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Id transcriptional regulators in adipogenesis and adipose tissue metabolism

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Abstract

Id proteins (Id1-Id4) are helix-loop-helix (HLH) transcriptional regulators that lack a basic DNA binding domain. They act as negative regulators of basic helixloop-helix (bHLH) transcription factors by forming heterodimers and inhibit their DNA binding and transcriptional activity. Id proteins are implicated in the regulation of various cellular mechanisms such as cell proliferation, cellular differentiation, cell fate determination, angiogenesis and tumorigenesis. A handful of recent studies also disclosed that Id proteins have critical functions in adipocyte differentiation and adipose tissue metabolism. Here, we reviewed the progress made thus far in understanding the specific functions of Id proteins in adipose tissue differentiation and metabolism. In addition to reviewing the known mechanisms of action, we also discuss possible additional mechanisms in which Id proteins might participate in regulating adipogenic and metabolic pathways.

Keywords

WAT; BAT; Thermogenesis; Insulin Sensitivity; Lipid Metabolism; Review

2. INTRODUCTION

Obesity is rapidly emerging as one of the most serious health problems worldwide since its prevalence has drastically increased over the past 3 decades, reaching epidemic proportions (1, 2). A number of factors such as energy rich diets, sugary drinks, lack of physical exercise and technology-driven sedentary life styles have contributed to the current levels of obesity. Obesity is associated with an array of diseases and is an established risk factor for a number of diseases such as hypertension, type 2 diabetes, dyslipidemia, chronic heart disease, nonalcoholic fatty liver disease (NAFLD), atherosclerosis, degenerative disorders, including dementia and airway diseases (3–6). Moreover, various epidemiological and clinical studies suggest that obesity is an independent risk factor for various cancers such as liver, colon, breast, gastric, gall bladder, endometrial, esophagus and pancreatic cancers and renal cell carcinoma (7–10). Therefore, understanding the development, maintenance and function of

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fat (adipose) tissue, and in particular, the molecular mechanisms that govern adipocyte differentiation and expansion of adipose tissues and their metabolic functions in normal and obese conditions have become increasingly essential for the treatment and intervention of obesity.

Two types of functionally distinct adipose tissue exist in the body: white adipose tissue (WAT) and brown adipose tissue (BAT) (11). The major function of WAT is to store excess energy in the form of triglycerides when energy intake exceeds expenditure. The adipocyte number, size, and the total amount of WAT are dynamically in proportion to the energy levels of the body (12). If the energy influx is consistently higher than expenditure, it leads to continuous synthesis of triglycerides, with a concomitant increase in adipocyte size (hypertrophy) due to triglyceride accumulation. In addition, these excessive triglyceride levels in the body force preadipocytes and/or adipocyte progenitors in the WAT to differentiate into adipocytes and store newly synthesized triglycerides. This leads to a steady increase in total body WAT, resulting in overweight and ultimately obesity. In recent years, significant progress has been made in understanding the development, differentiation, maintenance and specific functions of adipose tissues. A number of genes, such as members of the *PPAR* and *C/EBP*, and *KLF, STAT, SREBP-1c, FOXC2, E2F, Rb, Wnt*, and *GATA* have been identified to play essential roles in regulating white adipocyte differentiation and metabolic function (13–17). Deletion of some of these genes in mice resulted in a lean phenotype due to impaired adipogenesis, altered adipose tissue metabolism and reduction in total body WAT.

In contrast to WAT, BAT is specialized for energy expenditure by dissipating energy as heat, a process termed adaptive thermogenesis. This unique metabolic property of production of heat by BAT is attributed to its high mitochondrial density and its exclusive expression of uncoupling protein-1 (UCP1) in the inner mitochondrial membrane. During respiration, an electron-motive force established across the inner mitochondrial membrane is dissipated as heat by UCP1, rather than being used to drive the synthesis of ATP. For the past few years BAT has been receiving tremendous interest due to its ability to expend energy and function as a defense against hypothermia and possibly obesity. Subsequently, a number of genes such as *PGC1*α, *UCP1, PPAR*γ, *BMP, EBF2, Cidea, FOXC2, SRC2, PRDM16, Orexin, LXR, ARRDC3, Twist1*, and *TR*α*1* were discovered that play crucial roles in BAT development, maintenance and its thermogenesis function (18–24). PGC1α especially has emerged as the master regulator of various genes that are involved in thermogenesis, and it predominantly controls the entire BAT-mediated thermogenesis program (24). On the other hand, BMP and Ebf2 determine between the white versus brown adipose progenitor differentiation programs (25, 26). In addition to the above genes, recent studies revealed the involvement of another family of proteins, inhibitor of DNA binding (Id), in adipocyte differentiation and adipose tissue metabolism.

3. Id PROTEINS

Id proteins (Id1, Id2, Id3 and Id4) are a subfamily of helix-loop-helix (HLH) transcription factors that lack a basic DNA binding domain. Therefore, they function entirely by dimerization with other transcriptional regulators, mainly those of the basic-helix-loop-helix

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(bHLH) factors. The heterodimers (Id/bHLH) fail to bind to DNA, and hence Id proteins function as dominant negative regulators of bHLH proteins (27). The Id proteins (Id1-Id4) range in size from 120–160 amino acids and, despite the highly conserved HLH domain among all 4 Id proteins, they display extensive sequence divergence. Expression analysis of Id proteins revealed widespread and overlapping expression patterns in multiple tissues, suggesting that they play an essential role in many cell types with the existence of possible redundancy in their functions. A number of *in vitro* and *in vivo* studies implicated Id proteins in the regulation of multiple cellular processes such as cell cycle regulation, cell proliferation, cellular differentiation, cell fate determination, hematopoiesis, angiogenesis and tumorigenesis. As a general mechanism of action, Id proteins induce their inhibitory effect by acting as negative regulators of basic helix-loop-helix (bHLH) transcription factors, which control cell type-specific gene expression. They form heterodimers with DNA binding bHLH proteins and prevent their DNA binding and transcriptional activity. For example, Id1 regulates the transcription of the cell cycle inhibitor p16 by directly binding to its transcriptional activators E47 and Ets2 (Figure 1A). Some of the well-known targets of Id proteins include E proteins, Rb, p107, p130, PAX, Ets, MyoD and Myf-5 (28–31). The specific function of Id proteins and their mechanism of action in cell cycle control, cellular differentiation, hematopoiesis and tumorigenesis have been extensively reviewed elsewhere (28–34). Here, we review the progress made thus far in understanding the specific functions of the Id family of proteins in adipogenesis and adipose tissue metabolism. Since the mechanism of action of Id proteins in cellular metabolism is still an active area of investigation, in addition to reviewing their known mechanisms of action in adipose tissue metabolism, we also provide our views on possible additional mechanisms in which Id proteins might participate to regulate adipogenic and metabolic pathways.

3.1. Id1

A protein expression screen in different metabolic tissues of adult mice revealed higher expression of Id1 in both WAT and BAT. Id1 expression is especially highest in BAT compared to other metabolic organs (35). These observations suggest that Id1 could possibly play a role in adipogenesis, WAT metabolism and BAT-mediated thermogenesis. Subsequently, when wild-type mouse embryonic fibroblasts (MEFs) or 3T3-L1 preadipocytes were induced to differentiate into adipocytes, Id1 protein was strongly expressed before the induction of differentiation, but rapidly declined and completely disappeared during adipocyte differentiation (35). It appears that the clearing of Id1 protein from cells might be necessary before the cells start differentiating into adipocytes. Why is it necessary to clear Id1 from the cells to initiate differentiation? One possibility could be that Id1 promotes cell proliferation and inhibits cellular differentiation (28, 29). Id1 prevents the expression of the cell cycle inhibitor p16 through E47 and Ets2 (36, 37). Id1 can sequester the bHLH transcription factor E47, which is a transcriptional activator of p16. In addition, phosphorylation of the Ets family transcription factor Ets2 by Ras/Raf/MEK signaling leads to transcriptional activation of p16. However, expression of p16 by Ets2 can be effectively inhibited by Id1 through its direct interaction with Ets2 (27, 37, 38). In the absence of p16 the uninhibited Cdk/cyclin complexes continue to promote cell cycle progression by phosphorylating and keeping pRB in its inactive state, leading to constitutive activation of E2F and DP factors, which activate a number of genes that drive cell proliferation (Figure

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1A). It is undesirable to initiate cellular differentiation because in order for the cells to differentiate into adipocytes, they need to permanently exit from the cell cycle and reach a state of irreversible growth arrest for terminal differentiation. In the presence of Id1, cells may not reach this growth arrest stage due to its ability to block p16 expression, leading to constitutive activation of Cdk/cyclin complexes followed by pRB inactivation, which continues to promote cell proliferation. This is further supported by the observation that *pRB*$^{-/-}$ cells fail to differentiate into adipocytes, and repression of E2F transcription by pRB contributes to establishing a permanent exit from the cell cycle (39). Furthermore, it was demonstrated that C/EBPα promotes adipocyte differentiation by repressing E2F–dependent transcription. However, C/EBPα mutants defective in repression of E2F–dependent transcription have an impaired ability to suppress proliferation and thereby fail to induce adipocyte differentiation (40). This further indicates that inhibition of E2F–mediated cell proliferation is a pre-requisite for the initiation of adipocyte differentiation. This could be the primary reason why Id1 is targeted for degradation and cleared from the cells, which facilitates p16 expression, and ultimately, E2F–repression, which is required to initiate adipocyte differentiation (Figure 1B). In line with these studies, in the absence of Id1, the ability of *Id1*$^{-/-}$ MEFs to differentiate into adipocytes was significantly accelerated. It appears that p16-mediated inhibition of Cdk/cyclin complexes in the absence of Id1 helped cells to exit the cell cycle, which is essential for differentiation. As a result, earlier expression of PPARγ, the master regulator of adipocyte differentiation, and its downstream target, aP2 were detected in differentiating *Id1*$^{-/-}$ cells (35).

Alternatively, it is also possible that Id1 regulates PPARγ and operates upstream to PPARγ. Since PPARγ expression is induced in the absence of Id1, overexpression of Id1 could directly demonstrate whether PPARγ is really downstream to Id1. If that is the case, overexpression of Id1 should suppress the expression and/or activity of PPARγ and its downstream target genes such as aP2, CD36, perilipin, lipoprotein lipase (LPL) and phosphoenol pyruvate carboxykinase (15, 41), which in turn should delay or block adipogenesis. However, surprisingly, Id1-overexpressing 3T3-L1 preadipocytes showed the same degree of adipocyte differentiation as control cells. This is because, in addition to endogenous Id1, overexpressed Id1 was also rapidly cleared from cells during adipocyte differentiation. This further demonstrates how essential it is to clear all the Id1 from cells to facilitate initiation of adipocyte differentiation. Id1 was heavily ubiquitinated and targeted for proteasome-mediated degradation in Id1-overexpressing 3T3-L1 cells undergoing differentiation. As a result, the overexpressed Id1 was unable to inhibit adipocyte differentiation as expected (35). Therefore, it is unclear whether Id1 really operates upstream to PPARγ, and if so, how it regulates PPARγ. It is possible to modify ubiquitin-binding lysine sites in Id1 protein and reevaluate if the mutant Id1 suppresses adipogenesis. However, it was shown before that generation of a lysine-less Id1, where all the lysine residues were replaced to alanine, still could not prevent Id1 from degradation, and Id1 underwent N-terminus-dependent ubiquitination which is modulated by MyoD (42). During adipocyte differentiation, in addition to N-terminus-dependent ubiquitination and degradation, Id1 could also have undergone ubiquitin-mediated degradation by other E3 ligases such as Smurf2. A recent study showed that Smurf2 mediates Id1 degradation in senescent cells (43). Another potential problem with the approach of lysine residue

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replacement is that amino acid substitutions in Id1 protein could abolish Id1's transcriptional regulatory functions, and this mutant Id1 may no longer sequester its target bHLH factors as effectively as the native Id1. Nevertheless, the studies in *Id1*$^{-/-}$ cells at least established the basic function of Id1 in adipogenesis, and in the absence of Id1, adipocyte differentiation is accelerated with earlier expression of PPARγ and its downstream target genes.

Surprisingly, although adipogenesis is accelerated, Id1 knockout mice are leaner compared to wild-type controls. This lean phenotype is especially more striking in aged animals as the *Id1*$^{-/-}$ mice failed to gain fat mass during aging (35). Since adipogenesis is not defective in the absence of Id1, a failure to gain fat mass during aging in *Id1*$^{-/-}$ mice could be due to increased energy expenditure. This speculation is mainly fueled by the fact that Id1 expression is strongest in BAT compared to other metabolic tissues, and hence, Id1 might have a significant role in the regulation of BAT-mediated thermogenesis. Consistent with this prediction, the expression levels of PGC1α and its downstream target UCP1 are up-regulated in *Id1*$^{-/-}$ BAT at room temperature and in response to cold-induced thermogenesis, suggesting that *Id1* deficiency resulted in increased thermogenesis. This is further strengthened by the observation that *Id1*$^{-/-}$ mice exhibited higher O_2 consumption and lower respiratory exchange rate (RER), indicating that energy expenditure is increased in *Id1*-deficient mice and that they use a relatively higher proportion of lipid as an energy source compared to control mice (35). Thus, the failure to gain fat mass with age in *Id1*$^{-/-}$ mice could be explained, in part, by an increase in lipid oxidation and energy expenditure. PGC1α is the master regulator of thermogenesis and controls the entire BAT-mediated thermogenesis program (24). Although PGC1α levels are increased in the BAT in the absence of Id1, it is unclear how Id1 regulates PGC1α expression and/or its transcriptional activity. Since Id1 acts as a dominant negative regulator of other transcription factors, one possibility could be that Id1 may directly bind to PGC1α and suppress its transcriptional activity. Alternatively, Id1 may co-operate with other negative regulators of PGC1α such as Rb and Twist1, thereby controling the expression and activity of PGC1α. In the absence of Id1 where there is less inhibitory environment for PGC1α, it is relatively free to induce transcriptional activation of its downstream targets such as UCP1 at a higher rate, thereby increasing thermogenesis. However, additional *in vitro* and *in vivo* studies are required to more clearly understand the transcriptional inhibitory functions of Id1 in the thermogenesis signaling pathway. Especially since Id1 is one of the major regulators of E, Ets, Rb, and PAX proteins, these studies should be directed at understanding how Id1/E, Id1/Ets or Id1/pRB protein signaling regulate the expression and activities of the PGC1α network of proteins involved in thermogenesis.

3.2. Id2

Induction of adipogenesis in the 3T3-F442A preadipocyte cell line by the adipogenic compound harmine followed by transcriptional profiling identified Id2 as a potential regulator of adipocyte differentiation since its expression is induced during differentiation (44). Accordingly, overexpression of Id2 increased the ability of preadipocytes to differentiate into adipocytes with induced expression of PPARγ and its downstream target genes aP2, CD36, LPL, adiponectin, and C/EBPα. Conversely, knockdown of Id2 by siRNA inhibited the ability of preadipocytes to differentiate into adipocytes due to impaired

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induction of PPARγ and its downstream target genes (44). This suggests that Id2 might be directly regulating PPARγ. Accordingly, overexpressed Id2 increased the expression of PPARγ, whereas either overexpression or knockdown of PPARγ did not influence the expression of Id2. These studies indicate that PPARγ does not regulate Id2, but PPARγ expression is regulated by Id2, and PPARγ operates downstream to Id2. In contrast, inhibition of Id2 had no effect on the induction of other transcription factors such as C/EBPβ and C/EBPδ, which also function to activate PPARγ in a parallel adipocyte differentiation pathway. This is perhaps not surprising since Id2 is a direct downstream target of C/EBPβ, and C/EBPβ can directly bind to the Id2 promoter and induce its expression (45). This explains why inhibition of adipocyte differentiation is only partial when Id2 is knocked down since C/EBPβ and C/EBPδ are still able to activate PPARγ and drive adipocyte differentiation in the absence of Id2.

In this context, it is essential to understand which receptor/ligand signaling pathway regulates Id2, and subsequently, how Id2 controls PPARγ during adipocyte differentiation. It appears that Id2 expression could be regulated by Wnt-β-catenin signaling. Treatment of preadipocytes with Wnt-3a–conditioned media not only suppressed PPARγ expression but also Id2 expression, suggesting that Wnt signaling regulates Id2 expression during adipocyte differentiation (44). Wnt signaling is initiated when Wnt ligands bind to membrane receptors of the Frizzled family, leading to Dishevelled-mediated inhibition of kinase activity of a complex containing glycogen synthase kinase 3 (GSK3), β-catenin, Axin1 and APC (46, 47). Due to GSK3 inhibition in the presence of Wnt, β-catenin cannot be phosphorylated and targeted for degradation, and the hypophosphorylated, stabilized β-catenin translocates to the nucleus and binds to the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors. TCF/LEF-β-catenin complexes bind to TCF/LEF-binding sites or Wnt-response elements (WRE) in the promoters of target genes such as c-Myc and cyclin D1 leading to their transcriptional activation. Therefore, active Wnt signaling promotes the maintenance of a proliferative state and inhibits differentiation (Figure 2, Left) (46, 47). For example, Wnt-1-expressing cells fail to express C/EBPα and PPARγ, which are required for adipocyte differentiation (16). Does Wnt-β-catenin signaling directly regulate Id2 expression? Although such a mechanism was not investigated thus far in the differentiated adipocytes, it was demonstrated in human keratinocytes that β-catenin, indeed, directly suppresses Id2 expression. β-catenin directly binds to WRE at the Id2 promoter and recruits retinoic acid receptors (RARs) to the WRE at the Id2 promoter. This is followed by RAR-dependent recruitment of LSD1 demethylase to the WRE in the Id2 promoter, leading to a reduction in histone H3 and H4 acetylation and histone H3 K-4 methylation, resulting in transcriptional repression (48). Similar mechanism might operate in adipocytes and therefore blocking Wnt signaling is not only necessary for activation of Id2 but also for the C/EBPs, which collectively induce PPARγ and initiate adipocyte differentiation (Figure 2, Right). Consistent with this hypothesis, inhibition of Wnt signaling in 3T3-L1 cells by dominant-negative TCF4 (dnTCF4) induced adipogenesis, suggesting that blocking Wnt signaling is necessary to initiate adipocyte differentiation (16). Thus, the Wnt-β-catenin pathway appears to regulate both the C/EBP and Id2 parallel pathways that lead to activation of PPARγ and adipocyte differentiation. However, although how the C/EBPs regulate PPARγ is well

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established (49), the precise mechanism by which Id2 regulates PPARγ is largely unclear, and further studies are required to establish a molecular link between Id2 and PPARγ.

In accordance with these observations in the preadipocyte cell lines, 4–6-day-old $Id2^{-/-}$ neonates exhibited detectable deficiencies in interscapular and inguinal WAT tissues. Similarly, $Id2^{-/-}$ MEFs subjected to adipocyte differentiation exhibited a partial impairment in adipogenic potential with concomitant reduction in the levels of PPARγ and its downstream targets, LPL, adiponectin, and aP2 (44). A partial reduction in the adipocyte differentiation potential of $Id2^{-/-}$ cells indicate that although C/EBPβ and C/EBPδ can still activate PPARγ and drive adipocyte differentiation, Id2 operates in a parallel pathway and is required for complete activation of PPARγ, leading to normal differentiation. *In vivo*, Id2 expression was significantly elevated in the adipose tissues of mice fed a high-fat-diet (HFD) as well as genetically obese mice. However, whether impaired adipogenesis in the absence of Id2 can lead to an anti-obesity phenotype similar to that of *C/EBPα* and *PPARγ*-deficient mice is unknown. This is mainly because, although the gross phenotype of $Id2^{-/-}$ mice at birth was indistinguishable from wild-type litter mates, after day 6, $Id2^{-/-}$ neonates show severe growth defects and ~80% of the mice do not survive beyond 2–3 weeks of age. Only ~20% of mice escape from death but they are severely growth retarded (44, 50). These $Id2^{-/-}$ mice displayed several metabolic phenotypes such as increased glucose tolerance, insulin sensitivity, reduced gonadal WAT, altered daily and circadian rhythms of feeding and locomotor activity, and increased energy expenditure (51). Although some of the observed metabolic phenotypes could be attributed to severe growth defects in $Id2^{-/-}$ mice, it cannot be excluded that lack of Id2 could also have direct impact on adipose tissue metabolism. This is due to the fact that Id2 can directly regulate adipocyte determination and differentiation factor 1 (ADD1), also known as sterol regulatory element-binding protein 1c (SREBP-1c). SREBP-1c, a basic helix-loop-helix leucine-zipper (bHLH-LZ) transcription factor, regulates the expression of various genes involved in fatty acid and triglyceride metabolism and is a potent trans-activator of the fatty acid synthase (FAS) promoter. Id2 can directly bind to SREBP-1c and inhibit it from transactivating FAS, thereby regulating fatty acid synthesis and metabolism (52). Moreover, SREBP-1c can also regulate a number of genes such as glycerol-3-phosphate acyltransferase, S14, stearoyl CoA desaturase, adiponectin and leptin. Therefore, in the absence of Id2, regulation of SREBP-1c as well as its target genes could be altered, leading to dramatic changes in adipose tissue metabolism. However, additional studies, such as specific deletion of *Id2* in the adipose tissues by crossing $Id2^{fl/fl}$ mice with $aP2^{Cre}$ mice followed by metabolic analysis, are required in order to clearly understand the specific role of Id2 in adipose tissue maintenance and metabolism *in vivo*.

3.3. Id3

The role of Id3 in adipocyte differentiation and adipose tissue metabolism created considerable debate due to recent studies that conflicted with previous reports. One of the studies described that Id3 mRNA levels are abundant in proliferating 3T3-F422A preadipocytes but rapidly declined during adipocyte differentiation. Subsequently, forced expression of Id3 in preadipocytes prevented adipocytes from differentiation, as measured by adipsin and glycerol phosphate dehydrogenase activity, markers of adipocyte

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differentiation (53). However, a recent study demonstrated that overexpression of Id3 did not result in any detectable changes in the expression of GLUT4, another marker of adipocyte differentiation. They also did not detect any significant differences in Oil-Red-O lipid staining between control and Id3-overexpressing cells or between wild-type and *Id3*$^{-/-}$ MEFs that were induced to differentiate into adipocytes, suggesting that either overexpression or lack of *Id3* did not alter the adipocyte differentiation program (54). Timing of analysis and the choice of adipocyte markers might have partially contributed to these conflicting observations in these two studies. Further studies in *Id3*$^{-/-}$ mice also did not reveal any significant changes in the metabolic phenotypes when they were fed a regular diet compared to wild-type mice. Nevertheless, when the mice were fed a HFD, expansion of visceral adipose depots was significantly reduced in *Id3*$^{-/-}$ mice, whereas, such a reduction was not observed in other WAT locations (54). Accordingly, Id3 expression was more abundant in visceral compared to subcutaneous WAT, and Id3 expression was elevated in response to HFD only in visceral but not in subcutaneous adipose tissues of wild-type mice. Due to impaired visceral WAT expansion, *Id3*$^{-/-}$ mice were protected from HFD-induced obesity (54). However, no detectable differences were observed in the expression pattern of adipocyte differentiation markers such as aP2 or C/EBPα in visceral adipocytes, indicating that reasons other than defects in adipocyte differentiation are primarily responsible for the observed reduction in visceral adiposity in *Id3*$^{-/-}$ mice.

To explore the possible reason behind impaired visceral adipose tissue expansion in response to HFD, expression analysis of Id3 in adipocytes and stromal vascular fraction (SVF) of visceral adipose tissues in wild-type mice was performed. This analysis revealed induced expression of Id3 only in SVF but not in adipocytes in response to HFD, suggesting that a defect in visceral adipose tissue vascularization might have contributed to impaired visceral adipose tissue expansion in *Id3*$^{-/-}$ mice. Consistent with this hypothesis, expression of the angiogenesis factor VEGF is significantly induced in the visceral adipose tissue of wild-type mice in response to HFD but not in *Id3*$^{-/-}$ mice. This suggests that adipose tissue vascularization is impaired in the visceral adipose tissue of *Id3*$^{-/-}$ mice, leading to a failure in the expansion of adipose tissue (54). Previous studies have demonstrated that lack of Id3 leads to impaired angiogenesis (55), and therefore it is not entirely surprising that Id3 also plays a similar role in adipose tissue angiogenesis during its expansion. However, an important question that needs to be resolved is how does Id3 regulate VEGF expression? The VEGF promoter consists of several E-boxes where the bHLH factors bind and regulate its expression (56). The authors showed that transient expression of the E-protein E12 suppresses the expression of VEGF, indicating that E12 functions as a transcriptional repressor of VEGF. Id3 may promote VEGF expression by interacting with and preventing E12 from binding to the VEGF promoter (Figure 3A). Consistent with this idea, Id3 significantly antagonized the suppression of VEGF by E12 (54). This explains why there is a defect in the visceral adipose tissue vascularization and adipose tissue expansion in the absence of Id3. Taken together, these results suggest that loss of Id3 attenuates visceral fat expansion by inhibiting HFD-induced visceral fat VEGF expression and growth of blood vessels. However, E proteins often function as activators of gene expression, and it is somewhat intriguing that E12 functions as a transcriptional repressor of VEGF and Id3 binds to and releases this repression during adipose tissue vascular growth. It will be interesting to

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study if Id3 or its other family members are also involved in the regulation of other angiogenic factors during adipose tissue expansion.

In addition to Id3's role in adipocyte differentiation, its role in adipose-specific metabolism is further complicated by conflicting reports on the regulation of SREBP-1c by Id3. SREBP-1c is a member of the bHLH family of transcription factors but also contains a leucine zipper (bHLH-LZ). SREBP-1c functions as a transcriptional activator of a number of genes involved in fatty acid and adipokine metabolism, such as FAS, leptin and adiponectin (57). Based on their structure, whether HLH-LZ factors can directly bind to Id proteins is debatable. A previous study showed that Id3 and SREBP-1c interact with each other, thereby Id3 antagonizes SREBP-1c transactivation of the FAS promoter. The authors were able to co-immunoprecipitate Id3 and SREBP-1c in a cell-free system. Moreover, in an *in vitro* translated system they demonstrated that Id3 alters SREBP-1c binding to the FAS promoter (52). However, later studies in a mammalian 2-hybrid system or by co-immunoprecipitation were unable to identify such a direct interaction between Id3 and SREBP-1c (58). Instead, they discovered that Id3 inhibits SREBP-1c through the E-protein E47. E47 interacts with SREBP-1c, a positive regulator of adiponectin, and enhances SREBP-1c–mediated adiponectin promoter activation. Id3/E47 and E47/SREBP-1c were shown to interact, suggesting that the action of Id3 on SREBP-1c takes place through inhibition of the SREBP-1c interacting partner, E47. As a result, E47 binding to the adiponectin promoter was significantly reduced by the overexpression of Id3 *in vitro* and conversely, lack of Id3 resulted in increased adiponectin expression in *Id3*$^{-/-}$ adipose tissue (58). These studies contradict previous studies that Id3 directly binds to SREBP-1c (52). The latter studies instead propose that Id3 regulates SREBP-1c activity indirectly by interacting with E47, thus preventing E47 binding to the SREBP-1c leading to impaired activation of adiponectin promoter and its expression (Figure 3B). Adiponectin plays an important role in glucose metabolism but Id3 deficiency did not appear to affect glucose metabolism or insulin sensitivity (54). Moreover, a failure to expand visceral adipose tissue in *Id3*$^{-/-}$ mice in response to HFD should lead to ectopic accumulation of lipids in the liver, BAT, kidney and skeletal muscle, leading to insulin resistance. However, such a phenotype was not observed in *Id3*$^{-/-}$ mice. A possible explanation could be that energy expenditure is increased in *Id3*$^{-/-}$ mice, leading to increased thermogenesis and/or fatty acid oxidation, resulting in normal insulin sensitivity. However, whether Id3 has any significant role in BAT or fatty acid oxidation is largely unknown. Further *in vivo* studies are required to understand if Id3 has any specific function in energy expenditure, energy balance, fatty acid oxidation and insulin signaling.

3.4. Id4

A role for Id4 in adipocyte differentiation first emerged when the expression of Id4 mRNA was analyzed during *in vitro* differentiation of 3T3-L1 preadipocytes. Id4 mRNA level was low in confluent undifferentiated preadipocytes but rapidly increased during differentiation (59). Knockdown of Id4 by shRNA in 3T3-L1 cells resulted in significant reduction in intracellular lipid accumulation with a concomitant decrease in the expression levels of C/EBPα and PPARγ and its downstream target genes such as aP2, Glut4 and LPL, suggesting that adipocyte differentiation is impaired *in vitro* in the absence of Id4 (60). Expression

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analysis of Id4 in adult mice and human adipose tissues revealed abundant expression of Id4 in these tissues (59), suggesting a possible *in vivo* role for Id4 in adipose tissue metabolism. To dissect the specific functions of Id4 in adipose tissue differentiation and metabolism, later studies utilized *Id4*$^{-/-}$ mice and discovered a reduction in both WAT and BAT mass in *Id4*$^{-/-}$ mice compared to control mice (60). MEFs isolated from *Id4*$^{-/-}$ embryos and WAT harvested from *Id4*$^{-/-}$ mice have diminished expression of C/EBPα and PPARγ and its downstream target genes aP2, Glut4, and LPL, suggesting that adipocyte differentiation and WAT development are impaired in *Id4*$^{-/-}$ mice. As a result, *Id4*-deficient mice are leaner compared to wild-type controls, and this lean phenotype is especially more apparent when the mice were fed a HFD (60). Since genetic mouse models such as *C/EBP*α and *PPAR*γ-deficient mice with impaired adipose tissue development are protected from adipocyte hypertrophy when fed a HFD, the lean phenotype could be attributed to defective adipocyte differentiation in *Id4*$^{-/-}$ mice. However, it was proposed that the observed lean phenotype could be mainly due to a defect in fatty acid uptake or fat storage in the WAT of *Id4*$^{-/-}$ mice as they fail to efficiently take up, synthesize and/or store triglycerides (60). However, the molecular mechanism by which Id4 facilitates fatty acid uptake or triglyceride storage in the WAT is completely unknown. Insulin plays an essential role in both fatty acid uptake by adipocytes and adipocyte specific lipogenesis. Adipocyte-derived LPL is essential for efficient fatty acid uptake and storage, and insulin triggers PI3K–mediated induction of LPL activity. LPL hydrolyzes circulating triacylglycerols in lipoproteins into glycerol and fatty acids, whose entry into adipocytes is mediated by fatty acid transporter protein 1 (FATP1), which catalyses the conversion of fatty acids into fatty acyl-CoA. Insulin induces fatty acid uptake in adipocytes by stimulating translocation of FATP1 from intracellular vesicles to the plasma membrane (61). Subsequently, insulin promotes the binding of bHLH-LZ transcription factors, upstream stimulatory factor-1 and 2 (USF1 and USF2), to the FAS promoter. Insulin by signaling through protein phosphatase-1 dephosphorylates and activates DNA dependent protein kinase (DNA-PK), which in turn phosphorylates and activates USFs. USFs directly interact with SREBP-1c, resulting in a highly synergistic transcriptional activation of the FAS promoter, leading to lipogenesis (62). However, it is unclear which component of this fatty acid uptake and lipogenesis pathway is regulated by Id4. One possibility could be that Id4 activates the PI3K/Akt pathway similar to its other family member Id1. Although activation of PI3K/Akt by Id1 was not demonstrated directly in the adipocytes, such an activation mechanism was evident in oesophageal cancer cells (63). In the absence of Id4, impaired activation of the PI3K/Akt pathway might lead to impaired activation of LPL, leading to defective fatty acid uptake and storage. However, it is purely speculative, and further studies are required to test this hypothesis and pinpoint a molecular link between the Id4 and PI3K/Akt pathways.

In contrast to its role in white preadipocyte differentiation, Id4 appears to have quite an opposite function during mesenchymal stem cell (MSC) differentiation. MSCs are the progenitors from which osteoblasts, chondrocytes and adipocytes are derived, and MSC differentiation is a very precisely regulated process (64, 65). Knockdown of Id4 in the stromal cell line ST2 resulted in increased adipogenesis with induced expression of PPARγ and its downstream targets. Subsequently, histological analysis of *Id4*$^{-/-}$ tibia disclosed a drastic increase in the number of adipocytes in epiphyseal bone marrow of tibia and in the

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lateral calvaria compared to control mice. Consistent with this observation, PPARγ expression was increased in bone marrow cells of the tibia and femur of *Id4*$^{-/-}$ mice. These observations suggest that Id4 could function as a critical regulator in the lineage choice of MSCs differentiating into either osteoblasts or adipocytes. Conversely, overexpression of Id4 in ST2 cells partially inhibited adipogenesis with decreased lipid accumulation and increased osteogenesis, suggesting that Id4 alters the MSC differentiation program and suppresses adipogenesis and promotes osteogenesis (66). It appears that Id4 switches the direction of osteoblast and adipocyte differentiation by selectively regulating the transcriptional programing of MSC differentiation. Subsequently, an attempt was made to identify candidate bHLH transcription factors that bind to Id4, and discovered direct binding of Id4 with Hey2 (Hairy and Enhancer of Split-related with YRPW motif 2), a direct target of canonical Notch signaling that is involved in osteogenesis and bone formation. Hey2 forms heterodimers with Hes1, and the Hey2/Hes1 complex binds to the E-box motifs and represses transcription (67, 68). Therefore, a direct interaction of Id4 with Hey2 suggests that Id4 might regulate the transcriptional repression of Hey2/Hes1 during osteogenesis. Consistent with this hypothesis, Id4 reversed the transcriptional repression by Hey2/Hes1 heterodimer in a dose-dependent manner (66). However, the contrasting roles of Id4 in white adipocyte differentiation and MSC differentiation program highlight that additional studies are required to fully understand the transcriptional regulatory functions of Id4 during the adipogenesis and MSC differentiation programs in bone marrow.

4. CONCLUSIONS

All 4 Id proteins (Id1- Id4) are expressed in cultured preadipocytes but their differential expression patterns during adipogenesis are quite different, suggesting unique roles for each of the Id proteins in the regulation of adipocyte differentiation and adipose tissue development. The expression pattern of Id1 during adipocyte differentiation is exactly opposite to the expression of other members of the family, i.e., Id2 and Id4, which are induced during adipogenesis. Lack of either Id2 or Id4 resulted in defective adipogenesis. In contrast, Id1 is targeted for degradation during adipocyte differentiation and lack of Id1 accelerated adipogenesis although overall, it did not cause an abnormal increase in total adipose mass *in vivo*. Overexpression of Id2 and Id4 enhanced adipocyte differentiation, but the consequence of Id1 overexpression on adipocyte differentiation is inconclusive due to targeted degradation of overexpressed Id1 during adipogenesis. Therefore, Id1 appears to have a distinct function during adipocyte differentiation compared to Id2 or Id4. Overall, the Id proteins appear to have non-overlapping functions during adipocyte differentiation, and it would be interesting to investigate how the Id proteins coordinate with one another during adipogenesis. In this regard, future studies should be directed at generating and analyzing adipose tissue development in compound Id-null mice. In addition to their non-overlapping functions during adipogenesis, Id proteins also appear to have very distinctive functions in adipose tissue metabolism. From the studies conducted thus far, it appears that Id1 is mostly involved in BAT-mediated thermogenesis. In contrast, Id2 appears to regulate fatty acid metabolism, and Id4 controls fatty acid uptake, triglyceride synthesis/storage and also regulates the MSC differentiation program. Id3 also seems to have some overlapping function in fatty acid and adipokine metabolism but its role in adipose tissue vascularization

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is unique to other family members. Although studies performed in all 4 Id null mice provided some basic understanding of each of the four Id proteins in adipose tissue development and metabolism, it appears that the current available information is not quite sufficient to establish a clear connection between different Id proteins and their specific regulatory mechanisms in adipose tissue development and metabolism. Specific deletion of the Id proteins in adipose tissues by crossing Id conditional alleles with $aP2^{cre}$ mice will possibly provide more in-depth information regarding the involvement of individual members of the Id family of proteins in adipose tissue-specific metabolic pathways.

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Abbreviations

ADD1	Adipocyte determination and differentiation factor 1
Akt	AKT1 kinase
aP2	Fatty acid binding protein
APC	Adenomatous polyposis coli

ARRDC3	Arrestin domain containing 3
bHLH	basic Helix-loop-helix
bHLH-LZ	basic helix-loop-helix leucine zipper
BAT	Brown adipose tissue
BMP	Bone morphogenetic protein
c-Myc	Myelocytomatosis oncogene
C/EBP	CCAAT/enhancer binding protein
CD36	Thrombospondin receptor
CDK	Cyclin dependent kinase
Cidea	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A
Cre	Cyclization recombinase
DNA-PK	DNA dependent protein kinase
DP	E2F dimerization partner
E2F	E2F transcription factor
Ebf2	Early B cell factor 2
Ets2	Avian erythroblastosis virus E26 (v-ets) oncogene homolog 2
FAS	Fatty acid synthase
FATP1	Fatty acid transporter protein 1
FOXC2	Fork-head box C2
GATA	GATA binding protein
Glut4	Glucose transporter type 4
GSK3	Glycogen synthase kinase 3
Hes 1	Hairy and enhancer of split 1
Hey 2	Hairy and enhancer of split-related with YRPW motif 2
HFD	High fat diet
HLH	Helix-loop-helix
Id	Inhibitor of DNA binding
KLF	Kruppel-like factor
LPL	Lipoprotein lipase
LSD1 demethylase	Lysine specific demethylase 1
LXR	Liver-X-receptor

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MEFs	Mouse embryonic fibroblasts
MEK	Mitogen activated protein kinase kinase
MSCs	Mesenchymal stem cells
Myf-5	Myogenic factor 5
MyoD	Myogenic differentiation protein
NAFLD	Non-alcoholic fatty liver disease
PAX	Paired box gene
PGC1α	Peroxisome proliferator activated receptor gamma coactivator 1 alpha
PI3K	Phosphatidylinositol 3-kinase
PPAR	Peroxisome proliferator-activated receptor
pRB	Retinoblastoma protein
PRDM16	PR domain containing 16
Ras	Rat sarcoma viral oncogene homolog
Raf	Raf kinase
RARs	Retinoic acid receptors
RER	Respiratory exchange ratio
siRNA	Small interfering RNA
Smurf2	SMAD specific E3 ubiquitin protein ligase 2
ST2 cells	Stromal cell line
STAT	Signal-transducer and activator of transcription protein
SVF	Stromal vascular fraction
SREBP-1c	Sterol regulatory element-binding protein 1c
SRC2	Src tyrosine kinase 2
TCF/LEF	T-cell factor/lymphoid enhancer factor
TRα1	Thyroid hormone receptor alpha
UCP1	Uncoupling protein 1
USF1	Upstream stimulatory factor 1
USF2	Upstream stimulatory factor 2
VEGF	Vascular endothelial growth factor
WAT	White adipose tissue
Wnt	Wingless

WRE	Wnt response element

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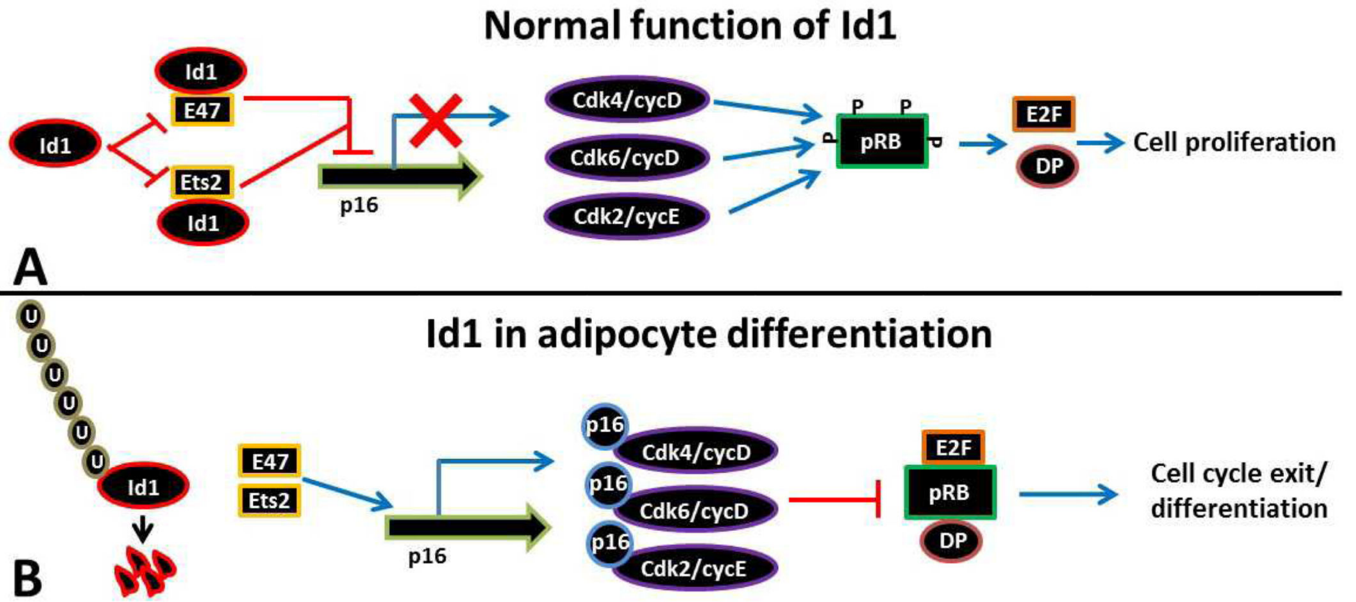

Figure 1.

(A). Transcriptional regulation of p16 by Id1 through E47 and Ets2, leading to cell proliferation. Id1 prevents the expression of the cell cycle inhibitor p16 through E47 and Ets2. In the absence of p16 the uninhibited Cdk/cyclin complexes continue to promote cell cycle progression by phosphorylating and keeping pRB in its inactive state. This leads to constitutive activation of E2F and DP factors, which activate a number of S phase genes that drive cell proliferation (29, 30, 33, 34). (B). During adipocyte differentiation, Id1 is ubiquitinated and targeted for degradation. We speculate that this facilitates expression of p16 through E47 and Ets2. Subsequently, p16 inhibits Cdk/cyclin complexes which fail to phosphorylate pRB leading to cell cycle exit and adipocyte differentiation.

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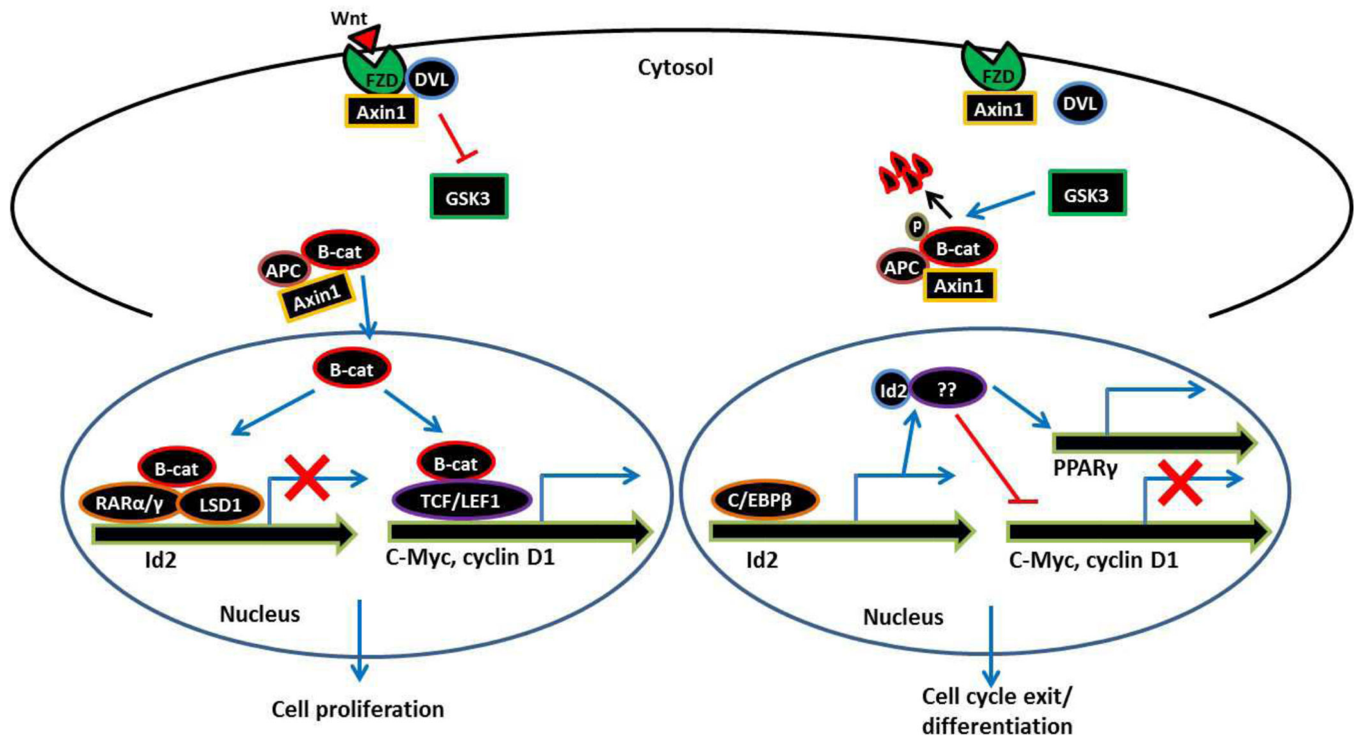

Figure 2.

Possible mechanism showing the regulation of Id2 in the presence or absence of active Wnt signaling and consequently how Id2 regulates adipocyte differentiation through PPARγ. (Left) In the presence of Wnt, β-catenin cannot be phosphorylated and targeted for degradation. β-catenin translocates to the nucleus and performs two tasks: 1. Binds to the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors. TCF/LEF-β-catenin complexes bind to Wnt-response elements (WRE) in the promoters of target genes such as c-Myc and cyclin D1 leading to their transcriptional activation. Thus, active Wnt signaling promotes cell proliferation and inhibits adipocyte differentiation (46, 47). 2. β-catenin could suppress Id2 expression by directly binding to WRE at the Id2 promoter and recruits retinoic acid receptors (RARs) to the WRE at the Id2 promoter. This is followed by RAR-dependent recruitment of LSD1 demethylase, leading to a reduction in histone H3 and H4 acetylation and histone H3 K-4 methylation, resulting in Id2 transcriptional repression and impaired adipogenesis (48). (Right) The precise mechanism by which Id2 regulates PPARγ is not known. One possibility could be that in the absence of Wnt, C/EBPs might gain access to the Id2 promoter and induce its expression. Subsequently, Id2 by directly binding to an unknown factor, on one hand induces PPARγ expression and on the other hand suppresses c-Myc and cyclin D1 expression leading to cell cycle exit and adipocyte differentiation.

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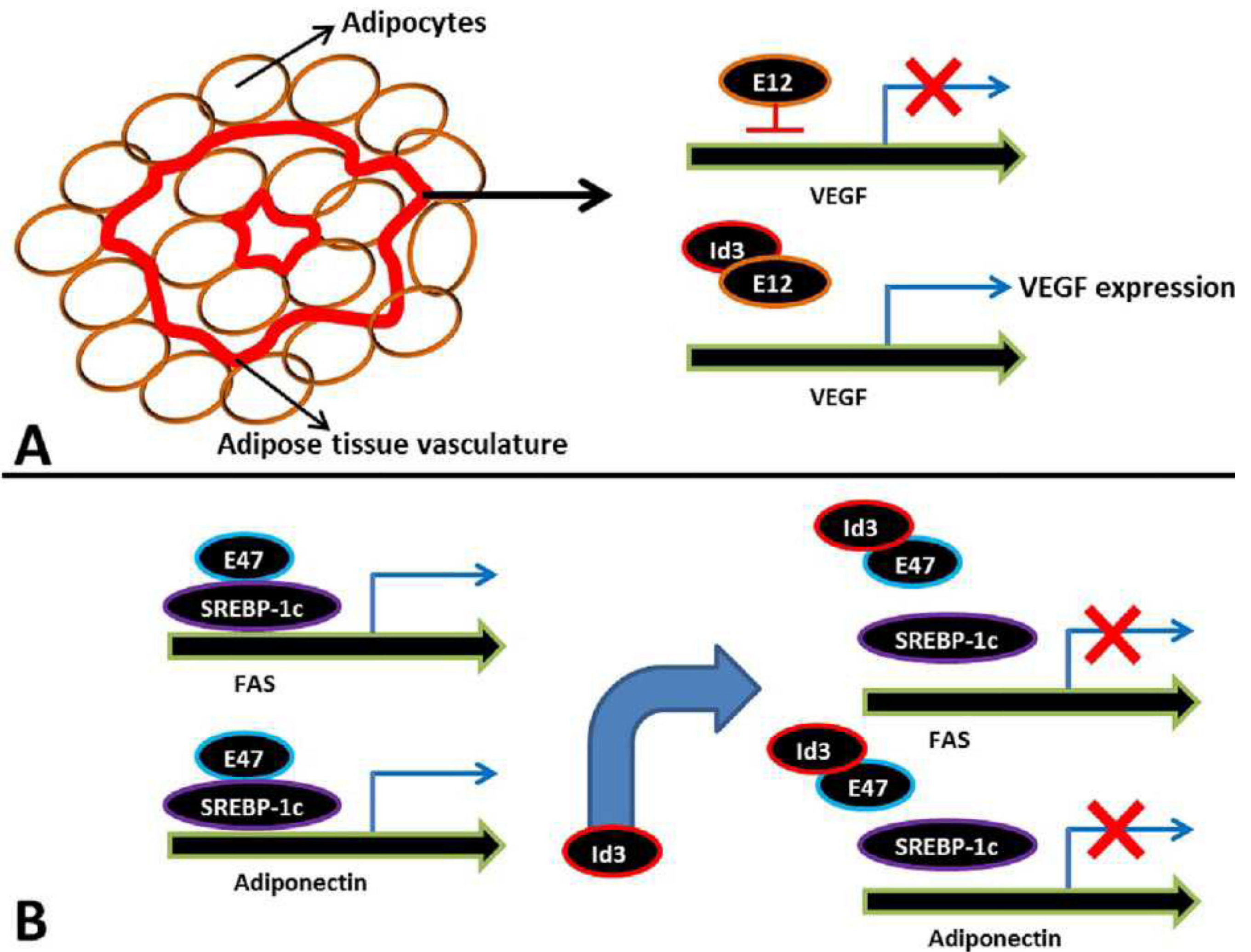

Figure 3.

(A) Possible mechanism describing the role of Id3 in adipose tissue vascularization during adipose tissue expansion. VEGF is essential for growth of blood vessels during visceral fat expansion. E12 functions as a transcriptional repressor of VEGF. Id3 promotes VEGF expression by interacting with and preventing E12 from binding to the VEGF promoter (54). (B). Possible mechanism showing the role of Id3 in adipose tissue-associated fatty acid and adipokine metabolism. E47 interacts with SREBP-1c, a positive regulator of FAS and adiponectin, and enhances SREBP-1c–mediated promoter activation. Id3 regulates SREBP-1c activity indirectly by interacting with E47. Id3/E47 interaction prevents E47 binding to the SREBP-1c leading to impaired activation of FAS and adiponectin promoters and their expression (58).