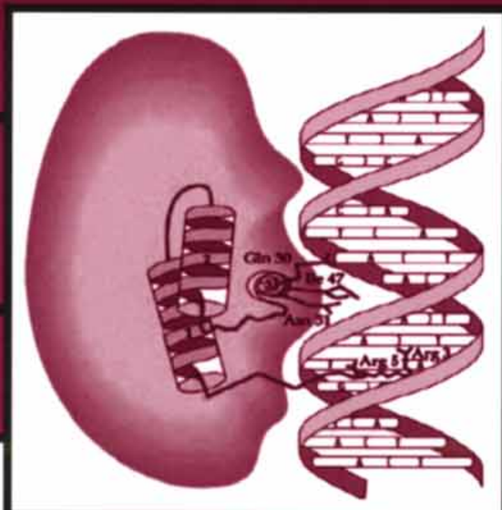
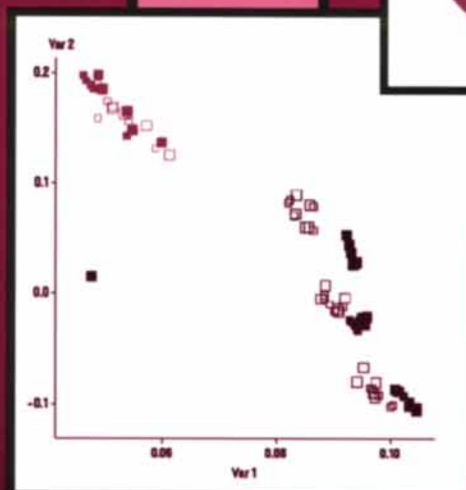
Unraveling Lipid Metabolism with Microarrays





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Microarray Analyses of SREBP-1 Target Genes

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INTRODUCTION

Microarray analyses are becoming increasingly prevalent and are being used in various research fields in life science. Application of this technique for the quantification of mRNA expression levels has enabled us to obtain vast amount of information on the expression levels of numerous genes at once. This is of great help especially for the elucidation of transcriptionally regulated pathways. In this chapter, we present our recent studies on a transcription factor, sterol regulatory element-binding factor (SREBP)-1, as an example where we applied the microarray technique for research on the nutritional regulation of gene expression.

ADVANTAGE OF MICROARRAY ANALYSES FOR THE RESEARCH OF LIPOGENIC GENE REGULATION

Mammals store their body energy in the form of glycogen and triglycerides. The former is stored chiefly in liver and skeletal muscle and the latter in liver and

adipose tissue. Whereas glycogen synthesis is regulated by protein phosphorylation cascades, the synthesis of fatty acids and triglycerides (lipogenesis) is mainly controlled at the transcriptional level.[1] This is presumably relevant to the differences in turnover rate between glycogen and triglycerides; glycogen is synthesized or degraded in minutes to hours while for triglycerides it is in hours to days. Transcriptional regulation is best suited for these relatively slow responses.

Another feature of lipogenic responses is that each enzyme involved in the metabolic pathway is coordinately regulated.[2] The fatty acid biosynthetic pathway is composed of about 25 enzymes. Among these enzymes, the following are of particular importance: fatty acid synthase (FAS), the main synthetic enzyme that catalyzes the condensation of malonyl-CoA to produce the 16-carbon saturated fatty acid palmitate; acetyl-CoA carboxylase (ACC), which synthesizes malonyl-CoA from acetyl-CoA; and ATP citrate lyase (ACL), which synthesizes acetyl-CoA from citrate provided through glycolysis. These enzymes are known to be coordinately upregulated 10–50-fold at the transcriptional level when lipogenesis is accelerated in hepatocytes and adipocytes, for instance, when fasted animals are refed.[1]

As lipogenic enzyme genes are coordinately upregulated upon refeeding, it has been postulated that these genes have regulatory sequences in their promoter that interact with common trans-acting factors. Currently, the most important regulator is considered to be SREBP-1.[3,4]

SREBPs ARE MEMBRANE-BOUND TRANSCRIPTION FACTOR OF bHLH SUPERFAMILY

SREBPs are transcription factors that belong to the basic helix–loop–helix (bHLH) leucine zipper family. Unlike other transcription factors, SREBP proteins are initially bound to the rough endoplasmic reticulum (rER) membrane and form a complex with SREBP-cleavage activating protein (SCAP), a sterol-sensing molecule. Upon sterol deprivation from the cells, SREBP/SCAP travels from rER to Golgi where SREBP is cleaved to liberate the amino-terminal portion containing a bHLH leucine zipper domain (nuclear SREBP), and enters the nucleus where it can bind to specific sterol response elements (SRE) in the promoters of target genes (reviewed in Brown and coworkers[5–7]). Meanwhile, many cellular sterols retain the SREBP/SCAP complex on the rER where insulin inducible genes (INSIGs) play an important role in the regulation of SCAP configuration and the cleavage of SREBP, and thus, cholesterol synthesis does not occur. To date, three isoforms of SREBP-1a, -1c, and -2 have been found and characterized. In the differentiated tissues, SREBP-2 plays a crucial role in the regulation of cholesterol synthesis[6,7], whereas SREBP-1c controls the transcription of lipogenic enzymes involved in the synthesis of fatty acids and triglycerides.[8–11] SREBP-1c is drastically induced when fasted animals are refed.[12] SREBP-1a is expressed in actively growing cells, and activates both lipogenic

and cholesterogenic genes. Thus, SREBP-1a has the most potent and global range of target genes among the isoforms.

SREBP-1a TARGET GENES ON A DNA MICROARRAY ANALYSIS

In an attempt to comprehensively understand SREBP targets and potentially identify new target genes, we performed microarray analysis (randomly selected some 9000 genes on an array manufactured by IncyteGenomics) for mRNA from livers of a transgenic mouse model that overexpresses a nuclear form of SREBP-1a protein in liver. This mouse exhibited markedly elevated expression of enzymes involved in fatty acid and cholesterol biosynthetic pathways, and thereby developed massive enlargement of the liver, owing to the engorgement of hepatocytes with cholesterol and triglycerides (hepatic steatosis).[13] From the microarray data we calculated the fold changes in mRNA expression of each gene in the transgenic mice livers compared with wild-type, and listed them in descending order in Table 10.1.

Consistency and Reliability of the Data

There has been much discussion about the problems in the quantification and reproducibility of the results from DNA chips. It could be technical issues related to cDNA or oligoprobes, or sample problems. However, fortunately, the data obtained from DNA microarrays of SREBP1a-transgenic/wild-type liver RNAs, exhibited perfect reliability and consistency; we had a chance to compare the extent of SREBP-1a-induction estimated by DNA microarrays and by Northern blot analysis in some known SREBP target genes such as FAS and ACL, and the fold-induction was highly consistent between the two different methods convincing us about the validity of the rest of DNA microarray data. Although rare, some EST clones used in microarrays were identical to provide us two results for the same gene, the data were very similar again. For example, secreted modular calcium-binding protein 2 listed in Table 10.1 had two independent EST clones (GenBank accession nos.: AA272826 and AA059909) on the array, and the fold-increase obtained from them were 9.3 and 8.2, respectively (only the former value is presented in Table 10.1).

Most of the Inducible Genes were Lipogenic and Cholesterogenic Genes, as Predicted

In the top 25 genes whose expression was the most strongly increased in transgenic mice, 9 were lipogenic genes and 5 were cholesterogenic. The most highly ranked gene was FAS and the second was ACL, both were well characterized as SREBP targets in the promoter analyses. 6-Phosphogluconate dehydrogenase (6PGD) was a key enzyme, so was glucose-6-phosphate dehydrogenase, an enzyme in the pentose pathway, which produces NADPH for reducing potential for lipid synthesis, and was considered as one of lipogenic enzyme members, although the promoter analysis was not yet done in the context of the SREBP target.

Table 10.1 Microarray Analysis of Mice Overexpressing SREBP-1a in Liver

Fold increase	Gene name	Function	Accession no.
19.5	Fatty acid synthase (*FAS*)	Lipogenesis	AA116513
19.5	Long chain fatty-acyl CoA elongase (*FACE*)	Lipogenesis	AA239254
16.0	Membrane-associated protein 17	Unknown	AA274146
15.1	ATP citrate lyase (*ACL*)	Lipogenesis	W33415
14.8	Lymphocyte antigen 6 complex, locus D	Unknown	AA163336
14.6	Diphosphomevalonate decarboxylase (*MVD*)	Cholesterol synthesis	AI510113
14.4	Secretory leukocyte protease inhibitor	Unknown	AA200339
13.7	6-Phosphogluconate dehydrogenase (*6PGD*)	Lipogenesis	AI893710
12.6	Squalene epoxidase (*SQLE*)	Cholesterol synthesis	AA268608
12.4	Acetyl-CoA synthetase (*ACAS*)	Lipogenesis	AA537637
12.1	Isopentenyl-diphosphate delta-isomerase 1 (*IDI1*)	Cholesterol synthesis	AA237469
11.1	Proline-serine-threonine phosphatase-interacting protein 2 mitochondrial glycerol-3-phosphate acyltransferase (*GPAT*)	Unknown Lipogenesis	AA390032 AA209041
10.4	HMGCoA synthase (*HMGCS*) 1	Cholesterol synthesis	AI892192
9.4	Acetyl-CoA carboxylase (*ACC*) 1	Lipogenesis	AA014384
9.3	Secreted modular calcium-binding protein 2	Unknown	AA272826
7.9	Delta-5 fatty acid desaturase (*D5D*)	Lipogenesis	AA068575
7.6	7-Dehydrocholesterol reductase (*DHCR7*)	Cholesterol synthesis	AA003001
7.3	UDP-glucose 4-epimerase	Conversion from galactose to glucose	AA386807
7.1	p21Waf1	Cell cycle arrest	W88005

Note: The fold changes in mRNA expression of each gene in the transgenic mice livers compared with wild-type are listed in descending order. Methods: PolyA RNAs were extracted from livers of transgenic or wild-type mice ($n = 4$) and pooled. The microarray analysis was performed by IncyteGenomics with their standard protocol. Briefly, each set of polyA RNA was labeled with fluorescence (Cy3 for wild-type and Cy5 for transgenic mice). Then they were mixed and hybridized onto the microarray plate containing approximately 9000 clones. After washing, the plate was scanned by fluorescence imager and signals were quantified. The same method was used for all microarray data shown in this chapter.

Mitochondrial glycerol-3-phosphate acyltransferase was also identified as a highly inducible gene. Many of cholesterolgenic genes were also detected, which, along with established genes, led us to conclude that SREBP can activate an entire pathway of cholesterol synthesis.[14]

SREBP-1 PLAYS THE CENTRAL ROLE IN REFEEDING RESPONSES

To clarify the positioning of SREBP-1 activation in the refeeding responses in liver, we also performed microarray analysis on gene expression profiles in refed animals in a similar way. First we quantified each gene expression level (for some 9000 genes on the IncyteGenomics microarray) in liver and adipose tissue from fasted or refed mice in order to obtain the entire list of genes that are nutritionally regulated at mRNA level. The calculated fold increases in refed state vs. fasted state were displayed in the scatter plot in logarithmic scale (data from liver are plotted along the horizontal axis and those from adipose tissue are along vertical axis) (Fig. 10.1). As shown here, both in liver and in adipose tissue, lipogenic enzymes such as FAS, ACL, and ACC are markedly induced upon refeeding and are revealed to be the representative genes of refeeding responses.

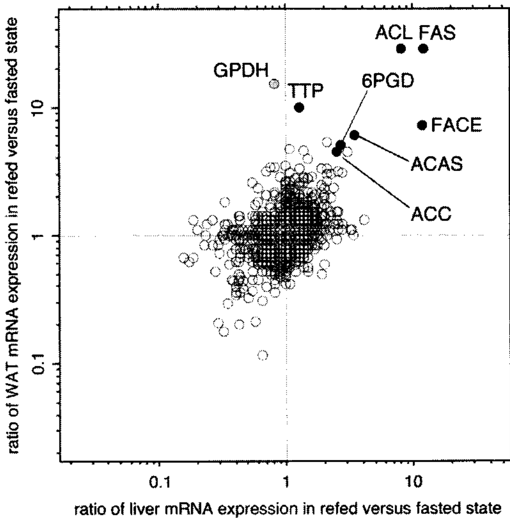

Figure 10.1 Lipogenic genes are the most strongly upregulated genes when fasted animals are refed. Increases in mRNA expression in liver (on horizontal axis) and adipose (on vertical axis) after refeeding fasted mice quantified by microarray analysis are plotted in two dimension in logarithmic scale. Lipogenic genes under SREBP-1 control (shown as solid circles) are coordinately elevated both in liver and in adipose tissue. GPDH does not seem to be an SREBP-1 target gene because it was not increased in SREBP-1 transgenic mice. Methods: Mice ($n = 8$ for each group) were 24 h fasted or 12 h refed on a high-sucrose fat free diet after 24 h starvation. PolyA RNA samples extracted from livers and white adipose were pooled within each group and analyzed with IncyteGenomics DNA microarray. *ACAS*, acetyl-CoA synthase; *ACC*, acetyl-CoA carboxylase; *ACL*, ATP citrate lyase; *FACE*, fatty-acyl-CoA elongase; *FAS*, fatty acid synthase; *GPDH*, glycerophosphate dehydrogenase; *6PGD*, 6-phosphogluconate dehydrogenase; *TTP*, tricarboxylate transport protein; WAT, white adipose tissue.

Then we compared these refeeding-responsive genes with SREBP-1 target genes. In Fig. 10.2, mRNA quantification results by microarray analysis including those of lipogenic and cholesterogenic genes are plotted in a 2D field of refed-vs.-fasted changes on the horizontal axis and transgenic changes on the vertical axis. This scatter diagram demonstrates that target genes of SREBP-1 are activated the most vigorously in refed mice livers and indicates that SREBP-1 plays the central role in refeeding responses.

SREBP-1 IS INVOLVED IN SYNTHESIS OF PHOSPHOLIPIDS

It has been reported that phosphocholine cytidylyltransferase, a rate-controlling enzyme in the phosphatidylcholine biosynthesis pathway is an SREBP target.[15,16] Thus, SREBPs could also be involved in phospholipid metabolism,

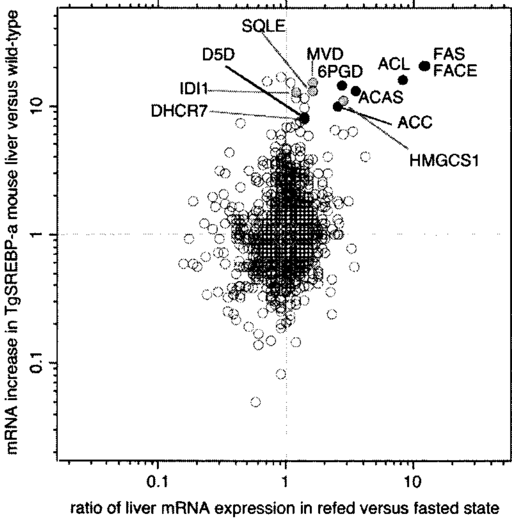

Figure 10.2 SREBP-1 plays the pivotal role in refeeding responses. Increases in mRNA expression in liver after refeeding fasted mice (on horizontal axis) and those in SREBP-1a transgenic mice compared with wild-type mice (on vertical axis) quantified by microarray analysis are plotted in 2D in logarithmic scale. Lipogenic and cholesterogenic genes are shown as black and gray circles, respectively. Methods: For the fasting–refeeding experiment, mice ($n = 8$ for each group) were 24 h fasted or 12 h refed on a high-sucrose fat free diet after 24 h starvation. PolyA RNA samples extracted from livers were pooled within each group and analyzed with IncyteGenomics DNA microarray. *ACAS*, acetyl-CoA synthase; *ACC*, acetyl-CoA carboxylase; *ACL*, ATP citrate lyase; *D5D*, delta-5 fatty acid desaturase; *FACE*, fatty-acyl-CoA elongase; *FAS*, fatty acid synthase; *6PGD*, 6-phosphogluconate dehydrogenase; *DHCR7*, 7-dehydrocholesterol reductase; *HMGCS1*, hydroxymethylglutaryl-CoA synthase 1; *IDI1*, isopentenyl-diphosphate delta-isomerase 1; *MVD*, diphosphomevalonate decarboxylase; *SQLE*, squalene epoxidase.

which potentially places SREBPs in the regulation of entire lipid synthesis. Intriguingly, in *Drosophila*, the SREBP/SCAP system is regulated by phosphatidylethanolamine,[17,18] suggesting that the SREBP/SCAP system conserved in a wide range of species plays a more diverse role in lipid synthesis by regulating physicochemical functions of biomembranes. Although it has been published that there are some SRE sites in the promoters of some phospholipid genes, fold changes of activation by SREBP-1a is not marked in those genes. SREBPs could influence, but not dominate, the expression of these genes in mammalians.

NEWLY IDENTIFIED SREBP-1 TARGET GENES WHOSE FUNCTIONS HAVE BEEN ELUCIDATED

The most intriguing and exciting part of DNA microarray analysis is identification of new target genes of a transcription factor. The listing of SREBP-1a-induced EST clones offered us an opportunity to characterize in detail these genes whose functions were not yet well documented. These are acetyl-CoA synthetase (ACAS),[19] fatty-acyl-CoA elongase (FACE),[20] and delta-5 fatty acid desaturase (D5D)[21] as discussed.

Acetyl-CoA Synthetase

One of the highly inducible genes (12-fold increase by SREBP-1 overexpression) was designated as a gene highly similar to bacterial ACAS. We and another group[22] cloned the whole cDNA and confirmed that it has activity for mammalian ACAS and that its regulation is in a manner similar to that of lipogenic genes, and controlled by SREBPs. This enzyme catalyzes production of acetyl-CoA from acetate and CoA. Acetyl-CoA is a hub molecule for energy metabolism and nutrition. SREBP regulation of this enzyme could implicate that mammalian lipogenic organs have a pathway for energy conversion from free acetate as well as glucose. ACAS activity has also been well known among researchers in ruminology to play a crucial role in energy production on ruminants as volatile fatty acids (also known as short chain fatty acids) produced through fermentation of cellulose and other fibers in the rumen are their main source of energy.[23]

Long Chain Fatty-Acyl-CoA Elongase; LCE

Pursuing another SREBP inducible EST helped us clone a novel elongase which was revealed to catalyze the last elongation step in mammalian fatty acid synthesis,[20,24] and was named FACE or LCE. The process was as follows: after sequencing a long 3′ UTR cloned by rapid amplification of cDNA ends (RACE), we found that this gene encodes an integral membrane protein similar to Cig30 and SSC1 in mammalians and ELO2 and ELO3 in yeast. It was well known that FAS covers synthesis from malonyl-CoA to palmitate (C16:0), and stearoyl-CoA desaturase (SCD)-1 desaturates stearate to oleate

(C18:0 to C18:1), but the enzyme responsible for the conversion of C16:0 to C18:0 was missing. So, we intensively investigated the enzymatic activity of this new protein on various substrates, and identified it as the missing elongase. This enzyme also has an activity for elongation of monounsaturated fatty acids and could be involved in regulation of ratio of saturated/monounsaturated fatty acids with a link to SCD-1, and might potentially play a role in obesity and insulin sensitivity.

Delta-5 Fatty Acid Desaturase

D5D was also identified as an SREBP target gene on the basis of our microarray data,[21] and so was D6D (delta-6 fatty acid desaturase). They are involved in the production of polyunsaturated fatty acids (PUFA). As PUFA inhibit SREBP-1c and lipogenesis, it is hard to explain the physiological meaning of SREBP induction of these PUFA relating enzymes. D5D and D6D are also regulated by peroxisome proliferator-activated receptor (PPAR) alpha, and hence the expression of D5D and D6D could be regulated by two distinct mechanisms; one through SREBP-1c, as is shown by its induction during refeeding, and the other through PPAR alpha, which is inducible by fasting. Dual regulation of D5D and D6D by the two factors SREBP-1 and PPAR alpha that possess opposing activation properties depending upon nutritional status caused no remarkable change in the overall expression of these enzymes between fasting and refeeding. This dual regulation of both desaturases contribute to a stable production of PUFA that are essential for cellular functions regardless of the energy state.

Other Potential SREBP Target Genes

SREBP and Other Carbohydrate Metabolism

UDP-glucose (galactose) 4-epimerase which involves conversion from galactose to glucose is a potential SREBP target gene. SREBP-1a transgenic liver express this enzyme 5.7-fold higher than wild-type, suggesting that SREBP-1 could be involved in galactose metabolism and possibly in lactose production. SREBP-1c could play a role in suckling mammary glands, in which FAS activity is high. UDP-glucose dehydrogenase also showed 5.0-fold induction in SREBP-1a overproduction. This enzyme catalyzes a step which is important for the uronic acid pathway. Physiological consequences of this pathway are the production of pentose and UDP-glucronate. UDP-glucuronate is used for production of proteoglycans and conjugates with steroid hormones, drugs, and bilirubin. These data could implicate roles of SREBP-1 in galactose, lactose, and glucuronate metabolism. Recently, we and another group found that hepatic SREBP-1c could be induced by ingestion of fructose as well as glucose. Considering that the pentose pathway is important in lipogenesis for production of NADPH, SREBP-1 is likely to be highly involved in sugar metabolism, in which transcriptional regulation of related genes are not fully understood.

However, further investigation on promoters of these and other enzymes in the pathways are required.

SREBP-1 AND LIPOGENIC GENES ARE DOWNREGULATED IN ADIPOCYTES OF OBESE ANIMALS

To gain insight into the pathogenesis of obesity, we examined, with the same microarray technique discussed earlier, the gene expression profile of *ob/ob* mouse, a genetically obese mouse model formed from a nonsense mutation in appetite-suppressing hormone leptin. Quite unexpectedly, as shown in Fig. 10.3, we found that the mRNA expression levels of lipogenic enzymes were lowered. Later we demonstrated that refeeding responses of SREBP-1 and its downstream lipogenic enzymes were markedly impaired in adipocytes

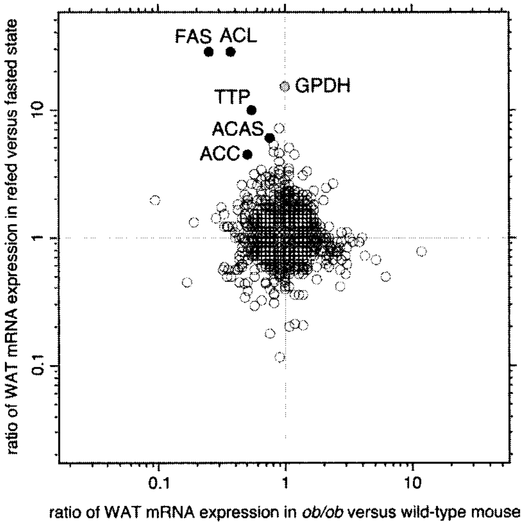

Figure 10.3 Lowered expression of SREBP-1-regulated lipogenic genes in *ob/ob* adipose. Increases in mRNA expression in adipose tissue after refeeding fasted mice (on vertical axis) and those in *ob/ob* adipose compared with wild-type (on horizontal axis) are plotted in 2D in logarithmic scale. The mRNA expression levels of lipogenic enzymes under SREBP-1 control (FAS, ACL, TTP, ACAS, and ACC; shown as solid circles) were lowered in *ob/ob* mice adipose tissue. GPDH that did not seem to be an SREBP-1 target gene because of no alteration in SREBP-1 transgenic mice was not suppressed in *ob/ob* adipose. Methods: For the *ob/ob* mice experiment, wild-type and *ob/ob* mice were sacrificed in a fed state and RNA was extracted from adipose tissue. RNA samples were pooled within each group and analyzed with IncyteGenomics DNA microarray. *ACAS*, acetyl-CoA synthase; *ACC*, acetyl-CoA carboxylase; *ACL*, ATP citrate lyase; *FAS*, fatty acid synthase; *GPDH*, glycerophosphate dehydrogenase; *TTP*, tricarboxylate transport protein; WAT, white adipose tissue.

of *ob/ob* mouse.[25] The similar results from microarray analyses performed elsewhere were reported by others.[26,27] Notably, this downregulation of SREBP-1 and lipogenic genes is also documented in humans[28–30] and seems to be an important characteristic of adipocytes in obesity.

In an attempt to search for the upstream regulator for this lipogenic gene suppression, we focused on the data by Soukas et al.[26] that $p21^{Waf1/CIP1}$ and Bax alpha were elevated two to threefold in *ob/ob* adipose tissue. Both of these genes are well-known p53 targets, so we hypothesized that p53 is activated in *ob/ob* adipocytes, leading to the activation of target genes such as p21 and at the same time to the suppression of SREBP-1 and its downstream lipogenic genes. The tumor suppressor p53 not only activates transcription of target genes through its response element, but also represses genes lacking the element by binding to and sequestering essential transcription factors such as TATA-binding protein.[31,32] To test this hypothesis, we intercrossed *ob/ob* and p53-knockout mice and demonstrated that the disruption of p53 in *ob/ob* mice completely suppressed the p53-regulated genes to wild-type levels and partially restored expression of lipogenic enzymes;[33] thus the hypothesis was proven.

This is an example where microarray analysis provides clues from which we can speculate upstream regulators. Microarray data can help us not only to search the downstream genes of a transcription factor but also to explore for upstream regulators. As we show here, gene expression profiling with microarray technique is of great assistance in the elucidation of transcriptionally regulated pathways.

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