the adipose cellulome for both *db/h* and *db/db* male and female white and brown adipose tissues (WAT and BAT, respectively). Each dot represents a nucleus and colours highlight distinct cell populations. Subplots show separation of WAT and BAT. C, Compositional bar chart showing proportion of each cell population in each experimental condition. D, Canonical markers of broad adipose tissue cell populations represented in heat map with relative expression being indicating by intensity of colour. E, Top 5 marker genes for each broad cell population with dot size indicating the proportion of cells within that population expressing the marker and dot colour indicating the average level of expression. Fibro, fibroblast, Multipot. prog., multipotent progenitors, Meso. cells, mesothelial cells, ECs, endothelial cells, LECs, lymphatic endothelial cells, SMCs, smooth muscle cells, Mac, macrophage, Prolif. macs, proliferating macrophages.

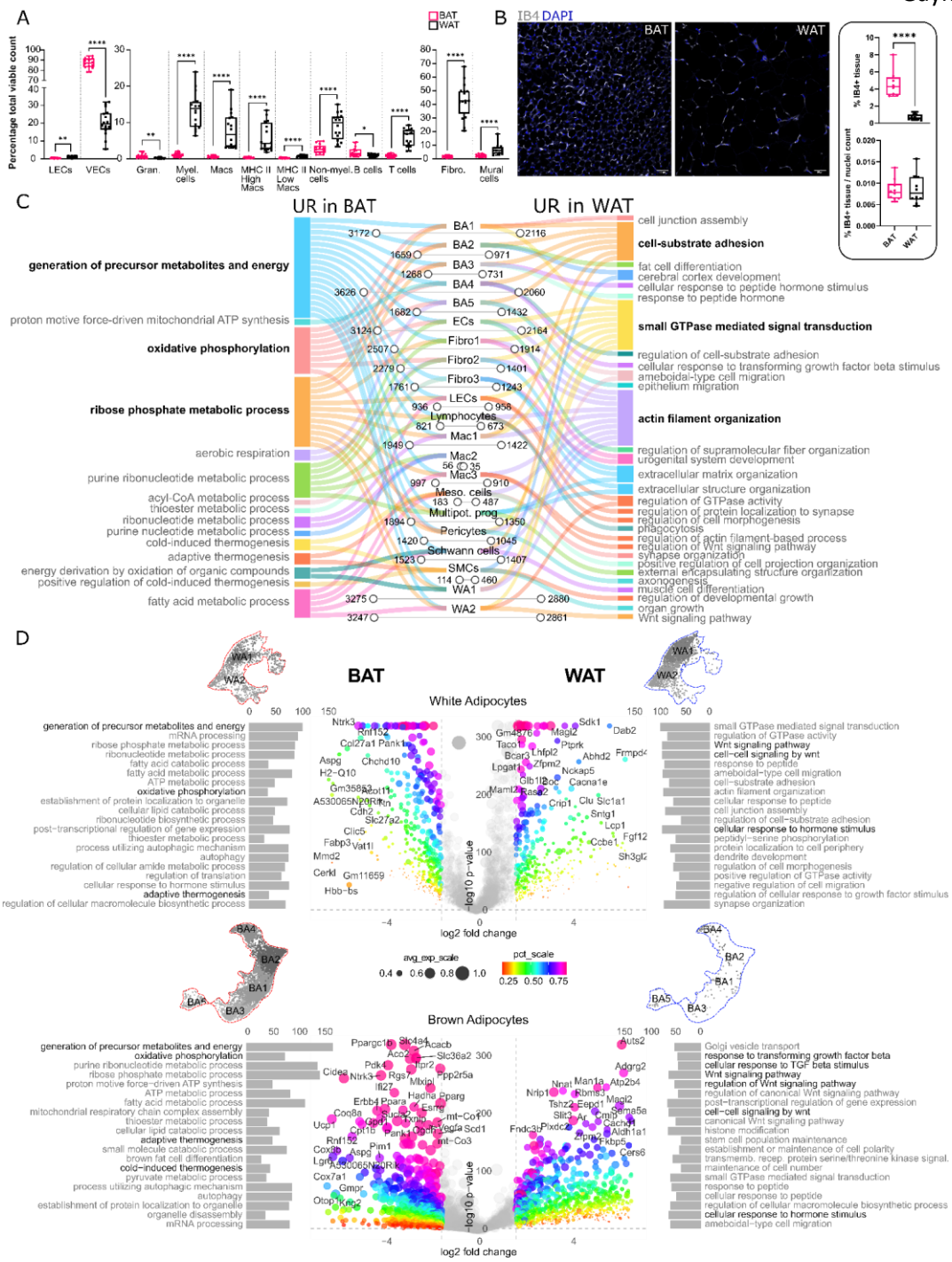


Figure 2. Cellularity and gene expression differences between disparate adipose depots. A, Box and whisker plots of flow cytometry data representing differences in adipose cell population contribution to total viable cell count across WAT (black) and BAT (pink). B, Histological validation of endothelial cell differences using isolectin B4 (IB4; grey) to stain vasculature and DAPI (blue) to stain nuclei. C, Differential gene expression analysis presented as lollipop plot of upregulated genes across each depot and top 3 linked gene ontology terms shown as Sankey plots. D, Volcano plots showing differentially expressed genes between white and brown adipocyte subsets in BAT and WAT. Genes to the right of the x axis indicate a positive fold change, or upregulation in WAT, while genes to the left indicate a negative fold change, or upregulation in BAT, dot colour represents the percent of cells expressing the gene, while dot size represents the average expression of each gene, finally, the relative y axis position indicates the p value with those highest having most significance. GO term analysis of DEGs presented in grey indicating key terms upregulated in BAT or WAT (left and right, respectively) for each subpopulation with the length of the bar representing the number of genes associated with the respective GO term. Statistical significance between males and females was assumed at $P<0.05$, * $P< 0.05$, ** $P< 0.01$, *** $P< 0.001$, **** $P< 0.0001$.

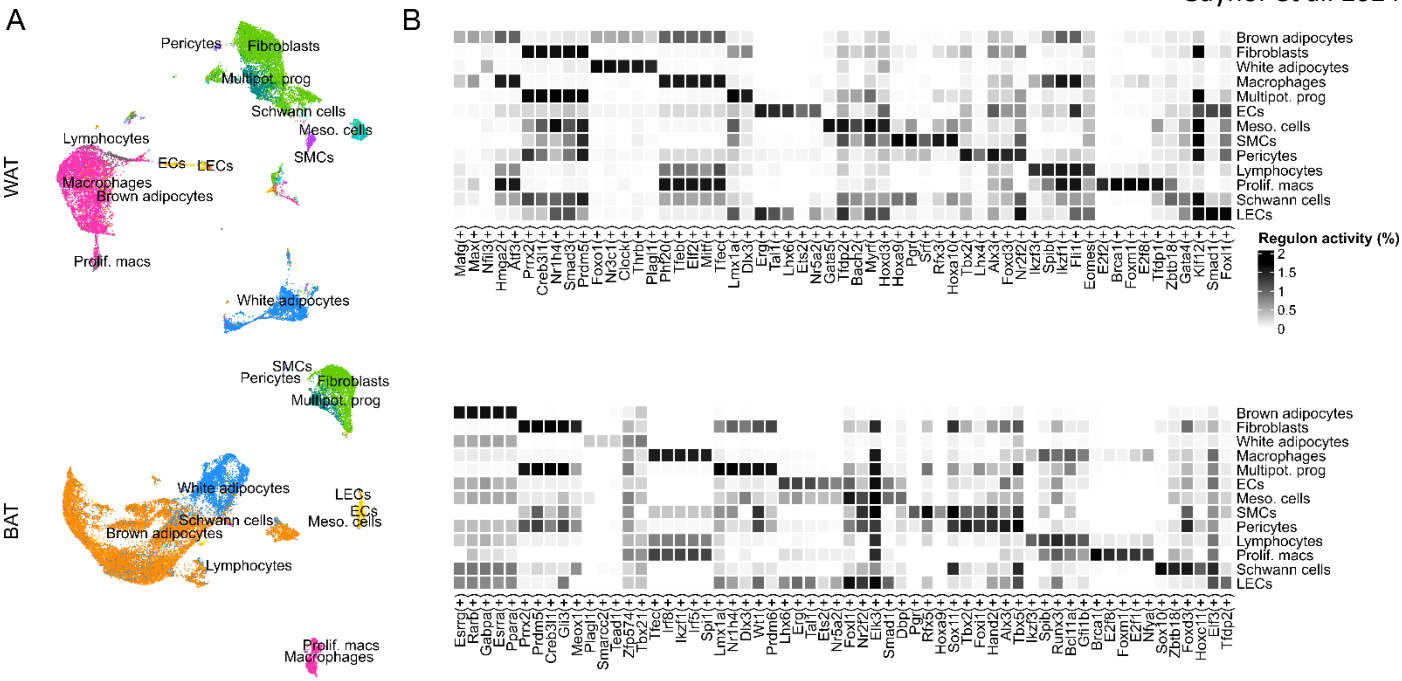


Figure 3. Cell abundance, gene expression and morphological differences in healthy depots driven by biological sex. A, UMAP clustering based on regulon-specific expression patterns (AUCBinary assay). Annotation of cell populations carried over from RNA-based clustering seen in Figure 1B. WAT and BAT separated B, Top 5 marker regulons for each cell population. Intensity of black indicates regulon activity for each cell population.

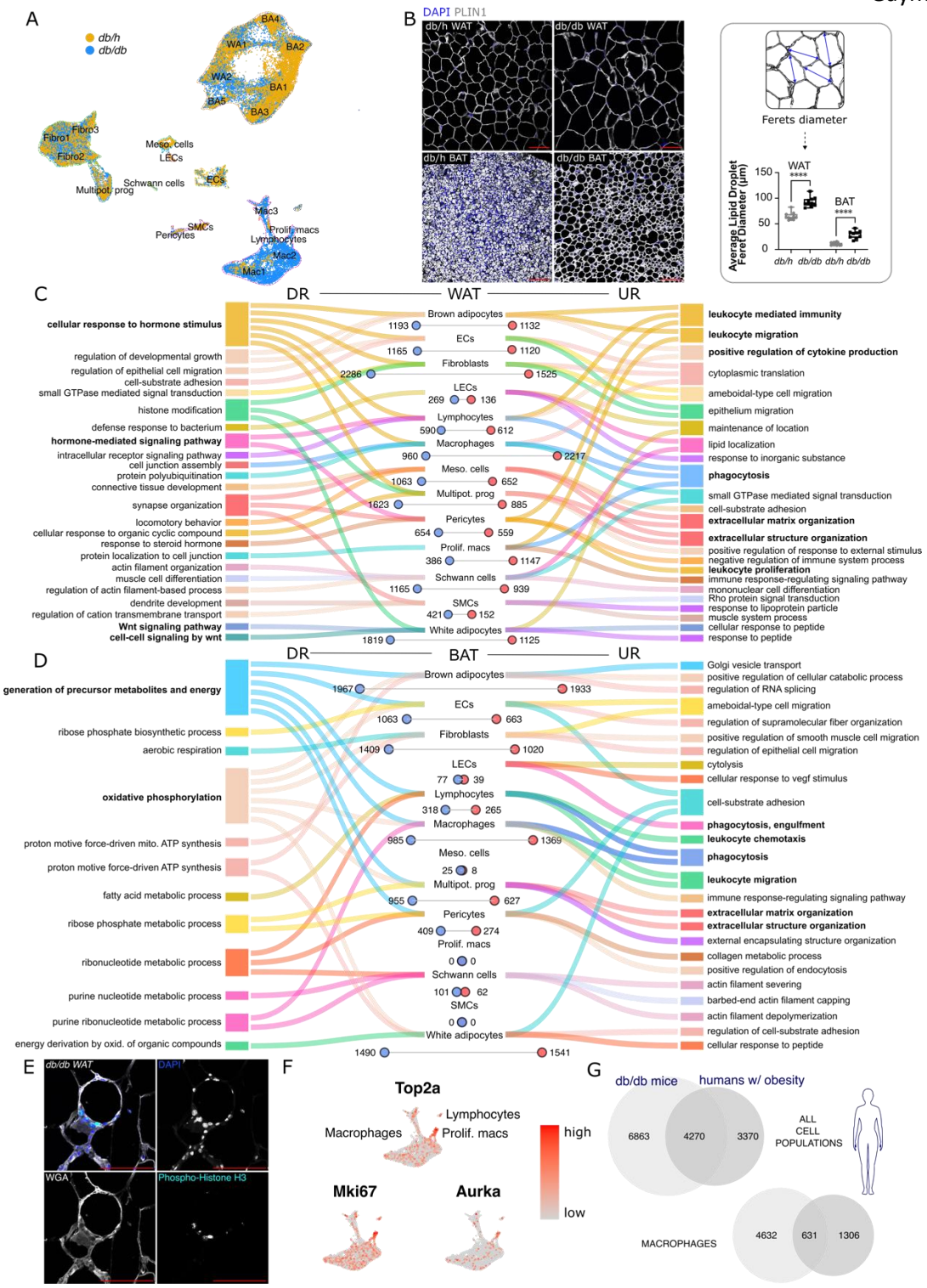


Figure 4. Concerted immune response and extracellular matrix remodelling in type-2 diabetic adipose depots. A, UMAP of both *db/h* nuclei (yellow) and *db/db* nuclei (blue) highlighting an abundance of macrophages in disease. B, adipocyte lipid droplet size measured using Feret's diameter in both *db/h* and *db/db* WAT and BAT. Lipid droplet stained with perilipin 1 (PLIN1; grey) and nuclei stained with DAPI (blue). Corresponding quantification presented. ****, *P*<0.0001. C, sankey plots for WAT demonstrating upregulated (right) and downregulated (left) Gene Ontology (GO) terms and overlayed lollipop plot for associated differentially expressed genes in T2D. D, sankey plots for BAT as described previously. E, immunofluorescence staining of proliferative marker, phosphohistone H3, in macrophage-crown-like structures in *db/db* WAT. F, Feature plots showing expression of proliferation markers *Top2a*, *Mki67*, and *Aurka* in macrophage subsets. G, Overlay of differentially expressed genes in all cell populations for visceral WAT or white adipocytes in visceral WAT between the present study and a human obesity study.

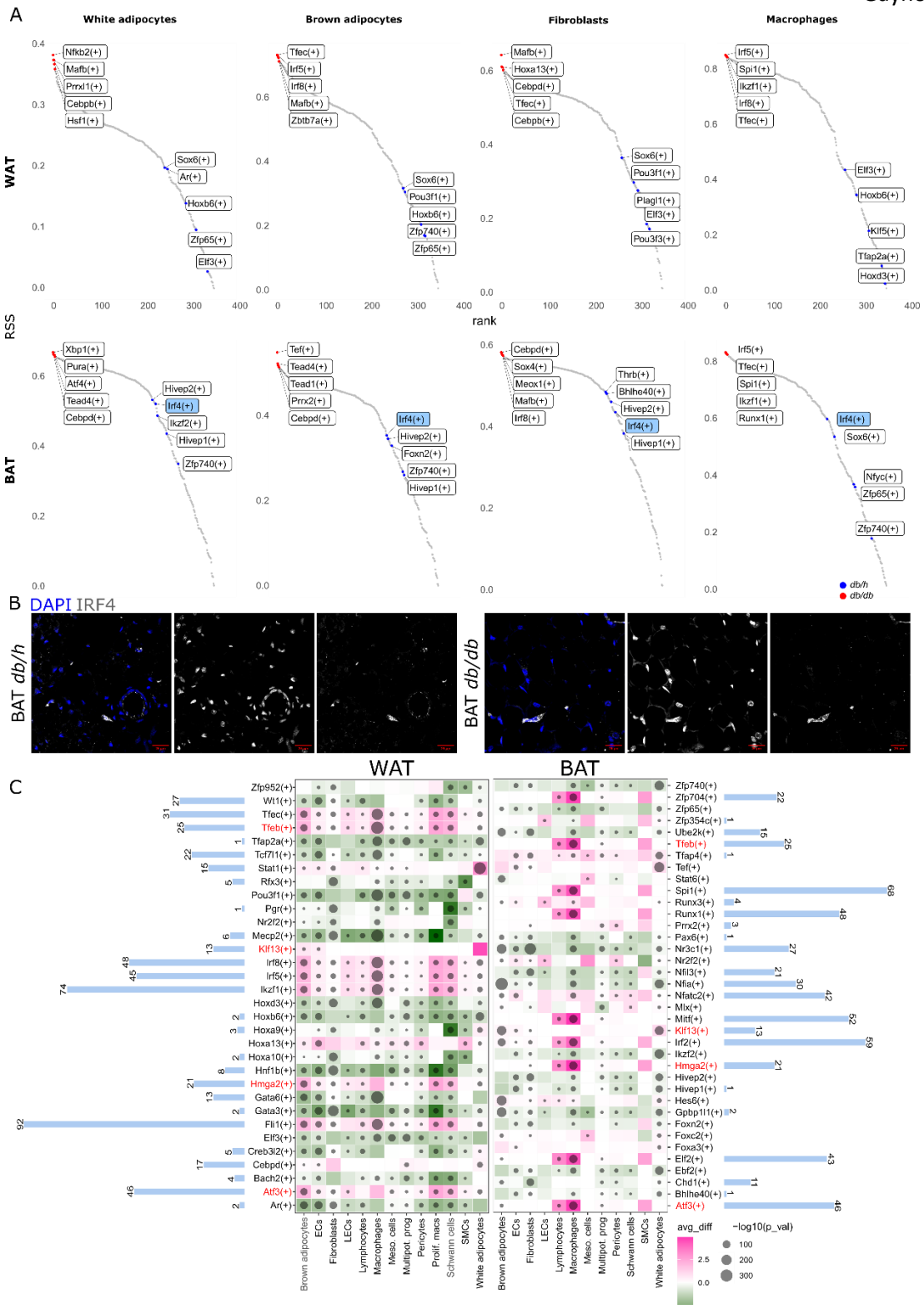


Figure 5. Gene regulatory network alterations in T2D adipose depots and contribution to leukocyte migration. A, RSS plots demonstrating the top 5 activated regulons for cell populations including white adipocytes, brown adipocytes, fibroblasts and macrophages for WAT (top panel) and BAT (lower panel) between *db/h* (blue dots) and *db/db* (red dots) samples. B, Immunofluorescence validation of transcription factor IRF4 (grey) in both *db/h* and *db/db* BAT tissue with nuclei stained with DAPI (blue). C, differentially expression of regulons with those upregulated being presented as pink and those downregulated presented as green. Intensity of colour indicates the average expression within that cell population while dot size represents statistical significance. Regulons highlighted in red represent those shared across both depots. Bar charts adjacent to regulons indicate the number of target genes within that regulon that are also linked to the GO term, *leukocyte migration.*

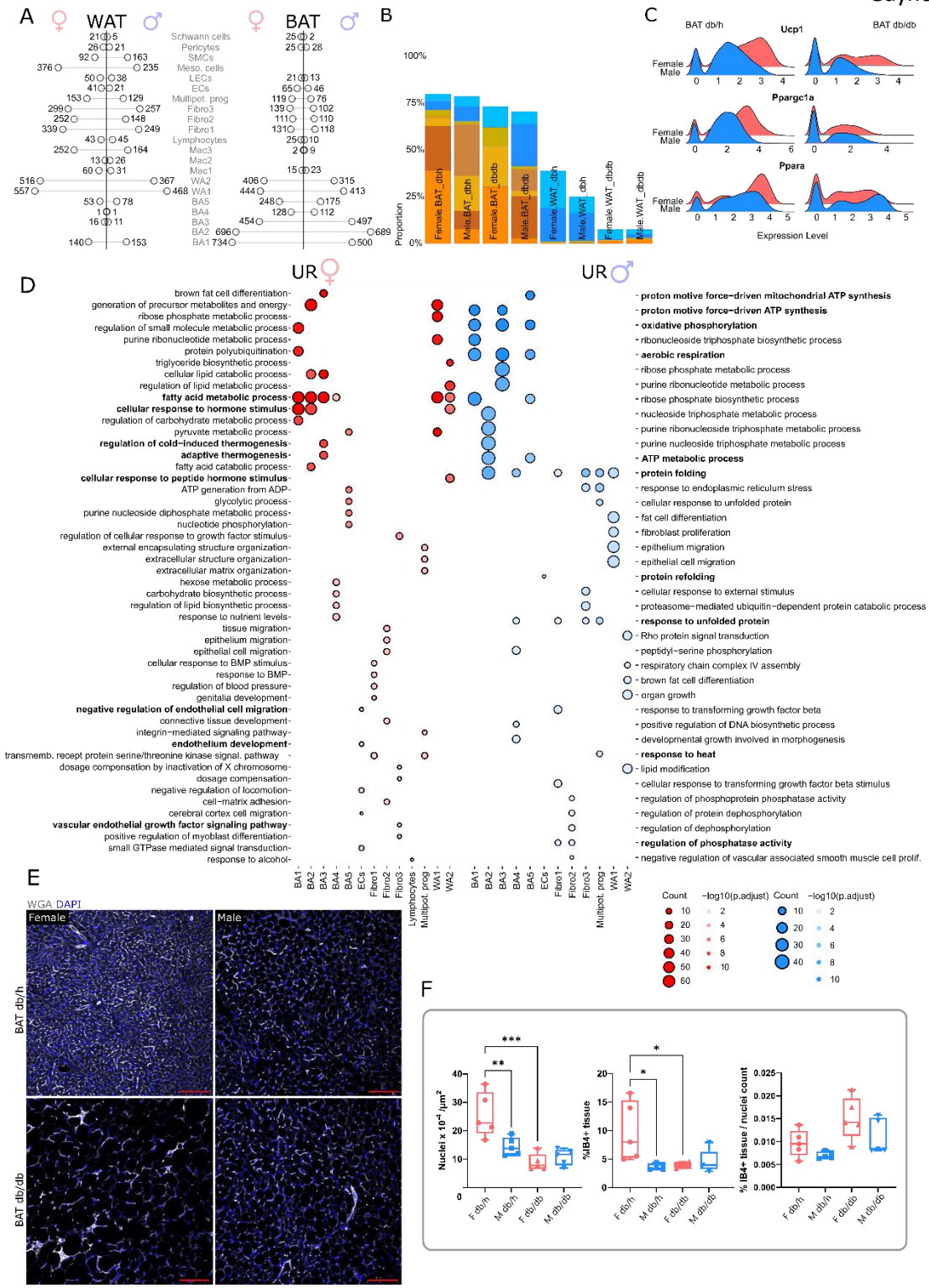
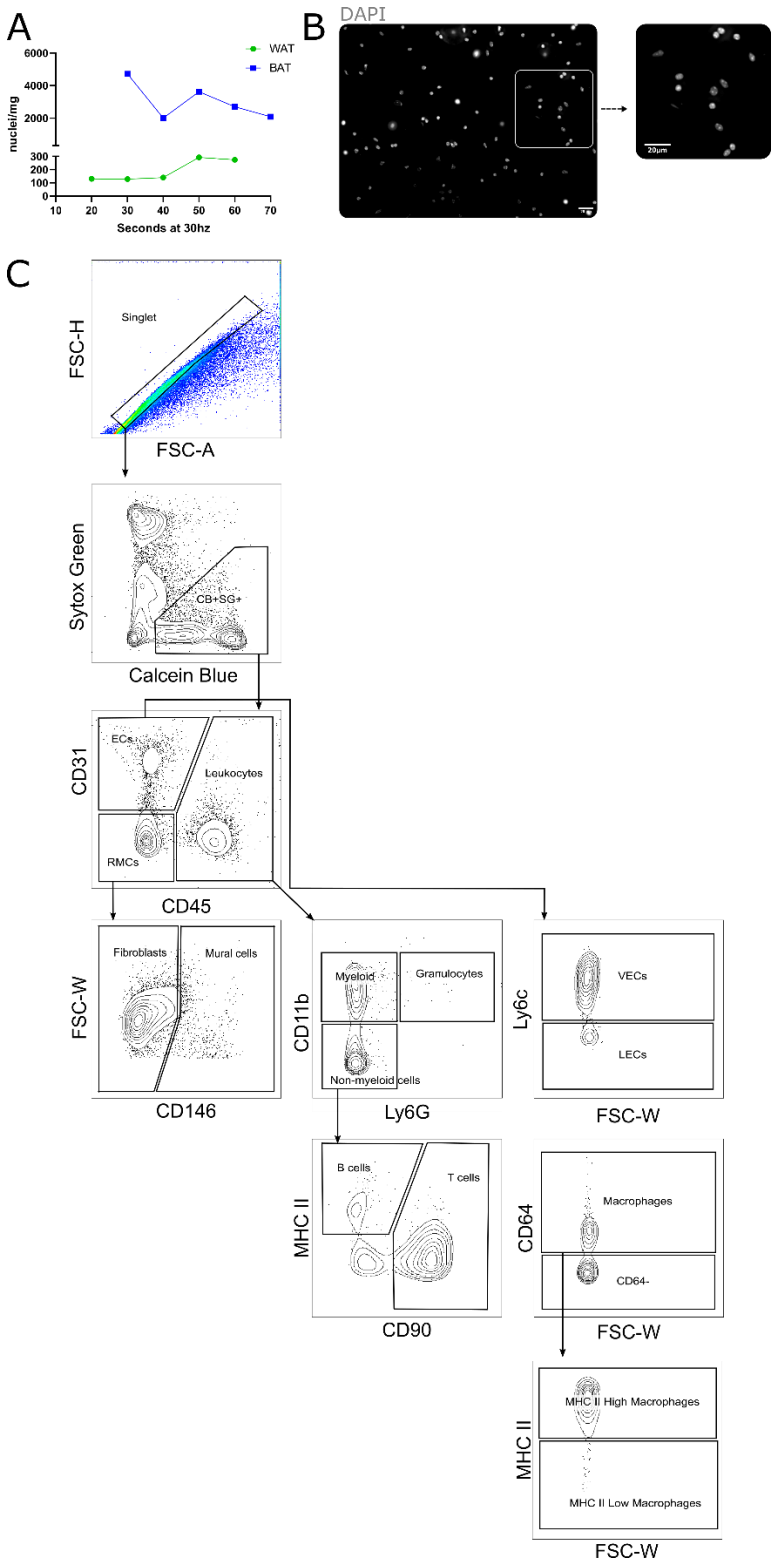
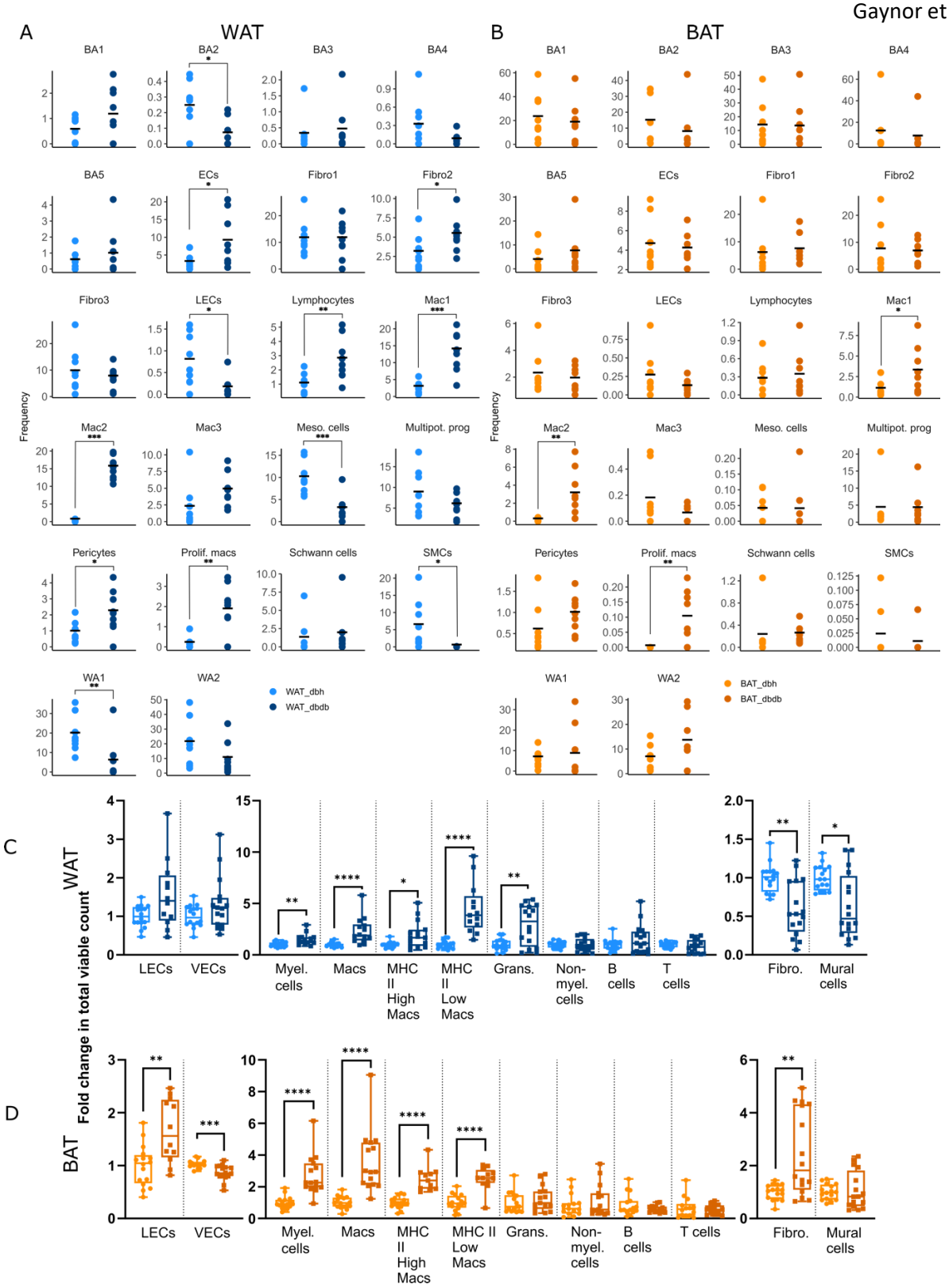


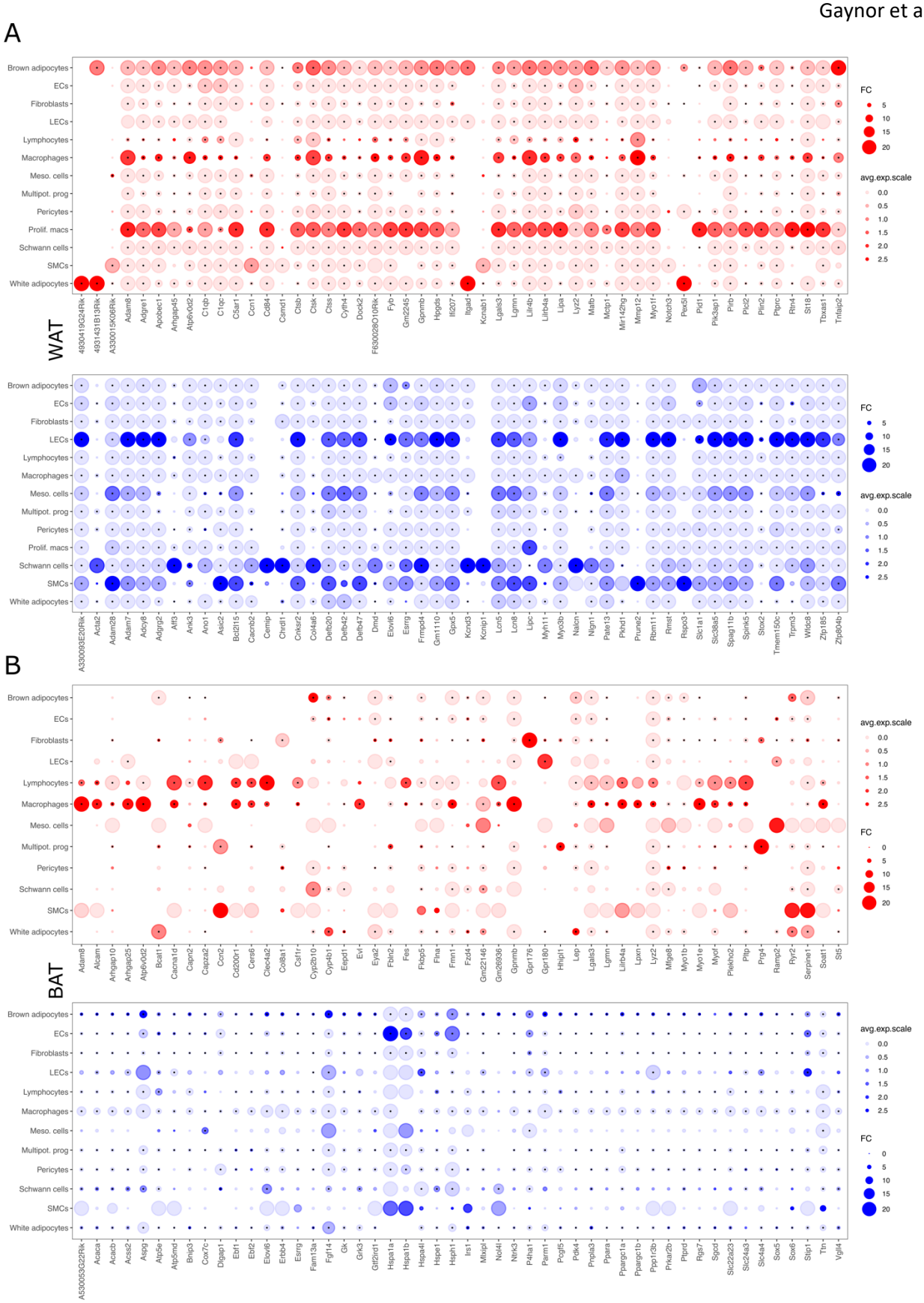
Figure 6. Sex differences in gene expression and tissue architecture across adipose depots. A, Differential gene expression analysis between females and males in *db/h* WAT and BAT. B, Composition bar chart demonstrating the contribution of adipose tissue cell populations to each depot and how they differ between conditions and biological sexes. C, Ridgeplots showing female and male sample expression of key thermogenic genes between *db/h* and *db/db* conditions in BAT. D, Top 5 gene ontology terms for each cell population where dot size represents the number of genes contributing to this GO term and dot colour represents the statistical significance. GO terms of interest are highlighted in bold. E, Immunofluorescence staining of BAT vascularisation using isolectin B4 (IB4; red) and nuclei stained with DAPI (grey) demonstrating differences in tissue architecture between females and males in both *db/h* and *db/db* conditions. F, Quantification of vascularisation and cell density histology with female data points presented in pink and males in blue. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.



Supplementary Figure 1. Qiagen optimisation and flow cytometry gating strategy. A, Flow cytometry-based optimisation of the time length required to mechanically digested at 30hz using the Qiagen tissue lyser to get optimal nuclei/mg of tissue yield. B, micrographs showing nuclei post Qiagen digestion and fluorescence-activated cell sorting to remove cellular debris. Scale bar represents 20uM. C, Flow cytometry gating strategy for SVF cell population analysis. C, Viable and metabolically active single cells were gated and cell populations were delineated based on staining by antibodies specific for cell surface markers. FSC-H, forward scatter height; FSW-A, forward scatter area; FSC, forward scatter width; CB, calcein blue; SG, sytox green; ECs, endothelial cells; RMCs, resident mesenchymal cells; VECs, vascular endothelial cells; LECs, lymphatic endothelial cells.



Supplementary Figure 2. Cell population abundance changes in T2D. A, Cell proportion differences between *db/h* and *db/db* samples based on nuclei count in WAT (blue) and BAT (orange). Y axis indicates frequency of total cell count. B, Contribution of adipose tissue cell populations to total viable cell count with differences between *db/h* and *db/db* samples being shown. BA, brown adipocyte; ECs, endothelial cells; meso. cells, mesothelial cells; multipot. prog, multipotent progenitors; prolif. macs, proliferating macrophages; SMCs, smooth muscle cells; WA, white adipocyte; LECs, lymphatic endothelial cells; VECs, vascular endothelial cells; myel. cells, myeloid cells; macs, macrophages; grans, granulocytes; non-myel. cells, non-myeloid cells; fibro, fibroblasts. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$.



Supplementary Figure 3. Top 50 upregulated and downregulated genes in T2D WAT and BAT. Differential gene expression presenting genes upregulated and downregulated across cell populations in *db/db* WAT (A) and BAT (B). Dot size represents the fold change (FC) difference to *db/h* controls and dot colour intensity indicates the expression level (avg. exp. scale). ECs, endothelial cells; LECs, lymphatic endothelial cells; meso. cells, mesothelial cells; multipot. prog, multipotent progenitors; SMCs, smooth muscle cells.