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Forming functional fat: a growing understanding of adipocyte differentiation

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PREFACE

Adipose tissue, which is primarily composed of adipocytes, is crucial for maintaining energy and metabolic homeostasis. Adipogenesis is thought to occur in two stages: commitment of mesenchymal stem cells to a preadipocyte fate and terminal differentiation. Cell shape and extracellular matrix remodelling have recently been described to regulate preadipocyte commitment and competency by modulating WNT and RHO GTPase signalling cascades. Adipogenic stimuli induce terminal differentiation in committed preadipocytes through the epigenomic activation of PPARγ. Coordination of PPARγ with C/EBP transcription factors maintains adipocyte gene expression. A better understanding of these mechanisms may identify therapeutic targets for the growing worldwide epidemic of metabolic disease.

INTRODUCTION

Adipose tissue is a complex organ that regulates and coordinates energy homeostasis[1]. It is primarily composed of adipocytes surrounded by fibroblasts, fibroblastic preadipocytic cells, endothelial cells, nerves and immune cells[2]. Although adipose tissue was originally thought to just be an energy storage site, studies in the past years have revealed that it carries out many key endocrine functions[1]. Indeed, dysfunction of the adipose compartment is central to the pathology associated with metabolic diseases such as obesity, type 2 diabetes, cancer cachexia and lipodystrophies[1].

There are two main types of adipose tissue, white and brown; these are primarily composed of white or brown adipocytes, respectively[2]. White adipose tissue (WAT), which is characterized by adipocytes containing large unilocular lipid droplets, is an active endocrine organ that regulates diverse activities such as insulin sensitivity, lipid metabolism and satiety[1]. WAT the main type of adipose tissue found in adult humans and is distributed throughout the body in subcutaneous regions, surrounding visceral organs and in the face. Interestingly, despite their histological similarities, subcutaneous and visceral adipose tissue are thought to have distinct depot-specific metabolic functions[3], possibly owing to different exposures to paracrine and endocrine signals[4] or to distinct developmental programmes[5,6]. Indeed, accumulation of visceral fat during the development of obesity is correlated with

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pathologic inflammation and insulin resistance[7], whereas subcutaneous adipose tissue is thought to offer improved glucose tolerance[8].

By contrast, brown adipose tissue (BAT) mainly participates in thermogenesis and is located in discrete pockets in the paravertebral, supraclavicular and periadrenal regions[9]. BAT is histologically distinct from WAT; it is composed of multiloculated adipocytes that contain large numbers of mitochondria, accounting for their 'brown' colouring upon visualization[9]. For many years, BAT was thought to be absent in human adults, but recent fluorodeoxyglucose positron emission tomography studies of normal humans has identified regions of high glucose uptake that represent metabolically active tissue[10–12].

Despite the functional, developmental and location differences between white and brown adipocytes, the two cell types share many common differentiation features. All adipocytes, along with osteoblasts, myocytes and chondrocytes, differentiate from mesenchymal stems cells (MSCs)[13] (Figure 1) in a process known as adipogenesis. Adipogenesis can be divided into two main phases: commitment and terminal differentiation. This Review focuses on the integration of underappreciated and novel mechanical and molecular cues governing the conversion of MSCs to committed preadipocytes and the epigenomic transition state that is required for the activation of the master adipogenic regulator peroxisome proliferator-activated receptor-γ (PPARγ). We also discuss the signalling cascades that lead to terminal differentiation and the conservation of these pathways between species. The Review focuses on white fat, but a discussion of brown fat is given where appropriate.

IN VIVO DEVELOPMENT OF FAT

White adipose compartments begins to develop in late gestation[14,15] and the rate of adipogenesis rapidly surges in response to increased nutrient availability, leading to a marked postnatal expansion of adipose compartments[15]. During this period, precursor cells undergo the morphological and functional conversion from spindly fibroblasts into round lipid-laden mature fat cells. By contrast, mammalian subscapular fat, which is mostly BAT, expands primarily *in utero*, possibly to maintain body temperature upon birth[9].

Whether adipogenesis occurs in the adult adipose tissue remains controversial. In animal studies, white adipocyte numbers increase through puberty but are relatively steady in the mature fat pad[16]. Similar findings have been obtained in humans[17]; however, within human adult WAT, adipocytes seem to undergo approximately a 10% annual turnover[17]. Thus, adipogenesis takes place in adults to maintain the adipose compartment. Additionally, adipogenesis probably has a role in the pathology of obesity; when animals are kept on a high-fat diet, adipocyte cell size initially increases, followed by an increase in fat cell number upon prolonged over-nutrition[18–20]. Human studies correlating fat mass with cell number have yielded conflicting results[17,21,22], but this may be attributed to differences in study protocols and basal characteristics of the studied populations. Moreover, short-term overfeeding studies in adults demonstrate increases in adipocyte cell numbers[21]. By contrast, preclinical and human studies have shown that weight loss is associated with decreased adipocyte cell size, but has no effect on adipocyte cell numbers[17,19]. Further work must be

carried out to determine whether increased adipogenesis has a crucial role in the development of obesity in humans.

Interestingly, lineage studies (Box 1) have revealed that BAT is more closely related to skeletal muscle than WAT, as both muscle and BAT have progenitors that express the early muscle marker MYF5 but WAT does not[23]. An inducible *Pax7-Cre* model shows that BAT and muscle share a common Pax7-expressing progenitor as late as embryonic day 10.5[24]. Knockdown of the zinc finger transcriptional co-regulator PR domain-containing protein 16 (Prdm16) in primary brown preadipocytes leads to increased myocyte gene expression and promotes skeletal muscle morphology[23]. This surprising finding means that in early development there is a divergence between white and brown precursor cells (Figure 2). In addition, upon prolonged cold exposure or in response to β-adrenergic signalling, WAT can display characteristics of BAT, such as expression of uncoupling protein 1 (UCP1)[25], which is associated with improved metabolic profiles in response to high fat diet[26,27]. The 'brown'-like adipocytes within WAT are developmentally distinct from brown adipocytes found in BAT[23]; thus, the plasticity of WAT in response to these stimuli may be due to transdifferentiation of mature white adipocytes into brown adipocytes[28] or the result of *de novo* adipocyte formation.

Despite advances in tracing adipocyte development *in vivo*, studies of factors that regulate adipogenesis have frequently been limited to *in vitro* models (in particular mouse 3T3-L1 cells) because of the inadequacy of current models to study adipocyte differentiation *in vivo* (Box 2).

ADIPOGENIC COMPETENCY AND COMMITMENT

Preadipocytes are defined as cells that are restricted to becoming adipocytes, but do not spontaneously undergo terminal differentiation in the absence of exogenous adipogenic stimuli. Here we distinguish between adipogenic competency and commitment as follows: adipogenic competency refers to the ability of cells to undergo adipocytic differentiation upon the addition of defined stimuli, whereas adipogenic commitment refers to the cell fate decision of a multipotent cell type to undergo adipocyte conversion.

Identification of preadipocytes in vivo.

Defining the characteristics distinguishing the morphologically similar adipogenic and non-adipogenic fibroblasts had proven difficult until recent years. Technological advances in flow cytometry, transgenic animals and identification of stem cell surface markers in other tissues have made it possible to isolate the subpopulation of fibroblasts in the stromal vascular fraction (SVF) of WAT that has adipogenic potential[14,29]. Such stem cell surface markers can be used to sort cell populations in the SVF and test them for adipogenic potential. Lineage tracing studies based on the expression of PPARγ, the master regulator of adipogenesis (see below), have identified dividing adipogenic precursor cells within the SVF that dominate the adipose compartment during the first month of postnatal development (Box 1)[14]. When transplanted into wild-type mice, these PPARγ-positive precursor cells could differentiate into adipocytes[14]. Both sorted SVF and genetically marked preadipocytes are perivascular within the developing adipose tissue and mature adipose organ[14,29].

Interestingly, transplantation of isolated adipogenic precursors (Lin $^{-}$CD29^{+}CD34^{+}Sca1^{+}CD24^{+} cells) rescued the diabetic phenotype in lipodystrophic A-Zip mice[29], serving as a proof of principle for using primary preadipocytes to ameliorate adipose-related metabolic disease.

Brown preadipocytes that are Sca^{+} have been isolated from BAT, subcutaneous WAT and muscle[30]. However, further studies are required to determine whether they also express low, but detectable, levels of PPARγ. The isolation of these adipogenic fibroblast populations from WAT and BAT will allow a more complete understanding of how the pathways described throughout this Review are regulated during the development of adipose tissue.

WNT signalling.

Wingless-type MMTV integration site family members (WNTs) are secreted glycoproteins that have key roles during development. Canonical WNT signalling is activated following the binding of WNT ligands to the heterodimeric cell surface receptors low density lipoprotein receptor-related protein 5 (LRP5) and LRP6 and Frizzled. This induces the family of TCF transcription factors to recruit a β-catenin-dependent co-activator complex to activate target gene transcription[31]. In the context of adipogenesis, these include cyclin D1 and the nuclear receptor COUP-TFII[32], although the role of COUP-TFII in adipogenesis is controversial[33,34].

Canonical WNT signalling has been shown to inhibit adipogenesis[31] (Figure 3): addition of a canonical WNT ligand to committed preadipocytes inhibits adipogenesis[35], and mouse embryonic fibroblasts (MEFs) lacking the canonical WNT receptor LRP6 display increased adipocyte differentiation[36]. Moreover, mice expressing WNT10b, the main WNT ligand expressed by preadipocytes, in adipocytes show decreases in white and brown fat tissue mass[37]. WNT10b promotes osteogenesis in MSCs[38], indicating that canonical WNT signalling also regulates brown adipogenesis and MSC cell fate. Repression of WNT10b and other canonical ligands by the histone methyltransferase EZH2 in white primary preadipocytes is required for adipocyte differentiation[39].

However, there is also evidence that the canonical WNT pathway is essential for survival of adipocyte precursors. WNT10b increases in confluent cultures of 3T3-L1 cells[35], and WNT1 can protect preadipocytes from apoptosis during serum starvation by regulating the expression of insulin growth factor 1 (IGF1) and IGF2[40] (which are known to protect preadipocytes from cell death upon serum starvation[41]).

WNT ligands can also signal through β-catenin-independent pathways, known as non-canonical signalling, by signalling through alternate cell surface receptors and activating different intracellular pathways. The non-canonical WNT ligand WNT5A activates the histone methyltransferase SET domain bifurcated 1 (SETDB1)[42]. SETDB1 forms a complex with chromodomain helicase DNA-binding protein (CHD7) and Nemo-like kinase (NLK) to inhibit the ability of PPARγ to transcriptionally activate its downstream metabolic target genes in the MSC cell line ST2 and 3T3-L1 cells[42,43]. Activation of this non-canonical WNT pathway can also promote osteogenesis in MSCs[42,44], indicating that this pathway is crucial for lineage determination in these multipotent cells. However, the non-canonical

WNT ligand WNT5B, which is associated with type 2 diabetes in Japanese populations[45], promotes adipocyte differentiation by inhibiting the canonical WNT pathway upon addition of adipogenic stimuli to committed preadipocytes by preventing nuclear translocation of β-catenin[46].

Activation of the canonical and non-canonical pathway in preadipocytes can be modulated by regulating expression of WNT ligands. For example, expression of the canonical ligand WNT10b can be repressed upon addition of cAMP agonists, which act as adipogenic stimuli to committed preadipocytes[47]. Interestingly, many of the cell structure pathways described below regulate adipogenesis in part by controlling the expression of pro- and anti-adipogenic WNT ligands.

TGFβ superfamily signalling.

Transforming growth factor-β (TGFβ) superfamily ligands are secreted morphogens, some which are crucial for MSC lineage decisions and adipogenic competency of committed preadipocytes cell lines[48]. The exact role of TGFβ, the canonical member of the superfamily, during adipogenesis has remained unclear. TGFβ expression positively correlates with obesity in humans and animal models[48], but paradoxically inhibits *in vitro* adipogenesis of 3T3-F442A cells by signalling through SMAD3[49]. However, SMAD3-null mice are resistant to diet-induced obesity, and MEFs from these mice have diminished adipogenic potential[50]. The discrepancy between the *in vitro* and *in vivo* studies may be due to differences between ectopic activation TGFβ–SMAD3 signalling and the nuanced regulation of endogenous TGFβ and SMAD3. Alternatively, TGFβ–SMAD3 signalling may promote adipogenesis in multipotent progenitor cells during early WAT expansion but inhibit adipogenesis in committed preadipocytes populations. Interestingly, SMAD3-null mice also display increases in BAT markers in WAT compartments when kept on a regular diet, suggesting this pathway may be involved in the transdifferentiation of WAT to BAT upon cold exposure[50].

Several bone morphogenetic proteins (BMPs), which are members of the TGFβ superfamily, have also been implicated in adipogenesis[48], and single nucleotide polymorphisms neighbouring a BMP receptor, BMPR1A, are associated with obesity in humans[51]. Overall, BMPs promote adipogenesis by activating various SMADs and by signalling through the p38 kinase pathway[52,53]. BMP2 stimulates adipogenesis when provided in conjunction with a PPARγ agonist[53,54], although it can also promote osteogenesis in committed preadipocytes when given with retinoic acid[55]. BMP2 activates SMAD1 and increases nuclear translocation of the transcriptional activator Schnurri2 (Shn2) to directly stimulate PPARγ expression during early adipocyte differentiation[56]. Shn2-null mice have decreased WAT, and Shn2-null MEFs cannot differentiate in the presence of BMP2, although BAT remains unaffected[56]. Similarly, BMP4 has been shown to increase the capacity of C3H10T1/2 cells to respond to adipogenic stimuli and specifically promote white adipocyte differentiation. By contrast, BMP7 induces brown adipogenesis[30,57]. Consistent with this, BMP7-null mice had less BAT than controls, and mice overexpressing BMP7 showed increases in brown adipocyte gene expression in BAT while other metabolic organs remained unaffected[57].

The composition and stiffness of the ECM can regulate adipogenesis.

The conversion of spindly fibroblasts to round adipocytes is in part characterized by major remodelling of intra- and extracellular structures[58,59], and studies have shown that this can be influenced by the composition of the extracellular matrix (ECM), by ECM stiffness and by tension. For example, the differentiation of 3T3-F442A cells into adipocytes is inhibited by fibronectin, and this can be rescued by chemical inhibition of actin stress fibre formation[60]. Integrin α5 binds fibronectin and is expressed by preadipocytes but not mature adipocytes. Ectopic expression of integrin α5 blocks adipocyte differentiation by maintaining high levels of the active form of the RHO GTPase Rac, which must be reduced in 3T3-L1 cells for adipogenesis to proceed[61] (Figure 4a).

The stiffness of the ECM has also been shown to have a role in specifying lineage commitment in human MSCs[62–64]. Indeed, human MSCs grown on polyacrylamide gels with low stiffness are more likely to become adipocytes than cells seeded on stiffer matrices[64]. Similarly, primary mouse preadipocytes embedded in stiffer matrices that also have a higher concentration of collagen I show reduced rates of differentiation into adipocytes than when grown in softer gels[65]. Together, these studies suggest that force imposed on the cell by the ECM is a key gatekeeper of adipogenic competency.

ECM stiffness causes tissue tension, and, consistently, tension, which leads to enhanced actin and myosin fibre formation and cell stretching, mediates cell fate decisions in MSCs[66,67]. Although myogenic cell lines can transdifferentiate into adipocytes when treated with adipogenic stimuli[68] (such as glucocorticoids and insulin) or by ectopically expressing PPARγ[69], stretched myoblasts cannot undergo adipogenesis, correlating with increased expression of anti-adipogenic WNT ligands (see above)[66]. Furthermore, MSCs exposed to mechanical strain show increases in nuclear β-catenin, consistent with WNT activation, and cannot differentiate[70]. Lung embryonic MSCs can differentiate into adipocytes if not stretched, but when stretched will become smooth muscle cells, in part owing to altered expression patterns of different tension-induced protein isoforms (transcription co-factors that may regulate the activity of nuclear receptors such as PPARγ)[67].

Cells can regulate the stiffness and composition of their environment by digesting the surrounding ECM with matrix metalloproteinases (MMPs), a family of secreted or membrane-bound zinc-dependent peptidases that cleave ECM components or other secreted factors[71]. Humans have 23 MMPs, which have a range of targets and functions, as well as a distinct family of four endogenous tissue inhibitors of MMPs (TIMPs)[71]. Complete inhibition of MMP activity blocks the differentiation of committed preadipocytes and impairs adipose tissue development *in vivo*[72–75], suggesting that the balance between MMPs and TIMPs can determine adipogenic potential. In support of this hypothesis, polymorphisms neighbouring *MMP14* (also known as MT1-MMP), a membrane-tethered collagenase known for its role in tumour metastasis[76], are associated with human obesity[77] and mice lacking MMP14, have reduced adipose tissue[65]. Interestingly, although preadipocytes lacking MMP14 can differentiate under two-dimensional growth conditions, they do not differentiate when embedded in three-dimensional collagen gels[65], which better mimic physiological tissue development. This defect in three-dimensional differentiation is

rescued by lowering the concentration of collagen surrounding the cells[65]. Whether other MMPs also regulate adipogenesis has yet to be described.

The MMP inhibitor TIMP3 is repressed during adipogenesis, and its ectopic expression inhibits 3T3-L1 adipocyte differentiation by blocking the expression of transcription factors involved in the early stages of terminal differentiation[78]. Although the mechanism of TIMP3-mediated repression of adipogenesis is unknown, TIMP3 strongly binds ECM proteins and may act as a pericellular regulator of MMP activity[79]. By contrast, TIMP1 is a secreted factor and does not seem to affect adipocyte differentiation, although it may have a role in regulating lipid droplet and blood vessel formation in mature adipose tissue[80,81].

One of the key remaining questions is how stiffness and tension are detected by preadipocytes. Many scaffolds are currently being developed for *ex vivo* adipose tissue development and cell culture[82]. These new tools may help to elucidate the molecules that detect matrix stiffness and how they modulate signalling pathways regulating adipogenic commitment.

Cell–cell contact and cell shape influence adipogenesis.

Cellular confluency — when all cells in culture are all physically in contact with one another — is a requirement for many *in vitro* models of adipogenesis[83,84]. Remarkably, seeding MSCs in a spread configuration can direct lineage commitment towards osteoblasts, whereas confluent MSCs become adipocytes[85]. Because confluent preadipocytes can no longer proceed through the cell cycle, this cell contact was primarily thought to inhibit cellular proliferation in preparation for adipogenesis[86].

However, in 3T3-L1 cells, preadipocytes undergo a period of mitotic clonal expansion upon addition of adipogenic stimuli[87], suggesting that cell cycle progression in early adipogenesis does not exclude their ability to terminally differentiate. Thus, confluency could be mediating adipogenic commitment by inducing other cellular changes, such as modifying cell structure. Indeed, cells embedded within a methylcellulose gel, where they no longer divide, can also undergo adipogenesis[83,86], but this is due to changes in cell shape[88] that mimic the phenotype of confluent cells. Furthermore, inhibition of actinomyosin fibre formation commits pre-confluent human MSCs to adipogenesis instead of osteogenesis[89]. Single human MSCs plated on small surfaces that lead to a rounded morphology express pro-adipogenic *WNT* genes (see above) and differentiate into adipocytes upon dual adipogenic–osteogenic stimulation[89,90]. By contrast, single cells grown on larger features retain a spindly fibroblastic morphology that favours osteogenesis[89,90].

These changes in cell shape regulate RHO GTPase–RHO-associated kinase (ROCK) signalling[89] (Figure 4b). The inactive form, RHO-GDP, is the predominant species in confluent or rounded human MSCs and promotes adipogenesis; consistent with this, ectopic addition of constitutively active RHO, RHO-GTP, inhibits adipocyte differentiation[89]. RHO-GTP in spread cells activates ROCK; this, in turn, promotes actinomyosin fibre formation, which inhibits adipogenesis[89,90]. Knockdown and genetic studies have shown that ROCK II, but not ROCK I, is the kinase downstream of RHO regulating cell structure in 3T3-L1 cells and MEFs[91]. A recent study has suggested that active RHO inhibits adipogenesis by

promoting the expression of the transcription factors YAP and TAZ; knockdown of these factors promotes adipogenesis even in MSCs grown under otherwise osteogenic conditions[92], although how these factors are regulated by RHO or how they may be inhibiting adipogenesis has not been elucidated.

Not surprisingly, factors that control RHO activity (GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs)) have also been found to participate in MSC lineage commitment to adipogenesis. Mice lacking p190-B RHOGAP have decreased adiposity, and MEFs from these mice have a decrease in adipogenic capacity that is rescued with the addition of a general ROCK inhibitor[93]. Furthermore, p190-B RHOGAP-deficient MEFs undergo increased myogenic differentiation[93], suggesting that RHO may not only determine MSC cell fate between the osteoblasts and adipocytes, but also distinguish adipogenic commitment from myogenesis. GEFT, a RHO-specific GEF that stabilizes active RHO-GTP, can also inhibit adipogenesis and promote myogenesis in cell culture models[94]. However, whether cell shape regulates RHOactivity in MSCs through this GAP or GEF remains unclear.

Transcriptional factors regulating adipogenic competency.

Identifying specific transcription factors that define the preadipocyte population provides additional insight into the signals required to transition from a multipotent MSC to an adipocyte progenitor cell. Expression profiling of known transcriptional regulators between adipogenic and non-adipogenic fibroblasts has shown that zinc finger protein 423 (ZFP423) is found almost exclusively in adipogenic cells[95] although how it is regulated has not been identified. ZFP423 is necessary for 3T3-L1 adipogenesis and can promote white adipogenesis in non-adipogenic NIH-3T3 cells[95]. Furthermore, BAT development was impaired in ZFP423-null mice[95], suggesting it is also involved in brown adipogenesis. However, the effect of ZFP423 on *in vivo* white fat development remains unclear, as ZFP423-null mice do not survive after birth, when most WAT expansion occurs[96]. Interestingly, ZFP423 has a SMAD-binding domain that is required for BMP4-dependent adipogenesis. However, ZFP423 mutants lacking this domain can still promoted adipocyte differentiation in the presence of glucocorticoids, cAMP agonists and insulin[95], suggesting ZFP423 may promote adipogenesis in both a BMP-dependent and BMP-independent manner.

Other stem cells populations, including embryonic stem cells, require cooperation between multiple transcription factors to maintain their precursor state[97]; it is likely that adipogenic precursors also subscribe to this paradigm and that other factors cooperate with ZFP423 to determine adipogenic competency. One such factor may be TCF7L1[98], which is downstream of the canonical WNT pathway. Unlike other TCFs, which function as transcriptional activators, TCF7L1 is primarily a repressor of the canonical WNT pathway and has been shown to regulate embryonic stem cell pluripotency, skin stem cell differentiation and neurological development[99]. Like ZFP423, TCF7L1 is present in adipogenic but not non-adipogenic fibroblasts, where ectopic expression promotes adipogenic competency[98]. TCF7L1 appears to function by repressing cell structure-related genes and inhibiting myosin fibre formation upon addition of adipogenic stimuli[98].

TERMINAL DIFFERENTIATION

Once preadipocytes have committed to the adipogenesis programme, a transcriptional cascade that induces the expression of metabolic genes and adipokines associated with the adipocyte phenotype, such as FABP4, GLUT4, leptin and adiponectin, is activated; this is known the terminal differentiation stage[100–102]. Many of the molecular mechanisms regulating this stage of differentiation have been determined by exploiting known targets of the adipogenic stimuli used to stimulate adipogenesis in confluent committed preadipocytes, in particular glucocorticoid activation of glucocorticoid receptor (GR) and cAMP agonist activation of both protein kinase A (PKA)-dependent and PKA-independent pathways[103–108], although the importance of these pathways *in vivo* has yet to be determined. While many transcription factors have a role in adipogenesis[100,102,109], it is the expression of PPARγ, C/CAAT enhancer binding protein-α (C/EBPα), C/EBPβ and C/EBPδ, as well as the epigenomic coordination between these factors, that are the primary drivers of adipocyte gene induction during terminal differentiation[110,111].

C/EBP activation by adipogenic stimuli.

C/EBP proteins are widely expressed transcription factors that have a role in the development of many diverse cell types. Genome-wide studies have shown that C/EBPβ in committed preadipocytes is present at low levels before the addition of adipogenic stimuli and is bound to quiescent ‘adipogenic hotspots’. These are regions of the genome that display marks of active enhancers and recruit other adipogenic transcription factors (C/EBPδ, signal transducer and activator of transcription 5A (STAT5A), GR and RXR) after the addition of the adipogenic cocktail[112]. C/EBPβ is required for the binding of the other ‘hotspot’ transcription factors, except for C/EBPδ[105,112].

In addition to acting as a marker for adipogenic enhancers in preadipocytes, C/EBPβ expression and function are extensively regulated upon addition of adipogenic stimuli (Figure 5a). C/EBPβ expression is markedly induced by cAMP agonists within hours of stimulation[103], and this is mediated by the transcriptional activator cAMP response element-binding protein (CREB), which is phosphorylated in response to cAMP agonists, allowing it to act as a direct activator of C/EBPβ during early adipogenesis[113]. A cascade involving Janus kinase 2 (JAK2) and STAT3 also directly promote C/EBPβ expression in committed preadipocytes[114,115], although the initial stimulus has not been determined. Krüppel-like factor 4 (KLF4) also promotes adipogenesis by directly activating C/EBPβ transcription in early terminal differentiation[116]. In addition, the transcription factor KROX20 (also known as EGR2) is present during the first 6 hours of differentiation and can promote C/EBPβ expression, but this seems to be an indirect effect, suggesting that KROX20 may induce another, as-yet-unidentified transcriptional activator of C/EBPβ[117]. Interestingly, depletion of C/EBPβ increases the expression of both KLF4 and KROX20 in 3T3-L1 cells, suggesting a negative feedback loop during early adipogenesis[116]. Of note, cAMP also promotes the expression of EPACs (exchange proteins directly activated by cAMP)[107,108], although whether this pathway directly induces C/EBPβ and δ activity has not been shown.

C/EBPβ is also regulated in adipogenesis by post-translational modifications. C/EBPβ phosphorylation by mitogen-activated protein kinases and glycogen synthase kinase 3β is

required for the ability of C/EBPβ to bind DNA during differentiation[118,119]. Glucocorticoids, probably in part through GR[120], have also been reported to have a role in regulating C/EBPβ transcriptional activity by directing the acetylation of C/EBPβ and interfering with the interaction of C/EBPβ with the transcriptional co-repressor histone deacetylase 1 (HDAC1)[121,122].

Much less is known about C/EBPδ regulation, which is also induced rapidly by glucocorticoids upon addition of adipogenic stimuli[103]. C/EBPδ is likely just as important to adipose development as C/EBPβ since both C/EBPβ- and C/EBPδ-null mice have mild adipose tissue developmental defects, MEFs and mice lacking both proteins have marked primary defects in adipogenesis and adipose tissue development[123]. By contrast, C/EBPα is expressed later in terminal differentiation and is a direct target of C/EBPβ[124].

Epigenomics provides insights into early PPARγ activation.

Terminal differentiation cannot occur in the absence of the nuclear receptor PPARγ[125]; consistently, thiazolidinediones (TZDs), which target PPARγ to treat diabetes, can promote adipogenesis in adipocyte progenitor cells both *in vitro* and *in vivo*[126,127]. There are two PPARγ protein isoforms: PPARγ1 is expressed at highest levels by adipocytes but also by other cell types, including preadipocytes and other MSC-derived cell types; PPARγ2 is adipocyte specific[128,129]. However, adipogenic conversion can be equally mediated by either isoform[102]. The endogenous PPARγ ligand has yet to be found; nevertheless, cAMP agonists contribute to the production of an endogenous PPARγ ligand within the first 48 hours of terminal differentiation in 3T3-L1 cells[106].

Recent studies using *in vitro* adipogenic models have revealed that the *PPARg* locus is dynamically and extensively regulated upon addition of adipogenic stimuli by C/EBPs and GR during an epigenomic transition state[105,112,130] (Figure 5b). Genome-wide mapping of histone marks associated with active transcription, such as His3 Lys9 acetylation (H3K9ac) or H3K27ac, have shown that the *PPARg* locus has multiple functional enhancers located as far at −122 kb upstream of the *PPARg1* transcription start site[105,130].

Given the dynamic changes in histone acetylation during adipogenesis, regulators of these marks have been implicated in adipocyte differentiation, although their role seems to be complicated. Exposure of preadipocytes to inhibitors of HDACs has led to conflicting results[131–134], indicating HDAC subtype-specific effects and potentially off-target effects of the chemical agents. Moreover, studies in which the class I HDAC, HDAC1, was manipulated have also yielded conflicting results[131,135]. The class II HDAC9 inhibits adipogenesis, although this does not require its deacetylase activity[134], and inhibitors of class II HDACs reportedly block adipogenesis[136]. Moreover, both sirtuin 1 and sirtuin 2, which are class III HDACs, are downregulated during adipogenesis and also inhibit white adipocyte differentiation, potentially by regulating the acetylation of transcription factors rather than histone acetylation[137,138]. Whether these factors are important for regulating acetylation at the *PPARg* gene remains unclear.

Regulation of histone methylation has also been implicated in adipogenesis. In addition to the SETDB1[42,43], the histone methytransferase SETD8 has been shown to be required for

committed preadipocyte differentiation by adding the activating histone mark His4 Lys20 monomethylation (H4K20me) at the promoters of *PPARg* and of PPARγ target genes upon addition of adipogenic stimuli[43]. MLL3, a His4 Lys3 methyltransferase, is also required for adipogenesis, and MLL3-null mice have small WAT but normal BAT[139]. Reciprocally, the histone demethylase jumonji domain-containing 2C (JMJD2C) inhibits adipogenesis *in vitro*, although this effect may be due to HDAC binding and not demethylase activity[140].

PTIP (Pax transactivation domain-interacting protein), which forms a complex with MLL3 and the related MLL4, increases H3K4Me3 at the *PPARg* and *CEBPa* promoters, and deletion of PTIP inhibits differentiation of both white and brown preadipocytes[141]. However, in contrast to MLL3-null mice, PTIP-null mice have defects in BAT formation, but normal WAT development[141]. Moreover, the transcriptional regulators ASXL1 and ASXL2, which can recruit histone methyltransferases to promoter regions, have opposite effects on adipogenesis: ASXL1 inhibits adipogenesis and recruits repressive histone marks to PPARγ target genes, whereas ASXL2 promotes adipogenesis and recruits activating histone marks to these same loci[142]. Thus, additional studies are clearly needed to better understand the role of histone methylation, as well as other epigenomic changes, in adipocyte differentiation.

Other factors regulating PPARγ.

PPARγ levels and activity are also regulated by circadian rhythm factors[109]. REV-ERBα, a circadian transcription factor, is regulated by adipogenic stimuli, and both knockdown and overexpression of this factor inhibit adipogenesis and PPARγ induction during terminal differentiation[143]. Nocturnin is a circadian rhythm-regulated protein that is required for adipogenesis by acting as a co-activator for PPARγ and enhancing its activity during terminal differentiation[144].

Other transcription factors also regulate PPARγ expression and its ability to cooperate with C/EBP proteins in terminal differentiation. Examples of such factors include GATA-binding proteins 2 (GATA2) and GATA3. These are expressed by committed preadipocytes, in which they inhibit C/EBPβ and C/EBPα function, and therefore PPARγ activation[145,146], and must be repressed for adipogenesis to proceed in the presence of adipogenic stimuli[145].

Maintenance of mature adipocyte gene expression.

Genome-wide studies of mature adipocytes have shown that PPARγ and C/EBPα are present at ~ 60% of all genes induced during terminal differentiation[110]. Knockdown studies have revealed that PPARγ, C/EBPα and C/EBPβ are all required for sustained expression of PPARγ and C/EBP target genes in mature adipocytes, such as adiponectin, FABP4, and hormone sensitive lipase[110]. PPARγ and these C/EBP proteins also regulate each other in a positive feedback circuit central to terminal[105,124,147].

The central role of PPARγ in maintaining mature adipocyte function has been shown *in vitro* and *in vivo*. Depletion of PPARγ in mature 3T3-L1 adipocytes with small interfering RNA leads to decreased expression of adipocyte metabolic genes[148] and reduced ability to respond to insulin[149] without any effect on adipocyte morphology. However, in a mouse model where PPARγ is deleted in mature adipocytes, this transcription factor was required

for cell survival[150,151]. These experiments suggest that mature adipocytes can survive and maintain their lipid content in the presence of minimal PPARγ (such as in the knockdown experiments), but that in the complete absence of PPARγ these differentiated cells cannot be sustained (as in the genetic mouse model).

Conservation of adipocyte gene activation across species.

PPARγ and many other metabolic genes are induced during adipogenesis in models from multiple species[130]. Comparison of genome-wide maps of histone marks, PPARγ binding and C/EBPα between human- and mouse-derived adipogenic cell lines has further developed our understanding of the conservation of developmental programmes[130,152,153]. First, these studies have revealed that there is conservation of adipogenic stimulus-dependent induction of PPARγ binding and histone modification near genes that are induced during adipogenesis[130]. Intriguingly, although many of the same metabolic genes are induced and epigenomically modulated in human and mouse adipogenesis, the precise genomic loci that are regulated during differentiation are not well conserved despite the conservation of these sequences in the genome of the other species[130,153]. Moreover, genes that were expressed in mature adipocytes of both species were more likely to have roles in pathways related to the metabolic function of these cells, whereas genes expressed only in one species did not[153], suggesting that comparison between expression profiles of different species can filter out species-specific artefacts and highlight the biologically important genes. These studies also suggested that conservation of a PPARγ binding site could be predicted in the syntenic region of the genome from the other species contained a C/EBPα-binding site[152], supporting a role for the cooperation of these factors in maintaining mature adipocyte function in evolution.

Brown adipocyte-specific transcriptional regulators.

Brown adipocyte differentiation also requires PPARγ and C/EBP activity[123,154]. To date no genome-wide studies of PPARγ or C/EBP binding have been done in brown adipocytes to compare the extent of overlap between these related, but distinct cells types. In addition, many transcription factors and signalling pathways, such as ZFP423 and WNT, have been shown to have crucial roles in BAT development[37,95]. However, brown adipocyte differentiation and mature adipocyte function have unique requirements for transcriptional co-regulators than their white adipocyte counterparts.

The transcriptional regulator PRDM16 is critical for brown fat adipogenesis. PRDM16 exists in a complex with C/EBPβ to induce the expression of genes common to white and brown adipocytes, such as PPARγ and FABP4, as well as brown fat-specific genes such as *UCP1*[23,155]. Activation of brown fat genes occurs following recruitment of PPARγ to a PRDM16 transcriptional complex[156]; PRDM16 also forms a repressive complex with C-terminal binding protein 1 (CTBP1) and CTBP2 to repress the expression of white adipocyte genes[156]. *In vitro* PRDM16 knockdown in primary brown preadipocytes promotes myogenesis, consistent with a role in brown adipocyte–myocyte cell fate specification[23]. However, although PRDM16-knockout mice do not express brown adipocyte-specific thermogenic genes, they still express adipogenic markers common to white and brown adipocytes in brown adipose depots[23]. This indicates that brown adipogenesis can still

proceed in part in the absence of PRDM16. Thus, other regulators of brown adipogenesis must exist, possibly including other PRDM family members. Although PRDM16 has no role in white adipocyte differentiation and is not expressed in visceral WAT, it is moderately expressed in subcutaneous WAT, where it promotes the metabolically favourable thermogenic properties[27].

Another key regulator of brown fat is PPARγ co-activator 1α (PGC1α), which was identified in a screen for proteins that bind PPARγ in brown fat[157]. Although PGC1α and the related PGC1β do not have a significant role in brown adipogenesis, they are required to maintain the expression of thermogenic genes in mature brown adipocytes[158]. Interestingly, PGC1α, along with thermogenic genes, is induced in WAT from mice or humans treated with thiazolidinediones (see above), contributing to the improved metabolic profile seen upon treatment with these compounds[159].

CONCLUSION AND PERSPECTIVES

Recent advances in adipogenesis have provided insights into precursor competency, remodelling of the genome and the relationship of those pathways to *in vivo* fat pad development. Cell structure and signalling pathways, as well as epigenomic remodelling, are potential targets of novel therapeutics targeting specific elements of adipogenic commitment and terminal differentiation. Indeed, compounds targeting epigenomic pathways such as histone acetylation are already used clinically for other disorders, such as cancer,[160] and may be exploited for the treatment of metabolic disease.

However, many important questions remain about adipogenesis and adipose tissue development. First, our understanding of adipocyte development *in vivo* is limited by the lack of candidate markers distinguishing different stages of precursor cell development. Second, although mouse models have shown that subcutaneous and visceral fat are functionally distinct[5,6,8,13] and express different developmental genes[5,6], it remains inconclusive whether these differences are due to adipogenic precursor potential. Third, the mechanisms that increase the ‘brown’ phenotype in white adipocytes with cold exposure have yet to be discovered but should offer an intriguing strategy for treating metabolic disease. Last, although many pathways described here and elsewhere have been shown to regulate adipogenic commitment and terminal differentiation, how these two stages of adipogenesis are physiologically integrated during development remains unknown.

The recent isolation of preadipocyte populations *in vivo*[14,29] should help to answer many of these questions by identifying specific markers for adipocyte precursor cells, allowing more advanced lineage tracing of WAT development in both the subcutaneous and visceral depots, and providing a pure population of more physiologically relevant cells for genome-wide studies of transcription factor binding and histone modifications to further understand the relationship between epigenomic regulation and gene expression in cellular differentiation. Ultimately, a better understanding of *in vivo* adipogenic commitment and triggers of terminal differentiation will be crucial for manipulating MSCs and adipose derived stem cell populations in ways that have promise above and beyond treating conditions of metabolic dysfunction[82,161].

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GLOSSARY

type 2 diabetes
chronic disease that is characterized by increased blood glucose related to insulin resistance and pancreatic failure

cancer cachexia
Weight, muscle atrophy, fatigue, weakness, and loss of appetite in the setting of cancer

lipodystrophies
Reduced or abnormally redistributed adipose compartments (acquired or genetic)

unilocular
One large lipid droplet

thermogenesis
The process of producing heat

fluorodeoxyglucose positron emission tomography
Molecular imaging technique using a labelled glucose analogue

uncoupling protein 1 (UCP1)
Mitochondrial protein that dissociates oxidative phosphorylation from energy production, leading to increased thermogenesis

Mesenchymal stem cell
Multipotent progenitor that can differentiate into adipocytes, osteoblasts, myocytes or chondrocytes

subscapular
region below the scapula, the shoulder blade

epigenomic
of the epigeome, i.e., chromatin modifications including DNA methylation and histone modification that regulate gene expression and function without a corresponding alteration in DNA sequence

A-Zip mice
mouse model in which a dominant negative transcription factor that interferes with C/EBP function is expressed by adipocytes, leading to lipodystrophy

Integrins
Heterodimeric, cation-dependent cell surface receptors that attach cells to their surrounding environment, connecting ECM cues to intracellular signalling

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Box 1:

Lineage tracing studies

Mouse models have been crucial for understanding the relationship between undifferentiated cells in a developing embryo and the mature differentiated cell types that develop from progenitor populations. Lineage tracing studies genetically and permanently identify cell populations that may express a precursor gene only for a short period of time by taking advantage of recombination systems such as Cre-lox, a system to knock out alleles, which have been engineered to contain *loxP* sequences that direct Cre recombinase-mediated recombination[162]. For example, mice expressing the *Rosa26R3-YFP*, a *loxP* flanked reporter gene, and expressing Cre recombinase from the *Myf5* promoter were used to permanently indicate all cells that at one point during development expressed MYF5, an early marker of skeletal muscle development[23]. All tissues in adult mice containing progenitors that currently or transiently expressed MYF5 would be marked with YFP expressed from the *Rosa26* promoter, which is active in all cells. Skeletal muscle and brown fat were both positive for YFP expression in this model, whereas white adipose tissue was not, suggesting that skeletal muscle and brown fat are more closely related in lineage than white and brown fat[23]. However, the *in vivo* precursor population that can differentiate into muscle or brown fat, but not white fate, has yet to be discovered.

Lineage tracing based on expression of *Pparg* in mice has provided insights into *in vivo* white adipogenesis, particularly with the discovery of committed preadipocyte precursors neighbouring blood vessels within the mature fat pad[14,127]. This lineage tracing method coupled with timed feeding of animals with nucleotide analogues[127] can be used to assess the kinetics of adipogenesis *in vivo*.

Box 2:

The case for developing tools to study *in vivo* adipogenesis.

Much of what is known about factors required for adipogenesis is based on studies of adipogenic cell lines such as mouse 3T3-L1 cells[83]. The differentiation of these cells into adipocytes can be achieved *in vitro* by adipogenic stimuli, including glucocorticoids, cyclic AMP agonists and insulin[163–165]. Other *in vitro* models of adipogenesis, such as mouse 3T3-F442A cells and human SGBS cells, have many similar properties with 3T3-L1 cells[84,166]. Although these *in vitro* models share many similarities with primary adipocytes, including triglyceride storage, insulin sensitivity, expression of adipocyte genes (such as *GLUT4* and *FABP4* (also known as *AP2*)) and factor secretion[167,168], there are some important differences. For example, triglycerides are stored in many droplets in the cell lines, whereas white adipocytes characteristically contain a single large droplet[83]. Also, some adipogenic cell lines, including 3T3-L1 and 3T3-F442A cells, express the adipocyte secreted factor leptin at much lower levels than primary adipocytes[167].

Ideal mouse models have yet to be developed for studying adipogenesis. Most studies use the *Fabp4* promoter-enhancer[169] to drive adipocyte-specific expression of factors of interest or the Cre recombinase. However, this model system has major limitations for studying adipogenesis. First, FABP4 is expressed during terminal differentiation[124,170] in a PPARγ-dependent manner[128]. Thus, PPARγ$^{lox/lox}$/FABP4-Cre mice (in which PPARγ has been conditionally deleted in adipocytes) develop WAT[151], despite significant evidence that PPARγ is required for *in vivo* adipogenesis[125] and is a marker for preadipocytes within the adipose compartment[14]. Second, the 5.4 kb upstream proximal promoter of *Fabp4* used in these models can drive transgene expression ectopically in macrophages[171], during embryonic development[172] and in the brain[173], raising questions about the specific involvement of adipocytes in the phenotypes of mouse models developed using *Fabp4* transgenes. *Adiponectin-Cre* models have also been developed and seem to be more adipocyte-specific than FABP4[174]; however, adiponectin is generally expressed in mature adipocytes and not precursor cells. The study of adipogenesis *in vivo* will require the development of models in which factors can be expressed or deleted in multipotent cells and committed preadipocytes. *Prx1-Cre* is activated mid-gestation and has recently been suggested as one such tool to study osteogenic and adipogenic cell-fate decisions *in vivo*[175].

ONLINE SUMMARY

- Adipogenesis is a highly regulated process that converts fibroblast-like precursor cells into round and lipid-laden adipocytes.
- White and brown adipocyte differentiation share many key important features, such as shared requirement for the master regulator PPARγ, but have important differences.
- Identification of committed precursor cells within adipose tissue has been important for understanding of adipogenesis *in vivo*.
- Adipogenic stimuli activate signalling pathways that coordinate transcription factors to promote stem cell commitment to an adipogenic fate.
- Extensive epigenomic modifications underlie the commitment and stability of adipocytic differentiation.

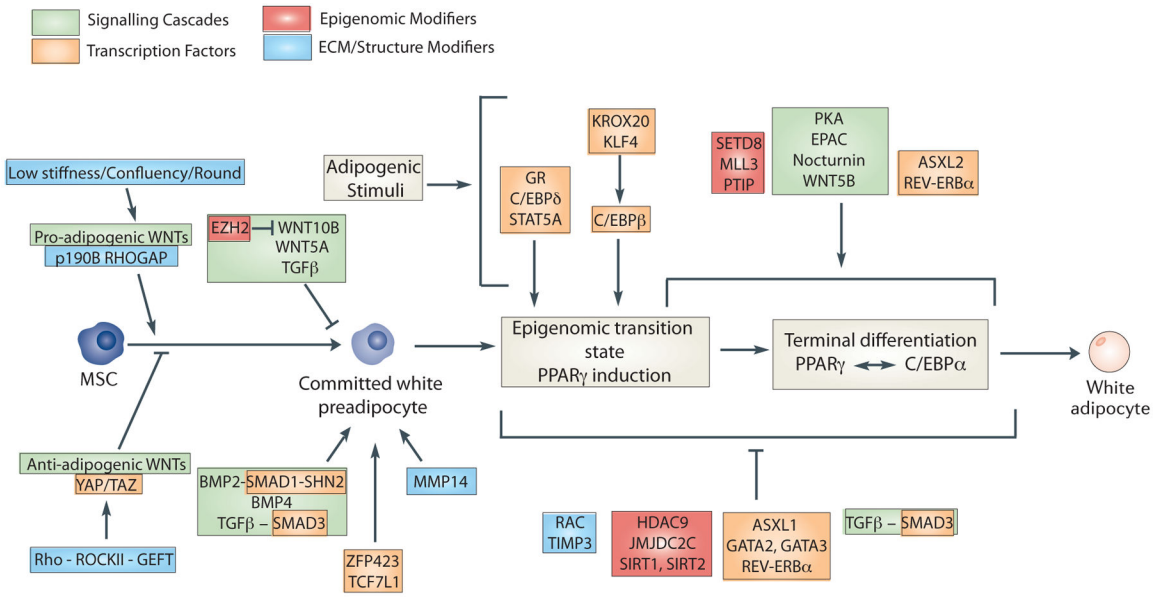

Figure 1: Cues influencing white adipogenic progression.

Differentiation of multipotent mesenchymal stem cells (MSCs) to mature adipocytes involves a complex integration of cytoarchitecture, signalling pathways, and transcriptional regulators. The first step of adipogenesis is the transition of embryonic stem cells to MSCs (not shown.) MSCs then transition to committed white preadipocytes (mediated by factors such as cell shape, confluency or matrix stiffness). Alternatively, MSCs can be stimulated to differentiate into myoblasts, chondrocytes or osteoblasts. Committed white preadipocytes can become mature white adipocytes upon addition of adipogenic stimuli, such as glucocorticoids, insulin and cyclin AMP. Factors emphasized in this Review are shown along the adipogenic progression at the stage where they act on precursor cells. Factors that have been shown to play a part during different phases of adipogenesis are listed multiple times.

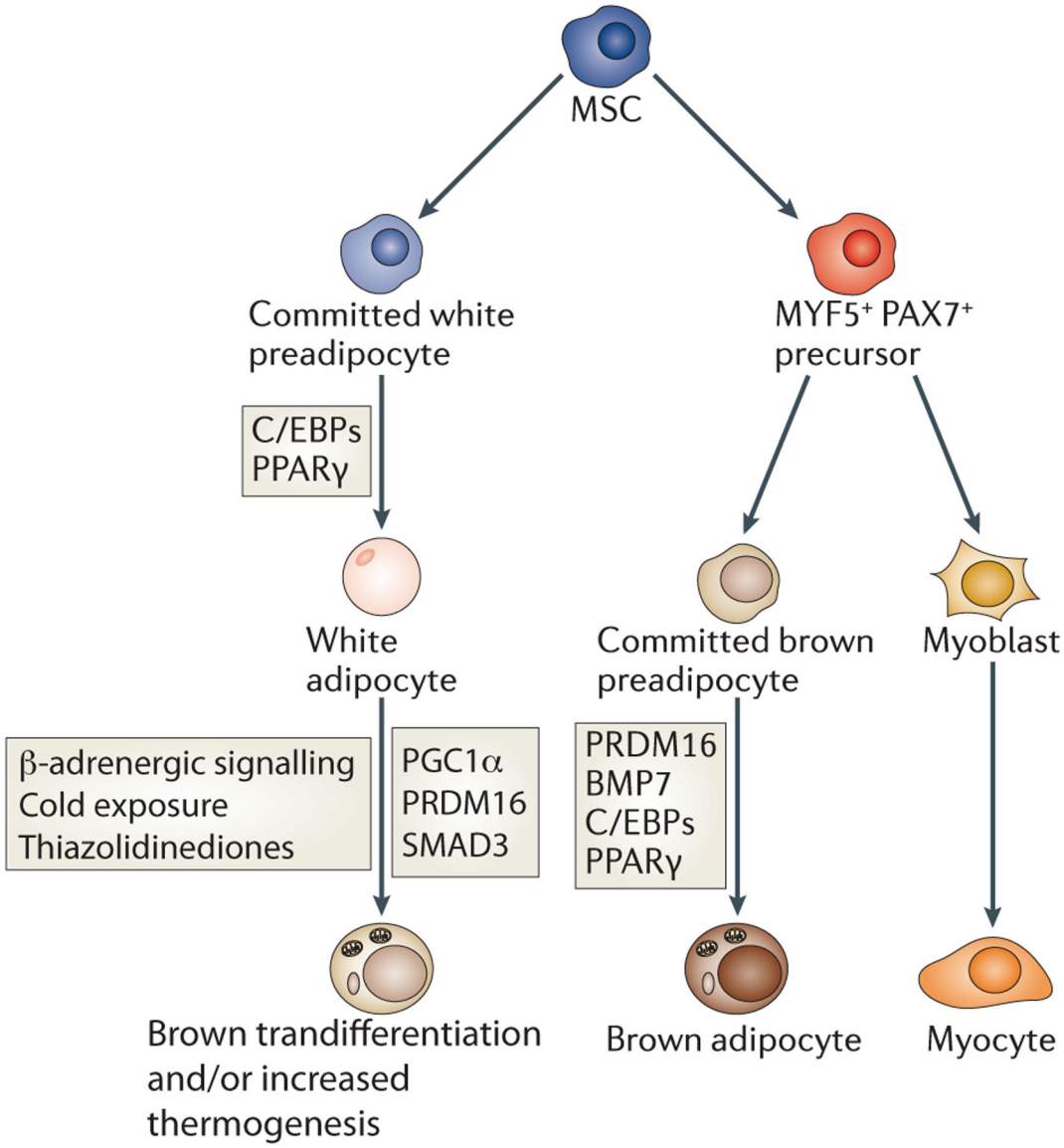

Figure 2: Relationship between white and brown adipogenesis.

Historically, white and brown adipocytes were thought to derive from the same precursor cell. However, brown adipocytes instead share a common MYF5/PAX7-positive precursor with muscle cells (Box 1). The MYF5/PAX7-positive precursor is driven to brown adipocyte terminal differentiation by PPARγ and C/EBPs cooperating with the transcriptional co-regulator PRDM16. By contrast, PRDM16 does not affect white adipogenesis. White adipocytes can also be stimulated to display characteristics of brown adipocytes by cold exposure, β-adrenergic signalling, and thiazolidinediones, which appear to function via the indicated factors.

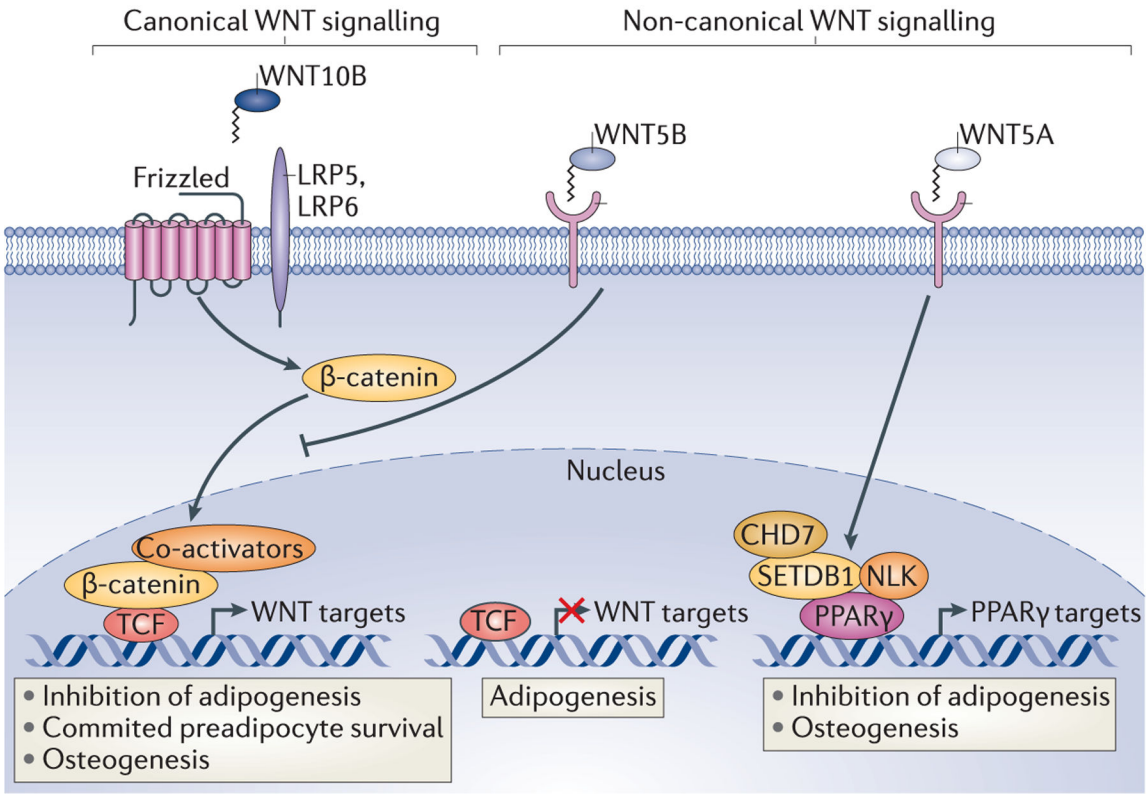

Figure 3: WNT signalling in adipogenesis.

In the presence of canonical WNT ligands, such as WNT10B, β-catenin translocates into the nucleus, where it recruits a co-activator complex to TCF transcription factors and activates WNT target genes. In committed preadipocytes this pathway promotes cell survival, but inhibits adipogenesis. However, the WNT targets that inhibit adipogenesis are not completely understood, it is known that activation of this pathway in MSCs promotes osteogenesis. WNT5B, a non-canonical WNT ligand, promotes adipogenesis by inhibiting β-catenin nuclear localization to these targets. The non-canonical ligand WNT5A also signals to inhibit adipogenesis and promote osteogenesis. This is achieved through the activation of the histone methyltransferase SET domain bifurcated 1 (SETDB1) following the assembly of a SETDB1–NLK–CHD7 complex, inhibiting target gene transcription. It remains unclear which receptors are critical to non-canonical WNT signalling in adipogenesis.

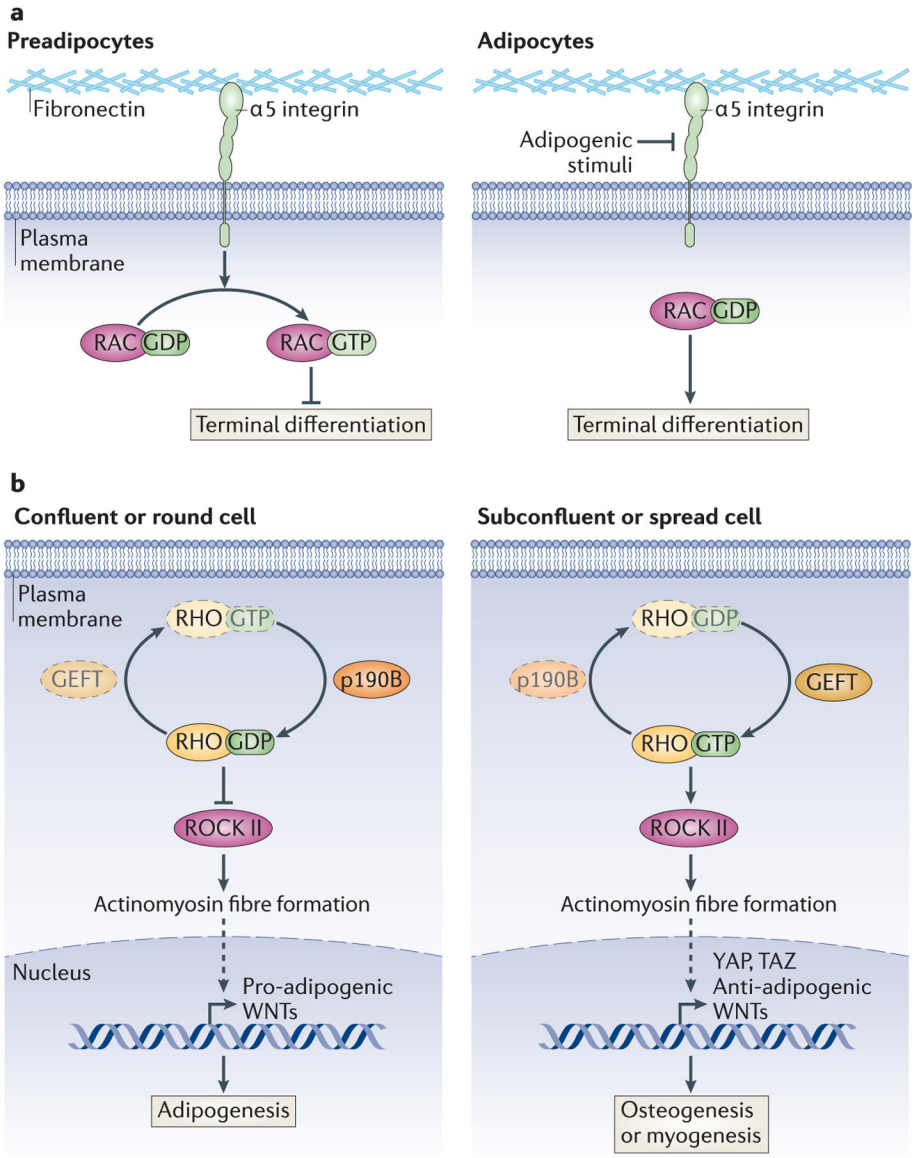

Figure 4: Rho-GTPase Family in Adipogenesis.

a | RAC-GTP inhibits adipogenesis. Integrins transduce extracellular structural signals into intracellular signalling cascades. Integrin α5, a fibronectin-binding protein, prevents the progression of preadipocytes to mature adipocytes in the absence of adipogenic stimuli by promoting the activation of RAC. Integrin α5 is repressed by adipogenic stimuli, leading to inactivation of RAC and terminal differentiation. **b |** Regulation of RHO determines MSC lineage fate. The shape of MSCs determines their ability to differentiate into adipocytes or alternate lineages by regulating RHO activity. Factors that favour the inactive form of RHO (RHO-GDP), such as p190-B RHOGAP, promote the adipogenic programme in these precursor cells, by inhibiting ROCK II activation of the actinomyosin cytoskeleton, which leads to the expression of pro-adipogenic WNTs. Conversely, factors that promote Rho-GTP lead to osteogenic or myogenic differentiation programmes, and this is mediated through the

expression of anti-adipogenic WNTs and YAP and TAZ. Whether shape directly regulates p190-B RHOGAP or GEFT is unknown. Dashed arrows indicate an indirect interaction.

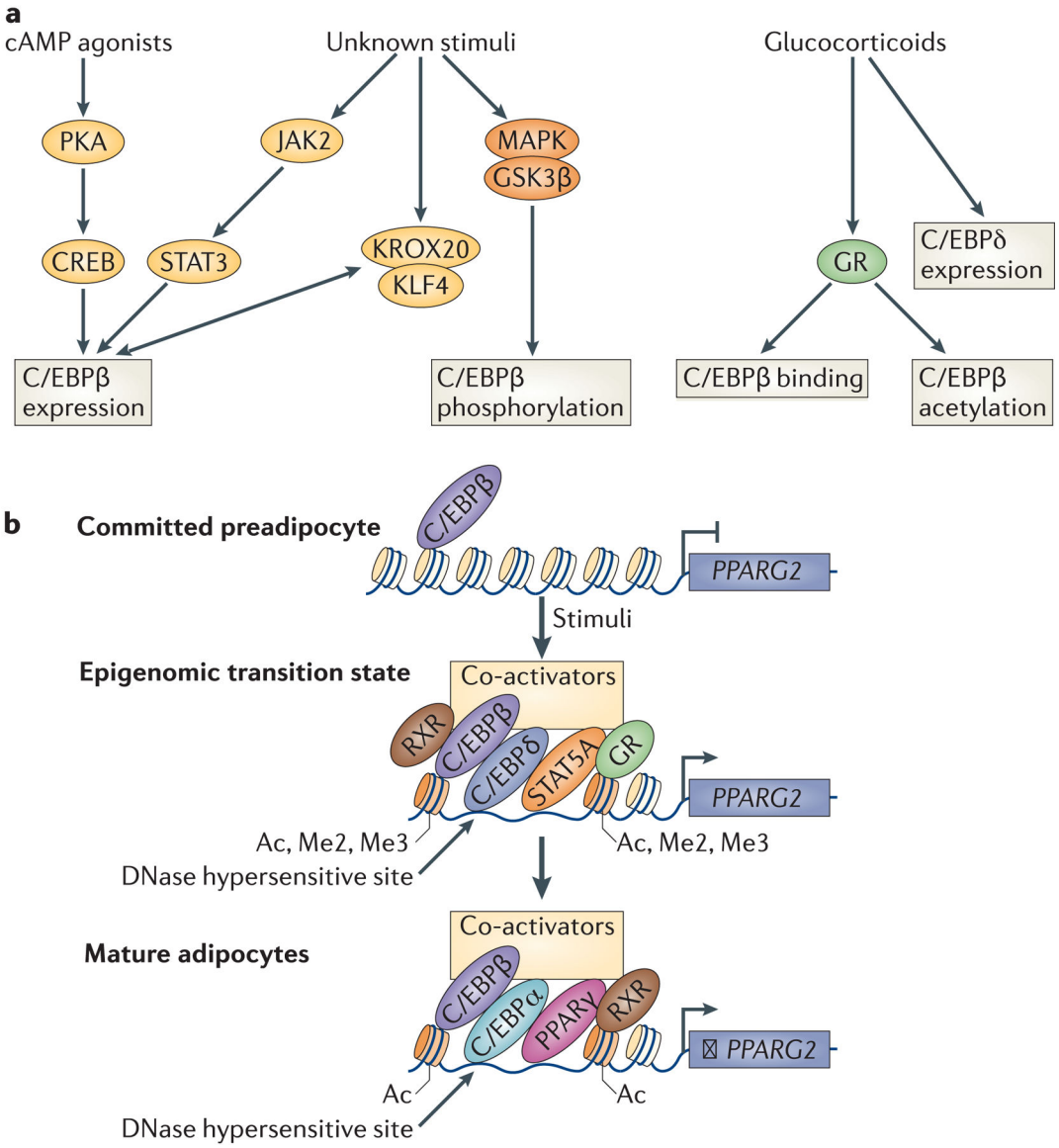

Figure 5: Activation of C/EBPs and PPARγ during terminal differentiation.

a | C/EBP activation by adipogenic stimuli. Glucocorticoids and cAMP agonists are common components of the adipogenic stimuli used to promote adipogenesis in both MSCs and committed preadipocytes. Experiments adding these compounds individually have elucidated some of the mechanisms through which C/EBPs, especially C/EBPβ, are induced during adipogenesis. C/EBPβ and C/EBPδ expression is induced upon addition of these adipogenic stimuli. C/EBPβ activity and binding are also regulated independently of its levels by glucocorticoids. In addition, C/EBPβ expression and phosphorylation are regulated by unknown components of the adipogenic cocktail, which may include insulin, growth hormone or BMPs. **b |** Recruitment of the transcription activation complex to *PPARγ*. Schematic of the recruitment of transcription factors to the PPARγ locus during adipogenesis. In preadipocytes, PPARγ enhancer regions are occupied by C/EBPβ and C/EBPδ, but are not accessible. Upon addition of adipogenic stimuli, levels of these

transcription factors increase and lead to the recruitment of a transcriptional activation complex, including the transcription factors GR, STAT5a and RXR and a co-activator complex. These ‘hotspots’ are also marked by an increase in DNAse I hypersensitivity and activating histone marks. Once PPARγ is robustly activated in differentiation, it can auto-regulate its expression in cooperation with C/EBPα and C/EBPβ.