The Helix–Loop–Helix Factors Id3 and E47 Are Novel Regulators of Adiponectin

Amanda C. Doran, Nahum Meller, Alexis Cutchins, Hamid Deliri, R. Parker Slayton, Stephanie N. Oldham, Jae B. Kim, Susanna R. Keller, Coleen A. McNamara

Abstract—Adiponectin is an adipocyte-derived cytokine with beneficial effects on insulin sensitivity and the development of atherosclerosis. Id3 is a helix–loop–helix factor that binds to E-proteins such as E47 and inhibits their binding to DNA. Although the helix–loop–helix factor sterol regulatory element binding protein (SREBP)-1c is a known activator of adiponectin transcription, this study provides the first evidence of a role for Id3 and E47 in adiponectin expression. Decreased Id3 in differentiating adipocytes correlates with increased adiponectin expression and forced expression of Id3 inhibits adiponectin expression. Moreover, Id3-null mice have increased adiponectin expression in visceral fat tissue and in serum. We demonstrate that E47 potentiates SREBP-1c–mediated adiponectin promoter activation and that Id3 can dose-dependently inhibit this action via interaction with E47. Mutation of a consensus E47 binding site results in nearly complete loss of promoter activation. Furthermore, we demonstrate E47 binding to the endogenous adiponectin promoter both in vitro and in vivo by chromatin immunoprecipitation analysis. Binding is not detected in undifferentiated cells which express Id3 but peaks during differentiation in parallel with Id3 decline. This promoter binding can be completely abolished by the overexpression of Id3 and is enhanced in adipose tissue null for Id3. These data establish Id3 and E47 as novel regulators of SREBP-1c–mediated adiponectin expression in differentiating adipocytes and provide evidence that Id3 regulates adiponectin expression in vivo. (Circ Res. 2008;103:624-634.)

Key Words: basic helix–loop–helix proteins ■ differentiation ■ gene regulation ■ adiponectin ■ adipocytes

Adiponectin is produced by adipocytes and is secreted into the circulation.1,2 Adiponectin levels are reduced in obese, insulin-resistant, and diabetic rodents,3 monkeys,4 and humans.5 Administration of adiponectin in rodent models increases insulin sensitivity and lowers plasma glucose.6,7 Low adiponectin levels are associated with the development of coronary artery disease8,9 and ApoE−/− mice overexpressing adiponectin demonstrate significantly less atherosclerosis in response to high-fat feeding.10,11 Although these studies implicate adiponectin as an important modulator of insulin sensitivity and the development of atherosclerosis, the transcriptional mechanisms that regulate adiponectin expression are incompletely understood.

Both the human and mouse adiponectin promoters contain binding sites for transcription factors including sterol regulatory elements (SREs), peroxisome proliferator-activated receptor (PPAR)–response elements, C/EBP sites, and E-boxes.12 Accordingly, many factors influence adiponectin expression either directly or indirectly, including sterol regulatory element binding protein (SREBP)-1c,12 PPARγ,12,13 and C/EBP.12,14 To date, no study has addressed the role of the 3 putative E-boxes present in the adiponectin promoter.12

SREBP-1c is a member of the basic helix–loop–helix (bHLH)–leucine zipper (bHLH-LZ) family of proteins that binds to SREs.15 Originally implicated in cholesterol-regulated gene expression,16 SREBP-1c is also a major regulator of adipogenic genes, including fatty acid synthase and adiponectin.12,17 Also known as adipocyte determination–and differentiation–dependent factor-1 (ADD1), SREBP-1c is expressed preferentially in adipose tissue and is upregulated as adipocyte differentiation progresses.12,15

The inhibitor of differentiation (Id) family of proteins contains 4 members, Id1 to -4, which have both redundant and unique functions.18 Ids are HLH proteins that function as dominant-negative transcription factors that are incapable of binding to DNA. Instead, the Ids bind to a subset of bHLH factors known as E-proteins, including E12, E47, ITF2, and HEB, thereby preventing their dimerization and DNA binding. Although the Ids bind preferentially to E-proteins, 1 report has suggested that Id3 binds to and inhibits SREBP-1c directly.19

Previous studies have proposed a role for Id proteins in adipocytes. The mRNAs for Ids 1 to 3 are expressed in 3T3-L1 preadipocytes and decrease to undetectable levels as they differentiate into adipocytes.19–21 Id3 has been shown to...
negatively regulate fatty acid synthase in vitro; however, no other adipocyte target genes have been identified.

In the present study, we examine the role of Id3 and E47 in the control of adiponectin transcription. We demonstrate that Id3 inhibits adiponectin expression in vitro and in vivo. In addition, we show that E47 and SREBP-1c bind the endogenous adiponectin promoter in vitro and in vivo and synergistically activate the adiponectin promoter. We refute previous suggestions that Id3 directly binds to SREBP-1c and propose instead that Id3 regulates SREBP-1c activity indirectly by interacting with E47, thereby preventing E47 binding to the adiponectin promoter and inhibiting adiponectin expression.

Materials and Methods
An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Transfections, adenoviral transductions, and promoter–reporter assays, Western blotting, and 2-hybrid analysis were performed as described previously.22,23 OP9 and 3T3-L1 cell culture and differentiation were carried out as described by Wolins et al.24 Chromatin immunoprecipitation (ChIP) methods were based on Francis et al and are described in the online data supplement.25

Results
Increased Levels of Adiponectin Protein and mRNA in Id3 Knockout Mice
To address the potential in vivo regulation of adiponectin by Id3 in an atherogenic model, total adiponectin levels in ApoE\(^{-/-}\) and Id3\(^{-/-}\) APOE\(^{-/-}\) mice were determined. Sera were collected from 8-week-old, weight-matched ApoE\(^{-/-}\) and Id3\(^{-/-}\) APOE\(^{-/-}\) mice, and adiponectin levels were determined by radioimmunoassay. Points represent serum measurements from individual animals; the bar represents the average. *P<0.005. B, Total protein was extracted from adipose tissue, separated by SDS-PAGE, and analyzed by Western blotting. Representative blot from 1 animal is shown. C, Densitometric analysis of the experiment described in B. Adiponectin levels were normalized to \(\beta\)-actin. Results are average values obtained from 4 animals per group presented relative to the value in the ApoE\(^{-/-}\) group. *P<0.01, **P<0.05. D, Total RNA was harvested from adipose tissue and analyzed by real-time PCR. Adiponectin was normalized to the corresponding cyclophilin signal. Three samples from each fat pad were analyzed, each in triplicate PCR measurements. Average adiponectin values from 3 animals per group are presented relative to adiponectin value in the ApoE\(^{-/-}\) group. *P<0.005, **P<0.01.

Figure 1. Increased adiponectin levels in serum and adipose tissue of Id3 knockout mice. Blood, epididymal, and mesenteric adipose tissues were harvested from 8-week-old ApoE\(^{-/-}\) and Id3\(^{-/-}\) APOE\(^{-/-}\) mice. A, Serum levels of adiponectin were determined by radioimmunoassay. Points represent serum measurements from individual animals; the bar represents the average. *P<0.005. B, Total protein was extracted from adipose tissue, separated by SDS-PAGE, and analyzed by Western blotting. Representative blot from 1 animal is shown. C, Densitometric analysis of the experiment described in B. Adiponectin levels were normalized to \(\beta\)-actin. Results are average values obtained from 4 animals per group presented relative to the value in the ApoE\(^{-/-}\) group. *P<0.01, **P<0.05. D, Total RNA was harvested from adipose tissue and analyzed by real-time PCR. Adiponectin was normalized to the corresponding cyclophilin signal. Three samples from each fat pad were analyzed, each in triplicate PCR measurements. Average adiponectin values from 3 animals per group are presented relative to adiponectin value in the ApoE\(^{-/-}\) group. *P<0.005, **P<0.01.
Adiponectin transcripts were quantified by real-time PCR. As compared to ApoE<sup>−/−</sup> mice, Id3<sup>−/−</sup>ApoE<sup>−/−</sup> animals expressed 2.7- and 3.1-fold more adiponectin mRNA in the epididymal and mesenteric fat pads, respectively (Figure 1D).

**Forced Expression of Id3 Inhibits Adiponectin Expression**

Throughout our study, we have confirmed many of our results in 2 adipocyte cell lines: 3T3-L1, a widely used adipocyte line; and OP9, a new alternative model.24 Wolins et
al have demonstrated that OP9 cells express the same adipocyte lineage markers as 3T3-L1 cells but differentiate more rapidly (3 to 7 days versus 2 weeks for complete differentiation after plating). We have confirmed that OP9 cells can be transiently transfected with high efficiency and are efficiently transduced with an adenovirus (95% transduction [data not shown] versus 50% or less with 3T3-L1 cells), enabling us to assay changes in expression of endogenous proteins in the total cell population. In addition, the shorter differentiation time allows OP9 cells to maintain expression of transfected genes over the course of differentiation.

We evaluated the expression of Id3 and adiponectin in both 3T3-L1 and OP9 cells. Undifferentiated (preadipocytes) or fully differentiated (adipocytes) cells were analyzed by Western blotting, revealing that Id3 is detected in undifferentiated but not in differentiated 3T3-L1 or OP9 cells. Conversely, adiponectin is present in differentiated but not undifferentiated cells (Figure 2A).

To determine whether Id3 modulates adiponectin expression, undifferentiated OP9 or 3T3-L1 cells were transduced with an adenovirus expressing either Id3 (Ad-Id3) or green fluorescent protein (Ad-GFP) and then differentiated for 3 days (OP9) or 5 days (3T3-L1). Expression of adiponectin, Id3, or GLUT4 (a marker of differentiated adipocytes) was analyzed by Western blotting. Exogenous Id3 expression significantly decreased adiponectin protein levels (Figure 2B) by approximately 3-fold in OP9 cells and 2-fold in 3T3-L1 cells (Figure 2C). GLUT4 expression in the Ad-Id3 and Ad-GFP groups was similar, indicating that the effect of Id3 on adiponectin expression is not attributable to inhibition of differentiation. To ascertain whether Id3 inhibits adiponectin expression independent of differentiation state, differentiated OP9 cells were transduced with Ad-GFP or Ad-Id3 and analyzed 72 hours later. Differentiated OP9 adipocytes transduced with Id3 showed a 2-fold decrease in adiponectin expression as compared to vehicle or Ad-GFP-treated cells (Figure I in the online data supplement).

To assess whether forced Id3 expression inhibits adiponectin at the transcriptional level, we also compared adiponectin mRNA levels in cells transduced with increasing amounts of Ad-GFP or Ad-Id3. Dose-dependent increases in Id3 mRNA expression in Ad-Id3 treated cells were confirmed by real-time PCR. Whereas increasing doses of GFP did not alter adiponectin expression, expression of Id3 in either OP9 or 3T3-L1 cells reduced adiponectin mRNA levels in a dose-dependent manner (Figure 2D).

**Id3 Inhibits Adiponectin Promoter Activation**

Because the level of Id3 expression affected adiponectin mRNA levels, regulation of the adiponectin promoter by Id3 was tested next. NIH3T3 fibroblasts were transfected with a 0.41-kb adiponectin promoter/luciferase reporter construct in combination with SREBP-1c (a potent activator of the adiponectin promoter) and/or Id3 (Figure 3). NIH3T3 cells were used to eliminate the potentially confounding effect of adipocyte differentiation state and to avoid background activity from the endogenous expression of SREBP-1c.12 Basal and SREBP-1c–induced adiponectin promoter activity were similar with either the proximal 0.41- or 0.98-kb promoter fragments (data not shown).12

**SREBP-1c Partners With E47 but Not Id3 in Adipocytes**

The established paradigm of Id function involves dimerization with E-proteins such as E47 and not a direct interaction with bHLH-LZ proteins such as SREBP-1c. A previous study demonstrated an Id3:SREBP-1c interaction using in vitro translated proteins outside of a cellular context.19 To determine whether SREBP-1c interacts with either Id3 or E47 in adipocytes, mammalian 2-hybrid analysis in differentiated OP9 cells was used (see methods). Large T antigen and p53, which are known to interact, were used as a positive control, and partners producing equal or greater signal than these were...
considered positive (Figure 4A). SREBP-1c bound to E47 but not to Id3, which is in contrast with the aforementioned study, but consistent with previous results demonstrating E47:SREBP-1c interaction in other tissues.30 To extend the 2-hybrid findings, coimmunoprecipitation of Id3 with E47 or SREBP-1c was tested. COS7 cells were transfected with HA-Id3 and either FLAG–SREBP-1c or FLAG–E47 before immunoprecipitation with anti-FLAG beads. HA-Id3 coimmunoprecipitated with FLAG–E47 but not with FLAG–SREBP-1c (Figure 4B), confirming the results of the 2-hybrid experiment. These findings provide evidence that, in adipocytes, SREBP-1c binds to E47 and not to Id3.

**E47 Potentiates SREBP-1c–Mediated Adiponectin Promoter Activation**

To determine whether E47:SREBP-1c interaction influences adiponectin promoter–reporter activation, NIH3T3 cells were transfected with the 0.41-kb adiponectin promoter–reporter construct together with SREBP1-c and varying amounts of E47. E47 alone did not activate the promoter, but coexpression of E47 with SREBP-1c augmented adiponectin promoter activation 6-fold more than the same dose of SREBP-1c alone (Figure 5).

To identify possible sites at which E47 acts, the 2 E-boxes within the proximal 400 bp of the adiponectin promoter were mutated. The most distal E-box was not tested because, as mentioned above, the 0.41-kb promoter–reporter construct, in which this E-box is deleted, responds to E47 and Id3 in the same manner as the 0.98-kb construct. Approximate positions of the E-boxes are shown in the schematic (Figure 6A). Mutations within E-box no. 1 but not E-box no. 2 inhibited SREBP-1c–mediated adiponectin promoter activity (Figure 6B). Moreover, the mutation of E-box no. 1, but not E-box no. 2, led to the loss of promoter activation by SREBP-1c and E47 (Figure 6C).

**E47 Binds to the Adiponectin Promoter During Adipocyte Differentiation**

To determine whether E47 binds the endogenous adiponectin promoter, ChIP was performed. OP9 and 3T3-L1 cells were harvested during differentiation, at days 3 and 5, respectively, and precipitated with anti-E47 antibody. Because SREBP-1c is known to bind the adiponectin promoter, it was used as a positive control. Adiponectin promoter recovery was determined by real-time PCR using primers spanning E-box no. 1 (Figure 7A). In OP9 cells, precipitation with E47 antibody led to a 4-fold enrichment of the adiponectin promoter compared to isotype control and in 3T3-L1 cells, 5-fold enrichment was obtained (Figure 7B). Precipitation with an antibody for E12, an alternate splice product of the E2A gene that also produces E47, did not lead to any detectable recovery of the adiponectin promoter in either cell line. Similar results were obtained using a second set of adiponectin promoter primers, whereas a negative set of primers gave no specific signal (supplemental Figure IIA and IIB). To investigate whether Id3 expression can interrupt binding of E47 to the promoter, OP9 cells were transduced with Ad-Id3 during differentiation (day 3), when endogenous Id3 had dropped to undetectable levels. OP9 cells transduced with Ad-GFP and precipitated with E47 antibody showed a 4.8-fold increase in adiponectin promoter recovery in the E47 immunoprecipitate compared to isotype control whereas cells transduced with Ad-Id3 had no detectable
promoter binding of E47 (Figure 7C). Immunoblotting of lysates transduced with either GFP or Id3 revealed that levels of E47 and SREBP-1c were similar between the 2 groups (Figure 7C), confirming that the observed decrease in binding was not attributable to a reduction in E47 or SREBP-1c expression. Transduction with Ad-Id3 also inhibited SREBP-1c binding to the adiponectin promoter. Results using a second set of primers were similar and no specific signal was obtained using negative control primers (supplemental Figure IIC).

To determine the temporal pattern of Id3, E47, SREBP-1c, and adiponectin expression in differentiating adipocytes, OP9 cells were harvested at day 0 (undifferentiated), day 3 (differentiating), and day 6 (fully differentiated). Immunoblotting revealed that E47 is expressed in undifferentiated preadipocytes and during differentiation, whereas SREBP-1c is expressed in differentiating and fully differentiated cells (Figure 7D). To test whether endogenous E47 and SREBP-1c binding to the adiponectin promoter is consistent with the temporal pattern of protein expression during differentiation, we performed ChIP analysis of samples harvested in parallel to those used for Western blotting (Figure 7E). Consistent with our data that Id3 levels are high in undifferentiated OP9 and 3T3-L1 cells, E47 did not bind the adiponectin promoter. At that stage, SREBP-1c is not expressed in undifferentiated cells and no detectable adiponectin promoter binding was observed. In OP9 cells undergoing differentiation, Id3 levels are declining, E47 levels remain high, and SREBP-1c is expressed. During this phase, both SREBP-1c and E47 bound the promoter with a 1.8- and 5.0-fold increase in binding compared to isotype, respectively. In mature adipocytes, Id3 is undetectable, E47 is reduced, and SREBP-1c is abundantly expressed. At this stage, E47 and SREBP-1c bound the promoter (1.5- and 5.9-fold increased binding, respectively). Consistent with Western blot data in Figure 7D, E47 binding peaks during differentiation when E47 levels are high, and SREBP-1c binding peaks later in differentiation when SREPB-1c levels are maximal (Figure 7E). To confirm that E47 and SREBP-1c bind the adiponectin promoter in vivo and that Id3 regulates this, in vivo ChIP was performed. Epididymal adipose tissue from ApoE<sup>−/−</sup> and Id3<sup>−/−</sup>ApoE<sup>−/−</sup> mice was harvested from 8-week-old animals and ChIP analysis was performed as above. Precipitation with an E47 antibody led to a 3.4-fold enrichment of the adiponectin promoter in ApoE<sup>−/−</sup> mice, whereas in Id3<sup>−/−</sup>ApoE<sup>−/−</sup> mice, the enrichment was 6.0-fold compared to isotype control. Precipitation with an SREBP-1c antibody resulted in an 8.8-fold enrichment of adiponectin promoter from ApoE<sup>−/−</sup> adipose tissue and 10.1-fold enrichment from Id3<sup>−/−</sup>ApoE<sup>−/−</sup> adipose tissue. Taken together, these data provide evidence that E47 promotes and Id3 inhibits the expression of adiponectin in adipocytes in vitro and in vivo.

Discussion

A strong link has been established between adiponectin levels and clinical atherosclerosis and type 2 diabetes; however, the molecular regulation of adiponectin is incompletely understood. The present study provides the first evidence that the HLH proteins Id3 and E47 regulate adiponectin transcription during adipocyte differentiation.

Here, we have shown that Id3 can negatively regulate adiponectin expression. Id proteins are unable to bind DNA; therefore, they regulate transcription by dimerizing with other HLH proteins, known as E-proteins, to prevent the DNA binding of these factors. We sought to identify an E-protein that may play a role in the regulation of adiponectin. A previous study demonstrated expression of E-proteins in 3T3-L1 cells at the mRNA level. The present study is the first to attribute a function to any E-protein in adipocyte biology. We show that E47 is expressed in preadipocytes and differentiating adipocytes and binds the adiponectin promoter. Furthermore, our data suggest that E47 interacts with SREBP-1c, a known positive regulator of adiponectin, to enhance SREBP-1c–mediated promoter activation. Consistent with our findings in adipocytes, a recent study reported
Figure 6. Mutagenesis of E-box no. 1 inhibits adiponectin promoter activation by SREBP-1c and E47. A, Schematic representation of the proximal 984 bp of the adiponectin promoter depicting the relative locations of 3 E-boxes. E-box no. 1 begins at −137 bp, E-box no. 2 begins at −396 bp, and E-box no. 3 begins at −679 bp. The nucleotide sequence appears above each E-box with asterisks marking the base pairs which were mutated. Specific mutations are described in the methods section. B, NIH3T3 cells were transiently transfected with wild type or mutant adiponectin promoters and an expression vector for SREBP-1c. Seventy-two hours after transfection, cells were harvested and assayed for luciferase activity. Luciferase values are relative to protein levels and are presented relative to promoter plus vector. C, NIH3T3 cells were transiently transfected with wild type or mutant adiponectin promoters, as well as with expression vectors for SREBP-1c, Id3, and E47. The experiment was performed as in B.
Figure 7. E47 binds the endogenous adiponectin promoter in a differentiation-state dependent manner. A, Schematic depicting relative locations of the primer sets used for ChIP analysis. Lines beneath the schematic represent the promoter area amplified by the indicated primer sets. B, OP9 and 3T3-L1 cells were differentiated for 3 and 5 days respectively and then cross-linked and precipitated with the indicated antibodies. Immunoprecipitated fragments were quantified by real-time PCR and normalized to an internal β-galactosidase control for recovery. Results are presented relative to IP with isotype control and are the average of triplicate PCR measurements from 3 independent experiments. C, OP9 cells were transduced with adenovirus encoding GFP (Ad-GFP) or Id3 (Ad-Id3) and then differentiated for 3 days. Cells were harvested for ChIP as in B. In parallel, transduced lysates were separated by SDS-PAGE and membranes were immunoblotted with the indicated antibodies. D, OP9 cells were harvested at day 0 (undifferentiated [U]), day 3 (differentiating [D]), or day 6 (fully differentiated [FD]). Total lysates were immunoblotted as in C. E, Undifferentiated, differentiating and fully differentiated OP9 cells were harvested in parallel to cells used for Western blotting as described in D and ChIP was carried out as described in B. F, Adipose tissue was harvested from 8-week-old ApoE−/− or Id3−/− ApoE−/− mice, snap-frozen, and homogenized. Homogenates were cross-linked and precipitated for ChIP analysis as in B. Left, Precipitation with E47 antibody. *P<0.05, as determined by paired t test. Right, Precipitation with SREBP-1c antibody. *P<0.05, as determined by paired t test.
Id3 on adiponectin is specific rather than via a global blockade of differentiation. This is true whether Id3 is expressed before or after the overexpression of Id3 inhibits adiponectin but does not reproduce these results in a cellular context using either mammalian 2-hybrid or immunoprecipitation techniques. Instead, both Id3:E47 and E47:SREBP-1c were shown to interact. This suggests that the action of Id3 on SREBP-1c is via inhibition of the SREBP-1c partner, E47. Accordingly, E47 binding to the promoter was dramatically reduced by the overexpression of Id3 in vitro and was increased in Id3−/−Apoe−/−adipose tissue. Although we demonstrate that Id3 inhibits SREBP-1c activity in NIH3T3 cells (Figure 3), we believe that this effect is not direct but, instead, attributable to the interaction of Id3 with endogenous E47 present in NIH3T3 cells.

Id3 has been proposed to regulate the differentiation of adipocytes because constitutive expression of Id3 in 3T3-F442A cells inhibits the expression of adipin and adipocyte lipid binding protein mRNAs. Our data show that acute overexpression of Id3 inhibits adiponectin but does not change the levels of GLUT4, another marker of differentiation. This is true whether Id3 is expressed before or after the cells undergo differentiation. This suggests that the effect of Id3 on adiponectin is specific rather than via a global blockade of differentiation.

The present study shows that E47 is highly expressed in undifferentiated cells and during differentiation, although expression is significantly decreased in differentiated cells. Correspondingly, E47 binding to the adiponectin promoter occurs in a differentiation state-specific manner (Figure 8). No binding is observed in undifferentiated cells when Id3 expression is high. Maximal binding occurs during differentiation when Id3 levels have fallen and a small amount of binding persists in fully differentiated cells that have reduced E47 levels. SREBP-1c expression and promoter binding increase throughout differentiation and are maximal in fully differentiated adipocytes. The expression of E47 and SREBP-1c overlap during differentiation when Id3 is undetectable, which may allow E47:SREBP-1c interaction and activation of the adiponectin promoter.

SREBP-1c is known to act at 2 distinct SREs within the mouse adiponectin promoter. This same study reported the existence of 3 putative E-boxes within the promoter, which we investigated here. These E-boxes are relatively conserved in the human adiponectin promoter, which shares 56% similarity to the mouse promoter. This similarity includes several E-boxes in the same region as mouse E-box no. 1, raising the possibility that at least 1 of these elements could function similarly in the human promoter. In our study, the elimination of E-box no. 3 (the 0.41-kb promoter construct) and the mutation of E-box no. 2 (Figure 5A) did not significantly change promoter activation by SREBP-1c and E47. Although these sequences can be identified as E-boxes based on their similarity to the “CANNTG” DNA sequence, neither of them represent the type of E-box that is considered to be the classic binding site (CACCTG or CAGCTG) for E-proteins such as E47. In addition, E-box no. 2 overlaps with a functional SRE site, making it less likely to be the location of E-protein binding. E-box no. 1 represents a classic E-box for E-protein binding and mutational analysis shows that this E-box is necessary for the activation observed with E47 and SREBP-1c (Figure 5). Interestingly, mutation of E-box no. 1 also decreases the effect seen with SREBP-1c.
alone. Although it is plausible that SREBP-1c binds to E-box no. 1 itself as SREBP-1c proteins can bind to both SRE and E-box elements, this latter scenario is less likely, given that SREBP-1c binding sites within the adiponectin promoter have been mapped by DNase footprinting and that SREBP-1c binding does not protect the region of the promoter containing E-box no. 1. Id3 overexpression during differentiation inhibits not only E47 but also SREBP-1c binding to the adiponectin promoter (Figure 7C). Id3 does not appear to bind to SREBP-1c (Figure 6), indicating that SREBP-1c binding at this stage may depend on E47 binding.

Other protein partners for E47 and SREBP-1c are likely to exist, particularly in the case of SREBP-1c, which continues to be expressed and to bind the adiponectin promoter later in differentiation even after E47 expression has been downregulated. Taken together, these data suggest that during differentiation, efficient adiponectin promoter activation by SREBP-1c is dependent on interaction with E47 and E47 binding to E-box no. 1. Little E47 and prominent SREBP-1c binding to the adiponectin promoter in differentiated cells suggest the existence of E47-independent mechanisms to activate adiponectin expression by SREBP-1c in mature adipocytes. Given the role for Id3 in regulating adiponectin expression both in vitro and in vivo, it will be important to determine what controls Id3 expression in adipocytes. Id3 is known to be an early response gene whose expression is dysregulated in obesity. Circ Res. 1996;79:1069–10703.

SREBP-1c binding at this stage may depend on E47 binding.

To date, no specific factor has been demonstrated to directly bind to E-box no. 1 itself as SREBP-1c proteins can bind to both SRE and E-box elements, this latter scenario is less likely, given that SREBP-1c binding sites within the adiponectin promoter have been mapped by DNase footprinting and that SREBP-1c binding does not protect the region of the promoter containing E-box no. 1. Id3 overexpression during differentiation inhibits not only E47 but also SREBP-1c binding to the adiponectin promoter (Figure 7C). Id3 does not appear to bind to SREBP-1c (Figure 6), indicating that SREBP-1c binding at this stage may depend on E47 binding.

Other protein partners for E47 and SREBP-1c are likely to exist, particularly in the case of SREBP-1c, which continues to be expressed and to bind the adiponectin promoter later in differentiation even after E47 expression has been downregulated. Taken together, these data suggest that during differentiation, efficient adiponectin promoter activation by SREBP-1c is dependent on interaction with E47 and E47 binding to E-box no. 1. Little E47 and prominent SREBP-1c binding to the adiponectin promoter in differentiated cells suggest the existence of E47-independent mechanisms to activate adiponectin expression by SREBP-1c in mature adipocytes. Given the role for Id3 in regulating adiponectin expression both in vitro and in vivo, it will be important to determine what controls Id3 expression in adipocytes. Id3 is known to be an early response gene whose expression is stimulated in response to mitogenic stimuli and decreased known to be an early response gene whose expression is dysregulated in obesity. J Biol Chem. 1996;271:10697–10703.

SREBP-1c binding at this stage may depend on E47 binding.

To date, no specific factor has been demonstrated to directly bind to E-box no. 1 itself as SREBP-1c proteins can bind to both SRE and E-box elements, this latter scenario is less likely, given that SREBP-1c binding sites within the adiponectin promoter have been mapped by DNase footprinting and that SREBP-1c binding does not protect the region of the promoter containing E-box no. 1. Id3 overexpression during differentiation inhibits not only E47 but also SREBP-1c binding to the adiponectin promoter (Figure 7C). Id3 does not appear to bind to SREBP-1c (Figure 6), indicating that SREBP-1c binding at this stage may depend on E47 binding.

Other protein partners for E47 and SREBP-1c are likely to exist, particularly in the case of SREBP-1c, which continues to be expressed and to bind the adiponectin promoter later in differentiation even after E47 expression has been downregulated. Taken together, these data suggest that during differentiation, efficient adiponectin promoter activation by SREBP-1c is dependent on interaction with E47 and E47 binding to E-box no. 1. Little E47 and prominent SREBP-1c binding to the adiponectin promoter in differentiated cells suggest the existence of E47-independent mechanisms to activate adiponectin expression by SREBP-1c in mature adipocytes. Given the role for Id3 in regulating adiponectin expression both in vitro and in vivo, it will be important to determine what controls Id3 expression in adipocytes. Id3 is known to be an early response gene whose expression is dysregulated in obesity. Circ Res. 1996;79:1069–10703.

SREBP-1c binding at this stage may depend on E47 binding.

To date, no specific factor has been demonstrated to directly bind to E-box no. 1 itself as SREBP-1c proteins can bind to both SRE and E-box elements, this latter scenario is less likely, given that SREBP-1c binding sites within the adiponectin promoter have been mapped by DNase footprinting and that SREBP-1c binding does not protect the region of the promoter containing E-box no. 1. Id3 overexpression during differentiation inhibits not only E47 but also SREBP-1c binding to the adiponectin promoter (Figure 7C). Id3 does not appear to bind to SREBP-1c (Figure 6), indicating that SREBP-1c binding at this stage may depend on E47 binding.

Other protein partners for E47 and SREBP-1c are likely to exist, particularly in the case of SREBP-1c, which continues to be expressed and to bind the adiponectin promoter later in differentiation even after E47 expression has been downregulated. Taken together, these data suggest that during differentiation, efficient adiponectin promoter activation by SREBP-1c is dependent on interaction with E47 and E47 binding to E-box no. 1. Little E47 and prominent SREBP-1c binding to the adiponectin promoter in differentiated cells suggest the existence of E47-independent mechanisms to activate adiponectin expression by SREBP-1c in mature adipocytes. Given the role for Id3 in regulating adiponectin expression both in vitro and in vivo, it will be important to determine what controls Id3 expression in adipocytes. Id3 is known to be an early response gene whose expression is dysregulated in obesity. Circ Res. 1996;79:1069–10703.

SREBP-1c binding at this stage may depend on E47 binding.

To date, no specific factor has been demonstrated to directly bind to E-box no. 1 itself as SREBP-1c proteins can bind to both SRE and E-box elements, this latter scenario is less likely, given that SREBP-1c binding sites within the adiponectin promoter have been mapped by DNase footprinting and that SREBP-1c binding does not protect the region of the promoter containing E-box no. 1. Id3 overexpression during differentiation inhibits not only E47 but also SREBP-1c binding to the adiponectin promoter (Figure 7C). Id3 does not appear to bind to SREBP-1c (Figure 6), indicating that SREBP-1c binding at this stage may depend on E47 binding.

Other protein partners for E47 and SREBP-1c are likely to exist, particularly in the case of SREBP-1c, which continues to be expressed and to bind the adiponectin promoter later in differentiation even after E47 expression has been downregulated. Taken together, these data suggest that during differentiation, efficient adiponectin promoter activation by SREBP-1c is dependent on interaction with E47 and E47 binding to E-box no. 1. Little E47 and prominent SREBP-1c binding to the adiponectin promoter in differentiated cells suggest the existence of E47-independent mechanisms to activate adiponectin expression by SREBP-1c in mature adipocytes. Given the role for Id3 in regulating adiponectin expression both in vitro and in vivo, it will be important to determine what controls Id3 expression in adipocytes. Id3 is known to be an early response gene whose expression is dysregulated in obesity. Circ Res. 1996;79:1069–10703.
The Helix–Loop–Helix Factors Id3 and E47 Are Novel Regulators of Adiponectin
Amanda C. Doran, Nahum Meller, Alexis Cutchins, Hamid Deliri, R. Parker Slayton, Stephanie N. Oldham, Jae B. Kim, Susanna R. Keller and Coleen A. McNamara

Circ Res. 2008;103:624-634; originally published online July 31, 2008;
doi: 10.1161/CIRCRESAHA.108.175893

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/103/6/624

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2008/07/31/CIRCRESAHA.108.175893.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/
**Materials and Methods:**

*Animals* – All animal protocols were approved by the Animal Care and Use Committee at the University of Virginia. Male ApoE<sup>−/−</sup> and Id3<sup>−/−</sup> ApoE<sup>−/−</sup> mice were given standard chow diet and water *ad libitum*. At eight weeks of age, mice were given an overdose of ketamine/xylazine and blood was collected by left ventricular puncture prior to perfusion with PBS. Epididymal and mesenteric fat depots were harvested for analysis.

*Cell Culture* – NIH3T3 cells were maintained in high-glucose Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% calf serum (Atlanta Biologicals) and 2 mmol/L L-glutamine (Invitrogen). 3T3-L1 cells were maintained as pre-adipocytes in DMEM supplemented with 10% fetal bovine serum (Zen-Bio), 1% newborn calf serum (Zen-Bio) and 2 mmol/L L-glutamine (Invitrogen). Differentiation of confluent cells was induced by conventional method, using the standard “adipogenic cocktail” consisting of DMEM with 10% fetal calf serum, 0.5 mmol/L 3-isobutyl methylxanthine (Sigma), 1 μmol/L dexamethasone (Sigma) and 5 μg/ml human recombinant insulin (Novo Nordisk)<sup>1</sup>. OP9 cells were a gift from Dr. Perry Bickel (Washington University, St. Louis). These cells were maintained in MEMα (Invitrogen) supplemented with 20% fetal calf serum (Zen-Bio) and 2 mmol/L L-glutamine. OP9 cells were differentiated using either the adipogenic cocktail method outlined above or an insulin oleate cocktail containing MEMα with 0.2% FBS, 5 μg/ml insulin and 900 μmol/L oleate bound to albumin (5.5:1 molar ratio, Sigma)<sup>2</sup>. The insulin oleate method was used to differentiate the OP9 cells for promoter-reporter and mammalian two-hybrid
assays in order to maintain transfection of the cells throughout differentiation. Cells used in all other experiments were differentiated by the adipogenic cocktail method. Day of differentiation is defined as the number of days cells were exposed to differentiation media after reaching confluence. Maximal differentiation was reached at eight days in OP9 cells and 14 days in 3T3-L1 cells.

**Real-Time PCR Analysis** – Total cellular RNA was collected from adipose tissue using TRIzol Reagent and from adipocyte cell lines using an RNeasy kit (Qiagen) as per the manufacturer’s instructions. cDNA was then synthesized using an iScript cDNA synthesis kit (BioRad). Total cDNA was diluted 1:5 in water and 2 μl were used for each real-time PCR reaction using a Bio-Rad iCycler and iQ SYBR Green Supermix (BioRad). Analysis of tissue samples and chromatin immunoprecipitation products was performed by normalizing to cyclophilin using the standard curve method to approximate the amount of starting material. The ΔCt method was used for *in vitro* cell samples in order to determine relative mRNA expression. Primers used for the detection of adiponectin were: 5’- TGT TGG AAT GAC AGG AGC TG -3’ and 3’- CGA ATG GGT ACA TTG GGA AC -5’. Primers for the detection of Id3 were: 5’- TGT CGT CCA AGA GGC TAA GAG GCT -3’ and 3’- TGC TAC GAG GCG GTG TGC TG -5’.

**Western Blot Analysis** – Cells were trypsinized, washed in PBS and lysed in buffer containing 50 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 1% Triton X-100 and protease inhibitors (Sigma, #P8340). Lysates were incubated on ice for ten minutes and centrifuged at 16,000 x g for ten minutes. Adipose tissue was homogenized in lysis
buffer (100 mmol/L Tris pH 6.8, 11% glycerol, 7.7% SDS), incubated on ice for ten minutes and centrifuged for five minutes at 1,310 x g. The infranatants were separated from the lipid layer and re-centrifuged at 1,310 x g for ten minutes. Cell supernatants were assayed for protein concentration, adjusted to equal concentration and supplemented with 5x loading buffer (0.3125 mol/L Tris pH 6.8, 10% SDS, 50% glycerol, 25% β-mercaptoethanol, 0.1% bromophenol blue) and adipose tissue samples were assayed for protein concentration, adjusted to equal concentration and supplemented with 11% β-mercaptoethanol and 0.5% bromophenol blue. Samples were resolved on 4-20% tris-glycine gels (Invitrogen) and transferred to polyvinylidene difluoride membrane (BioRad). Western blotting was carried out using antibodies to adiponectin (0.2 μg/ml, Chemicon, catalog #AB3269P), Id3 (0.1 μg/ml, CalBioreagents, catalog #M097), E47 (1 μg/ml, BD Pharmingen, catalog #554077), GLUT4 (5 μg/ml, previously described in Ross, et al ³) or β-actin (0.05 μg/ml, Santa Cruz, catalog #sc-47778) followed by horseradish peroxidase-linked secondary antibody (Jackson). Immunoreactive bands were visualized by chemiluminescence after incubation with ECL Plus reagent (Amersham Pharmacia Biotech).

**Transient Transfection** – Transfections were performed using FuGENE HD transfection reagent (Roche), according to the manufacturer’s instructions.

**Adenoviral Transduction** – The construction of the adenoviruses Ad-GFP and Ad-Id3 have been described previously ⁴. Both were purchased from the University of Iowa Viral Vector Core. Unless indicated otherwise, OP9 cells were infected with 4000
multiplicities of infection (MOI) in propagation medium and 3T3-L1 cells were infected with 2000 MOI in propagation medium supplemented with 0.3 μg/ml poly-L-lysine. Twenty-four hours post-infection, viral media was removed and replaced with propagation media. Cells were then differentiated and harvested as above.

Promoter-Reporter Analysis – Full length Id3, E47 and E12 were previously subcloned into pAdlox, pcDNA 3.1 and pXS expression vectors respectively. SREBP-1c (N-terminal amino acids 1-403) in pSV-SPORT1 and adiponectin promoter constructs containing 0.41 kb or 0.98 kb of proximal promoter sequence have been previously described. Mutant promoter constructs were generated with the 0.98 kb fragment of the adiponectin promoter using the QuikChange II site-directed mutagenesis kit (Stratagene). E-box #1 was mutated from CAGCTG to GTGCTC using the following primers: 5’- CCC TAA GGA GTC TTA AGG GTG CTC CCA GGA GCA AG -3’ and 3’- CTT GCT CCT GGG AGC ACC CTT AAG ACT CCT TAG GG -5’. E-box #2 was mutated from CATGTG to GGAGTG using the following primers: 5’- GAA AGA GTG GGA GTA TGG AGT GAC AAT TAG TGT TGT TGA C -3’ and 3’- GTC AAC AAC ACT AAT TGT CAC TCC ATA CTC CCA CTC TTT C -5’. Although E-box #2 overlaps with an SRE element, caution was taken to conserve the SRE consensus sequence while disrupting the E-box. NIH3T3 cells were transfected with 0.98 μg of expression plasmid along with 0.1 μg of adiponectin promoter. Seventy-two hours after transfection, luciferase activity was measured using the Luciferase Assay Kit (Promega) as per the manufacturer’s instructions.

Adiponectin Radioimmunoassay (RIA) – Serum was obtained from eight week old
ApoE\textsuperscript{-/-} and Id3\textsuperscript{-/-} ApoE\textsuperscript{+/-} mice at time of sacrifice. RIA was performed as per manufacturer’s instructions (Millipore).

**Mammalian Two-Hybrid Analysis** – The construction of vectors for two-hybrid analysis has been previously described\textsuperscript{4}. The Id3, SREBP-1c and E47 cDNAs were subcloned into the pM and pVP16 vectors containing the GAL4 DNA binding and GAL4 activation domains respectively, yielding the pM-Id3, pM-E47, pM-SREBP-1c, pVP16-Id3, pVP16-E47 and pVP16-SREBP-1c constructs. These plasmids were transfected in combination with the pGL5-CAT reporter plasmid (Boeringher Mannheim). Lysates were collected and assayed for chloramphenicol acetyltransferase (CAT) activity using a CAT ELISA kit as per the manufacturer’s instructions (Roche). If proteins partner, the GAL4 binding and GAL4 activation domains are brought into proximity, thus activating the pGL5-CAT promoter-reporter. Large T antigen and p53, which are known to interact, were used as a positive control, and partners producing equal or greater signal than these were considered positive results.

**Co-immunoprecipitation** – Full-length human E47 was subcloned into the pEF4-FLAG vector\textsuperscript{8} at KpnI and EcoRI sites. Rat SREBP-1c cDNA coding for amino acids 1-403 was subcloned into the pEF4-FLAG vector at the EcoRI site. Full-length rat Id3 was subcloned into the pEF4-HA vector\textsuperscript{9} using KpnI and SfuI sites. \(1 \times 10^6\) COS7 cells were plated in 10 cm dishes 24 hours prior to transfection. These cells were transiently transfected with HA-Id3 and either FLAG-SREBP-1c or FLAG-E47. Forty-eight hours after transfection, cells were washed in PBS and harvested in lysis buffer (50 mmol/L
Tris pH 7.5, 150 mmol/L NaCl, 1% Triton X-100) plus protease inhibitors (Sigma). 10 µl of anti-FLAG beads (Sigma) were added to 400 µl of whole cell lysate and were incubated with rocking for 3 hours at 4°C. Following the incubation, samples were washed three times in lysis buffer, then supplemented with SDS-PAGE sample buffer and boiled. Western blotting was carried out as described above. Membranes were probed with monoclonal antibodies against FLAG (Sigma, catalog #F3165) or HA (Covance, catalog #MMS-101P).

Chromatin Immunoprecipitation (ChIP) – The ChIP protocol was modified from a previously described version. $1 \times 10^7$ OP9 or 3T3-L1 cells were cross-linked with 1% formaldehyde. Cells were then suspended in 0.6 ml of lysis buffer (50 mmol/L Tris-Cl, pH 8.1, 1% Triton X-100, 0.1% deoxycholate, 150 mmol/L NaCl, and 5 mmol/L EDTA) plus protease inhibitors (Sigma). Adipose tissues were snap frozen in liquid nitrogen and 100 mg of tissue was ground using a mortar and pestle. Homogenates were resuspended in 1 ml of lysis buffer. Cell or tissue suspensions were then sonicated using 15 five second pulses with 15 second cool down intervals in order to shear chromatin to 1000 bp fragments. Approximately 100 µg of the clarified extracts were diluted to 1 ml in lysis buffer containing protease inhibitors and then incubated overnight at 4°C on a rocking platform with either 5 µg of SREBP-1c (Santa Cruz, catalog #sc-13551), E47 (Santa Cruz, catalog #sc-763) or E12 (Santa Cruz, catalog #sc-762) antibodies. A fraction of the extract was stored for later PCR analysis of the input extract. The following day, 40 µl of protein A or G agarose slurry (Santa Cruz or GE Healthcare) and 2 µg of herring sperm DNA (Sigma) were added prior to an additional one hour incubation. The agarose was
pelleted by centrifugation, and the pellets were washed consecutively with 1 ml of lysis buffer, lysis buffer plus 500 mmol/L NaCl, lysis buffer plus 0.25 mol/L LiCl, and 10 mmol/L Tris/1 mM EDTA. DNA and protein were eluted from agarose beads by incubating the pellets twice for 15 minutes in 0.25 ml of elution buffer (0.1 mol/L NaHCO3 with 1% SDS and 20 µg/ml herring sperm DNA). To increase recovery, samples were boiled for one minute in between elutions. β-Gal plasmid (1 µg/ml) was added to the elution buffer to allow for correction of unequal precipitation efficiencies. Protein-DNA cross-links were reversed by incubating at 65°C for three hours. DNA and protein were ethanol-precipitated overnight at -20°C. The precipitated samples were then pelleted, dissolved in proteinase K buffer (10 mmol/L Tris-Cl, pH 7.5 with 7% SDS) and incubated with 1 µg of proteinase K (Roche) for one hour at 55°C. The samples were extracted once with phenol/chloroform and ethanol-precipitated overnight at -20°C. Samples were pelleted, washed with 70% ethanol, and dissolved in 100 µl of 10 mmol/L Tris/1 mmol/L EDTA. 2 µl aliquots were used for each real-time PCR reaction, which was carried out as described above. Primer sets were designed to cover E-box #1 within the proximal adiponectin promoter (adiponectin primer set #1), a more distal portion of the promoter (adiponectin primer set #2) or a portion of intron 1 of the adiponectin gene approximately 10 kb from the promoter (negative primer set). Specific primers used to detect adiponectin promoter fragments were as follows: adiponectin primer set #1 5’-AGA AGC TCT ACT TGG CTT CCC -3’ and 3’- GCA GAC CCC AGC TTA CCA -5’; adiponectin primer set #2 5’- GTA TGG GAT CCG GTC TAG CA -3’ and 3’- ATT CCC AGC ACC CAC AGT AA -5’; β-Gal primer set 5’- TCA ATC GCG CGT TTG TTC CCA C -3’ and 3’- TCC AGA TAA CTG...
CCG TCA CTC CAA C '5'; negative primer set 5' - AGC CTG GAG AAG CCG CTT AG '3’ and 3’-
TTG CAG TAG AAC TTG CCA GTG C '5’.

Statistical Analyses – All experiments were performed in triplicate and unpaired t-tests
were used to determine significance unless noted otherwise. Error bars represent
standard deviation.
References for Supplemental Material


**Figure Legends for Supplemental Figures:**

**Online Figure I. Id3 expression decreases adiponectin in differentiated adipocytes.**

*A*, OP9 Cells were differentiated with an adipogenic cocktail for five days prior to transduction with adenovirus encoding GFP (Ad-GFP) or Id3 (Ad-Id3). Seventy-two hours after transduction, total lysates were collected and analyzed by Western blotting. Results shown are representative of two independent experiments. **B,** Densitometric analysis of the experiment described in A. Adiponectin levels were normalized to β-actin. Results are relative to the adiponectin signal in the vehicle group. **= p<0.01; Ad-GFP versus Ad-Id3.

**Online Figure II. E47 binds the endogenous adiponectin promoter.** *A*, OP9 were differentiated for three days, then cross-linked and precipitated with the indicated antibodies. Immunoprecipitated fragments were quantified by real-time PCR using the standard curve method and were normalized to an internal β-Gal control for recovery. Results are presented relative to IP with isotype control and are the average of triplicate PCR measurements from three independent experiments for each. Two independent adiponectin primer sets were used to confirm results (depicted in Figure 7A and described in detail in methods). In addition, a negative primer set, which recognizes a portion of the first intron of the adiponectin gene, did not detect any signal in immunoprecipitated samples. **B,** 3T3-L1 cells were differentiated for five days, then harvested for ChIP analysis and analyzed using three different primer sets as described in A. **C,** OP9 cells were transduced with adenovirus encoding GFP (Ad-GFP) or Id3 (Ad-Id3) and
then differentiated for three days. Cells were then harvested for ChIP and analyzed using three different primer sets as in A.

**Online Figure III. E47 binds the endogenous adiponectin promoter in a differentiation-state dependent manner.** OP9 cells were harvested at day zero (undifferentiated), day three (differentiating) or day six (fully differentiated). Lysates were then cross-linked and precipitated with the indicated antibodies. Immunoprecipitated fragments were quantified by real-time PCR using the standard curve method and were normalized to an internal β-Gal control for recovery. Results are presented relative to IP with isotype control and are the average of triplicate PCR measurements from three independent experiments for each. Two independent adiponectin primer sets were used to confirm results (depicted in Figure 7A and described in detail in methods). In addition, a negative primer set, which recognizes a portion of the first intron of the adiponectin gene, did not detect any signal in immunoprecipitated samples.
Online Figure I.

A.

B.
Online Figure II.

A.

B.

C.

Primer Set #1

Primer Set #2

Negative Primer Set

Primer Set #1

Primer Set #2

Negative Primer Set

Primer Set #1

Primer Set #2

Negative Primer Set

Primer Set #1

Primer Set #2

Negative Primer Set

Primer Set #1

Primer Set #2

Negative Primer Set
Online Figure III.

Primer Set #1

Primer Set #2

Negative Primer Set