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Abstract

Adipose morphology is defined as the number and size distribution of adipocytes (fat cells) within adipose tissue. Adipose tissue with fewer, but larger adipocytes is termed as having a 'hypertrophic' morphology, whereas adipose with many adipocytes, of a smaller size, is termed a 'hyperplastic' morphology. Hypertrophic adipose morphology is positively associated with insulin resistance, diabetes and cardiovascular disease. Contrastingly, hyperplastic morphology is associated with improved metabolic parameters. These phenotypic associations suggest that adipose morphology influences risk for cardiometabolic disease. Intriguingly, monozygotic twin studies have determined that adipose morphology is in part genetically determined. Therefore, identifying the genetic regulation of adipose morphology may help predict, prevent and ameliorate insulin resistance and associated metabolic diseases. Here we review the current literature regarding adipose morphology in relation to; (i) metabolic and medical implications, (ii) methods used to assess adipose morphology, and (iii) transcriptional differences between morphologies. We further highlight three mechanisms hypothesized to promote adipocyte hypertrophy and thus regulate adipose morphology.

Adipose tissue (AT) is a morphologically unique organ that accumulates lipid in response to an organism's energy status. During periods of caloric excess, AT sequesters circulating lipid which accumulates mainly as triacylglyceride (TAG) in cytoplasmic lipid droplets (LDs) within adipocytes (fat cells). Conversely, during periods of caloric need, AT mobilizes lipid from LDs into the circulation to act as an energy source for peripheral tissues. As such, AT functions as an energy buffer to protect an individual from adverse physiological demands. The ability to expand and contract to such extreme degrees, is unique to AT among other adult tissues. For example, in an individual whose weight increased from 70 to 150 kg, the AT mass quadrupled relative to changes in skeletal or muscle mass (Prins and O'Rahilly, 1997). Fluctuation in AT mass is largely due to changes in lipid volume and, to accommodate such dynamic variation, AT expands via increases in adipocyte size (hypertrophy) and adipocyte number (hyperplasia) and contracts via decreases in adipocyte size (hypotrophy) (Salans et al., 1973; Spalding et al., 2008). The balance between these growth and regression states establishes and maintains AT morphology: AT with fewer, but very large adipocytes is termed a hypertrophic morphology; whereas, AT with many, smaller adipocytes in termed hyperplastic **(Fig. 1A)**. In this review, we highlight how adipose morphology is associated with metabolic and physiological derangements, and present hypotheses for how adipose morphology may be regulated.

Obesity is not synonymous with metabolic dysfunction: a role for adipose morphology?

Overweight and obesity are characterized by increased lipid accumulation in adipocytes; whereas, weight loss is characterized by reduced lipid accumulation in AT (Eriksson-Hogling et al., 2015; Goodpaster and Sparks, 2017). Obesity is strongly correlated with metabolic disease – for every kg increase in body weight, diabetes rates increase linearly (Haffner, 2006). Although the rising prevalence of overweight and obesity has led to an increased occurrence of metabolic diseases including diabetes and cardiovascular disease (CVD) (Wilding, 2017), obesity is not synonymous with metabolic dysfunction. For example, in humans insulin resistance is a major underlying cause of CVD (Ginsberg, 2000), and is associated with dyslipidemia (Reaven et al., 1967), hypertension (Welborn et al., 1966) and atherosclerosis (Howard et al., 1996). However, huge variation exists in the degree of insulin resistance across all values of body mass index (BMI; a surrogate measure of adiposity) (McLaughlin et al., 2004). Indeed, the degree of insulin resistance can vary six fold at any given BMI (McLaughlin et al., 2004). Therefore, obesity *per se* is clearly not the sole driving force for metabolic dysfunction, and understanding which other

factors are responsible for the unexplained variance in insulin resistance will have important consequences for public health. Multiple related factors have been proposed to explain dysfunctional AT, and the variability in insulin resistance, during obesity; including, adipose inflammation, fibrosis, impaired angiogenesis, hypoxia and body fat distribution (Bluher, 2016; Crewe et al., 2017; Divoux et al., 2010; Khan et al., 2009; Sun et al., 2011; Trayhurn, 2013; Weisberg et al., 2003). Here, we present evidence from the literature that adipose morphology is an additional factor that influences susceptibility to metabolic disease.

Regional variation in adipose morphology

To assess the role of adipose morphology in metabolic disease, it is first essential to review how adipose morphology can vary between regionally-distinct ATs. Briefly, ATs are distributed throughout the human body, but are mainly categorized into subcutaneous ATs (SAT; AT situated between muscle and skin) and visceral ATs (VAT; AT associated with internal visceral organs) **(Fig. 1A)** (Shen et al., 2003). The subcutaneous and visceral sites of adipose accumulation appear conserved to mouse (Bartelt and Heeren, 2014; Cinti, 2012; Shen et al., 2003), and strikingly, most regional AT sites even appear conserved to zebrafish (Minchin and Rawls, 2017; Shen et al., 2003). The regional distribution of human AT is strongly associated with insulin resistance. A recent meta-analysis demonstrated that VAT was the strongest predictor of insulin resistance (measured by HOMA-IR) (Zhang et al., 2015); however, total fat mass, BMI, waist circumference, intra-abdominal fat, abdominal fat were also significantly associated with insulin resistance (Zhang et al., 2015). By contrast, lower-body SAT was not correlated with insulin resistance and has been shown to protect against metabolic dysfunction (Snijder et al., 2005; Snijder et al., 2004; Zhang et al., 2015). Many previous studies in humans have also linked accumulation of lipid within abdominal SAT to insulin resistance and metabolic disease, thus suggesting that upper body (or central adiposity) vs lower body (or peripheral adiposity) body fat distribution is an important factor in metabolic disease (Karpe and Pinnick, 2015; Porter et al., 2009).

Adipocytes from regionally distinct ATs can be significantly different sizes. For example, comparison of adipocyte size between three distinct human SATs (gluteal, anterior abdominal wall and triceps) revealed significant differences (gluteal > abdominal > triceps) (Salans et al., 1973; Salans et al., 1971). Indeed, intra-individual site-to-site variability in adipocyte size was greater than same site variability between individuals (Salans et al., 1971). In general, across

studies, SAT adipocytes were significantly larger than VAT adipocytes, irrespective of BMI or metabolic state (Liu et al., 2009; Tchernof et al., 2006), thus suggesting that SAT undergoes greater hypertrophy relative to VAT. However, previous studies have suggested that SAT is inherently more hyperplastic than VAT; although, these observations were based on in vitro data from mouse and humans showing that SAT-derived cells have greater adipogenic capacity than VAT-derived cells (Baglioni et al., 2012; Macotela et al., 2012; Tchkonia et al., 2006). More recent in vivo data using the transgenic AdipoChaser mouse line, have suggested the opposite; following diet-induced obesity, VAT (epidydimal) undergoes waves of hyperplastic growth, whereas SAT (inguinal) did not (Wang et al., 2013). Although these data conflict with the in vitro observations of higher SAT hyperplasia; these findings do conform to the larger adipocyte size, and presumed greater degree of hypertrophic growth in SAT. Thus, after exposure to a high-fat diet, SAT appears to preferentially undergo hypertrophic growth which leads to larger adipocytes relative to VAT. The contrasting growth dynamics of VAT and SAT is of biomedical importance as reduced expandability of SAT is associated with insulin resistance (Gealekman et al., 2011; Virtue and Vidal-Puig, 2008). In line with these observations, treatment of obese diabetics with thiazolidinidiones (TZDs) leads to greater weight gain, preferential lipid deposition in SAT and improved insulin sensitivity (Fonseca, 2003; Nichols and Gomez-Caminero, 2007). Thus, understanding the differential growth mechanisms of SAT may provide therapeutic targets for treating obesity-associated metabolic disease.

Using a ‘morphology value’ to quantify adipose morphology

To quantify adipose morphology, Arner et al. (2010) described a ‘morphology value’ – the difference between measured adipocyte volume and expected adipocyte volume (relative to total adipose mass) (Arner et al., 2010; Spalding et al., 2008) **(Fig. 1B)**. This metric was further utilized by Veilleux et al. (2011), and facilitated the categorization of individuals according to whether adipose exhibits a hypertrophic or hyperplastic morphology (Arner et al., 2010; Veilleux et al., 2011). To determine the morphology value, AT biopsies were first taken, then a single-cell adipocyte suspension was produced by collagenase digestion and buoyancy separation, and the size of individual adipocytes was then measured using image analysis software. A curvilinear line was then fitted to the data to best describe the relationship between adiposity and mean adipocyte size (Spalding et al., 2008). Each individual was then categorized in relation to the fitted line: individuals exhibiting a positive residual were hypertrophic (mean adipocyte volume larger than expected); whereas, individuals exhibiting a negative residual were hyperplastic (mean adipocyte

volume smaller than expected) (Arner et al., 2010; Veilleux et al., 2011) **(Fig. 1B)**. Importantly, lower morphology values (hyperplastic) were associated with an increased number of small adipocytes; whereas, high morphology values (hypertrophic) were associated with fewer, larger adipocytes (Arner et al., 2010; Veilleux et al., 2011). Within a population, adipose morphology appears highly variable. Arner et al. (2010) found that hyperplastic and hypertrophic morphologies were present at equal frequencies in both males vs females, and obese vs non-obese (Arner et al., 2010). Strikingly, at comparable BMI, women typically present with ~10% higher body fat, characterized with greater SAT in the abdomen and gluteofemoral regions (Camhi et al., 2011; Jackson et al., 2002; Karastergiou et al., 2012; Womersley, 1977). Body fat distribution is linked to health in both males and females; however, the protective peripheral distribution is mainly seen in females (Krotkiewski et al., 1983). Large inter-individual variation in adipocyte number and size within equivalent ATs was also observed, which was independent of adipose mass (Arner et al., 2011; Salans et al., 1973; Salans et al., 1971). Furthermore, estimates for adipocyte number varied by as much as 85% between individuals (Salans et al., 1973), suggesting a high-level of inter-individual variability in adipose morphology. Intriguingly, adipocyte number and size were highly similar in monozygotic twins concordant for BMI, suggesting a strong genetic basis (Heinonen et al., 2014). Therefore, understanding how genetics drives variation in adipose morphology with subsequent consequences for disease risk is a central research question.

Hypertrophic morphology is associated with insulin resistance and increased risk for cardiovascular disease

The association between SAT morphology and metabolic disease has been extensively characterized. In a cohort of 764 subjects exhibiting a wide adiposity range (BMI 18-60 kg/m^2), Arner et al. (2010) found that hypertrophic morphology was positively correlated with insulin resistance (measured by HOMA-IR) and fasting plasma Insulin in humans (Arner et al., 2010). Furthermore, in women, hypertrophic morphology was associated with a metabolic syndrome-like state, characterized by increased insulin resistance, and increases in circulating plasma Insulin, total cholesterol and TAG (Arner et al., 2010). Additionally, abdominal SAT adipocyte size by itself was positively associated with insulin resistance independent of BMI in non-diabetic humans (Lundgren et al., 2007). Further, SAT adipocyte size was positively associated with plasma Insulin, glucose, Insulin-induced glucose disposal and insulin sensitivity in humans (Hoffstedt et al., 2010). Finally, in humans, the average volume of adipocytes within abdominal SAT was

correlated with insulin resistance (Yang et al., 2012). By contrast, hyperplastic morphology was associated with significantly better blood glucose, Insulin and lipid profiles when compared to subjects with hypertrophic morphology (Hoffstedt et al., 2010). Taken together, these data demonstrate that hypertrophic SAT morphology is associated with metabolic dysfunction, and metabolic risk factors for diabetes and CVD. VAT morphology has also been implicated in metabolic disease. In a sample of 207 lean to severely obese females, subjects characterized by hypertrophic omental VAT had higher plasma TAG, higher very low density lipoprotein (vLDL)-TAG and higher vLDL-cholesterol when compared to subjects with hyperplastic VAT (Veilleux et al., 2011). It was also estimated that a 10% enlargement of VAT adipocytes increased the risk of hypertriacylglyceridemia 4-fold (Veilleux et al., 2011); whilst, a 10% increase in the number of VAT adipocytes increased the risk of hypertriacylglyceridemia by 1.55-fold (Veilleux et al., 2011). In morbidly obese females, and independent of age, BMI, body fat mass or body fat distribution, VAT adipocyte size was positively associated with plasma apolipoprotein B, total cholesterol, vLDL-cholesterol and triacylglycerides (Hoffstedt et al., 2010). Furthermore, large VAT adipocytes (>75 µm diameter) were associated with insulin resistance in canines (Kabir et al., 2011). These data demonstrate that hypertrophic VAT morphology is also associated with a metabolic-syndrome-like state.

Bi- and tri-modal adipocyte size distributions: a more complex relationship between adipose morphology and Insulin resistance?

Many studies have concluded that adipocytes comprise a complex population of cells that exhibit a bi- or tri-modal size distribution. In general, these studies have used osmium tetroxide fixation of adipocytes, followed by size analysis of adipocytes using a Coulter counter (Cushman and Salans, 1978; Etherton et al., 1977; Hirsch and Gallian, 1968). The advantages of this method include the ability to analyze large numbers of adipocytes (~6000 cells from each subject), and the automated and unbiased measurement of adipocyte size (Jo et al., 2012). To exclude the possibility that the small adipocytes (within a bimodal population) were not artefactual 'debris', it was confirmed by microscopy that these cells were comprised of intact, spherical small adipocytes (McLaughlin et al., 2007). In addition to osmium tetroxide fixation, measurement by microscopy has also indicated that adipocytes may form a bimodal size distribution (Fang et al., 2015). Comparison of the size distributions obtained from these methods revealed a peak of small adipocytes of ~25 µm diameter, and a peak of larger adipocytes of ~50 µm diameter **(Fig. 2)** (Jo

et al., 2012). Intriguingly, trimodal adipocyte size distributions in humans have also been observed with peaks at ~25, ~50 and ~100 µm diameters (Yang et al., 2012). Recently, 3D reconstruction of zebrafish VAT revealed a bimodal size distribution of adipocyte-localized LDs (Minchin et al., 2015). However, it is likely that the smaller population of LDs (~1 µm in diameter) correspond to additional LD 'locules' within multilocular adipocytes – a phenomenon also observed in human and mouse white adipocytes (Chau et al., 2014; Cushman, 1970). Together, these studies suggest that parametric statistics, such as mean adipocyte size and number, may not accurately represent the true population mean, and should be used with caution when assessing adipose morphology.

Multiple studies have confirmed that the size of larger adipocytes in a bimodal population is positively associated with metabolic dysfunction. First, McLaughlin et al. (2014) found that compared to BMI-matched insulin sensitive subjects, insulin resistant subjects had larger 'large' adipocytes within abdominal SAT. Second, in 35 subjects (with BMI ranging from 18-34 kg/m^2) the size of larger adipocytes within abdominal SAT was able to accurately predict insulin resistance (Yang et al., 2012). Third, in insulin sensitive obese individuals, an increase in size of the larger adipocyte fraction within abdominal SAT after feeding a hypercaloric diet, predicted a decline in Insulin-mediated glucose uptake (McLaughlin et al., 2016). In addition to insulin resistance, the size of the large adipocytes was also correlated with an increased VAT/VAT+SAT ratio (Kursawe et al., 2010), an increased proportion of small adipocytes in both VAT and SAT (Liu et al., 2009), and the normalization of insulin sensitivity in insulin resistant subjects after treatment with rosiglitazone (Eliasson et al., 2014). A summary of these findings is provided in **Table 1**. Taken together these studies show that hypertrophied adipocytes within the larger fraction of adipocytes in a bimodal population is also associated with insulin resistance.

The proportion and size of small adipocytes within a bimodal size distribution is also related to metabolic wellbeing. In moderately overweight/obese individuals, McLaughlin et al. (2007 & 2014) found an increased proportion of small adipocytes in abdominal SAT to be statistically associated with Insulin resistance, Further, an increased proportion of small adipocytes was found in both abdominal SAT and omental VAT of diabetics (Fang et al., 2015). Intriguingly, an increased proportion of small adipocytes in abdominal SAT also occurred in subjects with high VAT/VAT+SAT ratio (Kursawe et al., 2010), after insulin sensitive subjects were overfed (McLaughlin et al., 2016), or after diabetics were treated with rosiglitazone (Eliasson et al., 2014). In diabetics, the size of small adipocytes was also found to be inversely correlated with insulin sensitivity (Fang et al., 2015). Finally, an expanded nadir (the low point in frequency

between the small and large adipocyte populations) was found in insulin resistant subjects (McLaughlin et al., 2007). A summary of these findings is provided in **Table 1**. Together, these data show that insulin resistance is not only accompanied by hypertrophy of large adipocytes, but in studies that detect a bimodal adipocyte size distribution, insulin resistance is also associated with an increased proportion of small adipocytes. Related to the increased presence of small adipocytes, the expression of genes related to adipogenesis was also lower in insulin resistant individuals (McLaughlin et al., 2007). These findings are consistent with independent reports of reduced adipogenesis in insulin resistant patients (Goedecke et al., 2011; Yang et al., 2004). Further, these expression changes were also associated with modest increases in inflammatory activity in insulin resistant AT (McLaughlin et al., 2008). As the presence of small adipocytes in both VAT and SAT appears to be correlated with increased hypertrophy of larger adipocytes in abdominal SAT (Liu et al., 2009), these findings could be interpreted to suggest that SAT is the primary site for lipid accumulation; however, once a maximal SAT adipocyte size is reached, hyperplastic growth is initiated in both VAT and SAT. In support, adipocytes have been shown to expand to only a finite degree in both humans and rats (Faust et al., 1978; Kashiwagi et al., 1985). A summary of these findings is provided in **Table 1**. Therefore, these data suggest that insulin resistance is accompanied by an inability of small adipocytes to undergo hypertrophic expansion, suggestive of defective adipogenesis, resulting in a higher proportion of small adipocytes amongst a more general population of hypertrophied adipocytes.

Transcriptomic differences in distinct adipose morphologies

To study adipose hypertrophy and hyperplasia, and to identify potential molecular pathways that influence morphology, it is useful to analyze the transcriptional state underlying distinct morphologies. Strikingly, adipocytes of different sizes have distinct gene expression profiles. Jernas et al. (2006) fractionated human adipocytes into small (mean diameter 57.6 ± 3.54 μm) or large (mean diameter 100.1 ± 3.94 μm) groups. Subsequent microarray analysis of gene expression on the 2 groups of adipocytes revealed 14 genes with 4-fold higher expression in large adipocytes **(Table 2)** (Jernas et al., 2006). Strikingly, some transcripts exhibited 19-fold and 22-fold higher expression in large adipocytes, suggesting relatively large-scale differences between adipocytes based on size (Jernas et al., 2006). In an additional study, Heinonen et al. (2014) analyzed whether adipocyte size or number correlated with changes in the AT transcriptome. RNA was extracted from whole-adipose biopsies (including both adipocyte and stromal-vascular fractions), and gene expression changes positively correlating with adipocyte

size included, genes implicated in cell cytoskeleton and membrane modifications (*MSN*, *NHEDC2*, *KIF3B*, *PALLD*), oxidative stress and apoptosis (*MSN*), cell mediated immunity (*MIF*) and cancer (*NMES*) **(Tables 2 & 3)** (Heinonen et al., 2014). Genes inversely correlated with adipocyte size include *FDFT1* (mevalonate pathway, cholesterol biosynthesis), *ADH1B* (metabolism of a wide range of substrates, including hydroxysteroids and lipid peroxidation products), *EIF1B* (unknown function) **(Tables 2 & 3)** (Heinonen et al., 2014). Significant Gene Ontology (GO) terms, used to describe shared relationships between sets of genes, revealed that leukocyte migration and immune system processes were significantly enriched terms in genes upregulated in large adipocytes **(Table 4)**. Adiponectin mRNA was also found to be negatively associated with size in isolated adipocytes (Bambace et al., 2011); whereas, Leptin mRNA was positively associated with adipocyte volume in isolated adipocytes (Guo et al., 2004). However, Leptin mRNA per unit of fat mass decreased at more extreme levels of obesity (Guo et al., 2004). Skurk et al. (2007) analyzed the relationship between adipocyte size and secreted factors and found that Leptin, IL6, IL8, MCP1 and G-CSF were significantly increased in large adipocytes, supportive of altered immune signaling following hypertrophy (Skurk et al., 2007). Recently, Gao et al (2014) utilized adipose biopsies from a cohort of 56 healthy males and females, subdivided into obese or non-obese individuals with hyperplastic or hypertrophic morphologies (ie, obese hyperplastic, obese hypertrophic, non-obese hyperplastic and non-obese hypertrophic) (Gao et al., 2014). This analysis identified 619 genes differentially altered by morphology in non-obese subjects (Gao et al., 2014). Genes increased in non-obese hypertrophy were associated with pro-inflammatory pathways; whereas, genes increased n non-obese hyperplasic individuals where involved in carbohydrate and lipid metabolism (Gao et al., 2014). The transcriptome of adipocytes in bimodal size distributions have also been analyzed. Liu et al. (2010) characterized small adipocytes from epididymal AT (VAT) of Zucker Obese (ZO) and Lean (ZL) rats and found that small adipocytes had a 3-fold decrease in Adiponectin and Pparg in ZO versus ZL rats (Liu et al., 2010), along with a 2.5-fold increase in IL-6 (Liu et al., 2010). These data suggest that both hypertrophied adipocytes, and the small adipocytes from a bimodal population have pro-inflammatory characteristics. Altogether, these data support the conclusion that small and large adipocytes have distinct transcriptional profiles, and that large adipocytes are characterized by altered immune/inflammatory activity.

Cellular mechanisms hypothesized to regulate adipose morphology

Understanding the cell and molecular mechanisms that underpin adipose morphology is likely to provide new therapeutic targets for combating obesity-associated disease. For simplicity, we have separated the factors that are likely to influence adipose morphology into two categories: (i) factors that regulate adipocyte number and (ii) factors that regulate adipocyte size. As the regulation of adipocyte number (adipogenesis) is a well-studied subject with many in-depth reviews (Berry et al., 2016; Berry et al., 2014; Hepler et al., 2017), we will focus on mechanisms hypothesized to regulate adipocyte size. Surprisingly, relatively few studies have identified cellular mechanisms that regulate adipocyte size. Therefore, we first review highly conserved mechanisms for regulating cell size across multiple cell types, and investigate whether these conserved mechanisms may also regulate adipocyte size. We then focus on two interesting, adipocyte-specific pathways that regulate adipocyte size: how the phospholipid monolayer on the surface of LDs controls their expansion, and the emerging field of osmolarity sensors in regulating adipocyte hypertrophy.

A role for mTORC1 in regulating adipocyte size: a highly conserved signaling pathway that control cell size across multiple diverse cell types.

Highly conserved homeostatic mechanisms regulate cell size in eukaryotes (Lloyd, 2013). As adipocyte size is also highly regulated we reasoned that understanding the core pathways that maintain cell size, across multiple diverse cell types, has the potential to shed light on mechanisms controlling adipocyte hypertrophy. Central to the control of cell size across the animal kingdom is the Insulin Growth Factor (IGF), Phosphoinositide 3-kinase (PI3K), Protein kinase B (AKT), and Mechanistic target of rapamycin complex 1 (mTORC1) signaling pathway. IGF/PI3K/AKT/mTORC1 coordinates nutrition with cell growth, and acts as a node to integrate external signals, including Insulin signalling, with biogenic pathways (Edgar, 2006). mTORC1 responds to multiple inputs, including; amino acids, energy, stress, oxygen and growth factors, and regulates downstream anabolic processes that promote cell growth, including; protein and lipid synthesis (through SREBP1/2), mitochondria biogenesis, and ATP production (Cunningham et al., 2007; Duvel et al., 2010; Ma and Blenis, 2009; Porstmann et al., 2008). In addition, mTORC1 also promotes cell growth by negatively regulating autophagy (Hosokawa et al., 2009). Strikingly, artificial activation of the mTORC1 pathway promotes dramatic increases in cell size (Laplante and Sabatini, 2012). Altogether, these data suggest that activation of mTORC1 signaling may induce and augment adipocyte hypertrophy. In accordance, *Raptor* KO mice

(Raptor is an mTOR binding protein essential for formation, and activity of the mTORC1 complex) have smaller adipocytes (and a reduced number), suggesting that mTORC1 may promote adipocyte hypertrophy (Polak et al., 2008). Indeed, *Raptor* KO mice, with specific loss of Raptor and mTORC1 in adipocytes, develop lipodystrophy with age, suggesting that mTORC1 is essential for maintaining a hypertrophic state in mature adipocytes (Lee et al., 2016). Furthermore, adipocyte-specific *Raptor* KO led to the induction of a bimodal 'polarized' adipocyte size distribution, characterized by the addition of a small population of adipocytes, further suggesting that mTORC1 is essential for maintaining adipose morphology (Lee et al., 2016). Such a polarized adipocyte size distribution is reminiscent of fat-specific insulin receptor knockout (FIRKO) mice (Bluher et al., 2002), suggesting that Insulin signalling maybe critically important for maintaining adipose morphology. Elevated mTORC1 signaling, produced after deletion of tuberous sclerosis complex 2 (Tsc2) – a complex made up of Tsc1 & 2 proteins that inhibits mTORC1 signaling, led to increased adipogenesis in mouse fibroblasts and 3T3-L1 adipocytes (Zhang et al., 2009). However, in vivo constitutive activation of mTORC1 in adipocytes by tuberous sclerosis complex 1 (Tsc1) deletion, did not induce adipocyte hypertrophy but instead led to reduced VAT mass, VAT adipocyte number and diameter without affecting SAT, pointing to the complex nature of mTORC1 signaling in adipocytes (Magdalon et al., 2016). Taken together, mTORC1 depletion leads to adipose atrophy; however, conclusive evidence for a role for mTORC1 in adipocyte hypertrophy has not been fully elucidated.

The availability of lipid as a rate-limiting step for adipocyte hypertrophy

The single defining feature of white adipocytes is the presence of large cytoplasmic LDs that can reach ~200 µm in diameter (Walther and Farese, 2009). This feature is unique to white adipocytes and, therefore, we reasoned that understanding how LD growth is regulated may also allow us to elucidate cellular mechanisms underlying adipocyte hypertrophy. LD size reflects two processes: (i) lipid incorporation into LDs and (ii) lipid mobilization from LDs. However, each of these processes is highly complex and can be regulated at multiple levels. For example, at a minimum, lipid incorporation into LDs depends on (i) circulating levels of lipid (i.e. availability of lipid to adipocytes), (ii) lipid uptake into adipocytes, (iii) re-esterification of non-esterified fatty acids (NEFAs) into TAG, and (iv) de-novo lipogenesis in adipocytes, (v) incorporation of TAG into LDs **(Fig. 3A)**. Lipid synthesis, transport and metabolism in adipocytes is a large subject area beyond the scope of this review; however, we recommend the following reference for further reading on the subject (Large et al., 2004). Briefly, however, and as described above, hypertrophic

VAT was associated with higher plasma TAG, vLDL TAG and cholesterol, total plasma cholesterol, higher total-to-HDL cholesterol and increased plasma apolipoprotein B when compared to hyperplastic VAT (Hoffstedt et al., 2010; Veilleux et al., 2011). Together, these data suggest that increased circulating lipid may promote hypertrophic growth of adipocytes. Accordingly, treatment of 3T3-L1 adipocytes with saturated or monounsaturated NEFAs resulted in adipocyte hypertrophy (Kim et al., 2015). Regarding uptake of lipid into adipocytes, the first step is often hydrolysis of TAG from circulating lipoproteins by Lipoprotein lipase (LPL). In adipose tissue, LPL is expressed on vascular endothelial cells and adipocytes (Gonzales and Orlando, 2007; Merkel et al., 2002), and hydrolyses TAG (from lipoproteins) to form glycerol and NEFAs for uptake into adipocytes (Geldenhuys et al., 2017). In SAT, higher levels of LPL activity were associated with adipocyte hypertrophy (Serra et al., 2015). Further, LPL deficiency in mice results in lipodystrophy and elevated plasma lipid levels (Weinstock et al., 1995). Following their production by LPL, NEFAs are taken up by adipocytes using specialized fatty acid transporters, including Fatty acid transport proteins (FATPs), the scavenger receptor CD36, and the mitochondrial aspartate amino transferase (FABPpm). In particular, isolated adipocytes from CD36 KO mice have impaired NEFA uptake (Coburn et al., 2000). Further, CD36-deficient mice do not develop diet-induced obesity, suggesting that adipocyte hypertrophy is impaired (Hajri et al., 2007; Koonen et al., 2010; Vroegrijk et al., 2013). We speculate that increased circulating lipid causes adipocyte hypertrophy and adipose growth; however, there is currently limited evidence that circulating lipid levels induce a hypertrophic morphology as defined by Arner et al. (2010). Altogether, these data suggest that lipid availability, in the form of plasma lipid levels, LPL activity and lipid uptake into cells, is key to promoting adipocyte hypertrophy.

Specialized pathways for regulating lipid droplet size in adipocytes

Large cells, such as neurons and ova, often have specialized mechanisms that allow them to grow to extreme sizes (Lloyd, 2013). Therefore, it is likely that adipocytes have adipocyte-specific pathways that govern their hypertrophic capacity. Multiple intriguing mechanisms regulate LD growth. First, a genome-wide screen in yeast identified 10 mutants that produced “supersized” LDs, capable of forming LDs >50 times larger than wild-type LDs (Fei et al., 2011). The genes identified include yeast homologs of Seipin (Fei et al., 2008; Fei et al., 2011), regulators of phospholipid metabolism, and multiple subunits of casein kinase 2 (Fei et al., 2011). Phosholipid metabolism was a shared feature of the genes identified from the screen, and the surface layer of LDs are coated with a phospholipid monolayer (Walther and Farese, 2009). Phosphatidic acid

(PA), a cone-shaped lipid common in phospholipids which alters the curvature of membranes, and promotes membrane fusion events, was a key factor in formation of supersized LDs (Fei et al., 2011; Marchesan et al., 2003). Further, supersized LDs could be formed by PA-stimulated fusion of LDs (Fei et al., 2011), suggesting that the phospholipid monolayer of LDs is important for LD growth and may mediate LD fusion to facilitate LD hypertrophy.

Regulation of adipocyte size by osmolarity-sensing ion channels

Cells respond to changes in size by generating osmotic gradients using plasma membrane ion channels and transporters to manipulate the osmolarity of the surrounding environment. Utilizing of these on channels can create a hypotonic environment leading to cell swelling (RVI; regulatory volume increase), or a hypertonic environment leading to cell shrinkage (RVD; regulatory volume decrease) **(Fig. 3B)** (Hoffmann et al., 2009; Jentsch, 2016). As cell size regulation is central to the dynamic growth and regression of adipocytes, the role of such osmosensers and regulators is under intense investigation. Transient receptor potential cation channel subfamily V member 4 (TRPV4) is a $Ca2^{+}$-permeable, nonselective cation channel involved in the regulation of osmotic pressure (Harteneck and Reiter, 2007), and is activated by cellular swelling and stretch (Liedtke et al., 2000; Mochizuki et al., 2009; Strotmann et al., 2000; Thodeti et al., 2009). Adipocytes from TRPV4 KO mice do not undergo hypertrophy and underwent increased oxidative metabolism (Ye et al., 2012) **(Fig. 3C)**. Additionally, the TRPV4 KO mice were protected from diet-induced obesity, adipose inflammation and Insulin resistance (Ye et al., 2012). Thus, TRPV4 promotes adipocyte hypertrophy, and may contribute to the Insulin resistance inherent to hypertrophied adipocytes. Recently the voltage-regulated anion channel (VRAC), SWELL1 (LRCC8A), was shown to regulate adipocyte size, Insulin signaling and glucose homeostasis (Zhang et al., 2017). VRACs export chloride ions (Cl^{-}) and other small organic osmolytes, and thus generate a hypertonic environment that induces cell shrinkage (RVD) (Jentsch, 2016; Qiu et al., 2014). Zhang et al. (2017) utilized patch-clamp recordings of ionic currents in freshly isolated adipocytes to identify that hypertrophic adipocytes exhibit an increased 'swell-activated' Cl^{-} current relative to smaller adipocytes (Zhang et al., 2017). Furthermore, the increased current observed in hypertrophied adipocytes was dependent on SWELL1. Although activation of VRACs has generally been shown to induce RVD and decrease cell volume (Jentsch, 2016), the authors propose that SWELL1-mediated expansion acts as a feed-forward amplifier for further adipocyte hypertrophy **(Fig. 3C)**. Further, SWELL1 knockout (KO) adipocytes were Insulin resistant with reduced GLUT4 translocation to the adipocyte plasma membrane after

stimulation with Insulin (Zhang et al., 2017). This effect was found to be mediated by PI3K-AKT signaling, and SWELL1 KO adipocytes had reduced phosphorylation of AKT (Zhang et al., 2017). Thus, taken together, these data suggest that osmosensing is active in adipocytes during hypertrophy, and may modulate adipocyte hypertrophy and Insulin sensitivity. In addition to ion channel osmosensers, the adipocyte plasma membrane contains abundant caveolae, small flask-shaped invaginations of the plasma membrane enriched in cholesterol and sphingolipids, which disassemble in response to osmotic and mechanical stress (Sinha et al., 2011). Caveolae are present at a high density in cells that experience mechanical stress, and cover ~30% of the adipocyte surface (Le Lay et al., 2015). Caveolae formation is driven by the assembly of 3 distinct Caveolin proteins (Cav1-3), and deletion of individual Cav genes leads to loss of caveolae (Le Lay and Kurzchalia, 2005). Caveolae mediate the response of several cell types to mechanical stress (Boyd et al., 2003; Sedding et al., 2005), and intriguingly, loss of caveolae induces lipodystrophy in mice and humans (Kim et al., 2008; Razani et al., 2002). Further, overexpression of Cav1 in adipocytes induced an increase in caveolae density, but also stimulated the accumulation of larger LDs **(Fig. 3C)** (Briand et al., 2014). No role for caveolae as an osmosensor/regulator during adipocyte hypertrophy is known; however, it is clear that caveolae are essential for lipid storage fluctuations in adipocytes.

Conclusion

At a population level, adipose morphology is highly varied, genetically determined and associated with cardiometabolic disease susceptibility. However, the precise genetic determinants of adipose morphology are largely unknown. In this Review, we find strong evidence in the literature that a hypertrophic morphology, adipose characterized by few but large adipocytes, is associated with a range of metabolic perturbances including plasma glucose, lipid and Insulin levels, insulin resistance and susceptibility to disease. We further review whether distinct morphologies have unique transcriptomic signatures, and identify that hypertrophic morphology is characterized by a pro-inflammatory expression profile across multiple studies and methodologies. Finally, we explore some intriguing cellular mechanisms that are predicted to regulate adipocyte cell size and morphology; including (i) how the phospholipid monolayer covering lipid droplets regulates their growth, and (ii) how osmolarity sensing in adipocytes can stimulate hypertrophy.

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767

768 # Tables

769 **Table 1. Human adipose morphology and metabolic parameter associations – bimodal**
770 **adipocyte size distribution.**

Morphology trait	AT	Direction of association	Metabolic Trait	Study	PMID
Relative frequency of small adipocytes	Abdominal SAT	Positive	Insulin resistance	McLaughlin*	17549449
	Abdominal SAT	Positive	Increased VAT/VAT+SAT ratio	Kursawe**	20805387
	Abdominal SAT	Positive	Insulin resistance	McLaughlin****	23666871
	Abdominal SAT	Positive	Type 2 diabetics treated with rosiglitazone	Eliasson^	26317056
	Abdominal SAT	Positive	Diabetics vs non-diabetics	Fang^^	26451283
	Omental VAT	Positive	Diabetics vs non-diabetics	Fang^^	26451283
	Abdominal SAT	Negative	Overfed Insulin sensitive subjects	McLaughlin^^^	26884438
Change in diameter of small adipocytes	Abdominal SAT	Negative	Diabetics vs non-diabetics	Fang^^	26451283
	Omental VAT	Negative	Diabetics vs non-diabetics	Fang^^	26451283
Nadir diameter (n)	Abdominal SAT	Positive	Insulin resistance	McLaughlin*	17549449

Relative frequency of large adipocytes	Abdominal SAT	Negative	Increased VAT/VAT+SAT ratio	Kursawe**	20805387
	Abdominal SAT	Negative	Insulin resistance	McLaughlin****	23666871
	Abdominal SAT	Negative	Diabetics vs non-diabetics	Fang^^	26451283
	Omental VAT	Negative	Diabetics vs non-diabetics	Fang^^	26451283
Large adipocyte size (Cp)	Abdominal SAT	Positive	Increased small adipocytes in Omental VAT and abdominal SAT	Liu***	19711137
	Abdominal SAT	Positive	Increased VAT/VAT+SAT ratio	Kursawe**	20805387
	Abdominal SAT	Positive	Insulin resistance	Yang****	22240722
	Abdominal SAT	Positive	Insulin resistance	McLaughlin****	23666871
	Abdominal SAT	Positive	Type 2 diabetics treated with rosiglitazone	Eliasson^	26317056
	Abdominal SAT	No change	Overfed Insulin resistant subjects	McLaughlin^^^	26884438
	Abdominal SAT	Positive	Overfed Insulin	McLaughlin^^^	26884438

			resistant subjects		

*Cohort was 28 obese individuals (mean age = ~50 years) stratified according to Insulin sensitivity (Insulin resistant BMI = 30.6 kg/m^2; Insulin sensitive BMI = 29.4 kg/m^2). No statistical differences between Insulin resistant and sensitive groups were found for age, gender, reported levels of exercise, blood pressure, fasting glucose, total cholesterol, LDL-cholesterol. HDL-cholesterol were lower in Insulin resistant group.

**Cohort was 38 adolescents (~ 15 years old) with similar degrees of obesity (mean BMI = ~37 kg/m^2) were divided into 2 groups: low VAT/VAT+SAT ratio (<0.11) and high VAT/VAT+SAT ratio (>0.11). None of the participants were on any medication or had any known disease.

***Cohort was 11 obese (mean BMI = 45.3 kg/m^2) Insulin resistant, but non-diabetic women. Patients were excluded if they had coronary heart disease, hepatic or renal disease, cancer, or use medications for weight loss.

****Cohort was 35 subjects with a range of BMI (range = 18-34 kg/m^2; mean = 25.7 kg/m^2) and age (range = 28-49 years; mean = 41 years). The subjects were non-diabetic, but had a known family history of diabetes, with at least 2 first-degree relatives with type 2 diabetes.

^Cohort was 12 patients with type 2 diabetes (11 male, 1 female). Patients had a mean BMI of ~ 28 kg/m^2. Patients were on diet or oral hypoglycemic treatments including sulfonylurea, repaglinide and metformin). Rosiglitazone (8 mg QD) was added to the treatment regimen. Subjects were excluded if they exhibited clinically significant disease. Adipose biopsies were taken before and after rosiglitazone treatment.

^^Cohort was 30 subjects with morbid obesity. Adipose biopsies were taken from subcutaneous, omental and mesenteric locations.

^^^Cohort consisted of healthy overweight adults, aged 30-60 years. BMI = 25-35 kg/m^2.Subjects had a stable body weight during the prior 3 months, and fasting plasma glucose <126 mg/dL. Subjects were given a hypercaloric diet to induce 3.2 kg weight gain over 4 weeks, followed by 1 week of weight stabilization.

797 **Table 2. Genes positively correlated with adipocyte size.**

Gene symbol	Gene name	Study PMID
SELE	Selectin E	16754744
SPARCL1	SPARC-like 1	16754744
TM4SF1	Transmembrane 4 L six family member 1	16754744
DCN	Decorin	16754744
IL8	Interleukin 8	16754744
PALLD	Palladin	16754744, 24549139
SAA2	Serum amyloid A2	16754744
CLEC3B	C-type lectin domain family 3, member B	16754744
C1QR1	Complement component 1, q subcomponent, receptor 1	16754744
COL1A1	Collagen, type I, alpha 1	16754744
CXCL2	Chemokine (C-XC motif) ligand 1	16754744
COL1A2	Collagen, type I, alpha 2	16754744
FLJ14054		16754744
AQP1	Aquaporin 1	16754744
MSN	Moesin	24549139
NHEDC2	Na+/H+ exchanger domain containing 2	24549139
RP11-877E17.2	undefined	24549139
KIF3B	Kinesin family member 3B	24549139
NME5	Non-metastatic cells 5, protein expressed in (nucleoside-diphosphate kinase)	24549139
IFT20	Intraflagellar transport 20 homolog (Chlamydomonas)	24549139
MIF	Macrophage migration inhibitory factor (glycosylation-inhibiting factor)	24549139
SLC24A3	Solute carrier family 24 (sodium/potassium/calci um exchanger),member 3	24549139
C15orf59	Chromosome 15 open reading frame 59	24549139

CD248	CD248 molecule, endosialin	24549139
SLC46A3	Solute carrier family 46, member 3	24549139
XPO6	Exportin 6	24549139
FAT1	FAT tumor suppressor homolog 1 (Drosophila)	24549139
GNG2	Guanine nucleotide binding protein (G protein), gamma 2	24549139
LPCAT1	Lysophosphatidylcholine acyltransferase 1	24549139
TCTA	T-cell leukemia translocation altered gene	24549139
CLTB	Clathrin, light chain B	24549139
SPTAN1	Spectrin, alpha, non-erythrocytic 1 (alpha-fodrin)	24549139
CYBASC3	Cytochrome b, ascorbate dependent 3	24549139

798

799 **Table 3. Genes inversely correlated with adipocyte size.**

Gene symbol	Gene name	Study PMID
NPEPPS	Aminopeptidase puromycin sensitive	24549139
GLYCTK	Glycerate kinase	24549139
PKP2	Plakophilin 2	24549139
AZGP1	Alpha-2-glycoprotein 1, zinc-binding	24549139
CIDEA	Cell death-inducing DFFA-like effector a	24549139
FAM184A	Family with sequence similarity 184, member A	24549139
NUP98	Nucleoporin 98kDa	24549139
WHSC2	Wolf-Hirschhorn syndrome candidate 2	24549139
FAM161A	Family with sequence similarity 161, member A	24549139
SLC27A2	Solute carrier family 27 (fatty acid transporter), member 2	24549139
ZFAND1	Zinc finger, AN1-type domain 1	24549139
MACROD1	MACRO domain containing 1	24549139
PPARA	Peroxisome proliferator-activated receptor alpha	24549139
GPD1L	Glycerol-3-phosphate dehydrogenase 1-like	24549139
BBC3	BCL2 binding component 3	24549139
CYP3A7	Cytochrome P450, family 3, subfamily A, polypeptide 7	24549139
TOE1	Target of EGR1, member 1 (nuclear)	24549139
EIF1B	Eukaryotic translation initiation factor 1B	24549139
ADH1B	Alcohol dehydrogenase 1B (class I), beta polypeptide	24549139
FDFT1	Farnesyl-diphosphate farnesyltransferase 1	24549139

800

801

802 **Table 4. Significant GO terms shared among genes positively correlated with adipocyte**
803 **size (both studies).**

GO ID	Term	Corrected P-value	Annotated genes
GO:0048522	positive regulation of cellular process	0.003361691	CXCL2, IL8, KIF3B, MIF, NHEDC2, C1QR1, CD248, CLEC3B, COL1A1, AQP1
GO:0006928	movement of cell or subcellular component	0.004711785	CD248, CXCL2, IL8, NME5, KIF3B, MIF, COL1A1
GO:0050900	leukocyte migration	0.005651237	CXCL2, IL8, MIF, COL1A1
GO:0002376	immune system process	0.007058561	CD248, CXCL2, IL8, KIF3B, MIF, NHEDC2, COL1A1, C1QR1

804

805

806 ## Figure legends

807 **Figure 1. Schematic illustrating regional adipose morphology. A.** This review largely
808 concentrates on 3 human ATs; visceral (VAT; blue), abdominal SAT (yellow) and gluteofemoral
809 SAT (red). **B.** Adipose morphology can be categorized by finding a line-of-best-fit to describe the
810 relationship between fat mass (mg) and mean adipocyte volume (pl). Such a fitted line produces
811 a curvilinear relationship (dotted line). AT from individuals (black circles) is assessed relative to
812 the fitted line (dotted line). A positive residual (adipocyte volume greater than expected) indicated
813 hypertrophic morphology, whereas an adipocyte volume smaller than expected denotes
814 hyperplastic morphology. **C.** Adipose morphology can be hyperplastic characterized by many,
815 small adipocytes. Or, hyperptrophic, characterized by few, large adipocytes. Each morphology is
816 associated with distinct metabolic parameters (Arner et al. 2010).

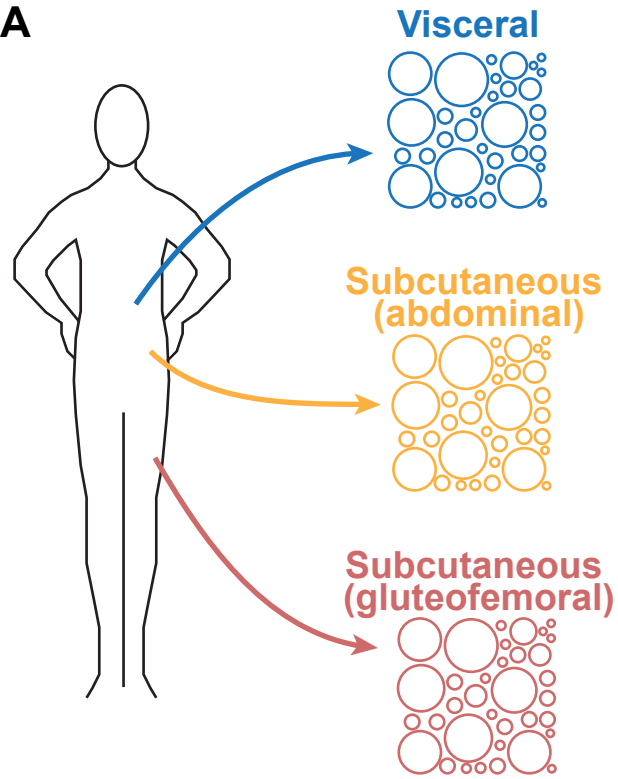
817

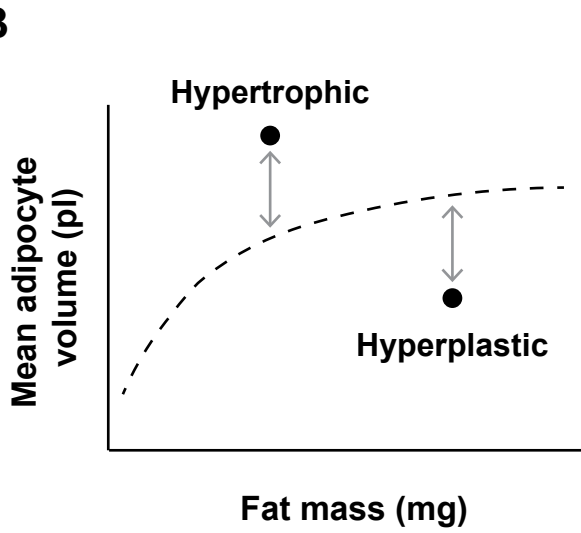
818 **Figure 2. Schematic illustrating common adipocyte size distributions. A,B.** A unimodal
819 adipocyte size distribution is often found and in obesity, or after exposure to a high-fat diet
820 (magenta) the population mean (μ) shifts to a larger size. **C,D.** A bimodal adipocyte size
821 distribution can be evaluated by; (i) the value for the nadir (n), (ii) centre of peak (Cp) of large
822 adipocytes. In obesity, the nadir and centre of peak for the large adipocytes is increased.

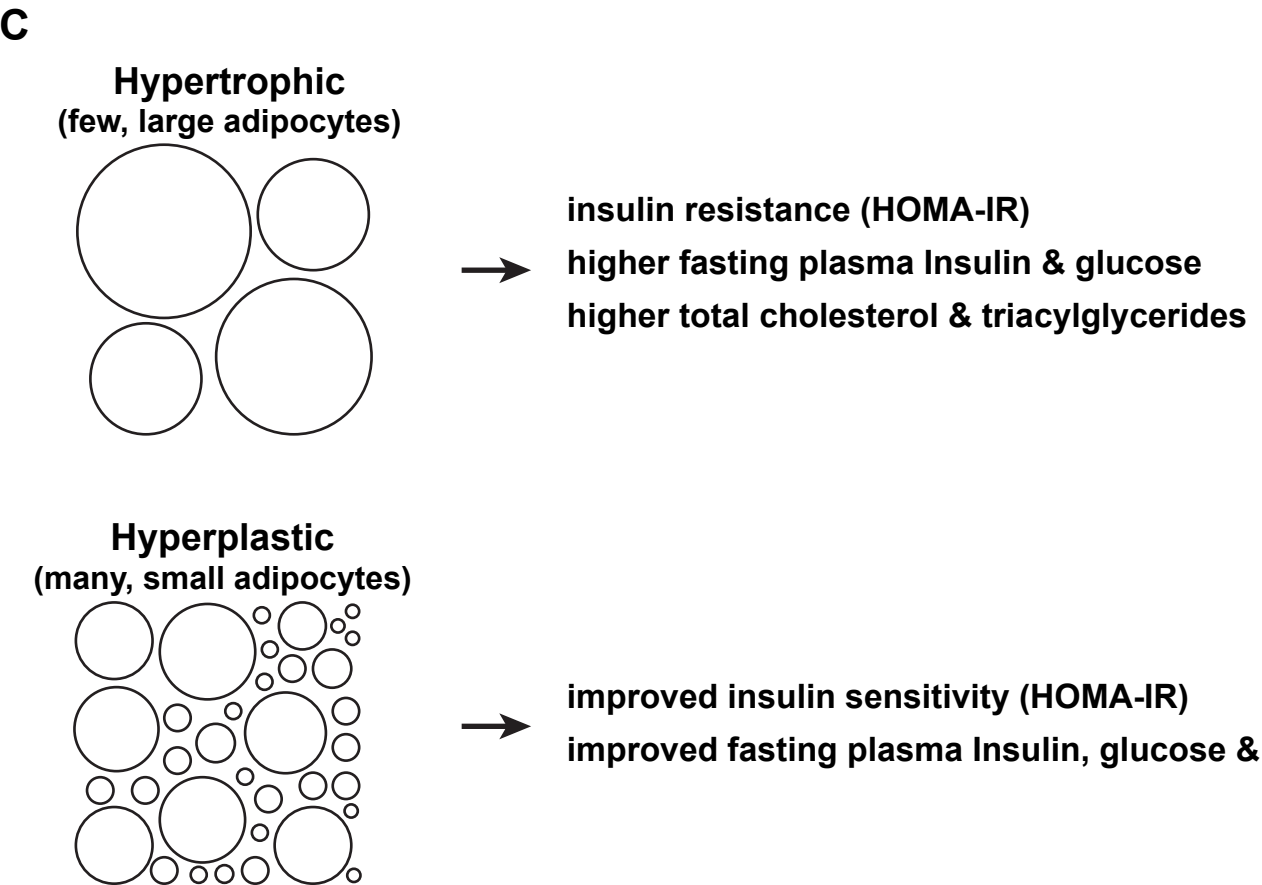
823

824 **Figure 3. Schematic illustrating putative mechanisms that regulate adipocyte hypertrophy.**
825 **A.** Lipid availability is critical to adipocyte hypertrophy, and can be split into multiple steps. 1)
826 circulating lipid in lipoproteins (for example, very Low Density Lipoprotein (vLDL)) can contain
827 TAG, and the plasma levels of lipoproteins is associated with adipocyte hypertrophy. 2) Hydrolysis
828 of TAG from lipoproteins is performed by LPL (and GPIHBP1) on the surface of adipocytes of
829 vascular endothelial cells and produces non-esterified fatty acids (NEFAs) and glycerol. Levels
830 and activity of LPL is associated with adiposity levels and plasma lipid levels. 3) NEFAs are taken
831 up into adipocytes by fatty acid transporters such as CD36 (and also FATPs). CD36 levels are
832 associated with adipocyte hypertrophy. 4) Intracellular NEFAs are re-esterified into TAG in
833 conjunction with the endoplasmic reticulum (6). De-novo lipogenesis within adipocytes also
834 contributes to the TAG pool (5). 6) Lipid droplets can grow large or small based on transfer of
835 lipogenic enzymes (eg, DGAT, diglyceride acyltransferase) from the endoplasmic reticulum
836 membrane to the LD membrane (Wilfling et al., 2013). 7) TAG within LDs is mobilized (lipolysis)
837 by lipases including HSL (hormone sensitive lipase). 8) NEFAs are released from adipocytes by

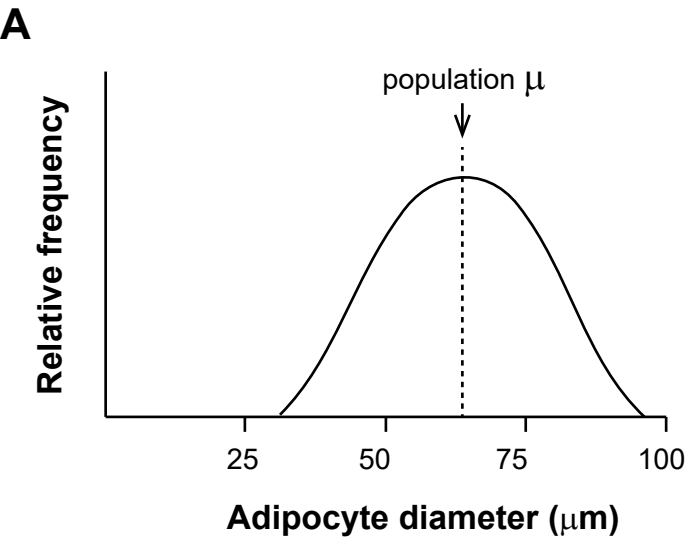
838 active transport mechanisms including by ABC transporters (Tarling et al., 2013). **B.** Osmolarity
839 is defined as either hypertonic which induces regulated volume decreases (RVD), or hypotonic
840 which induces regulated volume increases (RVI). **C.** 3 proposed mechanisms by which
841 osmosensing may regulate adipocyte hypertrophy.

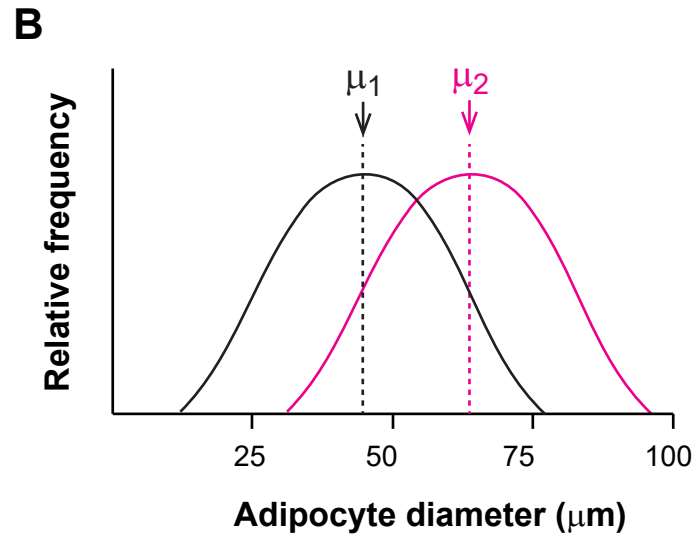

A
Visceral
Subcutaneous
(abdominal)
Subcutaneous
(gluteofemoral)


B
Hypertrophic
Hyperplastic
Mean adipocyte
volume (pl)
Fat mass (mg)

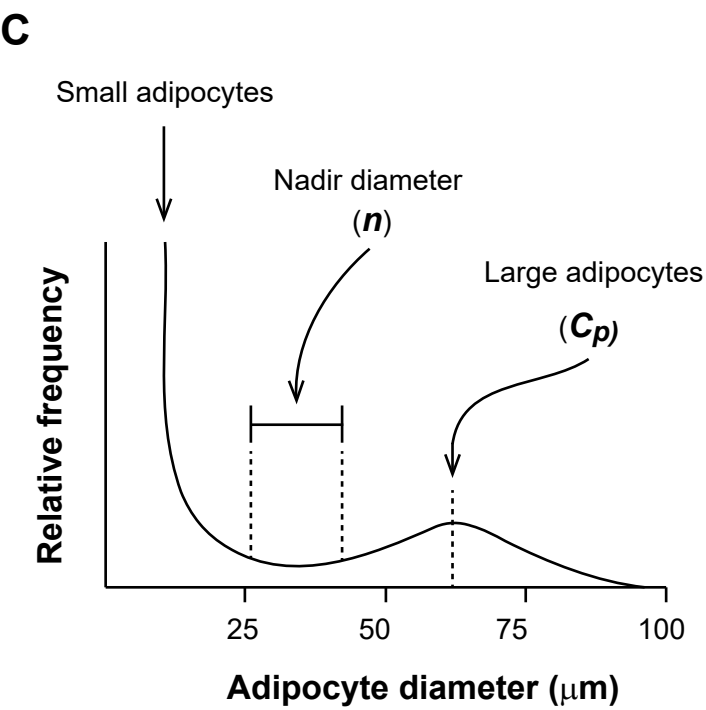

C
Hypertrophic
(few, large adipocytes)
insulin resistance (HOMA-IR)
higher fasting plasma Insulin & glucose
higher total cholesterol & triacylglycerides
Hyperplastic
(many, small adipocytes)
improved insulin sensitivity (HOMA-IR)
improved fasting plasma Insulin, glucose & lipid

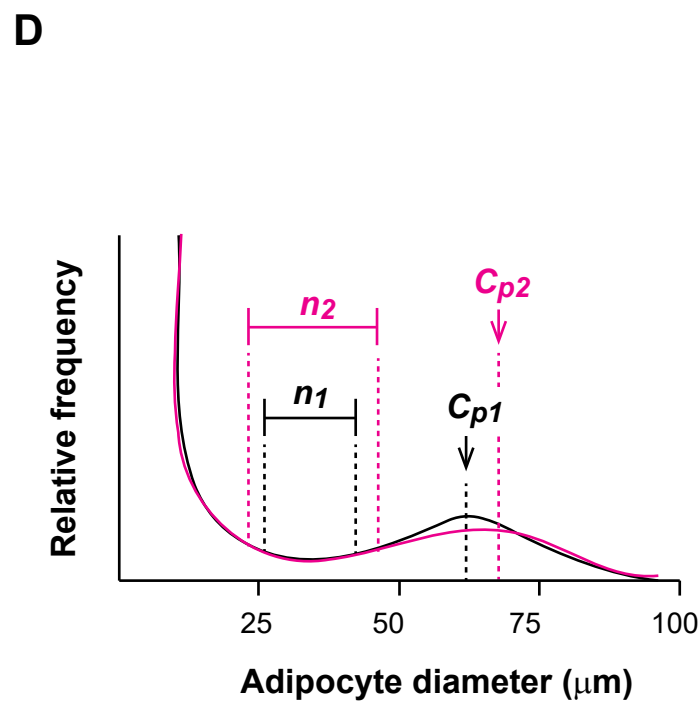
Unimodal adipocyte size distribution

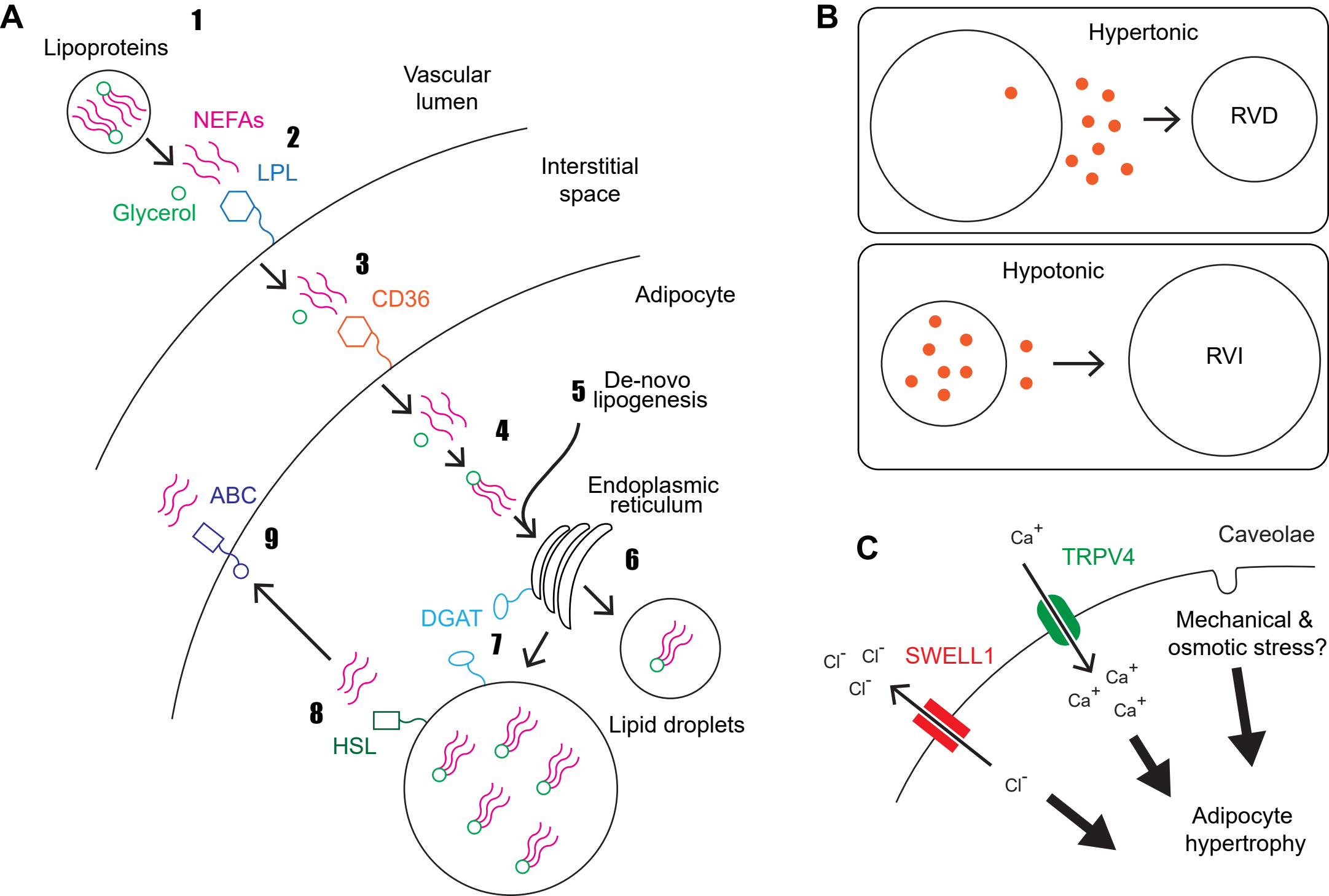




Bimodal adipocyte size distribution






A
1
Lipoproteins
Vascular lumen
NEFAs
2
LPL
Glycerol
Interstitial space
3
CD36
Adipocyte
5
De-novo lipogenesis
4
Endoplasmic reticulum
ABC
9
6
DGAT
7
8
HSL
Lipid droplets
B
Hypertonic
RVD
Hypotonic
RVI
C
Ca+
TRPV4
Caveolae
Mechanical & osmotic stress?
Cl- Cl-
Cl-
SWELL1
Ca+
Ca+
Ca+
Cl-
Adipocyte hypertrophy