Rapid depot-specific activation of adipocyte precursor cells at the onset of obesity

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Excessive accumulation of white adipose tissue (WAT) is the defining characteristic of obesity. WAT mass is composed primarily of mature adipocytes, which are generated through the proliferation and differentiation of adipocyte precursors (APs). Although the production of new adipocytes contributes to WAT growth in obesity, little is known about the cellular and molecular mechanisms underlying adipogenesis in vivo. Here, we show that high-fat diet feeding in mice rapidly and transiently induces proliferation of APs within WAT to produce new adipocytes. Importantly, the activation of adipogenesis is specific to the perigonadal visceral depot in male mice, consistent with the patterns of obesogenic WAT growth observed in humans. Furthermore, we find that in multiple models of obesity, the activation of APs is dependent on the phosphoinositide 3-kinase (PI3K)-AKT2 pathway; however, the development of WAT does not require AKT2. These data indicate that developmental and obesogenic adipogenesis are regulated through distinct molecular mechanisms.

The number of overweight and obese individuals continues to rise such that by the year 2030 it is projected that over half of the world’s population will be overweight or obese1. Despite the defining role of white adipose tissue (WAT) accumulation in this disease, our understanding of WAT growth in obesity is limited. WAT can expand via both an increase in adipocyte size (hypertrophy) and adipocyte number (hyperplasia)2–4, and recent studies have found that adipocyte hyperplasia plays an important role in human obesity5,6. Specifically, obese individuals have significantly more adipocytes than lean individuals, and this trend is maintained throughout adult life5. Even after obese individuals undergo severe weight loss, elevated adipocyte number is maintained7, indicating that increased adipocyte formation in obesity has lifelong effects on adipose tissue homeostasis and WAT mass. Another study found that variation in the size of the major omentum, a prominent visceral depot in humans, is primarily due to adipocyte number8. These data suggest that hyperplastic growth of WAT has important implications for metabolic health, given the risk of complications that accompany visceral obesity, including diabetes and cardiovascular disease9–11. Finally, several reports suggest that increased adipocyte number also contributes to obesity in rodents12–14. These studies point to a crucial role for adipocyte hyperplasia in the progression of obesity, yet the cellular and molecular mechanisms underlying the regulation of adipocyte number in vivo remain unclear.

RESULTS

The hyperplastic growth of WAT requires the formation of new adipocytes in vivo. Because mature adipocytes are post-mitotic, new adipocytes arise from the differentiation of adipocyte precursor (AP) cells residing within the adipose tissue stromal vascular fraction (SVF; refs 15,16). Importantly, the timing of AP activation and subsequent adipogenesis remains undefined, and the molecular cues regulating this process in vivo are not known. To quantitatively assess the formation of adipocytes in response to high-fat diet (HFD) feeding in male C57BL/6j mice, we performed an adipocyte pulse-chase experiment using an adipocyte-specific, tamoxifen-inducible Adiponectin-cre Estrogen Receptor (Adiponectin-creER) mouse model17 in combination with a dual fluorescent reporter. On expression of cre recombinase, the fluorescent reporter undergoes an indelible switch from expression of plasma membrane-targeted Tomato (mT, red fluorescence) to plasma membrane-targeted GFP expression (mG, green fluorescence) allowing clear identification of cre-expressing cells18,19. Tamoxifen treatment results in approximately 95% recombination efficiency in the adipocytes of both the inguinal subcutaneous (SWAT) and perigonadal visceral (VWAT) adipose depots (Fig. 1). To observe formation of new adipocytes, we pulsed Adiponectin-creER; mT/mG mice with tamoxifen and subsequently placed the mice on HFD or continued mice on standard

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low-fat diet (SD) for eight weeks. We then quantified the percentage of adipocytes labelled with mTomato, which indicates that they formed from mGFP-negative, mTomato-positive APs after the tamoxifen pulse (Fig. 1a). We observe significantly increased formation of adipocytes exclusively in VWAT of HFD-fed male mice, whereas adipocyte formation is not enhanced by HFD in SWAT (Fig. 1b,c). These data are consistent with recent qualitative findings using the Adipochaser mouse, which showed that new adipocytes form in male VWAT between five and eight weeks of HFD feeding; however the timing of AP activation and adipogenesis in response to HFD is unknown.

In many adult tissues, cellular differentiation supports tissue homeostasis and expansion, and this process requires proliferation to maintain precursor pools. Therefore, we reasoned that identifying the timing of AP proliferation in diet-induced obesity would allow us to determine when AP activation and subsequent differentiation is initiated. Whereas increased proliferation of total SVF cells in WAT after long-term HFD feeding has been reported, we focused on the first several weeks of HFD feeding to identify the cellular events that give rise to differentiated adipocytes by week 8 of HFD (Fig. 1b,c). We investigated the proliferation of APs (refs 16,19) in VWAT and SWAT depots by labelling with bromodeoxyuridine (BrdU) in vivo during a time course of HFD feeding in male mice (Supplementary Fig. 1A). Flow cytometry analysis of BrdU incorporation into Lin−:CD29+;CD34+:Sca-1+ APs (ref. 23; Fig. 2a and Supplementary Fig. 1B) shows increased proliferation of APs only in VWAT during the first week of HFD feeding (Fig. 2b). The initial burst of AP proliferation is followed by a return to background proliferation rates by the second week of HFD and remains low throughout the remainder of the time course (Fig. 2b). In contrast, we do not observe a significant increase in the proliferation of SWAT APs in response to HFD feeding (Fig. 2b).

We previously identified two subpopulations of APs, including Lin−:CD34+;CD29+:Sca-1+;CD24+ (CD24+) adipocyte progenitors and Lin−:CD34+;CD29+:Sca-1+;CD24− (CD24−) committed pre-adipocytes. After one week of BrdU treatment, these populations appear to proliferate at similar rates (Fig. 2c,d); however, a shorter BrdU treatment of 12 h reveals that AP proliferation is largely restricted to the CD24+ adipocyte progenitor population in both major depots of HFD-fed and SD-fed mice (Fig. 2c,d). Given that activated CD24+ adipocyte progenitors can rapidly differentiate into CD24− preadipocytes after only 24 h (refs 19,24), these data suggest that the increased proliferation of CD24+ cells is masked during longer BrdU treatments by their rapid differentiation into CD24− cells. As there is no difference in BrdU incorporation into the AP subpopulations with treatments longer than 12 h, we performed all further analyses on the total AP population, which includes both the CD24+ adipocyte progenitors and CD24− preadipocytes.
Figure 2 High-fat diet feeding induces adipocyte precursor activation. (a) Representative flow cytometry histogram showing gating for BrdU-positive AP cell populations in control (no BrdU treatment) and experimental samples. (b) Quantification of BrdU incorporation into APs from VWAT (left) or SWAT (right) of male mice at the indicated time points of diet treatment (see Supplementary Fig. 1A). (nD4 mice for wild-type male SD week 1, nD5 mice for all other groups). (c, d) Quantification of BrdU incorporation into CD24+ and CD24– subpopulations of APs in VWAT (c) or SWAT (d) after one week (left panel) or 12 h (right panel) of BrdU treatment at the onset of HFD feeding compared to SD controls. (For 1-week VWAT nD4 mice for SD and nD5 mice for HFD. For 12-h VWAT nD10 mice for SD and nD7 mice for HFD. For 1-week SWAT nD4 mice for SD and nD5 mice for HFD. For 12-h SWAT nD8 mice for SD and nD9 mice for HFD.) (e) 3D representation of confocal z-stack from VWAT of PdgfRα-H2B-GFP mice showing GFP-positive nuclei. Hash marks represent 50 μm increments. (f) Total number of GFP+ nuclei in the VWAT of PdgfRα-H2B-GFP mice after one week of HFD compared to SD controls was estimated using confocal microscopy (see Methods; nD5 mice per group). Significance in b was calculated using 2-way ANOVA with Bonferroni’s post-test for multiple comparisons. Significance between the indicated groups in c, d was calculated using a two-tailed Student’s t-test. Significance in f was calculated using a one-tailed Student’s t-test. Exact P-values are listed in Supplementary Table 1. Statistics source data for b can be found in Supplementary Table 2. Error bars represent mean ± s.e.m. * (P<0.05), ** (P<0.01), *** (P<0.001), **** (P<0.0001). AP, adipocyte precursor; HFD, high-fat diet; SD, standard diet; mT/mG, membrane tomato/membrane GFP; BrdU, bromodeoxyuridine.

To verify that HFD-induced AP proliferation leads to an increase in the number of APs in VWAT, we used PdgfRα-H2B-GFP mice. These mice express a nuclear GFP reporter from the endogenous PdgfRα promoter, which labels primarily APs in WAT (refs 19,25 and
Figure 3 Activated adipocyte precursors undergo adipogenesis in vivo. (a) Schematic depicting the time points for BrdU pulse-chase experiments. (b) BrdU incorporation into adipocyte nuclei after pulse chase from the first week of HFD feeding (n=5 mice for each group) (c) Quantification of BrdU incorporation into APs from VWAT of male mice at the indicated time points after BrdU treatment during the first week of diet (n=5 mice for each group). (d,e) Representative images (d) and quantification (e) of immunofluorescence staining for BrdU in adipocyte nuclei of VWAT from mice after one week of BrdU treatment and eight weeks on the indicated diet. Tissue is also stained for Caveolin-1 to visualize adipocyte plasma membranes, as well as DAPI to visualize nuclei. Adipocyte nuclei are indicated with arrowheads (yellow indicates BrdU-positive and white indicates BrdU-negative) are identified by their location inside the adipocyte plasma membrane. Multiple sections were analysed throughout the VWAT depot. (n=5 mice for SD, n=4 mice for HFD.) Significance in b was calculated using 2-way ANOVA with Bonferroni’s post-test for multiple comparisons. Significance between the indicated groups in c,e was calculated using a two-tailed Student’s t-test. Exact P-values are listed in Supplementary Table 1. Error bars represent mean ± s.e.m. * (P < 0.05), ** (P < 0.01), *** (P < 0.001). Scale bars in d are 25 μm. AP, adipocyte precursor; HFD, high-fat diet; SD, standard diet; BrdU, bromodeoxyuridine.

Supplementary Fig. 2C). As expected, proliferation of the GFP+ cells in PdgfRα-H2B-GFP VWAT is significantly enhanced with HFD feeding (Supplementary Fig. 2D). We used confocal microscopy to quantify total GFP+ nuclei in situ and found that the number of PdgfRα+ cells nearly doubles in VWAT after one week of HFD compared to age-matched SD-fed controls (Fig. 2e,f). These data indicate that a larger precursor pool is created within the WAT depot after only a short period of HFD feeding, which is consistent with the subsequent formation of new mature adipocytes over time (Fig. 1b,c).

Next we determined when proliferative APs give rise to mature adipocytes during the expansion of WAT in diet-induced obesity. For these experiments we treated mice with BrdU during the first week of HFD feeding and used BrdU as a marker to track activated APs in vivo. We then placed the mice on HFD for different lengths of time.
time and used two complementary quantitative methods to measure BrdU incorporation into mature adipocyte nuclei (Fig. 3a). First, we isolated adipocyte nuclei by collagenase digestion and differential centrifugation after 1, 4 and 7 weeks of diet and performed flow cytometry analysis for BrdU. We find that after one and four weeks of diet there is no significant differentiation of activated APs into mature adipocytes; however, after seven weeks of diet we detect BrdU labelling in mature adipocyte nuclei from all groups (Fig. 3b). Importantly, the percentage of BrdU-positive adipocyte nuclei is significantly higher in the VWAT of HFD-fed mice compared to SD controls (Fig. 3b), whereas there is no difference between SD-fed and HFD-fed groups in SWAT (Fig. 3b). These results are consistent with the depot-specific AP proliferation pattern that we observe at the onset of HFD feeding. Interestingly, when we analyse BrdU labelling in the SVF after seven weeks, we find a significant reduction in the percentage of BrdU-labelled APs in VWAT compared to the first week of HFD (Fig. 3c), consistent with the hypothesis that a significant subset of BrdU-labelled APs transition from the SVF to the adipocyte pool during this time. Finally, BrdU pulse chase from the second week of HFD feeding (Supplementary Fig. 2A), when AP proliferation has returned to SD levels (Fig. 2b), shows no difference in the rate of mature adipocyte labelling between SD-fed and HFD-fed groups (Supplementary Fig. 2B).

The second method we used to measure the differentiation of activated APs was immunofluorescence staining of adipose tissue sections after one week of BrdU and eight weeks of diet (Fig. 3a). VAT sections were stained for Caveolin-1 to identify adipocyte plasma membranes, and DAPI was used in combination with BrdU, bromodeoxyuridine; pAkt, phosphorylated Akt; MFI, mean fluorescence intensity.

Figure 4 Diet-induced proliferation of adipocyte precursors correlates with cell-intrinsic Akt phosphorylation. (a) Quantification of BrdU incorporation into APs from male VWAT after 24-h pulses of BrdU for each day at the beginning of HFD treatment. (n = 5 mice for each group.) (b) Representative flow cytometry histograms of APs stained for phosphorylated AKT (T308) on day 3 of HFD feeding compared to SD and fluorescence minus one control. (c) Quantification of mean fluorescence intensity (MFI) of pAKT staining by flow cytometry in AP cells on day 3 of HFD feeding and SD controls. (n = 5 mice for each group.) (d,e) Correlation between pAKT T308 (d) or S473 (e) MFI and AP proliferation in VWAT of wild-type mice on day 3 of HFD or SD feeding. (n = 10 mice for each group.) (f) Quantification of mean fluorescence intensity (MFI) of pAKT staining by flow cytometry in AP cells on day 14 of HFD feeding compared to SD controls. (n = 5 mice for each group.) Significance of each HFD group compared to SD in a was calculated using a two-tailed Student’s t-test. Significance in c was calculated using a two-tailed Student’s t-test. Significance in d,e was calculated using two-tailed correlation analysis. Exact P-values are listed in Supplementary Table 1. Error bars represent mean ± s.e.m. * (P < 0.05), ** (P < 0.01), *** (P < 0.001), **** (P < 0.0001). AP, adipocyte precursor; HFD, high-fat diet; SD, standard diet; BrdU, bromodeoxyuridine; pAkt, phosphorylated Akt; MFI, mean fluorescence intensity.
proliferation in response to HFD, we treated groups of mice with BrdU for individual days at the onset of HFD feeding. We find that AP proliferation in VWAT is highest at day 3 of HFD feeding and returns to SD levels by day 5 (Fig. 4a). This surprisingly rapid activation of APs on switching diets suggests that WAT cellular homeostasis is closely linked to nutrient sensing. One pathway that is involved in nutrient sensing mechanisms is the phosphoinositide 3-kinase (PI3K)-AKT pathway. To determine whether the central kinase in this pathway, AKT, is activated in VW AT at the height of the proliferation response, we analysed AKT phosphorylation at two critical activation sites in the activation of APs after three days of HFD feeding. There is no significant increase in AKT phosphorylation in protein lysates from whole adipose tissue (Supplementary Fig. 3A,B), which contains many cell types. However, when we analyse APs by flow cytometry on day 3 of HFD feeding (Supplementary Fig. 3C,D) we find that AKT phosphorylation at both sites is significantly elevated within APs in VWAT (Fig. 4b,c). Furthermore, when we combine AKT phosphorylation analysis with BrdU labelling on the third day of diet, we find a significant positive correlation between AP proliferation rate and the level AKT phosphorylation in APs within VWAT (Fig. 4d,e).

When we perform the same analysis on day 14 of diet, when AP proliferation rates have returned to background levels, we observe no difference in AKT phosphorylation in APs between SD-fed and HFD-fed groups (Fig. 4f). These data indicate that AKT signalling within APs is correlated with AP proliferation and therefore may play a role in the activation of APs at the onset of obesity.

The AKT kinases regulate several processes, including cellular growth, survival and metabolism. The most prominent mammalian isoforms are AKT1 and AKT2. Whereas AKT1 is widely expressed and promotes the growth of many tissues, AKT2 regulates metabolic flux within liver, muscle and adipose tissue. Because AKT1 and AKT2 are both expressed in APs isolated from both VWAT and SWAT (Fig. 5a), we isolated APs from VWAT after three days of HFD feeding and analysed AKT1 and AKT2 phosphorylation by western blot. Using
Figure 6  Akt2 is required for activation of adipocyte precursors in multiple models of obesity. (a, b) BrdU incorporation into VWAT (a) or SWAT (b) APs of the indicated groups after one week of treatment. (n=5 mice for wild-type SD, n=7 mice for wild-type HFD, n=6 mice for Akt1–/– SD, n=7 mice for Akt1–/– HFD, n=17 mice for Akt2–/– SD, n=15 mice for Akt2–/– HFD.) (c) Quantification of immunofluorescence staining for BrdU in adipocyte nuclei of VWAT from Akt2–/– mice after one week of BrdU treatment and eight weeks on the indicated diet. (n=4 mice for SD, n=5 mice for HFD). (d, e) Body weight (d) and fat mass (e) in young mice after weaning at three weeks of age. (n=7–8 mice for ob/ob; Akt2–/–, n=4 mice for ob/ob). Some error bars are obstructed by symbols. (f) BrdU incorporation into VWAT APs in the indicated groups after BrdU treatment from P35-40. (n=6 mice for wild-type, n=4 mice for ob/ob and n=3 mice for ob/ob; Akt2–/–.) (g) VWAT weights of the indicated groups at 12 weeks of age. (n=5 mice for wild-type, n=8 mice for ob/ob; Akt2–/–, n=3 mice for ob/ob.) Significance between indicated groups in a–c was calculated using a two-tailed Student’s t-test. Significance between indicated groups in d–e was calculated using 2-way ANOVA with Bonferroni’s post-test for multiple comparisons (P-values are adjusted P-values). Significance in f,g was calculated using a two-tailed Student’s t-test. Exact P-values are listed in Supplementary Table 1. Statistics source data for d,e can be found in Supplementary Table 2. Error bars represent mean ± s.e.m. “*” (P < 0.05), ** (P < 0.01), *** (P < 0.001), **** (P < 0.0001). AP, adipocyte precursor; HFD, high-fat diet; SD, standard diet; BrdU, bromodeoxyuridine.

antibodies specific for phosphorylated AKT2 at S474 and AKT1 at S473, we find significant elevation of phospho-AKT2 at the height of AP activation in HFD-fed mice whereas levels of phospho-AKT1 are low in APs and do not increase in response to HFD (Fig. 5b,c). We do, however, detect phospho-AKT1 in the non-AP cell population (Supplementary Fig. 3E). These data indicate that AKT2 is specifically activated in APs in response to HFD feeding.

To test whether signalling through the PI3K pathway is required for AP activation on HFD feeding, we performed daily injections of wortmannin, a PI3K inhibitor, during the first week of HFD feeding. This treatment blocks AP proliferation in VWAT (Fig. 5d) with no effect on SWAT AP proliferation (Supplementary Fig. 4A), indicating that PI3K signalling is required for HFD-induced AP activation. To focus our study on AKT2 signalling within adipose tissue, we generated a conditional knockout of Akt2 with the PdgfRα promoter, which targets adipocyte lineage cells19, but not liver or muscle27. We find that full excision of the Akt2 gene is achieved in APs from both SWAT and VWAT (Supplementary Fig. 4B), and that PdgfRα–cre; Akt2floxed/floxed mice exhibit normal body weight at six weeks of age (Supplementary Fig. 4C). When we place these mice on HFD to assess the activation of APs, we find a significant reduction in AP proliferation in VWAT of PdgfRα–cre; Akt2floxed/floxed mice compared to HFD-fed controls (Fig. 5e), despite normal food intake (Supplementary Fig. 4D), whereas SWAT AP proliferation levels remain low in these mice (Supplementary Fig. 4E). Furthermore, BrdU pulse-chase analysis shows that after eight weeks of HFD, adipocyte formation in VWAT of PdgfRα–cre; Akt2floxed/floxed mice is not increased compared to SD-fed wild-type mice (Fig. 5f), indicating a lack of AP activation and subsequent differentiation in response to HFD. These data show that AKT2 signalling within the adipocyte cellular lineage is required to support the activation of APs at the onset of HFD-induced obesity.

Consistent with the isomorf-specific role of AKT2 in AP activation, Akt1 global knockout mice placed on HFD for one week exhibit AP proliferation rates in VWAT similar to wild-type mice (Fig. 6a), whereas mice lacking Akt2 exhibit significantly reduced VWAT AP proliferation on HFD (Fig. 6a); however, we observe no difference in SWAT proliferation rates (Fig. 6b). When we analyse the production of new adipocytes in Akt2–/– mice by BrdU pulse labelling we find that, similar to PdgfRα–cre; Akt2floxed/floxed mice, there is no significant increase
in the percentage of BrdU-labelled mature adipocytes in Akt2−/− HFD-fed mice compared to SD-fed mice (Fig. 6c). These data indicate that Akt2 is indeed the AKT isoform required for AP activation in response to HFD feeding.

To determine whether the AKT2-dependent mechanism of adipocyte hyperplasia is restricted to HFD-induced obesity, we investigated the role of AKT2 in adipose tissue expansion of ob/ob mice, which lack functional leptin, exhibit hyperphagia, and become extremely obese14. At weaning, ob/ob; Akt2−/− mice have similar body weight and fat mass to ob/ob mice (Fig. 6d,e), and they exhibit a hyperphagic phenotype whether measured as total calories (Supplementary Fig. 5A) or calories normalized to body weight (Supplementary Fig. 5B). During this period of hyperphagia, ob/ob mice exhibit elevated rates of AP proliferation compared to wild-type mice, whereas ob/ob; Akt2−/− mice do not (Fig. 6f), which indicates a defect in AP activation in the absence of Akt2. Although ob/ob; Akt2−/− mice also exhibit reduced adipocyte hypertrophy compared to ob/ob mice (Supplementary Fig. 5C,D), these data suggest that their reduced VWAT mass at 12 weeks of age (Fig. 6g) is partially due to a defect in adipogenesis. Thus, Akt2 plays a role in AP activation at the onset of leptin-deficient obesity in addition to HFD-induced obesity.

Given the requirement for Akt2 in the obesogenic growth of WAT, we sought to determine whether Akt2 also plays a role in the normal growth of WAT by characterizing the development of WAT in wild-type and Akt2−/− mice. During the establishment of the SWAT depot before birth and the VWAT depot during the first week of postnatal life (Fig. 7a and ref. 35), APs within these depots undergo robust proliferation (Fig. 7b,c). This proliferation is followed by rapid hypertrophic growth of newly formed adipocytes35,36 (Fig. 7d,e and Supplementary Fig. 6A,B). AP proliferation rates in both SWAT and VWAT do not differ between wild-type and Akt2−/− mice at any observed developmental time point (Fig. 7b,c). Likewise, quantification of adipocyte size in both depots during development shows no difference in hypertrophic growth of adipocytes between wild-type and Akt2−/− mice (Fig. 7d,e and Supplementary Fig. 6A,B). Finally, total body fat mass on weaning into young adulthood is not significantly different between wild-type and Akt2−/− mice (Fig. 7f), and Pdgfrα−/−; Akt2+lox/lox mice also have normal fat mass at six weeks of age (Supplementary Fig. 6C), indicating that developmental WAT growth occurs normally in the absence of Akt2. Taken together, these data show that the regulation of adipogenesis occurs through distinct mechanisms in obesity and development.

**DISCUSSION**

Although the formation of adipose tissue in development and the expansion of adipose tissue in obesity are often viewed as temporal
variations on the same regulatory process, we show here that the formation of adipocytes in obesity and development are controlled by distinct molecular mechanisms. The existence of an aberrant mechanism of adipogenesis in obesity supports the American Medical Association’s classification of obesity as a disease and suggests that FAT regulation is more complex than previously appreciated. It has been hypothesized that once adipocytes reach their maximal size they stimulate the production of new adipocytes. Our data show, however, that at the onset of diet-induced obesity, adipogenesis is initiated long before existing adipocytes reach their maximum lipid-filling capacity. Nonetheless, it is possible that signal(s) from hypertrophied adipocytes promote the subsequent lipid filling of activated APs, given that the maturation of these cells takes several weeks in vivo (Fig. 3 and ref. 13).

We show here that Akt2 is required for the activation of APs in obesogenic adipogenesis. Although the precise role of Akt2 in this process remains to be determined, Akt2 is known to promote glucose uptake and insulin signalling within insulin-sensitive tissues such as liver, muscle and adipose. C57BL/6 mice with global knockout of Akt2 develop normally but exhibit a mild diabetic phenotype, and studies focused on liver metabolism have demonstrated that Akt2 is required for de novo lipogenesis. Given that insulin signalling and lipogenesis play important roles in adipogenesis, it is possible that Akt2 functions to promote these pathways during AP activation.

Furthermore, in response to obesogenic stimuli, mice lacking Akt2 are protected from weight gain, which our data indicate is partially due to a deficiency in hyperplastic WAT growth. Previous studies have also suggested that Akt2 is involved in regulating WAT mass in humans. Specifically, a dominant negative Akt2 allele results in reduced WAT mass, whereas an activating Akt2 mutation is associated with excess WAT accumulation. Data presented here, combined with the recent finding that human VAT expansion is primarily controlled by adipocyte hyperplasia, suggest that Akt2 plays a role in the expansion of WAT through increased adipocyte number in human obesity.

Our data also have important implications for the understanding of obesity-related disease. The accumulation of VAT is associated with higher risk of obesity-related diseases such as diabetes, cardiovascular disease, and cancer. Conversely, the accumulation of VAT may have beneficial effects on glucose homeostasis. In combination with the findings presented here, these data suggest that the metabolic consequences associated with visceral obesity may be linked to hyperplastic expansion in this depot. Although several studies have examined differences between subcutaneous and visceral adipose tissue, often focusing on gene expression, metabolic activity, or cellular lineage, it remains unclear which feature(s) of the VAT is responsible for the selective activation of visceral APs in HFD-induced obesity. The new adipocytes formed via obesogenic adipogenesis may have altered function, and thus directly contribute to endocrine dysfunction or inflammation in WAT. Alternatively, the production of more adipocytes may be beneficial, increasing the capacity of WAT to store excess lipid, thereby decreasing ectopic lipid accumulation in other tissues, which can promote metabolic disease.

This study provides a framework for understanding the effects of dietary nutrient sensing on cellular mechanisms of tissue homeostasis.

Our data suggest that even relatively short binges of altered eating behaviour may stimulate obesogenic adipogenesis, resulting in an intractable increase in adipocyte number that may make future weight loss more difficult. Future studies will determine the long-term effect of this transient induction of adipogenesis on adipocyte number, weight gain, and metabolic disease. Finally, identification of the precise nutrient signal(s) involved may lead to the development of therapeutic strategies to target the obesity-specific pathway, ameliorating WAT gain in human obesity without affecting normal WAT mass.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Note:** Supplementary Information is available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

E.J., C.D.C. and M.S.R. designed experiments. E.J., C.D.C., B.H., L.C. and M.S.R. performed experiments. E.J., C.D.C. and B.H. analysed data. E.J., C.D.C., B.H. and M.S.R. interpreted data. E.J. and M.S.R. wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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METHODS

Animals. All animal studies followed guidelines issued by Yale University’s Institutional Animal Care and Use Committee (IACUC). All mice used for these studies were on the C57BL/6J genetic background. Adult wild-type mice were purchased from Jackson Laboratories and bred in the Yale Animal Resource Center. Akt2
-mice were a generous gift from E. Rosen (Beth Israel Deaconess Medical Center, Boston, MA, USA) and Akt1
-mice were a generous gift from W. Sessa (Yale University, New Haven, CT, USA). Akt2
-mice were on the C57BL/6J genetic background. Adult wild-type mice were purchased from Jackson Laboratories. When possible, mice were randomized based on body weight to achieve similar average weight in different groups. For experiments involving developmental time points, wild-type mice were purchased from Jackson Laboratories and bred in the Yale Animal Resource Center. PdgfR
-mice, stock no. 007676, and Adiponectin-CreER mice were a generous gift from M. Birnbaum (University of Pennsylvania, Philadelphia, PA, USA) via K. Martin (Yale University, New Haven, CT, USA), and Adiponectin-CreER mice were a generous gift from E. Rosen (Beth Israel Deaconess Medical Center, Boston, MA, USA) and can now be purchased at Jackson Laboratories (stock no. 024671). For adipocyte pulse-chase experiments, m/lg
B6.129(Cg)-Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J, stock no. 007676 were purchased from Jackson Laboratories and bred to Adiponectin-CreER mice in the Yale Animal Resource Center. 

Biological, B2850) treatments lasting 12-24 h, mice or pregnant mothers were given -H2B-GFP mice, stock no. 007669, were purchased from Jackson Laboratories and bred in the Yale Animal Resource Center. When possible, mice were randomized based on body weight to achieve similar average weight in different groups. For experiments involving developmental time points, wild-type mice were purchased from Jackson Laboratories and bred in the Yale Animal Resource Center. PdgfR
-mice, stock no. 007676, and Adiponectin-CreER mice were a generous gift from E. Rosen (Beth Israel Deaconess Medical Center, Boston, MA, USA) and can now be purchased at Jackson Laboratories (stock no. 024671). For adipocyte pulse-chase experiments, m/lg

B6.129(Cg)-Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J, stock no. 007676 were purchased from Jackson Laboratories and bred to Adiponectin-CreER mice in the Yale Animal Resource Center. PdgfR
-mice, stock no. 007669, were purchased from Jackson Laboratories and bred in the Yale Animal Resource Center. Unless otherwise noted, mice were males 6-8 weeks of age at the start of experiments. In this study, YVAT refers to the perigonadal visceral adipose tissue in mice. SWAT refers to inguinal subcutaneous adipose tissue in mice. For BrdU (US Biological, B2850) treatments lasting 12-24 h, mice or pregnant mothers were given intraperitoneal injections of 50 mg kg
BrDU in sterile PBS every 6 h. For BrdU treatments lasting longer than 24 h, BrdU was administered in the drinking water at 0.8 mg ml
for experiments lasting one week or less, or 0.4 mg ml
for experiments lasting longer than one week. BrdU water was refreshed every two weeks. Where indicated, mice were given daily intraperitoneal injections of freshly made Wortmannin (1 mg kg
, Cayman Chemical, 1010591) in 1:8 DMSO:PBS. High-fat diet is from Harlan Laboratories (2018S). Briefly, adipose tissue was excised, minced, and incubated with CD34 Alexa Fluor 647 (BioLegend, 119314, clone MEC14.7, used at 1:400) or CD34 Brilliant Violet 421 (Biolegend, 119321, clone MEC14.7, used at 1:300) and CD24 PerCP-Cyanine 5.5 (eBioscience, 45-0242-80, clone M1/69, used at 1:400) or CD34 Brilliant Violet 421 (BioLegend 119321, clone MEC14.7, used at 1:300) and CD24 PerCP-Cyanine 5.5 (eBioscience, 45-0242-80, clone M1/69, used at 1:400), following antibody incubation, samples were washed and analysed on a BD LSRII analyser. Data analysis was performed using BD FACSDiva software (BD Biosciences).

Analysis of phosphorylated AKT by flow cytometry. AP cells were isolated as described above, however all buffers used before fixation contained Roche PhosStop phosphatase inhibitor cocktail and 15 μM Wortmannin (Cayman Chemical) to limit changes in pathway activation during isolation. Cells were fixed in BD Phosflow Lyse/Fix Buffer (BD Biosciences) and membranes were permeabilized in BD Phosflow Perm Buffer III (BD Biosciences) according to the manufacturer’s instructions. Cells were stained for pAKT with the following antibodies from Cell Signaling: phospho-AKT S473 PE conjugate (no. 5315, clone D9E, used at 1:100) and phospho-AKT T308 PE conjugate (no. 9088, clone C31E5, used at 1:100), at 4°C overnight. As a control for the ability of this isolation procedure to maintain the phosphorylation status of AKT, VAT was excised, minced and treated with varying concentrations of insulin in PBS at 37°C for 10 min, briefly washed to remove insulin, and AP cells were then isolated from the minced tissue as described above. Phospho-AKT staining with both antibodies exhibited a time-dependent increase in 1% IGEPAL with protease inhibitors (Roche) and PhosStop phosphatase inhibitors (Roche). Protein concentration for the resulting lysates was determined using the BCA (bicinchoninic acid) Protein Assay kit from Pierce, and lysates were run on 10% polyacrylamide gels from Invitrogen, followed by western blotting analysis.

Isolation of adipocyte nuclei. Adipose tissue was minced into approximately 3-4 mm pieces and gently digested in 1 mg ml
collagenase type II (Worthington Biochemical; LS004174) in Krebs Ringer Phosphate Buffer (KRP) for 80 min. Floating adipocytes were separated from stromal cells by centrifugation at 150g for 8 min, filtered through a 190 μm filter, and washed in KRP. Isolation of intact adipocytes was verified by staining for plasma membrane with Cell Mask Orange (Invitrogen, C10405) and nuclei with DAPI as described. Adipocytes were lysed for 5 min in 0.2% IGEPAL in KRP with vortexing every 1-2 min. Adipocyte nuclei were isolated by centrifugation at 2000g for 5 min. For BrdU analysis, nuclei were fixed, permeabilized, and treated with DNAse and stained for BrdU as described above for stromal cells. For identification, nuclei were stained with propidium iodide and analysed for BrdU incorporation on a BD LSRII analyser. Data is shown as the percentage of Propidium-positive events that were also BrdU-positive. Data analysis was performed using BD FACSDiva software (BD Biosciences).

Adiponectin-CreER; mT/mG pulse chase. Starting at eight weeks of age, mice were treated with 50 mg kg
tamoxifen (Sigma) dissolved in vegetable oil by intraperitoneal injection for five consecutive days, and then allowed to recover for one week. Mice were then euthanized for baseline analysis, or placed on HFD or remained on SD for the 8-week chase period. Inguinal subcutaneous and perigonadal visceral tissue were taken from several regions throughout the depot, and analysed by whole mount confocal microscopy for tdTomato and eGFP expression. For each data point, at least 150 (SW AT) or 1,300 (VW AT) adipocytes were counted from multiple images from each depot of each animal.

Immunofluorescence. Adipose tissue was prepared as described for paraffin-embedded tissue. Briefly, tissue was fixed in 1× zinc formaldehyde for 24-48 h at 4°C, then washed with PBS. Samples were then dehydrated in increasing concentrations of ethanol over 6 h, and embedded in paraffin wax. 5 μm sections of paraffin blocks were then deparaffinized and rehydrated, followed by antigen retrieval in 10 mM sodium citrate, pH 6.0 under pressure in a 2100 Retriever (PickCell Laboratories). Blocking and staining was performed in 2% BSA in PBS. Sections were incubated in primary antibodies including rat anti-BrdU (Abcam no. ab6326, clone no. BU1/75 (ICR1) used at 1:1350) and rabbit anti-Caveolin-1 (Cell Signaling no. 3238, used at 1:400) overnight at 4°C. Slides were then rinsed and incubated with Alexa Fluor 488 (Abcam, 111-295-144), both used at 1:250, were purchased from Jackson Immunoresearch, and incubated with tissue for 1-2 h at room temperature. Slides were mounted with DAPI Fluoromount-G mounting medium (Southern Biotech) and imaged by confocal microscopy. Several confocal images of each tissue section were acquired, and analysed for the presence of BrdU and adipocyte nuclei. Adipocyte nuclei were identified by their location inside adipocyte membranes as described.

Confocal microscopy. For mT/mG quantification experiments, confocal microscopy was performed as described previously. For developmental experiments, adipose tissue was excised and stained with HCS LIPIDTOX Green Neutral Lipid Stain (Invitrogen, H34475, used at 1:100) and Isoleucin GS-B4 Alexa 647 (Invitrogen, I32450, used at 1:200) for at least 30 min before being washed in PBS and mounted onto slides in Fluoromount-G (Southern Biotech; 0100-01). Images were taken on a Leica TCS SP5 confocal microscope. Size analysis of lipid droplets in developing WAT was performed on confocal images using Image J software.

For quantification of PdgfR-positive nuclei in PdgfR-H2B-GFP WAT, adult mice were either maintained on standard chow diet or fed high-fat diet for one week. WAT was excised, weighed, and confocal z-stacks were taken on a Leica TCS SP5 confocal microscope. A representative 3D image taken from a z-stack is shown in Fig. 2e. The nuclei were counted in ImageJ using the Object Counter3D (http://rsb.info.nih.gov/ij/plugins/track/objects.html) plugin. The volume of each z-stack was calculated using the dimensions of the stack (length: 387 μm x width: 387 μm x height: 0.7 μm x number of slices). Using the density of triolein (0.915 g ml
, ref. 7) with the volume of each z-stack, the weight of each z-stack was calculated. Then, the average number of cells per gram was calculated using the total number of PdgfR-positive nuclei in the stack and the weight of the z-stack. Last, the total number of cells in the depot was estimated by multiplying the number of cells per gram by the
weight of each fat pad. Five animals were used in the control group (SD) and five animals were used in the experimental group (HFD). For each animal, a total of six z-stacks (50–250 virtual slices per z-stack) were created from multiple regions throughout the depot.

**Food intake and body composition.** Analysis of body composition was performed by NMR using the Echo MRI whole body composition analyser (Echo Medical System, Houston, TX). For food intake experiments with ob/ob animals, mice were individually caged and food and mice were weighed weekly. Kilocalories of food intake were normalized to the average body weight of the mouse at the beginning and end of the week. For PdgfR–cre; Akt2flox/flox experiments, mice were individually caged and food intake was measured with a TSE PhenoMaster System for small laboratory animals. Kilocalories of food intake were normalized to the average body weight of the mouse at the beginning and end of the 4-day period.

**Western blotting.** Isolated AP cells were lysed in 1% IGEPA with protease inhibitors (Roche) and PhosStop phosphatase inhibitors (Roche). Whole fat was snap frozen in liquid nitrogen, and homogenized on ice in RIPA buffer including 10% glycerol, 1% triton-X100, 0.1%SDS, 0.5% sodium deoxycholate, protease inhibitors (Roche) and PhosStop phosphatase inhibitors (Roche). Lysates were centrifuged at 4°C at 13,000 r.p.m. and the lysate was transferred twice to remove lipid. Quantification of protein was performed using the BCA (bicinchoninic acid) Protein Assay kit from Pierce, and lysates were run on 10% polyacrylamide gels from Invitrogen. Protein was transferred to PVDF membrane using the Invitrogen NuPage system. Primary antibodies were purchased from Cell Signaling, including rabbit anti-AKT1 (no. 2938, clone C73H10 used at 1:1,000), rabbit anti-AKT2 (no. 3063, clone D6G4, used at 1:1,000), rabbit anti-phospho-AKT1 S473 (no. 9018, clone D7F10, used at 1:500), rabbit anti-phospho-AKT2 S474 (no. 8599, clone D3H2, used at 1:500), rabbit anti-β actin (no. 4970, clone 13E5, used at 1:2,000), and were diluted in tris-buffered saline with 0.1% Tween-20. Goat anti-rabbit-HRP secondary antibody was purchased from Jackson Immunoresearch (no. 211-032-171) and used at 1:10,000. Blots were developed using the SuperSignal West Pico Chemiluminescent Substrate from Pierce. Quantification was performed using Adobe Photosop CS6. For experiments in which both phospho-AKT and total AKT were analysed from the same blot, phospho-AKT was probed for first. The blot was then stripped with Thermo Scientific Restore Plus Western Blot Stripping Buffer for 5–10 min, washed, and incubated with secondary antibody for 30 min. The blot was then developed to check that there was no residual signal from phospho-AKT antibodies. The blot was then washed, and incubated in blocking buffer, and incubated with antibodies to detect total AKT.

**Statistical analysis.** Statistical tests used are indicated in the figure legends. All statistical tests were performed using GraphPad Prism version 6.02 for Windows (GraphPad Software). The investigators were not blinded to allocation during experiments and outcome assignment. Mice were genotyped before performing experiments. Data are expressed as mean ± s.e.m., and P < 0.05 was considered statistically significant. Samples were excluded from experiments if animals were not eating or were otherwise deemed unhealthy. Groups of at least five mice were used for experiments, unless statistical significance was reached with fewer animals. No statistical method was used to pre-determine sample size. Sample sizes for each group are listed in figure legends, and indicate individual animals (biological replicates).

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Supplementary Figure 1 Characterization of HFD-induced adipocyte hyperplasia. (A) Experimental scheme for BrdU time course in Figure 2B. (B) Representative flow cytometry dot plots indicating sequential gating used to identify AP populations, including CD24+ adipocyte progenitors (CD45-, CD31-, CD34+, CD29+, Sca1+, CD24+), CD24- preadipocytes (CD45-, CD31-, CD34+, CD29+, Sca1+, CD24-), and total APs (CD45-, CD31-, CD34+, CD29+, Sca1+). (C) Flow cytometry plots of SVF from VWAT of PdgfRα-H2B-GFP mice showing that the majority of GFP+ cells are CD29+; CD34+, of which 97.1 ± 1.5% are also Sca1+. (n = 3 mice) (D) Quantification of BrdU incorporation into GFP+ cells from VWAT of male PdgfRα-H2B-GFP mice during 1 week of SD or HFD feeding and BrdU treatment. (n = 5 mice for SD and 7 mice for HFD.) Significance between the indicated groups in (D) was calculated using a two-tailed student’s t-test. Exact p-values are listed in Supplementary Table 1. Error bars or values represent mean ± s.e.m. ** (p<0.01). HFD: high-fat diet, SD: standard diet, BrdU: bromodeoxyuridine.
Supplementary Figure 2 Adipocyte precursor proliferation during week 2 of HFD feeding does not lead to adipogenesis. (A) Schematic and depicting the second week BrdU pulse-chase in Supplementary Figure 2B. (B) BrdU incorporation into adipocyte nuclei after pulse-chase from the second week of HFD feeding. (n = 5 for all groups). Error bars or values represent mean ± s.e.m. HFD: high-fat diet, SD: standard diet, BrdU: bromodeoxyuridine.
Supplementary Figure 3  High-fat diet does not induce AKT phosphorylation in whole fat. (A) Western blot analysis for phosphorylated AKT at the indicated sites and total AKT, in lysates from whole VWAT on day 3 of diet. Each lane represents one animal. Uncropped blots are shown in Supplementary Figure 7I-L. (B) Quantification of pAKT western blots in (A), normalized to total AKT. (C-D) VWAT was treated with the indicated concentrations of insulin prior to tissue digestion, and APs were isolated in the presence of wortmannin and phosphatase inhibitors to limit further changes in pathway activation (see methods). APs were then fixed and analyzed by flow cytometry for levels of phosphorylated AKT at threonine 308 (C) or serine 473 (D). The same AP cell isolation procedure was used in the experiments in Figure 4B-F and 5B-C. (E) Western blot for phosphorylated AKT1 (S473) with lysates from APs enriched from SVF via Sca-1 bead pull down (Sca1-positive) and remaining unselected cells (Sca1-negative) (see methods) after 3 days of HFD or SD. Each lane represents pooled cells from 2 mice. Uncropped blots are shown in Supplementary Figure 7M-N. Significance between the indicated groups in (B) was calculated using a two-tailed student’s t-test. Error bars represent mean ± s.e.m. HFD: high-fat diet, SD: standard diet.
**Supplementary Figure 4** Deletion of AKT2 in the adipocyte lineage does not affect body weight or food intake. (A) Quantification of BrdU incorporation into SWAT APs after 1 week of HFD or SD with daily injection of wortmannin (Wort), or vehicle (Veh) (n = 5 mice for each group). (B) PCR analysis of DNA isolated from AP cells from the VAT and SWAT depots of female mice show successful excision of the floxed exons 4 and 5 from Akt2 gene, while Lin+ cells (CD45+ blood cells and CD31+ endothelial cells) contain the full-length Akt2 gene. Each lane represents cells isolated from the indicated depot of individual mice. (C) Body weight of Akt2fl/fl (n = 9 mice) and Pdgfrα-cre; Akt2fl/fl (n = 12 mice) at 6 weeks of age. (D) Quantification of food intake normalized to body weight in the indicated groups of mice on SD or during the first 4 days of HFD feeding. (n = 3 mice for Akt2fl/fl SD and Pdgfrα-cre; Akt2fl/fl SD, n = 4 mice for Pdgfrα-cre HFD, n = 2 mice for Akt2fl/fl HFD, and n = 3 mice for Pdgfrα-cre; Akt2fl/fl HFD. (E) BrdU incorporation into SWAT APs of the indicated groups during the first week of HFD feeding compared to SD controls. (n = 7 mice for Akt2fl/fl SD, n = 4 mice for Pdgfrα-cre; Akt2fl/fl SD, n = 14 mice for Pdgfrα-cre; Akt2fl/fl HFD, n = 8 mice for Akt2fl/fl HFD, n = 5 mice for wild-type and Pdgfrα-cre HFD). Significance in (D) was calculated using one-way ANOVA with Tukey’s test for multiple comparisons. Exact p-values are listed in Supplementary Table 1. Statistics source data for 4D can be found in Supplementary Table 2. Error bars represent mean ± s.e.m. ** (p<0.01), **** (P<0.0001).
Supplementary Figure 5 AKT2 is required for the activation of adipocyte precursors at the onset of obesity in ob/ob mice. (A-B) Weekly intake of SD in raw kilocalories (kCal) (A) or kCal normalized to body weight (B) for the indicated groups of young mice after weaning at 3 weeks of age. Week "3" denotes food intake between the ages of 3 and 4 weeks. (n = 4 for wild-type, n = 3 for ob/ob, n = 7-8 for ob/ob; Akt2−/−) (C-D) H&E-stained sections of VWAT (C) and corresponding adipocyte size measurements (D) for the indicated groups. (n = 7 for ob/ob; Akt2−/−, n = 4 for ob/ob). Significance between indicated groups in (A-B) was calculated using a two-tailed Student’s t-test. Significance in (D) was calculated using two-way ANOVA with Bonferroni’s test for multiple comparisons. Scale bars in (C) are 50μm. * or a (P<0.05), ** or b (p<0.01), *** or c (P<0.001), **** or d (P<0.0001). For (A-B), * indicates significance of ob/ob over wild-type, † indicates significance of ob/ob; Akt2−/− over wild-type, and ‡ indicates significance of ob/ob; Akt2−/− over ob/ob. Exact p-values are listed in Supplementary Table 1. Statistics source data for 5A-B can be found in Supplementary Table 2. Error bars represent mean ± s.e.m.
Supplementary Figure 6 AKT2 is not required for the normal development of white adipose tissue. (A-B) Confocal images (A) of developing wild-type and Akt2−/− SWAT stained with LipidTOX (a lipid stain) and Isolectin IB4 (an endothelial cell stain) and (B) corresponding lipid droplet size quantification based on LipidTOX staining (n = 3 mice for all groups except n = 4 mice for Akt2−/− P5). Each group includes mice from 2-3 different litters. (C) Echo MRI quantification of fat mass from the indicated groups of mice at 6 weeks of age. (n = 13 mice for Akt2fl/fl and n = 14 mice for PdgfRα-cre; Akt2fl/fl). Scale bars in (A) are 50μm. Error bars represent mean ± s.e.m. PX indicates postnatal day X.
Supplementary Figure 7 Uncropped western blots. (A-B) Western blots from the experiment in Figure 5A. (C-E) Western blots from the experiment in Figure 5B top panel. (F-H) Western blots from the experiment in Figure 5B bottom panel. (I-J) Western blots from the experiment in Supplementary Figure 3A top panel. (K-L) Western blots from the experiment in Supplementary Figure 3A bottom panel. (M-N) Western blots from the experiment in Supplementary Figure 3E.
**Supplementary Figure 7 continued**

- **i** Supplementary 3A top panel
- **j** Supplementary 3A top panel
  - 60 kDa
  - 50 kDa
  - 40 kDa
  - pAkt (T308)
  - 60 kDa
  - 50 kDa
  - 40 kDa
  - Akt
  - background band

- **k** Supplementary 3A bottom panel
- **l** Supplementary 3A bottom panel
  - 60 kDa
  - 50 kDa
  - 40 kDa
  - pAkt (S473)
  - 60 kDa
  - 50 kDa
  - 40 kDa
  - Akt
  - background band

- **m** Supplementary 3E bottom panel, short exposure
  - 160 kDa
  - 110 kDa
  - 80 kDa
  - 60 kDa
  - 50 kDa
  - 40 kDa
  - 30 kDa
  - membrane cut
  - bActin
  - pAkt (T308)

- **n** Supplementary 3E bottom panel, long exposure
  - 160 kDa
  - 110 kDa
  - 80 kDa
  - 60 kDa
  - 50 kDa
  - 40 kDa
  - 30 kDa
  - membrane cut
  - bActin
Supplementary Table 1

| Figure 1 | C | SD VWAT vs SD SWAT | 0.0373 |
| Figure 2 | A | HFD SD vs HFD | 0.0078 |
| Figure 3 | B | VWAT SD vs HFD for 7 weeks diet | 0.0007 multiplicity-adjusted p-value |
| Figure 4 | A | SD vs Day 1 HFD | 0.0007 |
| Figure 5 | C | pAkt T308 SD vs HFD | 0.013 |
| Figure 6 | A | WT SD vs HFD | 0.0045 |

**Supplementary Figure 1**

| | | 20-30µm ob/ob vs Akt2-/-;ob/ob | 0.0396 multiplicity-adjusted p-value |
| | | 30-40µm ob/ob vs Akt2-/-;ob/ob | 0.0002 multiplicity-adjusted p-value |
| | | 40-50µm ob/ob vs Akt2-/-;ob/ob | < 0.0001 multiplicity-adjusted p-value |
| | | 50-60µm ob/ob vs Akt2-/-;ob/ob | 0.0011 multiplicity-adjusted p-value |
| | | 60-70µm ob/ob vs Akt2-/-;ob/ob | 0.0236 multiplicity-adjusted p-value |
| | | 90-100µm ob/ob vs Akt2-/-;ob/ob | < 0.0001 multiplicity-adjusted p-value |
| | | 100-110µm ob/ob vs Akt2-/-;ob/ob | < 0.0001 multiplicity-adjusted p-value |
| | | 110-120µm ob/ob vs Akt2-/-;ob/ob | < 0.0001 multiplicity-adjusted p-value |
| | | 120-130µm ob/ob vs Akt2-/-;ob/ob | 0.006 multiplicity-adjusted p-value |

**Supplementary Table 1**

Exact p-values. Exact p-values for statistical tests that show significance.
Supplementary Table 2

Individual data points. Individual data points are reported for experiments with any group containing less than 5 mice, and results not plotted as individual data points in the figures.

| Week | SWAT AP SD |   |   |   |   |   |   |   |
|------|------------|---|---|---|---|---|---|---|
| 1    | 3.8        | 5.6 | 16.4 | 10.8 |   |   |   |   |
| 2    | 8.5        | 16.3 | 14 | 7.3 | 12.8 |   |   |   |   |
| 4    | 3.3        | 2.9 | 5.8 | 1.9 | 3.5 |   |   |   |   |
| 6    | 4.7        | 4.3 | 4.4 | 2.2 | 1.6 |   |   |   |   |

| Week | SWAT AP HFD |   |   |   |   |   |   |   |
|------|-------------|---|---|---|---|---|---|---|
| 1    | 17.6        | 8.6 | 13.7 | 16.4 | 18.1 | 9.2 | 10.5 | 18.5 |
| 2    | 16.8        | 9.1 | 10.8 | 11.4 | 19.2 | 10.6 | 11.3 | 15.5 | 16.6 |
| 4    | 6.3         | 5.8 | 5.6 | 4.7 | 3.3 | 1.6 | 3.5 |   |   |
| 6    | 2.5         | 4.5 | 4.3 | 2.5 |   |   |   |   |   |

| Week | SWAT AP SD |   |   |   |   |   |   |   |
|------|------------|---|---|---|---|---|---|---|
| 1    | 1.7        | 4.8 | 4.4 | 8.5 | 5.1 |   |   |   |   |
| 2    | 2.7        | 9.4 | 1.8 | 2.4 | 0.6 |   |   |   |   |
| 4    | 1.5        | 1.5 | 1.8 | 0.2 | 0.3 |   |   |   |   |
| 6    | 1.0        | 1.4 | 0.2 | 0.4 | 0.2 |   |   |   |   |

| Week | SWAT AP HFD |   |   |   |   |   |   |   |
|------|-------------|---|---|---|---|---|---|---|
| 1    | 0.37        | 0.52 | 0.29 | 0.14 |   |   |   |   |
| 2    | 0.39        | 0.47 | 0.16 | 0.19 |   |   |   |   |
| 4    | 0.44        | 0.52 | 0.10 | 0.23 |   |   |   |   |
| 6    | 0.36        | 0.47 | 0.16 | 0.19 |   |   |   |   |

Figure 5C

| pAkt2/Akt2 | pAkt1/Akt1 |
|------------|------------|
| SD         | HFD        |
| 0.37       | 0.29       |
| 0.36       | 0.47       |
| 0.44       | 0.32       |

Figure 6C

| Akt2fl/fl | Akt2fl/fl |
|-----------|-----------|
| SD         | HFD        |
| 2.22       | 2.02       |
| 2.07       | 2.02       |
| 2.03       | 2.02       |

Supplementary Figure 4D

| Akt2fl/fl | Akt2fl/fl |
|-----------|-----------|
| PDGFrα-cre; Akt2fl/fl | PDGFrα-cre; Akt2fl/fl |
| 2.22       | 2.02       |
| 2.07       | 2.02       |
| 2.03       | 2.02       |

Supplementary Figure 5A

| WT | ob/ob |
|----|------|
| 24.7 | 26.8 |
| 24.7 | 26.8 |
| 24.7 | 26.8 |

Supplementary Figure 5B

| WT | ob/ob |
|----|------|
| 25.3 | 23.5 |
| 25.3 | 23.5 |
| 25.3 | 23.5 |

Supplementary Figure 6A

| WT | ob/ob |
|----|------|
| 25.3 | 23.5 |
| 25.3 | 23.5 |
| 25.3 | 23.5 |

Supplementary Figure 6B

| WT | ob/ob |
|----|------|
| 25.3 | 23.5 |
| 25.3 | 23.5 |
| 25.3 | 23.5 |