Healthy adipose tissue remodeling depends on the balance between de novo adipogenesis from adipogenic progenitor cells and the hypertrophy of adipocytes. De novo adipogenesis has been shown to promote healthy adipose tissue expansion, which confers protection from obesity-associated insulin resistance. Here, we define the role and trajectory of different adipogenic precursor subpopulations and further delineate the mechanism and cellular trajectory of adipogenesis, using single-cell RNA-sequencing datasets of murine adipogenic precursors. We identify Rspo2 as a functional regulator of adipogenesis, which is secreted by a subset of CD142+ cells to inhibit maturation of early progenitors through the receptor Lgr4. Increased circulating Rspo2 in mice leads to adipose tissue hypertrophy and insulin resistance and increased Rspo2 levels in male obese individuals correlate with impaired glucose homeostasis. Taken together, these findings identify a complex cellular crosstalk that inhibits adipogenesis and impairs adipose tissue homeostasis.

Results
Integration of APC scRNA-seq data reveals heterogeneity of adipocyte progenitor cells. In a previous study7, we defined Lin−Sca1+CD142+ APCs as adipogenesis regulatory (Areg) cells and demonstrated that these cells are both refractory toward adipogenesis and control adipocyte formation of APCs through paracrine signaling. In contrast, Merrick et. al.4 observed that Lin−CD142+ cells could differentiate into adipocytes. To study these seemingly inconsistent observations, we re-examined 10X scRNA-seq of Lin− cells with the most recent computational algorithms12,13, which by unsupervised clustering of the transcriptomes revealed seven distinct subpopulations (Fig. 1a). The newly identified clusters P1-1, P1-2 and P1-3, which express Cd55 and Dpp4 (Fig. 1b) represent an early progenitor population and resemble the previously identified cluster G1/G4/Group 1 (ref. 4). The previously identified G2/G4/Group 2 (ref. 4) cells, which represent committed APCs, could be divided into two Parg-expressing subgroups (Extended Data Fig. 1a,b) P2-1 (Cd142−) and P2-2 (Cd142+) based on their Cd142 expression (Fig. 1b). The newly defined cluster P3 represents a subset of the previously identified Areg/Group 3 cluster4, with specific expression of Cd142, Clec11a and Fmo2 (Fig. 1b) and which is separated from the P2-2 cluster. The proliferating P4 cluster expresses high levels of cell-cycle genes (Extended Data Fig. 1c).

To define the relationships between these cells, we next predicted the cellular trajectories of these cell clusters through dynamical modeling of RNA splicing events by Velocyto12 and scVELO13.
Fig. 1 | Integration of different scRNA-seq data further reveals the heterogeneity of adipocyte progenitors. a, Uniform Manifold Approximation and Projection (UMAP) two-dimensional map of cells derived from 10X dataset in our previous study9 shows several distinct clusters, including Cd55+ progenitor cells (P1-1, P1-2 and P1-3), two subpopulations of committed pre-adipocytes (P2-1 and P2-2), P3 cells and dividing cells expressing cell-cycle genes of S phase (P4). b, Violin plots showing the expression of marker genes. Cd55 and Dpp4 (marker of cluster P1-1–P1-3); Vap1 and Icam1 (marker of cluster P2-1–P2-2); and Cd142, Clec11a and Fmo2 (marker of cluster P3). c, d, Cell trajectory analysis of Lin− cells by Velocyto and scVELO (c) and Monocle 3 (d). e, f, Single-nucleus RNA-seq (snRNA-seq) of human deep neck adipose tissue. Unsupervised clustering of pre-adipocyte populations shown as UMAP plot (e). P3 score (f) calculated as the sum of F3, CLEC11A, FMO2, GAS6, CYGB, PPL and STEAP4 for each cell. g, Feature plots of H1 (FMO2, FGF10, COL4A2 and PPARG), H2 (CD55, KCNAB1, KCNB2 and CREB5) and H3 (NR4A1, S100A10, S100A6 and CD81) markers in preadipocyte nuclei of human deep neck adipose tissue.
In-depth functional analysis of the different cell populations within mouse adipose tissue. Caution needs to be used when employing CD142 as a marker to isolate P3 cells, as adipogenic CD142-expressing P2-2 cells will also be collected. This fact might explain the divergent findings regarding the adipogenic potential of P3 cells.\(^2\) In our previous study, we observed a continuum of CD142-expressing cells within the Lin\(^-\)Scal\(^+\) (enriched pool of APCs) cell fraction (Extended Data Fig. 2a). This is supported by the finding that Lin\(^-\)Scal\(^-\)CD142\(^+\) cells are more similar to P3 cells compared to Lin\(^-\)Scal\(^+\)CD142\(^+\) cells based on P3 signature gene expression (Extended Data Fig. 2b). Furthermore, abundant P1 and P2 cells are admixed to the Lin\(^-\)Scal\(^+\)CD142\(^-\) fraction, while fewer are observed in the Lin\(^-\)Scal\(^-\)CD142\(^-\) population (Extended Data Fig. 2c), which was confirmed by the analysis of P1 and P2 marker gene expression (Extended Data Fig. 2d,e). Thus, for gating of P3-like Lin\(^-\)Scal\(^-\)CD142\(^+\) cells, CD142 staining within the Lin\(^-\) population could be used as a reference control (Extended Data Fig. 2a). Based on this strategy we next examined the adipogenic capacity of the following cell populations with different cocktails (Extended Data Table 2): Lin\(^-\)Scal\(^+\) cells, Lin\(^-\)Scal\(^+\)VAP1\(^+\) cells (P2-1), Lin\(^-\)Scal\(^-\)CD142\(^+\) (P2-2) and Lin\(^-\)Scal\(^-\)CD142\(^-\) cells (P3). We observed Lin\(^-\)Scal\(^-\)CD142\(^+\) cells were refractory toward adipogenesis similar to the previously described A\(_{ref}\) population upon adipogenic cocktail induction, whereas Lin\(^-\)Scal\(^+\)CD142\(^+\) cells could form adipocytes with previously used induction strategies (Extended Data Fig. 2fg).

To isolate P3 cells more reliably and to further investigate the function of cell populations defined in our combined analysis (Fig. 1a and Extended Data Fig. 1e), we established a new FACS strategy to purify the different subpopulations. As shown in Fig. 2a, enriched-P1 (eP1) composed of P1-1 to P1-3 cells, were isolated using a Lin\(^-\)Scal\(^-\)CD55\(^-\)VAP1\(^+\) CD142\(^-\) gating strategy. VAP1\(^+\) cells were further separated into P1-1 and enriched-P2 (eP2) cells, which are Lin\(^-\)Scal\(^-\)CD55\(^-\)VAP1\(^+\)CD142\(^-\); whereas enriched-P3 (eP3) cells were isolated using a Lin\(^-\)Scal\(^-\)CD55\(^-\)VAP1\(^+\)CD142\(^+\) strategy. Upon adipogenic cocktail induction, eP1, eP2 and VAP1\(^+\)CD142\(^-\) (P2-2) cells showed an adipogenic capacity and we furthermore observed that removal of P3 cells from Lin\(^-\)Scal\(^-\)VAP1\(^+\)CD55\(^-\) (or VAP1\(^+\)CD55\(^-\)) cells, Lin\(^-\)Scal\(^-\)VAP1\(^+\)CD55\(^-\) (or DN:CD142\(^-\)) showed markedly increased adipocyte formation, reminiscent of the fact that P3 cells are refractory toward adipogenesis (Fig. 2b,c). Moreover, gene expression analysis demonstrated that P3-specific genes are enriched in the eP3 population (Fig. 2d). In conclusion, we were able to isolate eP1, eP2, P2-2 and eP3 cells, which represent the P1, P2-1, P2-2 and P3 cells identified by the 10X scRNA-seq approach (Fig. 1a). EP1 cells, when treated with a minimal adipogenic cocktail (C cocktail; Extended Data Table 2) exhibited a lower adipogenic potential compared to eP2 cells, whereas no differences in adipogenesis were detected using robust adipogenic conditions (Fig. 1c). The models suggested that P1 cells can transit to P3 cells and further to either P2-1 or P2-2 cells. Another trajectory analysis using Monolc\(^2\) (Fig. 1d and Extended Data Fig. 1d) inferred that P1 cells can transition through the first branch point to become proliferating P4 cells or through the second branch point to become P2-1 cells. Some P1 cells continue transit through the third branch point to form the cluster of P2-1, P2-2 or P3 cells (Fig. 1d). These data are consistent with previous experimental findings\(^3\) that demonstrated that P1 cells define a group of early adipocyte progenitors, whereas Pparc-expressing P2 cells (Extended Data Fig. 1a,b) represent committed preadipocytes.

Given the newly identified separation of Cd142\(^+\) APCs into P3 and P2-2 cells, we hypothesized that the discrepancies reported by us and Merrick et. al. could be due to the fact that these two populations were analyzed as mixtures. To address this, we aligned data from the two mouse datasets from Merrick et. al. to our own data using canonical correlation analysis\(^4\) (Extended Data Fig. 1e) and performed clustering based on the aligned results. Indeed, we observed two distinct clusters of Cd142\(^+\) cells in the integrated analysis (cluster 0 and 2) (Extended Data Fig. 1f,g). Cluster 2, but not other Cd142\(^+\) cells, express Cd142, Clec11a and Fmo2, similar to the newly defined P3 cells. Cluster 0 was similar to P2-2, which expresses Cd142, Icam1 and Vap1 (Extended Data Fig. 1f,g).

We next wanted to extend our analyses to the human situation, as little is known about the presence of either early or late adipocyte progenitors. Therefore, we resolved the adipocyte heterogeneity in human deep neck subcutaneous adipose tissue (SAT), which allowed us to define 12 subpopulations\(^5\) (Extended Data Fig. 1j). The pre-adipocyte cluster featured by the pre-adipocyte marker PDGFR\(^A\) (Extended Data Fig. 1k) could be further sub-divided into three subsets, termed H1–H3 (Fig. 1e). We failed to correlate the mouse P3 cluster with either H1–H3 clusters, as the overlaps were not statistically significant (Extended Data Table 1).

Alternatively, using the P3 score as a sum of mouse P3 signature genes (Fig. 1f) and Cd142 expression (Extended Data Fig. 1i) indicated that mouse P3 cells were enriched in cluster H1 and H3. Based on these findings, it would be worthwhile to investigate, whether H1 or H3 cells are functionally similar to mouse P3 cells. The enrichment of PPARG in the H1 cluster, suggests that these cells might constitute the committed pre-adipocytes within human SAT (Fig. 1g). Enrich analysis of the H3 signature denotes this cluster as a smooth-muscle-cell-like population (Extended Data Fig. 1m) with enriched pathways such as VEGF\(~A\)–VEGFR2 signaling or the matrix metalloproteinase pathway, which might regulate adipose tissue microenvironment (Extended Data Fig. 1n) and the expression of known adipogenesis regulatory genes such as NR4A1 (ref. \(^11\)) and FSTL1 (ref. \(^22\)) (Extended Data Fig. 1o). Taken together, these data suggest that H3 might constitute a regulatory cell type within human SAT; however, more studies will be needed to delineate the function of H3 cells.

**Fig. 2 | Classification of different cell populations within the adipose tissue.** a. Flow cytometry dot plots show the new gating strategy used to sort eP1, eP2 and eP3. b. Quantification of adipogenesis (left) and cell number (right) of Lin\(^-\)Scal\(^+\) cells, eP1 cells, eP2 cells, VAP1\(^-\)CD142\(^+\) APCs, VAP1\(^-\)CD55\(^-\) cells, DN:CD142\(^-\) and eP3 induced by A Cocktail (1 \(\mu\)M dexamethasone, 0.5 \(\mu\)M isobutylmethylxanthine and 1 \(\mu\)M insulin). Data are shown as mean \(\pm\) s.e.m., n = 8 independent wells. Data were analyzed with one-way analysis of variance (ANOVA); F(6,49) = 69.7002, P < 0.0001. c. Microscopy images of different cell populations shown in b on differentiation day 6. h Photograph was repeated twice. d. Relative mRNA levels of P1 marker (Cd142, Pcsk6, Efd1, Pn16 and Sm23), P2 markers (Vap1, Col4a1, Sparc1 and Sclt1) and A\(_{ref}\) cell-specific marker (Cd142, Gdf10, Igfbp3, Fmo2 and Clec11a) genes in different cell populations; n = 3 biological replicates. Data show mean \(\pm\) s.e.m. e. Quantification of adipogenesis (left) and cell number (right) of Lin\(^-\)Scal\(^+\) cells, eP1 cells, eP2 cells and eP3 induced by A Cocktail. Data show mean \(\pm\) s.e.m.; n = 6 independent wells. Data were analyzed with one-way ANOVA; F(3,20) = 280.8, P < 0.0001 (left); multicomparison with Lin\(^-\)Scal\(^+\) group was performed by two-stage step-up method with false discovery rate (FDR) = 0.05. F(3,20) = 2.838, P = 0.064 (right). f. Quantification of adipogenesis (left) and cell numbers (right) of Lin\(^-\)Scal\(^+\) cells, eP1 cells, eP2 cells and eP3 induced by C Cocktail (1 \(\mu\)M insulin). Data are shown as mean \(\pm\) s.e.m., n = 6 independent wells. Data were analyzed with one-way ANOVA. F(3,20) = 48.27, P < 0.0001 (left), multicomparison with Lin\(^-\)Scal\(^+\) group was performed by two-stage step-up method with FDR = 0.05. F(3,20) = 0.189, P = 0.903 (right). g. Microscopy images of different cell populations shown in e and f on differentiation day 6. In all panels, nuclei were stained with Hoechst 33342 (blue) and lipids were stained with LDS540 (yellow). Scale bars, 100 \(\mu\)m.
(A cocktail) (Fig. 2e–g). These data imply that eP1 cells, which express low levels of Pparg (Extended Data Fig. 1a,b), are at an earlier stage of adipogenesis and might have not committed to the adipocyte lineage.

Identification of Rspo2 as a new marker of P3 cells. We previously could show that Areg/P3 cells regulate adipogenesis in a paracrine fashion through Spink2 and Rtp3 (ref. 9). To characterize P3 cells in more detail, we compared bulk RNA-seq of Areg (Lin–Sca1+CD142++) cells to Lin–Sca1+CD142− cells from mouse ingWAT7. A total of 216 differently regulated genes, of which 56 encoded secreted proteins, were enriched in Areg/P3 cells (Extended Data Fig. 3a). The list was reduced to 41 genes after exclusion of candidates expressed in mature adipocytes or other cell populations. When filtered for

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**Diagram Details**

- **Diagram a**: Shows a scatter plot with double-negative staining for Lin–Sca1+ eP1 eP2 eP3.
- **Diagram b**: Depicts a bar graph comparing mature adipocytes (%).
- **Diagram c**: Displays images of Lin–Sca1+ eP1 eP2, VAP1−CD55− DN:CD142− eP3.
- **Diagram d**: Features a graph comparing cell number per field.
- **Diagram e**: Illustrates mature adipocytes (%).
- **Diagram f**: Shows a graph comparing cell number per field.
- **Diagram g**: Illustrates A and C cocktail treatments.

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**Tables and Figures**

- **Table**: Contains cell number per field data for various cell populations.
- **Figures**: Show various markers and immune cell populations.
Recombinant Rspo2 protein inhibits adipogenesis through Lgr4 in primary SVF cells. Rspo2 could be detected in eP3 cell culture medium at 500–600 pg/mL (Extended Data Fig. 3h). To test whether Rspo2 inhibits adipogenesis in vitro, we added recombinant Rspo2 (rec.Rspo2) in an SVF differentiation assay starting from 2 d before until 2 d after cocktail induction (Fig. 3i) and observed that increasing amounts of Rspo2 led to a progressive decrease in adipocyte formation (Fig. 3j,k) without affecting cell numbers (Extended Data Fig. 3i). When rec.Rspo2 was inactivated by heat its inhibitory effect on adipogenesis was lost (Fig. 3j).

Among the three receptors of RSPO2 leucine-rich repeat-containing G protein-coupled receptor 4–6 (LGR4–6), we found that only Lgr4 was expressed at high levels in Lin− cells (Extended Data Fig. 3j). Depletion of Lgr4 expression induced more adipocyte formation in SVF, whereas knockdown of either Lgr5 or Lgr6 did not alter adipogenesis (Fig. 3m,n). Moreover, SVF adipogenesis was not inhibited by rec.Rspo2 when Lgr4 expression was ablated (Fig. 3m,n).

Rspo2 is an enhancer of the Wnt signaling pathway, which plays a key role in regulation of adipocyte commitment. As single-cell trajectory analysis (Fig. 1c,d) suggests a transition of P1 to committed P2 cells, we next aimed to identify mechanisms that regulate this transition. Enrichr analysis of differentially expressed genes of eP1 and eP2 cells suggested an enrichment of the Wnt signaling pathway in eP1 cells (Extended Data Fig. 3k,l) and adipogenesis genes in eP2 cells (Extended Data Fig. 3m,n). Notably, Rspo2 receptor Lgr4 was enriched in P1 cells compared to P2 cells (Fig. 3o and Extended Data Fig. 3j), which suggests that P1 and not P2 cells might be the target of Rspo2. In accordance with our hypothesis, when exposed to rec.Rspo2 during adipogenesis (Fig. 3i), eP1, but not eP2 cells, exhibited less adipocyte formation (Fig. 3p–q). Because eP1 cells are at an earlier stage of adipogenesis compared to eP2 cells, we assumed that Rspo2 might affect adipocyte commitment and late-phase adipocyte formation. This was confirmed by the finding that adipogenesis was unaltered when cells were exposed to rec.Rspo2 during differentiation from day 3 to 6 (late phase) (Extended Data Fig. 3r–s).

We found that rec.Rspo2 upregulated Wnt signals by inducing β-catenin levels in a time-dependent manner, independent of cell number changes (Extended Data Fig. 3t–w). Similarly, rec.Rspo2 upregulated Wnt signals in eP1 cells 24 h after treatment (Extended Data Fig. 3x,y) and the effect was blunted after ablation of Lgr4 by siRNA (Extended Data Fig. 3x–z). Collectively, these data demonstrate that Rspo2 inhibits P1 commitment during adipogenesis, possibly by regulation of the Wnt/β-catenin signaling pathway through Lgr4.

Rspo2 inhibits adipogenesis of eP1 cells in vivo. To extend our data to the in vivo situation, we first generated an adenovirus–adeno-associated virus (AAV) system to express Rspo2 under the chicken β-actin promoter (CAG), while pAAV–CAG–GFP was used as infection control. Next, eP1 or eP2 cells from ingWAT were resuspended in Matrigel, which contained either pAAV–CAG–Rspo2 or pAAV–CAG–GFP and was transplanted subcutaneously into mice (Fig. 4a). In addition, rec.Rspo2 was supplemented into the Matrigel to ensure that cells were exposed to Rspo2 during the initial phases of adipogenesis as AAV-mediated expression requires at least 5 d. Mice were exposed to a high-fat diet (HFD) for 4 weeks to induce adipocyte formation after transplantation. pAAV–CAG–Rspo2 significantly increased Rspo2 messenger RNA levels in Matrigel plugs (Fig. 4b) and reduced adipocyte formation of eP1 cells (Fig. 4c,d and Extended Data Fig. 4a). In accordance with the in vitro data, Rspo2 did not inhibit adipogenesis of eP2 cells in mice (Fig. 4e,f and Extended Data Fig. 4b).

To investigate adipocyte formation within ingWAT during obesity, 6-week-old AdipoCre-NucRed mice received either pAAV–CAG–GFP or pAAV–CAG–Rspo2 by tail-vein injection and 2 weeks after injection, mice were switched to HFD or continued on a chow diet for another 10 weeks (Fig. 4g). By using AdipoCre-NucRed transgenic mice we quantified adipocyte numbers using qPCR (Extended Data Fig. 4c,d). At 12 weeks after AAV injection, higher Rspo2 level were detected in liver and ingWAT (Fig. 4h,i). At 10 weeks after HFD, adipocyte numbers significantly increased in ingWAT (Fig. 4j; HFD CAG–GFP group versus Chow CAG–GFP group). Meanwhile, a reduced number of adipocytes was detected in Rspo2 overexpression mice (Fig. 4j; HFD CAG–GFP group versus HFD CAG–Rspo2 group). Higher levels of Rspo2 in chow-diet-fed mice did not alter adipocyte numbers in ingWAT. Moreover, in visWAT, reduced adipocyte numbers were detected in
**Rspo2 leads to unhealthy adipose tissue expansion and insulin resistance in vivo.** As Rspo2 inhibits adipocyte formation in vivo, we next investigated, whether Rspo2 influences adipose tissue expansion during obesity. Therefore, pAAV–CAG–Rspo2 was injected into 8-week-old diet-induced obese mice (Extended Data Fig. 5a), which led to a fivefold increase in Rspo2 protein levels in the liver (Fig. 6a–b) and plasma (Fig. 6c) and a twofold increase in ingWAT (Fig. 6d) and visWAT (Fig. 6e). We observed decreased weight gain in pAAV–CAG–Rspo2–infected mice (Fig. 6f), accompanied by reduced fat mass (Fig. 6g) both in ingWAT and visWAT (Fig. 6h and Extended Data Fig. 5b) independent of food intake (Extended Data Fig. 5c) or energy expenditure (Extended Data Fig. 5d). In addition, Rspo2 overexpression not only reduced adipocyte formation (Fig. 4j) but also led to adipocyte hypertrophy (Fig. 6i–k). Notably, even though Rspo2 overexpression reduced weight gain, higher levels of Rspo2 exhibited a worsened insulin sensitivity during an insulin tolerance test (ITT) (Fig. 6l–m), without affecting fast- ing blood glucose (Fig. 6n) or hepatic glucose secretion (Extended Data Fig. 5g,h). Fasting triglyceride (TG) levels were unaltered between the two groups (Fig. 6o), whereas less TG accumulated in the livers of Rspo2-overexpression mice (Extended Data Fig. 5e,f) independent of any changes in hepatic TG secretion (Extended Data Fig. 5i,j). These data suggest that adipocyte hypertrophy due to increased Rspo2 levels might be one factor contributing to the worsened metabolic phenotype.

Therefore, we next investigated whether Rspo2 overexpression might impair insulin sensitivity in mice whose ingWAT was targeted by pAAV–CAG–Rspo2 to increase intra-tissue Rspo2 levels (Extended Data Fig. 5k). Rspo2 overexpression in ingWAT (Extended Data Fig. 5l) did not affect body weight (Extended Data Fig. 5n) but slightly decreased ingWAT tissue weight (Extended Data Fig. 5o) without altering energy expenditure (Extended Data Fig. 5p). Furthermore, an increased number of large adipocytes was observed in ingWAT in Rspo2-overexpressing mice after 7 weeks of HFD feeding (Fig. 6p–q). However, the observed hypertrophy of the ingWAT did not impair insulin sensitivity (Fig. 6r). Taken together, we show that Rspo2 might affect systemic insulin sensitivity, possibly in part by regulation of de novo adipocyte formation and adipose tissue expansion.

**Serum Rspo2 correlates with insulin resistance in individuals with obesity.** Given the fact that higher circulating Rspo2 in obese mice led to insulin resistance, we queried this association using serum from obese metabolically healthy and unhealthy individuals.

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**Fig. 4 | Rspo2 inhibits adipogenesis of eP1 cells in vivo.** a–f. Experimental scheme (a) for cell transplantation in Matrigel. Rspo2 expression in eP1 Matrigel plugs and in eP2 Matrigel plugs (b). Quantification of adipocytes and cell number in eP1 Matrigel plugs (c) and eP2 Matrigel plugs (d). Representative hematoxylin and eosin (H&E) staining of eP1 Matrigel plugs (e) and eP2 Matrigel plugs (f). Data show mean ± s.e.m., n = 3 biological replicates (b), n = 5 biological replicates (c,e). Data analysis was performed using a two-tailed Student’s t-test. Scale bar, 100 mm. g–k. Experimental scheme for overexpression of Rspo2 in AdipoCre-NucRed mice fed with HFD or Chow diet. Western blot images (h) and quantification (i) of Rspo2 protein in liver and ingWAT; Hsp90 bands were used as loading control. Quantification of adipocyte numbers in ingWAT (j) and visWAT (k) of mice shown in g. Data are shown as mean ± s.d., n = 6 mice. Data analysis was performed by two-tailed Student’s t-test and one-way ANOVA (j,k). In j, Total cell number, F(3,20) = 14.4, P < 0.0001; adipocyte, F(3,20) = 15.50, P < 0.0001; non-adipocyte, F(3,20) = 14.1, P < 0.0001. In k, total cell number, F(3,20) = 14.4, P < 0.0001; adipocyte, F(3,20) = 15.50, P < 0.0001; non-adipocyte, F(3,20) = 14.1, P < 0.0001. l–o. Experimental scheme (l) for overexpression of Rspo2 in ingWAT by injection of AAV into ingWAT of AdipoCre-NucRed mice. Western blot images (m) and quantification (n) of Rspo2 protein in ingWAT of mice shown in l. Hsp90 bands were used as loading control. Quantification of cell numbers by quantitative PCR in ingWAT (o). Data show mean ± s.d., n = 5–6 mice. Data were analyzed using a two-tailed Student’s t-test.
**Figure Legend**

**a** 10 days after transplantation.

- **b** Scatter plot: TDTomato^+ cells vs. VAP1^+ cells.
  - CD142-APC.
  - Donor: mTmG mice, Recipient: WT mice.

**c** Bar chart: CAG-RSP2/HSP90.
  - eP1, dP4, p16, P53.
  - eP1^+ cells (derived from eP1).

**d** Bar chart: eP1 from donor.
  - VAP1^+ cells (derived from eP1).

**e** Bar chart: eP2 from donor.
  - VAP1^+ cells (derived from eP1).

**f** Bar chart: eP3 from donor.
  - VAP1^+ cells (derived from eP1).

**g** Injection of AAV into ingWAT.

**h** Graph: kD.
  - CAG-GFP, CAG-RSP2.
  - HSP90, RSPO2.

**i** Graph: Pparg/cebp a.

**j** Graph: eP1/SVF (%).

**k** Graph: eP2/SVF (%).

**l** Graph: Pparg/cebp a.

**m** Graph: CD55/VAP1/SVF (%).

**n** Injection of AAV into sub.

**o** Graph: VAP1^+ cells (tdTomato^+).

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**Key Figures**

- **a** 10 days after transplantation.
- **b** Scatter plot: TDTomato^+ cells vs. VAP1^+ cells.
- **c** Bar chart: CAG-RSP2/HSP90.
- **d** Bar chart: eP1 from donor.
- **e** Bar chart: eP2 from donor.
- **f** Bar chart: eP3 from donor.
- **g** Injection of AAV into ingWAT.
- **h** Graph: kD.
- **i** Graph: Pparg/cebp a.
- **j** Graph: eP1/SVF (%).
- **k** Graph: eP2/SVF (%).
- **l** Graph: Pparg/cebp a.
- **m** Graph: CD55/VAP1/SVF (%).
- **n** Injection of AAV into sub.
- **o** Graph: VAP1^+ cells (tdTomato^+).
Fig. 5 | Rsop2 inhibits transition of eP1 cells to eP2 cells. a-f. Experimental scheme (a) for transplantation of tdTomato+ eP1 cells into inguinal adipose tissue of wild-type (WT) mice. FACS analysis (b) of VAP1 and CD142 expression in tdTomato+ eP1 cells 10 d after transplantation. Expression of P1 marker genes (c) (Cd55, Ddap, Pit6 and PscK6), P2 marker genes (d) (Vap1, Icam1, Col4A1 and Sparcl1), Pparg and Ceiba (e) and P3 marker genes (f) (Cd142, Gdf10, Clec11a and lgfbp3) in eP1 cells (from donor mice), eP2 cells (from donor mice), eP3 cells (from donor mice), VAP1+ cells (derived from implanted eP1 cells) and VAP1+ cells (derived from implanted eP1 cells). Data are shown as mean ± s.e.m., n = 4 biological replicates. g-m. Experimental scheme (g) for injection of AAVs into ingWAT for overexpression of RSPO2. Western blot images (h) and quantification (i) of RSPO2 protein and Rsop2 mRNA (j) in ingWAT. FACS analysis of eP1/SVF (k), eP2/SVF (l), Cd55+VAP1+ (m) in ingWAT. Data are shown as mean ± s.e.m., n = 6 mice (k,l), n = 5–6 mice (j), n = 5 mice (k–m). Data were analyzed using two-tailed Student’s t-test. n–o. Experimental scheme (n) for transplantation of tdTomato+ eP1 cells into RSPO2 overexpression mice. FACS analysis of (VAP1+tdTomato+) cells in tdTomato+ eP1 cells (o). Data are shown as mean ± s.e.m., n = 5 biological replicates. Data were analyzed using a two-tailed paired Student’s t-test.

Sixty patients (body mass index (BMI) = 45.6 ± 5.6 kg m⁻²) were divided into an insulin-sensitive group (HOMA-IR = 0.9 ± 0.4) and an insulin-resistant group (HOMA-IR = 3.8 ± 0.9). In men, RSPO2 levels were significantly higher in the insulin-resistant group and we observed the same trend in women (Fig. 6). Similar to the mouse study, circulating RSPO2 levels exhibited an inverse correlation with the glucose infusion rate (Fig. 6) in men but not women. In line with the observation from mice, we noted that circulating RSPO2 levels correlated with the visceral fat area (Fig. 6a) and maximal adipocyte volume (Fig. 6b) in men but not women.

Single-nucleus sequencing revealed Rsop2 inhibit adipocyte formation in vivo. To comprehensively evaluate the effects of Rsop2 on different APC populations we performed 10X snRNA-seq on nuclei isolated from ingWAT of pAAV–CAG–GFP- and pAAV–CAG–Rsop2-infected mice. Unsupervised clustering identified seven clusters of cells (Fig. 7a), which were annotated on the basis of known cell marker genes (Fig. 7b). Among all clusters, adipocyte markers (Adipoq, Lep, Plin1, Cidec and Dgat2) were found in the adipocyte clusters (Fig. 7b and Extended Data Fig. 6c), while pre-adipocyte markers (Ly6a and Pdgfra) were found in the APC clusters (Fig. 7b). 10X snRNA-seq analysis revealed reduced adipocyte numbers (Fig. 7c) in pAAV–CAG–Rsop2-infected ingWAT, which underscores our previous findings and suggests that RSPO2 can inhibit adipocyte formation. Besides reducing adipocyte numbers, RSPO2 facilitated macrophage recruitment into ingWAT (Fig. 7a,c), which might contribute to the observed insulin resistance. RSPO2 overexpression also affected many of the identified clusters with regards to their gene expression profile (Fig. 7d and Extended Data Fig. 6e–g).

Next, we clustered pre-adipocyte nuclei into five subpopulations named PA–1–PA–5 (Fig. 7e). PA-1 represent noncommitted APCs (P1) with expression of P1 marker genes (Fig. 7f and Extended Data Fig. 6h), Sema3e, Pit16 (Extended Data Fig. 6h) and low Pparg expression (Fig. 7f). PA-2 represents the P2-2 population. Even though P2-2 marker Vap1 or Icam1 were barely detectable in PA-2 nuclei (data not shown), the committed preadipocyte marker Pparg was highly expressed in PA-2 nuclei (Fig. 7f). Similar to P2-2, we observed that some PA-2 nuclei expressed P3 markers such as Fmo2 and Cd142 (Fig. 7f and Extended Data Fig. 6h). The PA-3 population represents the P3 population based on expression of marker genes Cd142, Fmo2 and Meox2 (Fig. 7f and Extended Data Fig. 6h). The PA-4 population defines a cluster of proliferating cells with high levels of cell-cycle genes such as Top2a and Mki67 (Extended Data Fig. 6k). PA-5 represents another cluster of committed pre-adipocytes, which expresses Pparg (Fig. 7f and Extended Data Fig. 6j). Overexpression of RSPO2 led to more active Wnt signaling indicated by higher expression of Ctnnb1 (Extended Data Fig. 6d) and led to a higher proportion of adipocyte progenitors (PA-1) and reduced committed pre-adipocytes PA-5 (Fig. 7g,h). These data are in line with our cell transplant experiments, which suggest that RSPO2 inhibits P1 transition into committed preadipocytes (Fig. 5n,o). Collectively, our data suggest that Rsop2 inhibits adipocyte formation during obesity, which leads to adipocyte hypertrophy and macrophage infiltration into adipose tissue. We propose that a combination of these factors contribute to the development of insulin resistance in RSPO2-overexpressing mice.

Discussion

Adipogenesis follows a highly ordered process that initiates during embryogenesis and persists throughout life, requiring coordination of multiple regulatory signaling pathways and cell types. Recent studies using scRNA-seq have reported mouse and human adipocyte tissue cell-type composition. While our study mainly focuses on SAT, adipogenesis regulates satiety and visceral adiposity

Fig. 6 | Circulating RSPO2 leads to unhealthy expansion of adipose tissue and insulin resistance in vivo. a–o. RSPO2 overexpression in mice by tail-vein delivery of pAAV–CAG–Rsop2. Representative immunoblot (a) and quantification of RSPO2 and HSP90 in liver (b), circulation (c), ingWAT (d) and visWAT (e) in RSPO2-overexpression mice. Body weight curve (f), lean mass and fat mass (g) and ingWAT and visWAT tissue weight (h) of AAV-infected mice. Representative H&E staining images (i), average of adipocytes size (μm²) and adipocyte size frequency distribution of ingWAT. Blood glucose normalized to initial blood glucose after insulin injection (j) in ITT and area under the curve (AUC) was quantified as shown in k. Fasting blood glucose (n) and triglycerides (o) in AAV-infected mice. Data are shown as mean ± s.e.m., n = 6 mice. Data were analyzed using a two-tailed Student’s t-test. Scale bar, 100 μm. p–r, RSPO2 overexpression by injection into ingWAT. Adipocyte size frequency distribution (p) and representative H&E staining of ingWAT. Data are shown as mean ± s.e.m. Glucose levels in blood in ITT and glucose was normalized to time point 0 (r). Data are shown as mean ± s.d. Comparison of AUC (s) in ITT. Data are shown as mean ± s.e.m., n = 5 mice (CAG–GFP), n = 6 mice (CAG–Rsop2). Data analysis was performed using a two-tailed Student’s t-test. s, Circulating RSPO2 levels in insulin-sensitive and insulin-resistant individuals. Data are shown as mean ± s.d., n = 11 (male, insulin sensitive), n = 10 (male, insulin resistant), n = 18 (female, insulin sensitive), n = 21 (male, insulin resistant). Data analysis was performed using a two-tailed Student’s t-test. t–v, Spearman correlation coefficient analysis of circulating RSPO2 and glucose infusion rate (t), visceral fat area (u) and maximum adipocyte volume (v). P values are corrected by two-stage step-up method of Benjamini, Krieger and Yekutieli with an FDR = 0.05.
Fig. 7 | snRNA-seq reveals Rspo2 reducing adipocytes number in vivo. a, Integrated analysis of snRNA-seq, including 14,303 nuclei from ingWAT in mice fed on HFD with chronic expression of GFP or Rspo2 by AAV, yielding 2,218 genes (median). Unsupervised clustering shown as a UMAP plot, seven populations were identified, including adipocytes (adipo) (red), pre-adipocytes (PreA) (blue), macrophages (macro) (green) and natural killer (NK) cells (orange). b, Dot plots for representative markers of each cluster. Expression level (indicated by red color) refers to the log normalized ratio of gene expression reads, normalized to the sum of all reads within each nucleus. Percent expressed refers to the ratio of cells within each cluster that express the genes listed in x axis. c, Cluster compositions in CAG–GFP \( n = 7,190 \) nuclei and CAG–Rspo2 \( n = 7,143 \) nuclei conditions. d, Violin plots for Acss2, Nkain2, Sntg1, S100a6, Mrc1 and Gpx1, which are differentially expressed between CAG–GFP and CAG–Rspo2 conditions. e, Subclustering analysis of preadipocyte populations. Unsupervised subclustering of 6,411 preadipocyte nuclei from ingWAT, yielding 2,577 (median) genes. Five subpopulations of preadipocytes (PA-1–PA-5) were identified. f, Feature plots for Dpp4, Pparg and Fmo2, shown as separated plots by conditions. g, h, Pre-adipocyte cluster compositions in CAG–GFP \( n = 3,539 \) nuclei and CAG–Rspo2 \( n = 2,872 \) nuclei conditions.
formation from precursor cells in the context of muscular dystrophy\(^\text{32}\). Hepler et al.\(^\text{11}\) furthermore identified an anti-adipogenic PDGFR\(^\beta\)-population within visWAT, which exerts a proinflammatory phenotype named fibroinflammatory progenitor cells (FIPs). Shao et al.\(^\text{1}\) recently described the adipogenic differences of PDGFR\(^\beta\)-cells derived from ingWAT and visWAT, which indicate that both depots utilize different cellular and molecular mechanisms to regulate adipogenesis. A study on spatially resolved transcriptional profiling with scRNA-seq of human WAT\(^\text{19}\) suggests the presence of P3-like cells (C09 cluster), which were enriched in areas close to macrophages and linked to fibrotic/vascular structures, similar to the mouse P3 population\(^\text{1}\), suggesting that other regulatory cell types exist that might share some characteristics of ingWAT P3 cells.

De novo adipocyte formation within ingWAT is associated with age and gender. \(^\text{\textsuperscript{12}}\)N-thymidine-labeled newly formed adipocytes were detected in both ingWAT and visWAT in mice fed with HFD for 4 weeks of age\(^\text{35}\). Robust adipogenesis was reported in ingWAT of female mice exposed to HFD, but not in male mice. When discussing these data, we should keep in mind that newly formed adipocytes might be considered as existing adipocytes due to incomplete tamoxifen washout or newly formed adipocytes derived from precursor cells, which did not proliferate possibly due to a cell conversion from P1 to P2, which might not rely on proliferation. Therefore, other methods to examine adipogenesis within ingWAT during obesity will be required. Quantification of the absolute adipocyte numbers of the complete depot by qPCR in this context could pose an unbiased strategy to investigate adipocyte formation during adipose tissue expansion\(^\text{17}\).

HFD mice have more eP3 cells (Extended Data Fig. 7a,b) in ingWAT and express higher levels of RSPO2 in circulation and adipose tissue (Extended Data Fig. 7c,d), suggesting that eP3 or RSPO2 might regulate adipose tissue expansion during obesity. Another member in the R-spondin family, Rspos3 (ref. \(^\text{2}\)), which has also been detected in ingWAT (Extended Data Fig. 7e), was shown to be associated with reduced lower body fat, enlarged gluteal adipocytes and insulin resistance in humans and regulates adiposity in zebrafish, which is in line with our observations. However, these data have to be treated with caution as the findings from Rspos3 as well as our work on Rspos2 are based on changes in circulating levels, which could be due to other adipose tissue-independent mechanisms, even though we did not observe changes in liver function.

Overexpression of RSPO2, specifically in ingWAT, confirmed our finding that RSPO2 can inhibit adipocyte formation and cause adipocyte hypertrophy, with a trend toward development of insulin resistance. As RSPO2 is a secreted protein, expressed also in non-adipose tissue\(^\text{35}\), loss of RSPO2 in WAT might be compensated by circulating RSPO2.

Circulating RSPO2 correlated with insulin sensitivity and fat distribution in men but not in women. The detected sex differences may reflect the previous notion that adipocyte size and function are distinctly associated with parameters of metabolic health in women and men\(^\text{46,47}\). In this context, it has been suggested that SAT of premenopausal women has a greater capacity for adipose expansion via hyperplasia and hypertrophy; although larger, these glucose–femoral adipocytes remain insulin sensitive\(^\text{48}\). Moreover, sex differences have been reported in adipogenesis and metabolism\(^\text{19,40}\).

Inherently a study such as this has many limitations. Besides the ones outlined above it should be noted that without a transgenic mouse model that can be used to deplete P3 cells or induce loss of RSPO2 specifically within adipose tissue, it will be difficult to determine to which degree ingWAT derived P3 cells or RSPO2 contribute to metabolic adaptations. Also in human studies, RSPO2 expression within adipose tissue and its relation to metabolic diseases will require a fractionation of adipose tissue in larger patient cohorts. Last, while our in vitro experiments demonstrate that RSPO2 inhibits adipogenesis of P1 cells through Lgr4, a model with specific loss of Lgr4 in P1 cells will be required to delineate the effects on metabolism.

In conclusion, here we delineate additional regulatory mechanisms that control adipose tissue plasticity governed by a cellular and molecular crosstalk of different cell types within the SVF of adipose tissue. We identify RSPO2 as a new functional effector of the P3 population that controls adipocyte formation by modulating maturation of P1 to P2 cells and might therefore play an important role in regulating adipose tissue plasticity.

**Methods**

**Animals.** C57BL/6 mice were obtained from Charles River Laboratories and C57BL/6;Rosa26\(^\text{cre}\)\(^\text{lox}\) mice (stock no. 007686), AdipoCre mice (stock no. 028020), Nfatc1\(^\text{cre}\)\(^\text{lox}\) mice (stock no. 026806) were obtained from JAX. Mice were kept on a 12-h:12-h light/dark cycle and 20–60% (25 °C) humidity in a pathogen-free animal facility of SLA ETH Zurich. The HFD used contained 60% (kcal%) fat (diet no. 3346, Provimi Kliba SA). All animal experiments were approved by the cantonal veterinary office Zurich.

**Human cohort.** Sixty individuals were selected from the Leipzig Obesity Biobank to define age-, BMI- and sex-matched groups of insulin-sensitive (n = 30) and insulin-resistant (n = 30) individuals with obesity. Definition of the metabolic healthy obese participants was based on the glucose infusion rate (GIR) during the last 30 min of the steady state in euglycemic–hyperinsulinemic clamp. GIR > 60 μmol kg\(^{-1}\) min\(^{-1}\); insulin-sensitive; GIR < 60 μmol kg\(^{-1}\) min\(^{-1}\); insulin-resistant. All individuals fulfilled the previously reported inclusion and exclusion criteria\(^\text{4}\). BMI was calculated as weight divided by squared height. Waist circumference was measured at the midpoint between the lower ribs and iliac crest. Percentage body fat was measured by bioimpedance analysis. Abdominal visceral and subcutaneous fat areas were calculated using computed tomography or magnetic resonance imaging scans at the level of L4–L5. Insulin sensitivity was assessed using the euglycemic–hyperinsulinemic clamp method\(^\text{4}\).

The study was approved by the ethics committee of the University of Leipzig (approval nos. 159-12-21052012 and 017-12-23012012) and all participants gave written informed consent before taking part in the study.

**Re-processing scRNA-seq datasets.** The datasets from Schwalie et.al.\(^\text{13}\) and Merrick et al.\(^\text{3}\) were used. Briefly, we first used STARSolo (2.7.3a) to map reads and de-multiplex cell barcodes with parameters: -wimAnchorMultimapNmax 2,000–outFilterMultimapNmax 2,000–outSAMMultiplematchFlag AllBestScore–outSAMmultNmax 1–limitOutStoneRead 10,000–limitOutSflcollapsed 3,000,000–outSAMattrHs MATtributes NH nM AS CR UR CB UB GX GN sS sQ sM–soloType CB_UMI_Simple–soloCBwhitelist 737K-april-2014_rc.txt–soloCBlen 14–soloUMIstart 6,000 genes detected and with more than 30% of Unique molecular identifiers (UMIs) derived from mitochondrial RNAs. Raw UMI counts were normalized and log-transformed per 10,000 UMIs. When scaling the normalized count for calculating principal components (PCs), the number of UMIs per cell and the percentage of mitochondrial RNAs were regressed out. We selected top 30 PCs for graph-based clustering with resolution parameter 0.3 (snn), which has been implemented in the FindNeighbors and FindClusters functions of the Seurat R package. We used the RunUMAP functions of Seurat package to generate UMAP with n.neighbors = 50. For data integration, we first pooled individual datasets from both studies and selected top 2,000 most-variable genes from each dataset for canonical correlation analysis (CCA). The top 60 dimensions were used for identifying anchors (FindIntegrationAnchors). With these anchors, datasets were integrated with IntegrateData function. We scaled the integrated data and computed top 50 PCs for cell clustering (with resolution 0.3) and UMAP plot.

For re-clustering of mouse ingWAT cells (Fig. 1c), we applied unsupervised clustering on sctransform\(^\text{3}\) based on the 6,500 most variable genes. Cluster P5 was removed from subsequent analysis owing to its low gene expression (Extended Data Fig. 1b). The top ten markers for each cluster (logfc.threshold = 0.5) were plotted on the heat map (Extended Data Fig. 1i).

**Single-cell trajectory analysis.** Cellranger v.3.0 (by 10X Genomics) was applied for RNA analysis. We used STAR (v.2.6.0) as the mapping tool and a k-mer of 40 was used for mapping. 5,000 genes were retained for the analysis. Biclustering with UMAP was used to find biological meaningful clusters within the SPAn dependence. The top 60 clusters were identified, and the top ten markers for each cluster were plotted on the heat map (Extended Data Fig. 1i).

**Gene expression and differential gene analysis.** For differential gene analysis between different datasets, the DeSeq2 package was used. The top 600 highly variable genes were retained for the analysis. The top 150 genes were plotted on the volcano plot of the heat map (Extended Data Fig. 1i).

**Statistical analysis.** All statistical analyses were performed using GraphPad Prism 8 and R version 3.6.2. Significant levels were set at *p* < 0.05 and all error bars represent the mean ± SEM. The data obtained were analyzed using one-way ANOVA with post-hoc tests. The *p* values presented in the text are from the post-hoc tests. For the statistical analysis of the metabolic parameters (GWG, LPL activity, circulating RSPO2, etc.), the data were analyzed using the Kruskal-Wallis test followed by Dunn’s multiple comparisons test. For the analysis of the protein expression levels, the data were analyzed using the Student’s t-test.
Gene overlapping analysis between human and mouse preadipocyte. The R package biomaRt v2.48.2 was used to map the overlapping genes between human and mouse single-cell data based on positive cluster markers. Significance of overlap was calculated based on hypergeometric distribution and simulations using the R packages vst v.4.1 and purr v.0.3.4.

snRNA-seq of adipose tissue. Nuclei were isolated from mouse ingWAT infected with either pAAV–CAG–GFP or pAAV–CAG–Rspo2, following a previously established protocol. Around 10,000 nuclei were loaded to 10X Genomics Chromium and libraries were prepared with Single Cell 3′ (v3) RNA kit. Sequencing was performed with a NovaSeq 6000 (Illumina). Raw reads were mapped to GENCODE Release M26 (GRCm39). Cellbender was applied to remove ambient RNA and empty droplets; scrublet was applied to remove doublets. Seurat was applied for unsupervised clustering and CCA was applied for integrative analysis.

Mouse SVF isolation and culture. For SVF isolation, adipose tissues were minced with scissors and incubated in 1 mg/ml collagenase (C6866-1G, Sigma-Aldrich) in collagenase buffer (25 mM NaHCO3, 12 mM KH2PO4, 1.2 mM MgSO4, 4.8 mM KCl, 120 mM NaCl, 1.4 mM CaCl2, 5 mM glucose, 5.2% BSA and 1% Pen/Strep) was added and samples were centrifuged at 500 × g at 300g. The SVF pellet was resuspended in 2 ml erythrocyte lysis buffer (154 mM NH4Cl, 10 mM KHCO3 and 0.1 mM EDTA) and pH 7.4 adjusted to 7.0 and then incubated at room temperature for 10 min, filtered through 40-μm cell strainer. After centrifuging for 5 min at 200g, the pellets were resuspended in FACS buffer (PBS with 3% FBS, 1 mM EDTA and 1% P/S). Cells were then centrifuged at 1000g for 5 min and cell pellets were resuspended in FACS buffer for antibody staining or in culture medium for seeding. Lin−Sca1−CD142−CD142+ cells were purified by staining with anti-mouse CD31–PECy7 (1:600 dilution), anti-mouse TER119–PECy7 (1:600 dilution) (all from Beckman Coulter) and anti-mouse CD142+ antibody were conjugated with fluorescein (FITC) antibody and analyzed by Sony SH800 cell sorter. siRNA-mediated knockout experiments. A pool of 3–4 individual siRNA probes were used to knockdown targets at a final concentration of 100 nM. siRNA probes were dissolved in 1.5% Lipofectamine RNAiMAX (Invitrogen, 13778510) in Opti-MEM I reduced serum medium (Invitrogen, 31985062). 3–6-well plates, 100 μl primary cells (25 × 104 cells/ml) were reverse-transfected with 20 μl 100 nM of corresponding siRNA. At 48h after transfection, cells were changed to culture medium without siRNA. For the Transwell co-culture experiment, eP3 cells were cultured in inserts with corresponding siRNA for 48h with a blank receiver plate. At 48 h after transfection, the inserts were washed with warm PBS twice and co-cultured with Lin−Sca1−CD142+ cells growing on receiver plates until the end of the experiment. For the β-catenin experiment, 48 h after transfection, eP1 cells were changed to culture medium supplemented with 0.5 μg/ml recombinant RSPO2 for 24 h.

In vivo differentiation of SVF. A total of 200,000 sorted cells (eP1 or eP2 cells from male mice) were resuspended in 200 μl of Matrigel (Corning, 356234) supplemented with AAV and 5 μg rec.RSPO2 (heat inactivation for control group), then injected subcutaneously in the abdomen of the same 4-week-old mouse. After 4 weeks of HFD, Matrigel plugs were excised and fixed in 4% paraformaldehyde overnight, dehydrated and embedded in paraffin. Sections of 5 μm were stained with H&E and examined. From each plug, pictures of at least three full sections were taken and adipocyte numbers as well as the number of nuclei were determined automatically with Cell Profiler software.

AAV production. Adenoviral particles carrying overexpression constructs under control of the CAG promoter to express either GFP or RSPO2. The coding transcript sequence for RSPO2 was obtained from OriGene (MR216699) and insert into vector pAAV–CAG–GFP (Addgene, 37825) to replace GFP. The direction of insertion and nucleotide sequences of the insert was verified by sequencing analysis. Microsynth. Virus was produced in HEK 293A cells (Invitrogen) and purified with the AVANCE Cell Line Reconstitution System (Gene Bioscience, AAV100A-1) following the manufacturer’s recommendations for a standard-yield reaction (2 h of amplification time).

Adipocyte number quantification by qPCR. AdipoRe-NucRed® mice were used for quantification of adipocyte numbers in adipose tissue. Adiponectin−/− adipoocytes express tdTomato as a result of Cre-recombination of tdTomato allele (Extended Data Fig. 4c). Briefly, a known quantity of plasmid carrying ApoB sequence and recombined tdTomato sequence was used to build standard curves in a qPCR assay (Extended Data Fig. 4d). To quantify adipocytes (Adipoq+/−), genomic DNA was extracted from ingWAT or visWAT and the number of recombined tdTomato alleles and ApoB alleles (total cell number) were quantified by qPCR.

Mouse surgery. For cell transplantation, tdTomato+ eP1 cells were isolated from ingWAT of ROSA26tdTomato mice by FACS. eP2 cells from ROSA26tdTomato mice were collected in RLT+lysis buffer for RNA extraction. The eP1 cell pellets were resuspended in Matrigel for injection. The 3–4 week-old mice were anesthetized with isoflurane and abdominal hair was removed. A small opposing Y-shaped cutaneous incision was made following the abdominal midline to expose inguinal fat pads. A total of 100,000 cells in 20 μl of Matrigel was injected into the subcutaneous region. Ten days after injection, ingWAT was dissected and cells were isolated as described above. Cells were stained with VAP1+ or anti-CD44 antibody or other markers following the manufacturers recommendation. Stained samples were analyzed by Sony SH800 cell sorter. tdTomato+ eP2 cells and tdTomato+ VAP1+ cells were collected in RLT+lysis buffer for RNA extraction. For AAV injections, 40–50 μl of AAV were distributed in each side of ingWAT. Three weeks after surgery, mice were fed on HFD for another 3 weeks. ingWAT was dissected for AAV analysis and protein extraction. To investigate whether RSPO2 inhibits eP1 to e2, mice were first infected with pAAV–CAG–Rspo2 or pAAV–CAG–GFP in ingWAT. Three weeks after AAV injection, a suspension of 100,000 tdTomato+ eP1 cells (in 20 μl of Matrigel, with or without 5 μg rec.RSPO2) was injected into ingWAT. Ten days after surgery, the injected ingWAT was dissected and cells were isolated for FACS. Cells were stained with VAP1–allophycocyanin antibody and analyzed by Sony SH800 cell sorter.

Protein extraction and western blot. Protein samples were extracted from adipose tissue and in vitro cultured cells with RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS and 1% glycerol) supplemented with protease inhibitor cocktail (11697/498001, Sigma-Aldrich) and Halt Phosphatase Inhibitor (78420, Thermo Fisher). Protein levels were quantified using the DC Protein Assay (Bio-Rad). For immunoblotting, protein samples were separated by SDS–PAGE on 12% polyacrylamide gels and transferred onto a nitrocellulose membrane. Membranes were probed using the indicated antibodies (anti-RSPO2, 1:1,000 dilution, Bioryt orb1859866; anti-HSP90, 1:1,000 dilution, Cell Signaling 4877S; β-catenin, 1:1,000 dilution, Abcam ab232075; and β-actin, 1:1,000 dilution, Sigma-Aldrich A5441) and chemiluminescent signals of the HRP-conjugated secondary antibodies (1:5,000 dilution, Calbiochem) were detected by a LAS 4000 mini-Image Quant System (GE Healthcare). Band intensity was quantified using ImageJ.

Adipogenesis quantification. Differentiated cells were fixed with 4% formaldehyde before staining with LDS540 (2 μM) for lipid droplets, Hoechst 33342 (4 μM) for nuclei...
and Syto60 (5µM) for cytostatic staining (all Invitrogen). Twenty pictures per well were taken with an automated microscope imaging system (Operetta, PerkinElmer). Pictures were analyzed using Harmony software or Cell Profiler software.

H&E staining images of Matrigel plugs were analyzed by Cell Profiler software v3.1. The pipeline was modified from online pipeline file ‘AdipocyteSize.cpt’ from Cell Profiler Forum (https://forum.image.sc/adipocyte-h-e-cell-profiler-pipeline/12490). Briefly, by adjusting diameter of objects the pipeline could identify cell membrane and export ‘Count_Membrane’ which equals cell numbers. By adjusting diameter of adipocytes, the pipeline could identify all the adipocytes and export count number.

β-catenin staining. SVF cells was isolated from ingWAT as described above. A total of 20,000 cells were seeded in a 96-well plate (Greiner Bio-One, 655900) per well. At 24 h after SVF cells attachment, 0.5 µg mL⁻¹ rec.RSPO2 was added in medium for 0–2 h. Cells were washed with PBS before fixed with 4% formaldehyde. Cells were incubated with 5% donkey serum supplement with 0.1% Triton in room temperature for 1 h, followed with anti-β-catenin antibody (1:200 dilution, Abcam, ab223075) incubation overnight at 4°C. Cells were washed three times with PBS and Alexa Fluor Plus 488 donkey anti-rabbit IgG secondary antibody (1:500 dilution, Invitrogen, A32790) for 1 h at room temperature protected from light. Nuclei were stained in parallel using Hoechst 33342 (1:10,000 dilution, Cell Signaling, 4082). Cells were washed three times with PBS, before imaging.

Adipocyte size quantification. IngWAT paraffin blocks were sectioned at 6µm and stained with H&E. IngWAT sections were examined by light microscopy using Axiophot microscope equipped with AxiosCam MR (Zeiss). Adipocyte size was determined using the Fiji Adiposoft plugin. At least six fields of view were analyzed for each sample. Adipocyte size frequency distribution was calculated using GraphPad Prism 8.

Insulin tolerance test. Mice were fasted for 8 h and then injected i.p. with human insulin (Actrapid Penfill, Novo Nordisk) at 0.25 U kg⁻¹. Blood glucose levels were measured by a blood glucometer (Accu-Chek Aviva, Roche) before and at 15, 30, 60 and 120 min after insulin injection. For data analysis, glucose levels versus time after injection were plotted using GraphPad Prism 8. Area under curve was calculated as a surrogate of insulin sensitivity.

Pyruvate tolerance test. Mice were fasted overnight (>12 h) and then injected with pyruvate (1g kg⁻¹ body weight; i.p.: Sigma-Aldrich, P5280). Blood glucose was measured before and 15, 30, 60 and 120 min after pyruvate injection. For data analysis, glucose levels versus time after injection were plotted using GraphPad Prism 8. AUC was calculated to estimate hepatic gluconeogenesis.

Hepatic triglyceride production rate. After 6 h fasted mice were injected with Triton (Sigma) in saline (400 mg kg⁻¹ i.v.). Immediately before injection and at 1, 2, 3 and 4 h following injection, blood samples were collected in heparin capillary tubes and TG concentrations in plasma were determined. The TG production rate was calculated from the difference in plasma TG levels over a given interval following Triton injection and was expressed as mgTG dl⁻¹ plasma h⁻¹.

In vivo overexpression of RSPO2 by tail-vein delivery of pAAV–CAG–RSPO2. pAAV–CAG–GFP and pAAV–CAG–RSPO2 (5 x 10⁹ vg kg⁻¹) were administered into the tail vein of mice at 8 weeks of age. Mice were fasted with HFD from 4 weeks of age. TG in plasma and liver was measured by Cobas TRIGB kit (Roche/Hitachi) following the manufacturer’s instructions.

Triglyceride measurement. Total lipids were extracted from up to 50 mg tissue using chloroform:methanol (2:1) mixture and normalized to tissue weight. TG in plasma and liver was measured by Cobas TRIGB kit (Roche/Hitachi) following the manufacturer’s instructions.

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**Author contributions**

H.D. and C.W. designed the study; H.D., W.S., L.D., M.B. and L.B. performed all the experimental work. W.S. performed 10X scRNA-seq. 10X snRNA-seq and bulk RNA-seq data analysis. Y.S., M.L., B.H., H.N. and H.K. performed integration of 10X scRNA-seq data analysis. M.B. and N.K. contributed to the human study. All authors reviewed and edited the manuscript.

**Competing interests**

The authors declare no competing interests.

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### Table: Gene Expression Profiles

| Gene | Expression |
|------|------------|
| Pcas6 | 2          |
| Ace | -1         |
| Ehho1 | 0         |
| Smol2 | -1        |
| Pror | 2          |
| P1b18 | -2        |
| Ckb | -1         |
| Sftp2 | 2          |
| Ifi14 | -1        |
| Sbsn | 0          |
| Sema3c | -1        |
| Lrm4d | 1          |
| Dapk | -1         |
| Sftp4 | 2          |
| Akr1c18 | 1        |
| Plet8 | -1        |
| Irf7 | 2          |
| Steap4 | 1         |
| Cull4a1 | -1        |
| Gsn | 2          |
| Cxcl14 | -1        |
| Nrcp | 1          |
| Sppar1 | 2         |
| Zfp330 | 1         |
| Socs3 | -1        |
| Sncoc2 | 2         |
| Cct7 | -1         |
| Hmcl1 | 2          |
| Sdc1 | -1         |
| Cdh11 | 1          |
| Cull1a1 | -2        |
| Peg3 | 2          |
| Podo | -1        |
| Palid | 1          |
| Mdk | 2          |
| Snp3 | -1         |
| Fabp4 | 1          |
| Tgm2 | -1         |
| Igf15 | 2          |
| Zfp1 | -1         |
| Bcd | 2          |
| Ifi3 | -1         |
| Gng12 | 1          |
| Com8 | -1        |
| Thbs2 | 2          |
| Gas6 | 1          |
| Fmo2 | -2         |
| Clec11a | 2        |
| Igfip7 | -1        |
| Gdf10 | 1          |
| Cygb | -2         |
| Cat3 | 1          |
| Mpg | -2         |
| Tmnt | 2          |
| Igtp3 | -2        |
| Pcaa1 | 1          |
| Top2a | -2        |
| Birc3 | 1          |
| Stim1 | -1        |
| Cks2 | 2          |
| Cnma | -2        |
| Hmgb2 | 2          |
| H2af2 | -1        |
| Tubat1b | 2        |
| Tubb5 | -2        |

### Diagram: Heatmap

- **P1-1**: Red
- **P1-2**: Yellow
- **P2-2**: Green
- **P2-1**: Cyan
- **P1-3**: Blue
- **P3**: Purple
- **P4**: Orange

The heatmap represents the expression levels of genes across different conditions or groups, with color intensity indicating the magnitude of expression.
Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | Integration of two scRNAseq of mouse Lin- cells from ingWAT and delineation of the heterogeneity of preadipocytes in human adipose tissue by single-nuclei sequencing. a) Feature plots of Pparg and Adipoq in mouse preadipocytes clusters. b) Adipoq and Pparg relative expression along the pseudo trajectory. c) Cell cycle related genes expression of mouse preadipocytes clusters. d) Cell trajectories reconstruction. e) UMAP of aligned cells derived from study Schwalie et. al10 and Merrick et. al11. Cluster 0: Cd142+ committed preadipocytes; Cluster 1: Cd55+ progenitors; Cluster 2: Aregs; Cluster 3: Vap1+ committed preadipocytes. f) Feature plots of gene expression (log2 CPM) of marker genes for progenitor cells: Cd55, Dpp4; for committed preadipocyte: Vap1, Icam1; and for Aregs: Cd142, Clec11a and Fmo2. g) Integrated dot plots of gene expression (log2 CPM) of marker genes for progenitor cells: Cd55, Dpp4; for committed preadipocyte: Vap1, Icam1; and for Aregs: Cd142, Clec11a, and Fmo2. h) Violin plots of number of genes and reads detected in the unsupervised clustering of mouse SVF cells9 (Methods). i) Heatmap of signature genes expression across mouse preadipocytes clusters. j) Unsupervised clusters of preadipocytes from human deep neck adipose tissue. k) Feature plots of PDGFRA in nuclei isolated from human deep neck adipose tissue. l) Violin plots showing the expression of DPP4, VAPI, ICAM1, CD142, CLEC11A in the clusters colored in Fig. 1a, m) Cell type analysis was performed by Enrichr on significant genes of H3 cluster. Bars were sorted by p-value ranking. P-value was computed using the Fisher exact test. n) WikiPathway (WikiPathway 2021 human database) enrichment analysis was performed by Enrichr on significant genes of H3 cluster. Bars were sorted by p-value ranking. P-value was computed using the Fisher exact test. o) Heatmap of signature genes expression across human preadipocytes clusters H1-H3. This figure is related to Fig. 1.
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | Discrepancies of the two studies\(^\text{1, 9}\) regarding the sorting and culture of Aregs. a) Representative flow cytometry dots plots showing the gating strategy used to identify CD142-, CD142 +, and CD142 + + cells. b) Relative mRNA levels of P3 specific marker genes (Cd142, Gdf10, Igfbp3, Clec11a) in different cell populations. n = 4 biological replicates. Data show the mean ± SEM. c) Histogram plots shows expression level of CD142, CD55, and VAP1 in CD142- cells, CD142 + cells and CD142 + + cells. d) Relative mRNA levels of P1 specific marker genes (Cd55, Pcsk6, Efhd1, P16, Smpd3) in different cell populations. n = 4 biological replicates. Data shown as mean ± SEM. e) Relative mRNA levels of P2 specific marker genes (Vap1, Col4a1, Sparc1, Colla1, Sdc1) in different cell populations. n = 4 biological replicates. Data shown as mean ± SEM. f) Cells were sorted from ingWAT. Adipogenesis was induced by A Cocktail (1 μM dexamethasone, 0.5 mM isobutylmethylxanthine, 1 μM insulin) or B Cocktail (1 μM dexamethasone, 0.5 mM isobutylmethylxanthine, 125 nM Indomethacin, 1 nM T3, and 20 nM insulin). Quantification of adipogenesis (top) and cell numbers (below) in culture well on differentiation day 6. Data shown as mean ± SEM. \(F(3,30) = 14.78, P < 0.0001\) by two-way ANOVA. For Cocktail difference, \(F(1,10) = 290.5, P < 0.0001\). For cell type difference, \(F(1.929,19.29) = 148.8, P < 0.0001\). g) Microscopy images of different cell populations shown on differentiation day 6 in f. In all panels, nuclei were stained with Hoechst 33342 (blue) and lipids were stained with LD540 (yellow). Scale bars, 100 μm. This figure is related to Fig. 2.
a) CD142++ vs CD142-APC

58 significant DE genes encode secret factors (FDR<0.05, FC>2)

15 genes high expressed in adipocytes
(Nsgr1, Cas3, Acad, illgm, Fogg, Cxcr12, Cnri1, Nav2, C2, Kit, Gas6, Rgn, Cpe, Rapo, CD9)

23 genes high expressed in eP1 and eP2 cells
(Prod, Wnt1, Mamct2, Ang4, Cxcl2)

Sphk2 and 12 genes

b) Adipogenesis on day 6 (ngWAT SVF)

Adipogenesis fold change

f) Adipogenesis fold change

Adipogenesis on day 6 (transwell)

i) FACS

m) WikiPathway 2019 mouse

To be continued
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | Selection and identification of Aregs’ effectors. a–c) Scheme of identification of Aregs marker genes candidates (a), and their expression in eP1 and eP2 cells bulk RNAseq data, n = 3–5 biological replicates (b). mRNA expression in eP3 and eP3-depleted SVF (c). n = 6 biological replicates, data show the mean ± SEM, and analyzed by two-tailed Student’s t-test. d) Adipocyte ratio in ingWAT SVF after knocking down eP3 marker genes by siRNA. n = 3 biological replicates; data shown as mean ± SEM and analyzed by two-tailed Student’s t-test (compared to Ctrl group). e) Adipocyte ratio in CD142-cells co-cultured with eP3. n = 3 biological replicates; data shown as mean ± SEM and analyzed by two-tailed Student’s t-test (compared to Ctrl group). f) Comparison of adipogenesis of CD142- cells after knocking down of Spink2, Rspo2, Cgref1 and Serpinb6c in eP3 cells in transwell co-culture experiments. Adipocyte ratio normalized to ctrl group. n = 3 biological replicates; data is presented as mean ± SEM and analyzed by one way-ANOVA test. F(3,20)=2.025, P = 0.143. g) Adipocyte ratio in eP3 after knocking down Rspo2 by siRNA, n = 3 biological replicates. Representative images of adipocytes on differentiation day 7. h) RSPO2 conc. in cell culture medium, n = 4 biological replicates. Data is presented as mean ± SEM and was analyzed by one way-ANOVA test. F(3,12)=75; P < 0.0001. i) Quantification of cell number in Fig. 3j. n = 6 independent wells; data show the mean ± SEM, analyzed by one way-ANOVA test. j) Feature plots of Lgr4 in 10xscRNAseq of ingWAT Lin- cells. k) Pathway enriched in eP1 cells by Enrichr analysis. P-value is computed using the Fisher exact test. l) Heatmap of log2 fold changes of Wnt signaling related genes in eP1 and eP2 cells. Each row represents 1 gene; each column represents one replicate. m) Pathway enriched in eP2 cells by Enrichr analysis. P-value was computed using the Fisher exact test. n) Heatmap of log2 fold changes of adipogenesis related genes in eP1 and eP2 cells. Each row represents 1 gene; each column represents one replicate. o) Quantification of cell number per field in Fig. 3n. Data show the mean ± SEM, n = 6 independent wells. p) Lgr4-6 mRNA level in cells in Fig. 3n. Data shown as mean ± SEM, n = 6 independent wells. Statistical analysis was performed by two-tailed Student’s t-test. q) Quantification of cell numbers per field in Fig. 3p. Data shown as mean ± SEM, n = 6 independent wells. r) Experimental scheme of treatment cells with rec.RSPO2 during adipogenesis day3 to day6. s) Quantification of adipocytes per well (left) and cell number (right) ± rec.RSPO2 during day3 to day6. Data shown as mean ± SEM, n = 6 independent wells. t–w) SVF cells treated with 0.5ug/ml rec.RSPO2 for 0-24 h. Western blot images (t) and quantification (u) of beta-Catenin and beta-Actin in SVF. Data shown as mean ± SEM, n = 3 biological replicates. F(3,8)=3.85, P = 0.057 by one way ANOVA. Multiple comparison between groups was performed using Tukey test with FDR = 0.05. Quantification of cells number/field (v) and microscopy images (w) in each well treated with rec.RSPO2. Data shown as mean ± SEM, n = 3 biological replicates. Data was analyzed by one way-ANOVA. Experiment was repeated twice. x–z) After knocking down Lgr4 by siRNA, eP1 cells were treated with rec.RSPO2 (0.5ug/ml) for 24 h. Western blot images (x) and quantification (y) of Beta-Catenin protein in eP1 cells. Beta-actin protein levels were used as loading control. F(3,10)=52.68, P < 0.0001 by one way ANOVA test. Multiple comparison between groups was performed with Tukey FDR = 0.05. Lgr4 mRNA level (z) in eP1 cells 48 h post siRNA transfection. Data shown as mean ± SEM, n = 3–4 biological replicates (x, y), n = 6 independent wells (z). Data analysis was performed using two-tailed Student’s t-test (z). Nuclei were stained with Hoechst 33342 (blue). Scale bars, 100μm. This figure is related to Fig. 3.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Rspo2 inhibits adipogenesis of eP1 cells in vivo. a, b) Microscopy images of H&E staining of matrigel plugs with eP1 cells (a) and eP2 cells (b) without Rspo2 overexpression (top) and with Rspo2 overexpression (lower). Scale bar, 100 μm. Experiment was performed once. c, d) pUC57 plasmid insertion with ApoB and recombined tdTomato sequence. ApoB and recombined tdTomato standard curve generated with plasmid (d). e) Plasma Rspo2 level in CAG-GFP and CAG-Rspo2 ingWAT infection mice, n = 6 mice. Data are presented as mean values +/- SEM. Data analysis was performed using two-tailed Student’s t-test. f) Western blot images of Rspo2 in liver of ingWAT AAV infection mice. HSP90 served as a loading control. g) Immunohistochemistry staining of cleaved caspase-3 in ingWAT. Data are presented as mean values +/- SEM, n=6 mice. This figure is related to Fig. 4.
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | pAAV-CAG-GFP and pAAV-CAG-Rspo2 infection mice on HFD. a–j) Overexpression of RSPO2 by tail vein delivery of AAVs in mice (a). Images of ingWAT and visWAT (b), food intake per day (c), Time-resolved oxygen consumption (d), quantification of triglyceride per gram of liver (e), representative images of H&E staining of liver (f) from pAAV-CAG-GFP and pAAV-CAG-Rspo2 infection mice. Blood glucose (g) and area under curve (AUC) (h) shown by intraperitoneal pyruvate tolerance test. Triglyceride levels in blood (i) and hepatic triglyceride secretion rate (j) after tyloxapol injection. Data shown as mean ± SD (i, j), n = 6 mice. Data analysis was performed using two-tailed Student’s t-test. k–p) Experimental scheme (k) for overexpression of RSPO2 in ingWAT by injection AAV into ingWAT. Quantification of RSPO2 in ingWAT by western blot (l) and in circulation (m). Body weight (n), tissue weight (o), and time-resolved oxygen consumption (p) of pAAV-CAG-GFP and pAAV-CAG-Rspo2 ingWAT infection mice. Data shown as mean ± SD, n = 5 mice (CAG-GFP); n = 6 mice (CAG-Rspo2). Data analysis was performed using two-tailed Student’s t-test. q–r) Spearman correlation of serum RSPO2 level with HOMA-IR (q), mean adipocyte volume (r) in male and female subjects. The correlation coefficient was calculated using a Spearman’s Correlation Test. This figure is related to Fig. 6.
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | snRNA-seq of ingWAT in mice with HFD and pAAV-CAG-GFP or pAAV-CAG-Rspo2 AAV. a) Feature plot for the origin of pAAV-CAG-GFP or pAAV-CAG-Rspo2 conditions. b) Feature plot for the number of genes expressed in each nucleus. c) Feature plots for adipocyte markers Adipoq, Lep, Plin1, and Adrb3. d) Heat map of signature genes for each population. e–g) Scatter plots for gene expression analysis between pAAV-CAG-GFP and pAAV-CAG-Rspo2 within adipocytes (e), macrophages-1 (f), and macrophages-2 (g). h) Heat map of signature genes for preadipocytes population PA-2-PA-5. i) Feature plots for Ctnnb1 in preadipocytes clusters in pAAV-CAG-GFP and pAAV-CAG-Rspo2 ingWAT, separated by conditions. j) Feature plots for Dpp4, Pparg, Fmo2 in preadipocytes clusters in pAAV-CAG-GFP and pAAV-CAG-Rspo2 ingWAT. k) Dot plots for representative markers of each cluster PA1-PA-5, separated by conditions. l) Scatter plots for gene expression analysis between pAAV-CAG-GFP and pAAV-CAG-Rspo2 within PA-1-PA-5. This figure is related to Fig. 7.
Extended Data Fig. 7 | See next page for caption.
Extended Data Fig. 7 | other markers expression in subpopulations of adipogenitors. a) Scheme for HFD induced obesity. b) eP3 percentage in ingWAT quantified by FACS. Data show as mean ± SD, n = 6 biological replicates. Data was analyzed using two-tailed Student’s t-test. c) Circulating RSPO2 levels in chow and HFD fed mice. Data shown as mean ± SD, n = 6 biological replicates. Data was analyzed using two-tailed Student’s t-test. d) RSPO2 expression quantified by western blot in ingWAT, visWAT and liver. HSP90 served as loading control. Data show the mean ± SD, n = 6 biological replicates. Data was analyzed by two-tailed Student’s t-test. e) Rspo1-4 mRNA levels in Lin-Sca1+ cells and eP3 cells of ingWAT. Data are presented as mean ± SEM, n = 4 biological replicates. Data was analyzed by one-way ANOVA, in Lin-Sca1+ group, F(3,12)=602, P < 0.0001; in eP3 group, F(3,12)=326, P < 0.0001.
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BD FACSAria III flow cytometry and Sony SH8000 cell sorter software was used to collect flow cytometry data. Zen 2012 software was used to collect HE staining data. Operetta software 3.0 was used for in vitro adipogenesis imaging. LAS 4000 mini Image Quant software were used to collect western blot images.

Data analysis
Flow cytometric analysis were performed with FlowJo analysis software (FlowJo10). Cell Profiler software (CellProfiler 3) was used for quantifying adipogenesis in vivo. Harmony 3.5.2 was used for quantifying adipogenesis in vitro. The statistical analysis were performed with the Graphpad Prism 8 software. We used Seurat package (V 3.1.2) for downstream QC, cell clustering and generating 2D UMAP cell plots. Cellranger V3.0 (by 10x genomics) was applied to map the spliced and unspliced transcripts. Velocyto was applied to reconstruct the RNA states trajectory. scVelo was applied to model the cellular dynamics. Monocle 3 was used to estimate the pseudotime trajectory.

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RNA sequencing data has been deposited in ArrayExpress (www.ebi.ac.uk/arrayexpress). Accession codes E-MTAB-6677 for scRNAseq of ingWAT Lin cells.
Accession codes E-MTAB-5787 for bulk RNA seq of CD142++ cells and Lin-CD142- cells. Accession codes for Bulk RNA seq of eP1 and eP2 cells is E-MTAB-9827. Accession codes single nucleus RNAseq of mouse IgWAT with Rspo2 or GFP AAV injection is E-MTAB-11104. Please address correspondence and requests for materials to C.W. and requests for bioinformatic information to W.S. (Wenfei-sun@ethz.ch or Wenfei-sun@stanford.edu). The datasets can be explored interactively at https://batnetwork.org/. Source data are provided with this paper.

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| ☑ Clinical data                 |         |
| ☑ Dual use research of concern  |         |

Antibodies

| Antibodies used | | |
|-----------------|---|---|
| anti-CD16/CD32 (Biolegend, clone 93, Cat# 101302, Lot# B282595), anti-mouse CD31-PECy7 (BioLegend, clone 390, Cat# I02418, Lot# B264590), anti-mouse CD45-PECy7 (BioLegend, clone 30-411, Cat# 203114, Lot# B2485760), anti-mouse TER119-PECy7 (Biolegend, clone TER-119, Cat# 136222, Lot# B251241), anti-mouse Sca1-Brilliant Violet 711 (Biolegend, clone 07, Cat# 108131, Lot # B241586), anti-mouse CDS5-PE (BD BIOSCIENCES, clone RK0-5, Cat# 558037, Lot# 9121503), anti-mouse VAP1 (Abcam, clone 7-88, Cat#ab81673, Lot# GR3232949-2), anti-mouse CD142 (Sinobiological, clone 001, Cat# H50413-ROPO, Lot# HA100CD2402-B), anti beta-catenin (Abcam, clone IG4794R-3, Cat#ab223075, Lot# GR3178277-1), anti Rspo2 (Biorybt, Cat# orb185986, Lot# R85703), Alexa Fluor 488 donkey anti-rabbit IgG secondary antibody (Invitrogen, Cat# A32790, Lot# U1293143; anti-HSP90, 1:1000, Cell Signaling #48775, HRP-conjugated secondary antibodies (1:5000, Calbiochem). |

Validation

These are commercial antibodies with validations available in the manufacturers' website.

Animals and other organisms

Policy information about studies involving animals, ARRIVE guidelines recommended for reporting animal research

| Laboratory animals | |
|--------------------|---|
| CS7Bl/6 mice were obtained from Charles River Laboratories, and C57Bl/6j/RO5AmT/mG mice (stock: 007676), AdipoCre mice (stock: 028070), NucRed mice (stock: 025006) were obtained from the Jackson Laboratory. Mice were kept on a 12-h/12-h light/dark cycle and 20-60%(25°C) humidity a pathogen-free animal facility of SLA ETH Zurich. The HFD used contained 60% (ka%) fat (diet No. 3436, |
The age and sex of the animals were described in method part in detail.

Wild animals
This study did not involve wild animals.

Field-collected samples
This study did not involve samples collected from field.

Ethics oversight
All animal experiments were approved by the Animal Ethics Committee of Zurich.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Clinical data
Policy information about clinical studies
All manuscripts should comply with the ICMJE guidelines for publication of clinical research and a completed CONSORT checklist must be included with all submissions.

Clinical trial registration
For the purpose of this study, we selected 60 individuals from the Leipzig Obesity Biobank to define age-, BMI, and sex-matched groups of insulin sensitive (n=30) and insulin resistant (n=30) patients with obesity. The study was approved by Ethics committee of the University of Leipzig (approval numbers: 159-12-21052012 and 017-12-23012012).

Study protocol
The study was approved by the Ethics committee of the University of Leipzig, and detailed protocol could be found in Leipzig Obesity Biobank and related study.

Data collection
Definition of the M1/IO subgroups was based on the glucose infusion rate (GIR) during the last 30min of the steady state in euglycemic-hyperinsulinemic clamps (IS: GIR > 70μmol/kg/min; IR: GIR < 60μmol/kg/min). All individuals fulfilled the previously reported inclusion and exclusion criteria. BMI was calculated as weight divided by squared height. Waist circumference was measured at the midpoint between the lower ribs and iliac crest. Percentage body fat was measured by bioimpedance analysis. Abdominal visceral and subcutaneous fat areas were calculated using computed tomography or MRI scans at the level of L4–L5. Insulin sensitivity was assessed using the euglycemic-hyperinsulinemic clamp method as described in method.

Outcomes
The correlation of RSPO2 level in plasma with different clinical measurements listed in paper was calculated by Pearson’s Correlation Tests in Prism B.D.

Flow Cytometry
Plots
Confirm that:

☒ The axis labels state the marker and fluorochrome used (e.g. CD4–FITC).
☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
☒ All plots are contour plots with outliers or pseudocolor plots.
☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology
Sample preparation
For SVF isolation, dissected adipose tissues were minced with scissors and incubated in 1 mg/ml collagenase (1C6885-1G, Sigma-Aldrich) in collagenase buffer (25 mM NaHCO3, 12 mM KH2PO4, 1.2 mM MgSO4, 4.8 mM KCl, 120 mM NaCl, 1.4 mM CaCl2, 5 mM glucose, 2.5% BSA, 1% Pen/Strep, pH=7.4) for 50 min at 37°C under agitation. Equal volume of culture media (high glucose DMEM medium [#19965026, Gibco] supplement with 10% FBS and 1% Pen/Strep) was added and samples were centrifuged for 5 min at 300 g. The SVF pellet was resuspended in 2 ml erythrocyte lysis buffer (154 mM NaCl), 10 mM KHCO3, 0.1 mM EDTA, pH 7.4) and incubated for 4 min at room temperature. Samples were diluted with 10 ml culture media and filtered through 40 μm cell strainers. After 5 min of centrifugation at 200 g, the supernatant was removed and the pellets were resuspended in FACS buffer (PBS with 3% FBS, 1 mM EDTA, 1% P/S). Centrifuge cells at 200g for another 5 min, then remove supernatant and resuspend cell pellets in FACS buffer for antibody staining.

Instrument
BD FACS Aria III, and Sony SH800 cell sorter

Software
Flowjo

Cell population abundance
After sorting, a aliquot of collected cells were run through the same sorter, and the same gating strategy was applied to check the purity. A general purity of higher than 95% were achieved for all the sorted population.

Gating strategy
The gating strategies were described in Fig. 2a, Fig. 5b, extended data Fig. 2a. FSC/SSC gating was used to display all the events detected. FSCA/SSCH was used to exclude doublets, and Sytox Blue staining was used to exclude dead cells.

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.