Ad4BP/SF-1 regulates cholesterol synthesis to boost the production of steroids

Takashi Baba1,2, Hiroyuki Otake1, Miki Inoue2, Tetsuya Sato3, Yasuhiro Ishihara4, Ju-Yeon Moon5, Megumi Tsuchiya6, Kanako Miyabayashi1, Hidesato Ogawa6, Yuichi Shima1,2,7, LiXiang Wang8, Ryuichiro Sato9, Takeshi Yamazaki4, Mikita Suyama3, Masatoshi Nomura8,10, Man Ho Choi5, Yasuyuki Ohkawa11 & Ken-Ichirou Morohashi1,2

Housekeeping metabolic pathways such as glycolysis are active in all cell types. In addition, many types of cells are equipped with cell-specific metabolic pathways. To properly perform their functions, housekeeping and cell-specific metabolic pathways must function cooperatively. However, the regulatory mechanisms that couple metabolic pathways remain largely unknown. Recently, we showed that the steroidogenic cell-specific nuclear receptor Ad4BP/SF-1, which regulates steroidogenic genes, also regulates housekeeping glycolytic genes. Here, we identify cholesterogenic genes as the targets of Ad4BP/SF-1. Further, we reveal that Ad4BP/SF-1 regulates Hummr, a candidate mediator of cholesterol transport from endoplasmic reticula to mitochondria. Given that cholesterol is the starting material for steroidogenesis and is synthesized from acetyl-CoA, which partly originates from glucose, our results suggest that multiple biological processes involved in synthesizing steroid hormones are governed by Ad4BP/SF-1. To our knowledge, this study provides the first example where housekeeping and cell-specific metabolism are coordinated at the transcriptional level.
Steroid hormones vital for diverse biological processes are synthesized from cholesterol through multi-step reactions\(^1\sim^4\). Steroidogenesis is active only in functionally specialized steroidogenic cells, such as adrenocortical and testicular Leydig cells. Multiple studies have established that all cholesterogenic genes are regulated by the steroidogenic cell-specific transcription factor Ad4BP/SF-1 (NR5A1), which binds them directly\(^5\sim^8\). Recently, we showed that this transcription factor also directly regulates nearly all glycolytic genes, and thus also governs the expression of genes involved in housekeeping metabolism\(^9\). At that time, however, we could not determine why this steroidogenic cell-specific transcription factor regulates two apparently independent metabolic pathways, steroidogenesis and glycolysis. Because cholesterol is an essential component of all cells, its cellular levels are maintained within a narrow range by a complex control system that balances incorporation, excretion, de novo synthesis, and consumption\(^10\sim^12\). This control machinery is unique in that sensing of intracellular cholesterol concentration regulates proteolytic cleavage of the membrane-bound inactive form of a transcription factor, SREBP-2 (sterol regulatory element binding protein-2), into its active soluble form. Following proteolysis, the soluble form of SREBP-2 translocates into the nucleus to directly activate many cholesterogenic genes\(^11\).

In addition to its role as a key component of cellular membranes, cholesterol is used as the starting material for steroidogenesis in steroidogenic cells\(^1\sim^3\). Therefore, it is reasonable that steroidogenic cells would have a unique mechanism for meeting their augmented need for cholesterol. Indeed, it was reported that steroidogenic cells primarily use intracellular cholesterol stores for steroidogenesis in response to steroidogenic stimulation. Then, after the cholesterol stores decreased rapidly, de novo cholesterologenesis is activated and the synthesized cholesterol is used for steroidogenesis\(^13\). As mentioned above, Ad4BP/SF-1 regulates the genes implicated in both glycolytic and steroidogenic pathways. Given that pyruvate, the final product of glycolysis, can be converted to acetyl-CoA, the starting material for cholesterologenesis, we hypothesize that the glycolytic and steroidogenic pathways might be connected via the cholesterogenic pathway. Given the interconnections among these three metabolic pathways, we predict that Ad4BP/SF-1 would also regulate cholesterogenic genes, thereby collectively governing all metabolic processes from the one that utilizes glucose to the one that produces steroid hormones (Supplementary Fig. 1).

**Results**

**Indirect regulation of cholesterogenic genes by Ad4BP/SF-1.** To explore the possibility that Ad4BP/SF-1 is involved in cholesterogenic gene regulation, we obtained the transcriptomes of Y-1 cells, which are derived from a mouse adrenocortical tumor. The mRNA-seq data revealed that expression of 16 out of 20 cholesterogenic genes decreased below 70% of control levels in Ad4BP/SF-1 knockdown cells (Supplementary Fig. 2a). Consistent with the transcriptome data, quantitative RT-PCR (qRT-PCR) confirmed that the cholesterogenic genes, with the exception of Tm7sf2, were down-regulated by the knockdown (Fig. 1a, Supplementary Fig. 2b). Cyclic AMP (cAMP) stimulates steroidogenic gene expression\(^6\). Hence, we investigated whether cholesterogenic gene expression is activated by cAMP. Indeed, we found that expression of many cholesterogenic genes was increased by cAMP stimulation (Fig. 1a). In Ad4BP/SF-1 knockdown cells, the cAMP-induced up-regulation of cholesterogenic genes (except for Tm7sf2 and Hsd17b7) was reduced (Fig. 1a).

We next investigated whether similar knockdown effects would be observed in steroidogenic Leydig cells prepared from mouse testes. When these cells were cultured in the absence of cAMP, transcriptome analysis and qRT-PCR revealed that cholesterogenic genes were not collectively down-regulated by Ad4BP/SF-1 knockdown (Supplementary Fig. 3a and b). However, when the expression of these genes was activated by cAMP, they were mostly down-regulated by the knockdown (Supplementary Fig. 3a and b). Together, these findings suggest that Ad4BP/SF-1 is implicated in transcriptional regulation of nearly all cholesterogenic genes in adrenocortical Y-1 and cAMP-stimulated testicular Leydig cells.

SREBP-2, encoded by Srebf2, is a key regulator of cholesterogenic gene transcription\(^14\). Because a chromatin immunoprecipitation-sequencing (ChiP-seq) data set for SREBP-2 obtained from hepatocyte was available\(^14\), we investigated whether SREBP-2 ChIP peaks are present in cholesterogenic genes. Indeed, SREBP-2 ChIP peaks were detected in all cholesterogenic genes (Supplementary Fig. 4). Furthermore, Srebf2 was down-regulated by Ad4BP/SF-1 knockdown (Fig. 1b, Supplementary Fig. 5a and b). Cholesterogenic genes are up-regulated by SREBP-2 activated in response to a lowered intracellular cholesterol level\(^10\sim^12\). Thus, we expected that the decreased expression of Srebf2 leads to a reduction in active SREBP-2 protein, irrespective of intracellular cholesterol contents, and as a consequence, cholesterogenic genes are down-regulated. Indeed, Srebf2 knockdown (Supplementary Fig. 5c) resulted in down-regulation of many (17 out of 20) cholesterogenic genes in Y-1 cells (Fig. 1c, Supplementary Fig. 5d). These results raise the possibility that Ad4BP/SF-1 controls cholesterogenic genes indirectly by regulating Srebf2 transcription.

**Cooperative regulation by Ad4BP/SF-1 and SREBP-2.** The locations of the ChiP peaks of SREBP-2 and Ad4BP/SF-1 exhibited distinct features: the former accumulated near the transcription initiation sites (putative promoter regions) of all cholesterogenic genes (Supplementary Fig. 4), whereas the latter tended to accumulate far from the promoters. Hence, we investigated the functional correlation between Ad4BP/SF-1 and SREBP-2. Similar to Ad4BP/SF-1, SREBP-2 alone activated the reporter gene transcription (Fig. 3a). Moreover, these two factors cooperatively activated the reporter genes (Fig. 3a). In regard to
the mechanism underlying the cooperative action of Ad4BP/SF-1 and SREBP-2, we hypothesized that the two transcription factors physically interact. When purified FLAG-tagged SREBP-2 was incubated with Y-1 cell nuclear extract, and the mixture was subjected to immunoprecipitation with an anti-FLAG antibody, the immunoprecipitates contained Ad4BP/SF-1 (Fig. 3b). Moreover, direct protein–protein interaction was confirmed by an in vitro binding experiment using purified FLAG-Ad4BP/SF-1 and HA-SREBP-2 (Fig. 3c). Together, these findings indicate that Ad4BP/SF-1 regulates nearly all cholesterogenic genes in two ways: direct regulation in cooperation with SREBP-2, and indirect regulation through transcriptional induction of the Srebf2 gene (Fig. 3d).

Cholesterogenesis reduced by Ad4BP/SF-1 knockdown. These observations raised the possibility that Ad4BP/SF-1 knockdown decreases cholesterogenic activity. To measure activity, we cultured Y-1 cells in medium containing 14C-acetate, and then determined the amount of de novo synthesized 14C-cholesterol. As expected, the amount of 14C-cholesterol was decreased to 66% of control levels in Ad4BP/SF-1 knockdown cells (Fig. 4a). Consistent with this, the amount of cellular cholesterol was mildly (~15%) decreased by Ad4BP/SF-1 knockdown (Fig. 4b). The levels of the intermediate products lanosterol, desmosterol, and 7-dehydrocholesterol were decreased by the knockdown, whereas the level of lathosterol was unaffected. The level of cholesteryl-arachidonate, the major cholesteryl ester in the adrenal cortex, also decreased, whereas the level of cholesteryl-myristate was not changed. Together, these findings indicate that Ad4BP/SF-1 knockdown reduced cholesterol synthesis through the expression of cholesterogenic genes.

Cholesterogenesis and steroidogenesis bridged by Ad4BP/SF-1. If the glycolytic and steroidogenic pathways are connected via the cholesterogenic pathway, Ad4BP/SF-1 may govern multiple metabolic pathways from glycolysis to steroidogenesis. To test this hypothesis, two gaps between the metabolic pathways remained to be bridged.

The first of these gaps is between cholesterogenesis and steroidogenesis. In steroidogenic cells, cholesterol is supplied via de novo cholesterogenesis and receptor-mediated endocytosis of low-density and high-density lipoproteins13,16–18. Ldlr and Scarb1 encode the receptors responsible for the endocytosis of low-density and high-density lipoproteins, respectively. Similar to
the cholesterogenic genes, mRNA-seq studies showed that the expression of these receptor genes was decreased by Ad4BP/SF-1 knockdown, whereas that of genes related to excretion of cholesterol (Abca1 and Abcg1)19,20 was not altered (Supplementary Fig. 6a). The endocytosed lipoproteins are hydrolyzed in lysosomes, and then cholesterol is transported to endoplasmic reticula21, where the final reaction of cholesterogenesis takes place. Because CYP11A1, which catalyzes the initial reaction of steroidogenesis, is localized in the mitochondrial inner membrane22, cholesterol must be transported from endoplasmic reticula to the mitochondrial inner membrane. This transport consists of two steps: first from endoplasmic reticula to mitochondrial outer membrane, and the second from the outer membrane to inner membrane22. The STAR protein mediates the transport of cholesterol from mitochondrial outer membrane to inner membrane, and the encoding gene is regulated by Ad4BP/SF-123.

Importantly, HUMMR (hypoxia up-regulated mitochondrial movement regulator, also called MGARP or OSAP) was identified recently as a plausible candidate for the mediator of cholesterol transport from endoplasmic reticula to mitochondrial outer membrane24. Therefore, we investigated whether Ad4BP/SF-1 regulates the Hummr gene. qRT-PCR and mRNA-seq revealed that Hummr expression was decreased by Ad4BP/SF-1 knockdown in both Y-1 and Leydig cells, irrespective of cAMP treatment (Fig. 5a, Supplementary Fig. 6b, c). Furthermore, ChIP-seq revealed a clear Ad4BP/SF-1 peak at the Hummr locus in both Y-1 and Leydig cells (Fig. 5b). The accumulation of Ad4BP/SF-1 on the Hummr locus was confirmed by ChIP-qPCR (Fig. 5c). In addition, activity of a luciferase reporter gene carrying the ChIP-
peak and promoter region of *Hummr* was suppressed by Ad4BP/SF-1 knockdown (Fig. 5d, Supplementary Fig. 6d) and activated by ectopically expressed Ad4BP/SF-1 in a dose-dependent manner (Fig. 5e), indicating that the *Hummr* gene is regulated by Ad4BP/SF-1 via the ChIP-peak region.

Given that Ad4BP/SF-1 regulates *Hummr* gene, Ad4BP/SF-1 knockdown should reduce the amount of mitochondrial cholesterol. Accordingly, we determined the amount of mitochondrial cholesterol in knockdown Y-1 cells, and found that it was indeed reduced to approximately 60% of control levels in the knockdown cells (Fig. 5f). Furthermore, overexpression of HUMMR in Ad4BP/SF-1 knockdown cells markedly increased mitochondrial cholesterol (Fig. 5f), suggesting that the decrease was primarily due to reduced expression of *Hummr*. These results suggest that Ad4BP/SF-1 bridges the gap between the cholesterogenic and steroidogenic pathways by regulating *Hummr*, which has been implicated in cholesterol trafficking from endoplasmic reticula to mitochondria.

**Glycolysis and cholesterogenesis bridged by Ad4BP/SF-1.** Next, we examined the involvement of Ad4BP/SF-1 in the regulation of the genes bridging the second gap between glycolysis and cholesterogenesis. The end product of glycolysis, pyruvate, is converted to cytosolic acetyl-CoA, which is a starting material for cholesterogenesis. At least nine genes encoding enzymes or transporters are involved in this conversion (Supplementary Fig. 7a). According to the transcriptome data, the expression of the genes newly identified as potential targets of Ad4BP/SF-1 (Supplementary Fig. 7b). Among them, ACLY (ATP citrate lyase) has been established as a key enzyme for the synthesis of cytosolic acetyl-CoA, and its expression is regulated by SREBP-2. Hence, we investigated *Acly* as a potential target of Ad4BP/SF-1 in steroidogenic cells. The ChIP-seq data demonstrated that Ad4BP/SF-1 accumulated in the *Acly* locus (Supplementary Fig. 8a), which was confirmed by ChIP-qPCR (Supplementary Fig. 8b). Consistent with this, *Acly* expression was decreased by Ad4BP/SF-1 knockdown (Supplementary Fig. 8c). We then examined the transcription activity of the peak region using reporter gene assays. Reporter gene activity was suppressed by Ad4BP/SF-1 knockdown (Supplementary Fig. 8d) and activated by overexpression of Ad4BP/SF-1 in a dose-dependent manner (Supplementary Fig. 8e). Together, these results suggest that *Acly* is a target gene of Ad4BP/SF-1. Given that ACLY is crucial for producing cytosolic acetyl-CoA, these results suggest that Ad4BP/SF-1 bridges the second gap from glycolysis to cholesterogenesis.

**Discussion**

Cell-specific transcription factors have been studied in regard to their cell-specific functions, and thought to be specialized to regulate cell-specific genes. However, we previously reported that Ad4BP/SF-1 regulates housekeeping glycolytic genes, as well as cell-specific steroidogenic genes. At that time, we could not explain why a tissue-specific transcription factor would be involved in housekeeping metabolic regulation. However, the results of this study suggest a novel mechanism by which this tissue-specific transcription factor integrates three distinct metabolic pathways—glycolysis, cholesterogenesis, and steroidogenesis—into a combined regulatory unit (Supplementary Fig. 1). ERα (NR3B1) regulates genes involved in housekeeping energy metabolism, including glycolysis, the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation, whereas Nrf2 regulates genes involved in the pentose phosphate pathway. Considering their preferential expression in certain types of cells, these factors might also link housekeeping and cell-specific metabolism.

Given that Ad4BP/SF-1 is the predominant regulator of cholesterogenic genes in steroidogenic cells, we expected that expression of Ad4BP/SF-1 and cholesterogenic genes would be synchronized under specific physiological conditions. We recently reported that during the differentiation of mouse fetal Leydig cells from their progenitors, the expression of Ad4BP/SF-1 increases. At the same time, the genes involved in glycolysis, the TCA cycle, oxidative phosphorylation, and steroidogenesis are activated. Therefore, we investigated whether the genes newly identified as Ad4BP/SF-1 targets in this study are activated during differentiation. Similar to the metabolic genes above, the mRNA-seq data revealed that all cholesterogenic genes, as well as *Hummr* and *Acly*, were markedly up-regulated (Supplementary Fig. 9a). The up-regulation of cholesterogenic genes was confirmed by qRT-PCR (Supplementary Fig. 9b). Interestingly, Srebf2 expression was only slightly increased, by less than 2-fold, whereas expression of cholesterogenic genes was increased by 2- to 22-fold (Supplementary Fig. 9a and b). Ad4BP/SF-1 itself was increased by 17-fold, suggesting that the elevated expression of cholesterogenic genes during differentiation of fetal Leydig cells is at least in part due to increased expression of Ad4BP/SF-1.
Although the liver is the principal organ involved in de novo synthesis of cholesterol from acetyl-CoA, most cell types, including adrenal cortex and testicular steroidogenic cells, have the ability to synthesize cholesterol. The whole cholesterogenic pathway consists of multiple reactions executed by 20 enzymes. All genes encoding these enzymes have been regulated by SREBP-2.12 Our investigation of the ChIP-seq data reported by Seo et al.14 revealed the accumulation of SREBP-2 near the transcription initiation sites (putative promoter regions) of all cholesterogenic genes, arguing in favor of the orchestrated regulation of all cholesterogenic genes by SREBP-2.

Our study implicates Ad4BP/SF-1, originally identified as the principal transcriptional regulator of steroidogenic genes, in control of cholesterogenic gene expression via regulation of Srebf2 (encoding SREBP-2) and/or direct binding to the cholesterogenic genes themselves. Together, unlike hepatocytes, in which Ad4BP/SF-1 is not expressed, in steroidogenic cells cholesterogenic genes are regulated both by Ad4BP/SF-1 and SREBP-2. Because the demand for cholesterol is augmented in steroidogenic cells upon stimulation by pituitary hormones, these cells may be uniquely equipped to meet this demand. Indeed, it was reported that Scarb1, which encodes high-density lipoprotein (HDL) receptor responsible for cholesterol incorporation, is directly regulated by Ad4BP/SF-1.15 Our miRNA-seq result also suggested that low-density lipoprotein as well as HDL receptor genes, but not the genes required for cholesterol excretion (Abca1 and Abcg1), are regulated by Ad4BP/SF-1. Taken together, in the regulation of cholesterogenesis and perhaps cholesterol incorporation, Ad4BP/SF-1 provides an efficient mechanism for meeting the particular demand of cholesterol in the steroidogenic cells.

Steroidogenic cells do not store large amounts of steroids. Therefore, to secrete steroids upon stimulation, they must rapidly synthesize steroids by coordinating multiple routes that supply the materials for synthesis. Steroidogenic reactions consume NADPH, which is synthesized by multiple enzymes. Among them, malic enzyme and malyltetrahydrofolate dehydrogenase make a significant contribution to NADPH synthesis.24,25 Our recent study demonstrated that the genes encoding the enzymes mentioned above are direct target genes of Ad4BP/SF-1.26 In addition, our previous study implicated Ad4BP/SF-1 in glycolytic gene regulation. Glycolysis produces ATP, which is required for cholesterogenesis and for synthesis of cytosolic acetyl-CoA, mediated by ATP citrate lyase. Therefore, it is likely that glycolysis and cholesterogenesis are coupled by Ad4BP/SF-1 to balance the supply and the demand for ATP. Steroidogenesis is the most critical and specific function of steroidogenic cells. Together with our previously published results, the findings of this study suggest that Ad4BP/SF-1 orchestrates distinct metabolic pathways to supply the building blocks for steriodogenesis so that steroidogenic cells can accomplish their particular function (Supplementary Fig. 1).

In light of the possibility that Ad4BP/SF-1 might regulate glycolysis, cholesterogenesis, and steroidogenesis, as a unit, we noticed two gaps between these metabolic pathways. The first gap is between cholesterol trafficking from the endoplasmic reticulum, where cholesterogenesis terminates, to the mitochondria, where steroidogenesis starts. Although not all components of the machinery have been identified, a recent study found that a principal component, HuMRR (also referred to as MGARP or OSAP), is critical for cholesterol transfer to the mitochondrial outer membrane.24,25 Here, we showed that HuMRR is regulated by Ad4BP/SF-1, suggesting that Ad4BP/SF-1 bridges the gap between cholesterogenesis and steroidogenesis.

As for the second gap, between glycolysis and cholesterogenesis, we identified Acly as a target gene of Ad4BP/SF-1. Acetyl-CoA synthesized by AGLY can be used for lipogenesis, as well as cholesterogenesis. Therefore, Acly may act downstream of both lipogenic and cholesterogenic regulation. Indeed, SREBP-1a and SREBP-2, which predominantly regulate the genes responsible for lipogenesis and cholesterogenesis, respectively, regulate Acly by binding to an SREBP-binding sequence in the promoter.26 Therefore, Ad4BP/SF-1 and SREBP-2 together could bridge the gap between glycolysis and cholesterogenesis.

To our knowledge, our study provides the first example, where a single transcription factor can orchestrate housekeeping and cell-specific metabolism, enabling cells to effectively perform their particular functions.

Methods

Cell culture. Y-1 cells derived from a mouse adrenocortical tumor were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Wako, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) and 1x penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA) on collagen type I coated dishes (Iwaki, Tokyo, Japan). Mouse testicular Leydig cells were cultured in DMEM supplemented with 10% FBS and 1x penicillin–streptomycin on collagen type I coated dishes. HeLa cells derived from human cervical adenocarcinoma were grown in DMEM supplemented with 10% FBS and 1x penicillin–streptomycin.

Isolation of Leydig cells. Leydig cells were isolated from mouse testes by Percoll density gradient centrifugation as previously described.27 Briefly, testes harvested from ten 9-week-old male ICR mice were decapsulated and incubated for 10 min at 34 °C with gentle stirring with DMEM supplemented with 0.1% bovine serum albumin (BSA) (Roche, Basel, Swiss), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.025% soybean trypsin inhibitor (Sigma-Aldrich, St. Louis, MO, USA), and 0.5 mg/ml collagenase (Invitrogen), followed by filtration through a 70-μm cell strainer (BD Bioscience, Franklin Lakes, NJ, USA). The testicular cells were centrifuged in a discontinuous density gradient of Percoll (20, 30, 50, and 60%), GE Healthcare, Little Chalfont, UK) at 25,000 × g for 45 min at 4 °C. Leydig cells forming a thick band between the 30 and 50% layers were recovered and seeded onto collagen type...
**Fig. 5** Regulation of *Hummr* by Ad4BP/SF-1. a, The expression of *Hummr* in Ad4BP/SF-1 knockdown or control Y-1 cells was examined by qRT-PCR. *p < 0.05, n = 3. b, Ad4BP/SF-1 ChIP peaks at the *Hummr* gene in Y-1 and Leydig cells in the presence or absence of cAMP are shown. The ChIP peaks are enclosed by a red square. c, Accumulation of Ad4BP/SF-1 to the ChIP peak of *Hummr* gene was confirmed by ChIP-qPCR. *p < 0.05, n = 3. a, c, Average values and SDs are shown. The average value of the control was normalized to 1.0. d, A luciferase reporter gene, *Hummr-Luc*, was constructed with the promoter of *Hummr* (white square). Ovals represent potential Ad4BP/SF-1-binding sites. The reporter was transfected into Y-1 cells with siAd4BP/SF-1 or siControl. Average values and SDs of the luciferase activities are indicated. The average value in the absence of siRNA was normalized to 1.0. *p < 0.05, n = 3. e, Effects of Ad4BP/SF-1 overexpression on *Hummr-Luc* was examined. The reporter was transfected into HeLa cells with increasing amounts of the expression vector for Ad4BP/SF-1. Average values and SDs of luciferase activities are indicated. The average value for pGL3 basic in the absence of the expression vector was normalized to 1.0. *p < 0.05, n = 3. f, Levels of mitochondrial cholesterol in control, Ad4BP/SF-1 knockdown, and Hummr-overexpressing Ad4BP/SF-1 knockdown Y-1 cells. Average values and SDs are shown. *p < 0.05, n = 5

I coated dishes. Unattached cells, such as germ line cells, were washed out with phosphate-buffered saline (PBS) 4 h after seeding.

**Knockdown of Ad4BP/SF-1 and Sreb2.** For knockdown of Ad4BP/SF-1, we used two distinct small interfering RNAs (siRNAs) to exclude a possible off-target effect. Y-1 cells were transfected for 6 h with siRNA duplexes (Mission siRNA: Mm_Nr5a1_1635, 5′-CCUUUACUCCAUUGUGATT-3′ (#1635); Mm_Nr5a1_1636, 5′-CAUAACAGUGACCGAGATT-3′ (#1636); Sigma-Aldrich) using Lipofectamine RNAiMAX (Invitrogen) for 6 h. The medium was replaced, and the cells were cultured for an additional 48 h. The results obtained with siRNA #1635 are provided in the main text (Fig. 1a, b, Fig. 2c, Fig. 3a, b, and Fig. 4a, d, j), whereas those obtained with siRNA #1636 are provided in Supplementary Fig. 2b, 4b, 6b, 6c, and 6d. A control siRNA (Stealth RNAi Negative Control Medium GC Duplex, Invitrogen) was used as a negative control.

For knockdown of Ad4BP/SF-1 in Leydig cells, siRNA duplexes (stealth RNAi); Nr5a1-MS2240945, 5′-ACAAGGUGUAUCCCAAGGGCCG-3′; Invitrogen) was transfected for 4 h after the cells recovered by Percoll centrifugation were plated. The culture medium was replaced, and the cells were cultured for an additional 39 h. A control siRNA (Stealth RNAi Negative Control Medium GC Duplex, Invitrogen) was used as a negative control.

For the knockdown of Sreb2, Y-1 cells were transfected with siRNA duplex (stealth RNAi); Sreb2-MS277288, 5′-GGGTTTCCTTTGCTAGCTACTTTTFAA-3′; Invitrogen) for 6 h, and then cultured for 48 h.

**RNA-seq.** Poly-A+ RNA was prepared from siAd4BP/SF-1-treated or siControl-treated Y-1 cells using oligo(dT) magnetic beads. The mRNA was prepared using NEBNext Ultra Directional RNA Library Prep kit for Illumina (NEB, Ipswich, MA, USA). After the quality of the library was validated on an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA), the samples were subjected to sequencing with a next-generation sequencer (HiSeq500, Illumina, San Diego, CA, USA).

Total RNA was prepared from siAd4BP/SF-1-treated or siControl-treated Leydig cells using the RNeasy Mini kit (Qiagen, Hilden, Germany). Then, ribosomal RNAs were removed from the total RNA using the Ribo-zero RNA removal kit (Illumina). RNA-seq libraries were prepared from the RNA-depleted RNAs using the Tru-Seq RNA sample prep kit v2 (Illumina). After the library quality was validated on an Agilent Bioanalyzer 2100, the RNA-seq libraries were subjected to sequencing on an Illumina GAIIx. The RNA-seq reads were aligned to the reference mouse genome sequence, UCSC mm10, using TopHat version 2.0.13 with the default parameters. Cufflinks (version 2.2.1) was then used with default parameters to assemble the transcripts and calculate fragments per kilobase of transcript per million mapped fragments.

**Quantitative RT-PCR.** Total RNAs were prepared from Y-1 and Leydig cells treated with siAd4BP/SF-1 or siControl. cDNAs were synthesized from the RNAs using M-MLV reverse transcriptase (Invitrogen), and then subjected to qRT-PCR. Real-time PCR was performed on a CFX96 instrument (Bio-Rad, Hercules, CA, USA) using SYBR Select master mix (Applied Biosystems). Primers used for qRT-PCR are listed in the Supplementary Data.

**Chromatin immunoprecipitation-quantitative PCR.** ChIP was performed according to the previously described procedure. Briefly, 2.0 × 10⁶ Y-1 cells or Leydig cells fixed with 1% formaldehyde for 5 min at room temperature, and then glidine was added to a final concentration of 125 mM to stop the crosslinking reaction. Then, the fixed cells were lysed with 2 ml ChIP lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% sodium dodecyl sulfate (SDS)), followed by sonication (UCD-300 Bioruptor, Diagenode, Belgium) at 4°C. After dilution of SDS by addition of 9 volumes of dilution buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 150 mM NaCl, 1% Triton X-100), the sheared chromatin was subjected to immunoprecipitation with 2 μg of anti-Ad4BP/SF-1 antibody previously produced by ourselves. Normal rabbit IgG was used as a negative control for the immunoprecipitation. The immunoprecipitates were collected using Dynabeads with Protein A (Life Technologies, Carlsbad, CA, USA), and then sequentially washed with 1 ml of high-salt ChIP-RIPA buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 500 mM NaCl, 0.1% SDS, 0.5% NP-40), three times with 700 μl high-salt ChIP-RIPA buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 500 mM NaCl, 0.1% SDS, 0.5% NP-40), three times with 700 μl high-salt ChIP-RIPA buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 500 mM NaCl, 0.1% SDS, 0.5% NP-40), three times with 700 μl high-salt ChIP-RIPA buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 500 mM NaCl, 0.1% SDS, 0.5% NP-40), and once with 700 μl TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), all at room temperature. Finally, the chromatin fragments were eluted from the beads with 100 μl elution buffer (0.1 M NaHCO₃, 1% SDS, 10 mM dithiothreitol). After the crosslinks were reversed by heating at 65°C for 16 h, DNA fragments were purified using the QIAquick PCR Purification kit (Qiagen). The purified DNAs were subjected to real-time PCR using primer sets listed in Supplementary Data 1. Because ChIP-seq showed that Ad4BP/SF-1 does not bind to exon 7 of the Star gene, this region was used as a negative control in ChIP-qPCR.

ChIP-sequence. The DNA fragments prepared as described above were used to construct a ChIP-seq library using the NEBNext DNA sample prep master mix set 1.
Luciferase reporter gene assay. Luciferase reporter genes were constructed using the pGL3 basic vector (Promega, Madison, WI, USA) with upstream regions and the regions where Ad4BP/SF-1 was found to be accumulated by ChIP-seq (ChIP peak regions). For construction of reporter plasmids, fragments of Fdps-Luc, Fdft1-Luc, Msmo1-Luc, Memmo-Luc, Fdft1-Luc, SUMF1-Luc, Mom-1-Luc, Sf21 cells according to the manufacturer, were used. Structures of the reporters are illustrated in Fig.2c, Fig.4d, and Supplementary Fig.6c and 8d. In the experiments shown in Fig.2d and Supplementary Fig.6d, uniquely mapped reads were analyzed using MACS (Model-based Analysis for ChIP-Seq) program version 1.4.2 with the option “–nomodel”. The threshold P values for enriched regions of Ad4BP/SF-1 were set to 10−5.

Preparation of nuclear extract from Y-1 cells. Y-1 cells (4.0 × 107) were harvested and suspended in 500 μl of hypotonic buffer (10 mM HEPES-KOH, pH 7.9, 1.5 mM MgCl2, 10 mM KCl) supplemented with phenylmethylsulfonyl fluoride (PMSF) (Nacalai Tesque, Kyoto, Japan) and benzamidine (Nacalai Tesque). The cells were pelleted by centrifugation at 900 × g for 15 min at 4 °C. The pellet nuclei were suspended in 200 μl of 50 mM HEPES, pH 7.8, 10 mM NaCl, 0.1 mM EDTA, 5 mM MgCl2, 2% glycerol, and then the same volume of 50 mM HEPES, pH 7.8, 800 mM NaCl, 0.1 mM EDTA, 5 mM MgCl2. 2% glycerol was added. To extract nuclear proteins, the sample was kept on ice for 30 min. Then, the mixture was centrifuged at 15,000 rpm for 5 min at 4 °C, and the supernatant was collected as nuclear extract. The nuclear extract was dialyzed overnight with 1 L of binding buffer (see next paragraph for details) supplemented with PMSF and benzamidine, and then used for the subsequent experiments.

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Author contributions

T.B., M.O., M.I. and K.-I.M. conceived and designed the experimental approach and performed experiments. T.B. and K.-I.M. prepared the manuscript, T.S. and M.S. contributed to the computational analyses for mRNA-seq and ChIP-seq. K.M. and Y.S. constructed the mRNA-seq libraries and performed gene expression analyses. Y.I. and T.Y. measured 14C-cholesterol level. J.-Y.M. and M.H.C. performed GC/MS analyses. M.S. and T.B. prepared purified proteins. L.W. and M.N. performed the isolation of mitochondria. R.S. prepared expression plasmids of SREBP-2. Y.O. performed deep sequencing of the ChIP-seq and mRNA-seq libraries.

Additional information

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