

Role of Ad4-Binding Protein/Steroidogenic Factor 1 in regulating NADPH production in adrenocortical Y-1 cells

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<https://doi.org/10.15017/1806838>

出版情報：九州大学, 2016, 博士（理学）, 課程博士
バージョン：
権利関係：全文ファイル公表済



**Role of Ad4-Binding Protein/Steroidogenic Factor 1 in regulating
NADPH production in adrenocortical Y-1 cells**

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Table of Contents

SUMMARY	4
INTRODUCTION	5
MATERIALS AND METHODS	
<i>Knockdown of genes</i>	7
<i>Western blot</i>	7
<i>RT-qPCR</i>	8
<i>Dataset description</i>	8
<i>Chromatin immunoprecipitation (ChIP)-qPCR</i>	9
<i>Luciferase reporter assay</i>	10
<i>Measurement of NADPH levels</i>	10
RESULTS	
<i>Expression of genes involved in NADPH generation</i>	12
<i>Accumulation of Ad4BP/SF-1 in the gene loci involved in NADPH generation</i>	13
<i>Transcriptional activity of Ad4BP/SF-1 accumulated region</i>	14
<i>Intracellular NADPH concentration regulated by Ad4BP/SF-1 through target gene expression</i>	15
DISCUSSION	
<i>Genes involved in NADPH production as the direct targets of Ad4BP/SF-1</i>	16
<i>Significance of NADPH production regulated by Ad4BP/SF-1 in steroidogenic cells</i>	17
REFERENCES	20
ACKNOWLEDGEMENTS	25
LIST OF FIGURES	
<i>Fig. 1</i>	26
<i>Fig. 2</i>	27
<i>Fig. 3</i>	28

<i>Fig. 4</i>	29
<i>Fig. 5</i>	30
<i>Fig. 6</i>	31
<i>Fig. 7</i>	32
<i>Fig. 8</i>	33
<i>Fig. 9</i>	34

SUMMARY

Ad4BP/SF-1 (Ad4-Binding Protein/Steroidogenic Factor 1), a member of the nuclear receptor superfamily, is expressed in steroidogenic cells and regulates all steroidogenic gene expression. Based on the mRNA and chromatin immunoprecipitation sequencing carried out in the lab, it was demonstrated that Ad4BP/SF-1 directly regulates the expression of nearly all glycolytic genes. It's generally believed that the pentose phosphate pathway mainly contributes to the production of nicotinamide adenine dinucleotide phosphate (NADPH). Although the expression of pentose phosphate pathway genes and intracellular NADPH were decreased by Ad4BP/SF-1 knockdown, these genes were not the direct targets of Ad4BP/SF-1. My study therefore investigates whether Ad4BP/SF-1 directly regulates genes implicated in NADPH production. Examination of previously published data sets of mRNA sequence (mRNA-seq) and chromatin immunoprecipitation sequence (ChIP-seq) strongly suggested a possibility that other NADPH-producing genes, such as *malic enzyme 1 (Me1)* and *methylenetetrahydrofolate dehydrogenase 2 (Mthfd2)*, are the direct targets of Ad4BP/SF-1. Reporter gene assays and determination of intracellular NADPH concentration supported the notion that Ad4BP/SF-1 regulates NADPH production by regulating these genes. NADPH is required for macromolecule synthesis of compounds such as steroids, and for detoxification of reactive oxygen species. When synthesizing steroid hormones, steroidogenic cells consume NADPH through enzymatic reactions mediated by steroidogenic P450s. NADPH is also consumed through elimination of reactive oxygen species produced as the byproducts of the P450 reaction. Overall, Ad4BP/SF-1 potentially maintains the intracellular NADPH level through cooperative regulation of genes involved in the biological processes both NADPH consumption and supply in Y-1 cells.

INTRODUCTION

NADPH is a crucial reductive power in a variety of cellular biosynthetic reactions, such as the synthesis of fatty acids, cholesterol, and steroid hormones. In addition to these reactions, NADPH is consumed during the metabolism of xenobiotic (toxic) compounds (including drugs), leading to their detoxification and then excretion. NADPH is also required to maintain reduced glutathione (GSH) pools to uphold cellular redox homeostasis. Interestingly, NADPH is consumed by NADPH oxidase to produce superoxide and reactive oxygen species, which are subsequently used for defense against pathogens (1-3). Considering its involvement in these pivotal functions, NADPH is regarded as a critical compound for cellular activity, proliferation, and survival (4). The cellular NADPH pool is maintained through multiple reactions. Of the enzymes involved in the pentose phosphate pathway (PPP), glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) are well known to generate NADPH. Among other metabolic reactions, Isocitrate dehydrogenase (IDH), malic enzyme (ME), and methylenetetrahydrofolate dehydrogenase (MTHFD) also mediate NADPH production (5-7).

Ad4BP/SF-1, a member of the nuclear receptor superfamily, was identified as the key transcription factor regulating nearly all steroidogenic genes via direct binding to the gene loci (8-13). Consistent with this function, it is expressed in steroidogenic cells such as the adrenal cortex and gonads (14). In addition to the functional involvement of Ad4BP/SF-1 in steroidogenic gene regulation, our recent knockdown and ChIP-seq studies demonstrated that the transcription factor contributes to glycolytic gene regulation by direct binding to the gene loci (15). Indeed, knockdown of Ad4BP/SF-1 decreased the intracellular ATP concentration, as well as the concentration of NADPH (15). However, it remained unclear which genes are directly

regulated by Ad4BP/SF-1 and thus responsible for the decrease of NADPH after Ad4BP/SF-1 knockdown.

In the present study, I investigated whether Ad4BP/SF-1 in steroidogenic cells is functionally implicated in NADPH production through transcriptional regulation of genes related to NADPH production. The results show that Ad4BP/SF-1 regulates intracellular NADPH concentration via transcription of *Me1* and *Mthfd2* genes.

MATERIALS AND METHODS

Knockdown of genes

Y-1 cells (mouse adrenocortical tumor cell line) were cultured on TC-coated 24-well plates (Greiner Bio One, Kremsmünster, Austria) in DMEM (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum and 1X penicillin-streptomycin (Thermo Fisher Scientific, MA, USA). The cells were transfected with si*Ad4BP/SF-1* (Mm Nr5a1_1635: 5'-CCUUUAUCUCCAUGUCGATT-3'; Sigma, St. Louis, MO, USA), si*Me1* (Mm_Me1_4288: 5'-CAGAUGUACAGCACUAAUUTT-3'; Sigma), or si*Mthfd2* (Silencer® Select siRNA; s70134: 5'-CAUUAUUGCCGCAAAGAAA-3'; Thermo Fisher Scientific) using lipofectamine RNAiMAX reagent (Thermo Fisher Scientific) according to the manufacture's instructions for 24 or 48 h. A control siRNA (Stealth RNAi Negative Control Medium GC Duplex; Thermo Fisher Scientific) was used as a negative control.

Western blot

Whole-cell lysates were prepared from Y-1 cells treated with si*Ad4BP/SF-1*, si*Me1*, si*Mthfd2*, or si*Control* using a lysis buffer containing 50 mM Tris-HCl (pH8.0), 50 mM NaCl, 1 mM EDTA, and 1% SDS, followed by sonication. The protein concentration was determined using a BCA Protein Assay Kit (Thermo Fisher Scientific), and 10 µg of whole-cell lysate was used for SDS-polyacrylamide gel electrophoresis, followed by immunoblotting. The following primary antibodies were used: anti-Ad4BP/SF-1 antiserum (1:1000) (14), anti-ME1 antibody (ab97445, 1:500; Abcam, Cambridge, UK), anti-MTHFD2 antibody (ab151447, 1:500; Abcam) or anti- α -Tubulin antibody (T-9026, 1:1000; Sigma-Aldrich). Anti-rabbit donkey IgG (1:1000) and anti-mouse donkey IgG (1:1000; both GE Healthcare) were used as the

secondary antibodies. Bound antibodies were detected using Chemi-Lumi One L reagent (Nacalaitesque, Kyoto, Japan)

RT-qPCR

Total RNA was extracted from Y-1 cells treated with si*Ad4BP/SF-1* or si*Control*. cDNA was then synthesized using M-MLV reverse transcriptase (Thermo Fisher Scientific) and subjected to quantitative RT-PCR (qRT-PCR). qRT-PCR was performed with an ABI 7500 real-time system (Thermo Fisher Scientific) using THUNDERBIRD SYBR qPCR mix (Toyobo, Osaka, Japan). The following primers were used for RT-PCR:

5'- TATGCTAGGCCCACCCAACCTGGAC -3' and
5'- ATTCTTGTCTTGGGGCTGTGGGT -3' for *Ad4BP/SF-1*,
5'- TCTCATCTCTGAGCAACCCCTGGC -3' and
5'- TTGTCTGGGAACCACTGTCTGGCA -3' for *Me1*,
5'- GCGGGTGCTTTGAGGCCTATTTGT -3' and
5'- CCAACGGGGTACGTGAAAGACGTG -3' for *Mthfd2*,
5'- TTCTCTTCTCCTGTGACCCAGCCT-3', and
5'- ATGCAGCAGGTCAGCAGAAATCCA-3' for *Idh1*, 5'-
ATCCTGGCCTCACTGTCCACCTTC-3', and
5'- AAACGCAGCTCAGTAACAGTCCGC -3' for *Actb*.

Data sets used in this study

The mRNA-seq data sets of Y-1 adrenocortical cells treated with control siRNA and si*Ad4BP/SF-1*, and the data sets of ChIP-Seq with the antibodies for Ad4BP/SF-1 and H3K27Ac from Y-1 cells, have both been published previously, as has a

description of data processing (15). ChIP-Seq and mRNA-Seq data sets were deposited in DDBJ/EMBL/GenBank under the accession code DRA000853.

ChIP-qPCR

ChIP was performed according to the method described by Winnay *et al.* (16). Briefly, Y-1 cells were fixed with 1% formaldehyde for 5 min at room temperature, and the crosslinking reaction was stopped by glycine at a final concentration of 125 mM. The fixed cells were lysed with ChIP lysis buffer (50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% SDS), followed by sonication (UCD-300 Bioruptor; Diagenode, Belgium). SDS was diluted 10 times with dilution buffer (20 mM Tris-HCl (pH 8.0), 2 mM EDTA, 150 mM NaCl, 1% Triton X-100). The sheared chromatin was then subjected to immunoprecipitation with anti-Ad4BP/SF-1. The immunoprecipitates were collected using Dynabeads with Protein A (Thermo Fisher Scientific), and were sequentially washed with ChIP-RIPA buffer (50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.1% SDS, 0.5% NP-40), 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 LiCl buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 250 mM LiCl, 0.1% NP-40). Finally, the chromatin fragments were eluted from the beads with elution buffer (0.1 M NaHCO₃, 1% SDS, 10 mM DTT). After the crosslinks were reverted by heating at 65°C for 16 h, DNA fragments were purified using the QIAquick PCR Purification kit (Qiagen, Netherlands). Purified DNAs were subjected to real-time PCR using the following primer sets: 5'- CCGGCCCAGGCTTCAAGGAC -3' and 5'- AGCTCCTCCCCCGCAACCAT -3' for *Aldoa* locus, and 5'- CCCACTGCATAGCTGTGAAGGCT -3' and 5'- TCCCCTGTTCGTAGCTGCTGGT -3' for *Star* EX.VII. 5'-

AGAGTCAGTGTATGGTGCCC -3' and 5'- GGCTGACCTTGAAGTCACGA -3' for *Me1*, 5'- CCCAGCACTGTGGGAGTC -3' and 5'- GACAAGGTCTTATATAGTCCAGGATG -3' for *Mthfd2*.

Luciferase reporter assay

The reporter genes *Me1*-luc and *Mthfd2*-luc were constructed using the promotorless vector pGL3-basic (Promega, Madison, WI, USA). *Me1*-luc carries -322 to +47 and +10208 to +10635 as the promoter and enhancer, respectively, while *Mthfd2*-luc carries -1192 to +36 and +4059 to +5049 as the promoter and enhancer, respectively. Possible binding sites of Ad4BP/SF-1(13) in the enhancer regions were mutated to lose the ability to be recognized (15). After the original enhancer was substituted by the mutated DNA sequences synthesized (IDT, Coralville, IOWA, USA), reporter plasmids *Me1*-mut-luc and *Mthfd2*-mut-luc were produced. Y-1, HEK293 (human embryonic kidney), and HeLa (human cervical adenocarcinoma) cells were transfected with the reporter plasmids above using lipofectamine 2000 reagent according to the manufacture's instruction (Thermo Fisher Scientific). For HEK293 and HeLa cells that have no endogenous Ad4BP/SF-1 expressed, I co-transfected various amounts of Ad4BP/SF-1 expressing plasmids (17). pCMV-SPORT-βgal (Thermo Fisher Scientific) was used as the internal control for transection efficiency. Cells were harvested 48 h after transfection to determine luciferase and β-galactosidase activities.

Measurement of NADPH levels

After treatment with *siAd4BP/SF-1*, *siMe1*, *siMthfd2*, or *siControl* for 48 h, Y-1 cells were harvested and NADPH levels were determined using a NADPH Quantification Kit (BioVision, CA, USA) according to the manufacturer's instruction.

RESULTS

Expression of genes involved in NADPH generation

Previous study showed that the intracellular NADPH level was decreased by Ad4BP/SF-1 knockdown in adrenocortical Y-1 cells, and consistent with this, the expression of PPP genes decreased. However, the ChIP-seq study could not find any accumulation of Ad4BP/SF-1 at these gene loci, strongly suggesting that the PPP genes are not the direct targets of Ad4BP/SF-1 (15). We therefore again examined the mRNA-seq data sets obtained previously, focusing on the genes involved in NADPH production. In addition to PPP, ME that mediates the conversion from malate to pyruvate, IDH that mediates the conversion from isocitrate to α -ketoglutarate, and MTHFD that mediates the conversion from 5,10-methylenetetrahydrofolate to 5,10-methenyltetrahydrofolate have been known to perform major enzymatic roles in NADPH regeneration.

As described previously, Ad4BP/SF-1 knockdown (si*Ad4BP/SF-1* treatment) led to a decrease of PPP genes. Both *G6pd2* and *G6pdx* genes encoding G6PD decreased to approximately 45%, and *Pgd* gene encoding 6PGD to 70% (Fig. 1). Among the three *Me* genes (*Me1*, *Me2*, and *Me3*) encoded in the mouse genome, the expression of cytosolic *Me1* in control Y-1 cells (si*Control*) was dominant and much higher than the expression of mitochondrial *Me2* and *Me3* (Fig. 1). Ad4BP/SF-1 knockdown resulted in a decrease of *Me1* expression to approximately 50%. The expression of *Me2* was not affected, while that of *Me3* increased. There are five *Idh* genes (*Idh1*, *Idh2*, *Idh3a*, *Idh3b*, and *Idh3g*) encoded in the mouse genome. The cytosolic IDH1 and mitochondrial IDH2 require NADP⁺/NADPH for their enzymatic reactions, whereas IDH3, composed of *Idh3a*, *Idh3b*, *Idh3g* three subunits, requires NAD⁺/NADH for its enzymatic reactions. Ad4BP/SF-1 knockdown decreased the

expression of *Idh1* to approximately 80%, but did not decrease that of *Idh2*.

Noticeably, the expression of cytosolic *Mthfd1* and mitochondrial *Mthfd2* decreased to 70% and 45%, respectively.

Considering the levels of expression and down-regulation by the siAd4BP/SF-1 treatment, following studies were performed with a special focus on *Me1* and *Mthfd2*. qRT-PCR was performed to confirm the down-regulation of the two genes in siAd4BP/SF-1-treated Y-1 cells. Treatments for 24 and 48 h successfully decreased both protein and mRNA levels of Ad4BP/SF-1 to 20% or less (Fig. 2). And *Me1* and *Mthfd2* mRNAs were reduced significantly to approximately 60% after 24 h and even more strongly to 40-50% after 48 h (Fig. 3). Taken together, these results suggest that these two genes are the target genes of Ad4BP/SF-1. Consistent with mRNA-seq results, the expression of *Idh1* was also down-regulated to less than 80% of control sample (Fig. 3).

Accumulation of Ad4BP/SF-1 in the gene loci involved in NADPH generation

As described previously, the genome-wide ChIP-seq study revealed that Ad4BP/SF-1 accumulates in glycolytic genes in adrenocortical Y-1 cells. Interestingly, an active enhancer mark, H3K27Ac (acetylated H3 at K27), was accumulated in many, if not all, of the Ad4BP/SF-1 accumulated regions, suggesting that these regions in the glycolytic genes act as active enhancers through the binding of Ad4BP/SF-1. Since the results obtained in the present study suggest that *Me1* and *Mthfd2* are the possible target genes of Ad4BP/SF-1, we examined the ChIP-seq data sets to investigate whether Ad4BP/SF-1 accumulates at the gene loci. As expected, Ad4BP/SF-1 binding peaks were observed in both the *Me1* and *Mthfd2* gene loci (Fig. 4). The regions in question in *Mthfd2* were subjected to H3K27Ac modification, whereas in *Me1* they were not. Accumulation of Ad4BP/SF-1 in these regions was

validated by a conventional ChIP assay followed by quantitative PCR (ChIP-qPCR). As shown in our previous study, Ad4BP/SF-1 accumulated in the glycolytic *Aldoa* (*Aldolase a*) gene (15). Likewise, accumulation was observed in *Me1* and *Mthfd2* genes. Exon VII region of *Star* (steroidogenic acute regulatory protein) gene was examined as a negative control (15) (Fig. 5) .

Transcriptional activity of Ad4BP/SF-1 accumulated region

To examine the transcriptional activities of the ChIP-peak regions in *Me1* and *Mthfd2* genes, two luciferase reporter genes, *Me1-luc* and *Mthfd2-luc*, carrying the ChIP-peak regions were constructed (Fig. 6A). 5'-upstream regions from the transcription start site of *Me1* and *Mthfd2* genes were used as promoters in the reporter plasmids. The nucleotide sequences of the ChIP-peak regions and promoters are conserved among mammalian species. The reporter constructs or pGL3-basic (backbone plasmid) together with an expression plasmid for *Ad4BP/SF-1* (17) were transfected to HEK293 and HeLa cells, in which endogenous *Ad4BP/SF-1* gene expression cannot be detected. As expected, Ad4BP/SF-1 activated both *Me1-luc* and *Mthfd2-luc* transcription dose-dependently (Fig. 6B).

To evaluate the function of Ad4BP/SF-1 binding sites, I mutated all potential Ad4BP/SF-1 binding sites in the ChIP-peak regions to produce *Me1-mut-luc* and *Mthfd2-mut-luc* (Fig. 7). The transcriptional activities of these mutated reporters were significantly lower than those of the wild type reporters in Y-1 cells (Fig. 8). The activities of the mutated reporters were further examined in HEK293 cells.

Transcription of the mutated reporters failed to be activated by cotransfection with the Ad4BP/SF-1 expression vector (Fig. 8B). Taken together, these results strongly

suggest that Ad4BP/SF-1 directly regulates *Me1* and *Mthfd2* genes through binding to the ChIP-peak regions.

Intracellular NADPH concentration regulated by Ad4BP/SF-1 through target gene expression

To further determine the contribution of Ad4BP/SF-1 in the production of NADPH, I determined the cellular NADPH concentration in Y-1 cells treated with *siControl*, *siAd4BP/SF-1*, *siMe1*, or *siMthfd2*. Treatment of Y-1 cells with *siAd4BP/SF-1*, *siMe1*, or *siMthfd2* successfully decreased the protein levels of Ad4BP/SF-1, ME1, and MTHFD2, respectively. Moreover, to my expectation, treatment with *siAd4BP/SF-1* led to a detectable decrease of ME1 and MTHFD2 proteins (Fig. 9A).

NADPH concentration in the total cell lysate was determined under these experimental conditions. Treatment of Y-1 cells with *siMthfd2* decreased the cellular NADPH level to approximately 70% of *siControl*-treated cells. Likewise, treatment with *siMe1* decreased the level to 80%, although this decrease was not statistically significant. Interestingly, treatment with *siAd4BP/SF-1* led to the most severe decrease in NADPH level, to about 50% of *siControl*-treated cells (Fig. 9B). Considering that both *Me1* and *Mthfd2* are possibly the downstream genes of Ad4BP/SF-1, it stands to reason that NADPH in the *siAd4BP/SF-1* treated cells was significantly lower than in the cells treated with *siMe1* or *siMthfd2*.

DISCUSSION

Genes involved in NADPH production as the direct targets of Ad4BP/SF-1

In addition to the functional involvement of Ad4BP/SF-1 in steroidogenic gene regulation, our recent studies demonstrated that the transcription factor is implicated in glycolytic gene regulation through binding directly to the gene loci. Indeed, knockdown of Ad4BP/SF-1 decreased the intracellular ATP concentration, and interestingly, the concentration of NADPH also decreased (15). Consistent with this finding, expression of genes in the pentose phosphate pathway (PPP), one of the main NADPH generating pathways, decreased. Among the PPP enzymes, the rate-limiting glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) are responsible for producing NADPH. Nevertheless, a chromatin immunoprecipitation sequence (ChIP-seq) analysis could not find any accumulation of Ad4BP/SF-1 in the PPP gene loci, strongly suggesting that PPP genes are not the direct targets of Ad4BP/SF-1.

In the present study, I therefore investigated whether other genes that encode enzymes mediating NADPH production, such as isocitrate dehydrogenase (IDH), malic enzyme (ME), and methylenetetrahydrofolate dehydrogenase (MTHFD), are the targets of Ad4BP/SF-1. As expected, some but not all of these genes' expression was decreased by Ad4BP/SF-1 knockdown.

Of the three isoforms of MEs present in mammals, cytosolic NADP⁺ dependent ME1 is most abundantly expressed. Mitochondrial ME2 is another main isoform dependent on NAD⁺/NADP⁺ (5,18), while ME3 is an NADP⁺ dependent minor mitochondrial isoform. Based on the research of Fan *et al.* (19), the folate-intermediated one-carbon metabolism also contributes significantly to NADPH production. In the folate pathway, two isoforms of MTHFD (cytosolic MTHFD1 and

mitochondrial MTHFD2) convert methylenetetrahydrofolate to 10-formyl-tetrahydrofolate and concomitantly generate NADPH (6,20,21). Knockdown of either *Mthfd1* or *Mthfd2* decreased NADPH significantly, suggesting an essential role of the folate pathway in maintaining the cellular NADPH pool (19,21,22). Among the *Me* and *Mthfd* genes, cytosolic *Me1* and mitochondrial *Mthfd2* were most severely affected by siAd4BP/SF-1 treatment. Moreover, accumulation of Ad4BP/SF-1 was confirmed in the *Me1* and *Mthfd2* gene loci by the ChIP-seq study. These ChIP-peak regions contain multiple binding sites for Ad4BP/SF-1 and exhibit Ad4BP/SF-1 dependent transcriptional activity, strongly suggesting that the genes are direct targets of Ad4BP/SF-1. This assumption was supported by reporter gene assays.

Significance of NADPH production regulated by Ad4BP/SF-1 in steroidogenic cells

Ad4BP/SF-1 is expressed in steroidogenic cells such as the adrenal cortex and testicular Leydig cells. These cells synthesize and secrete a variety of steroid hormones. All steroids are synthesized from cholesterol via multiple enzymatic reactions, in which six forms of cytochrome P450s (CYP11A1, CYP17A1, CYP21A1, CYP11B1, CYP11B2, and CYP19A1) and two forms of steroid dehydrogenase (HSD3B and HSD17B) are involved. In addition to these enzymes, steroidogenic acute regulatory protein (STAR), which facilitates cholesterol transport from outer to inner mitochondrial membrane, is required for steroidogenesis (23,24). Importantly, mitochondrial and cytosolic NADPH is required for the mono-oxygenase reactions mediated by P450s localized in the mitochondrial inner membrane (CYP11A1, CYP11B1, and CYP11B2) and microsomal membrane (CYP17A1, CYP21A1 and CYP19A1), respectively.

Ad4BP/SF-1 was originally identified as the transcription factor regulating *CYP11B1* and *CYP11A1* (8,25). Many studies have since shown that all steroidogenic genes are regulated by Ad4BP/SF-1 (26-28), indicating that the transcription factor orchestrates steroidogenesis via regulating steroidogenic gene expression. It should be noted that a consistent supply of NADPH is essential for efficient steroidogenesis. In the present study, I showed for the first time that genes responsible for NADPH production are the direct target genes of Ad4BP/SF-1. Overall, it is likely that Ad4BP/SF-1 coordinates expression of steroidogenic and NADPH-producing genes to accomplish efficient steroidogenesis.

Steroidogenic P450s mediate their specific reaction through activating molecular oxygen by consuming NADPH. Concomitantly, they produce reactive oxygen species (ROS) as a byproduct; therefore, ROS levels are higher in steroidogenic cells (29). As far as has been established to date, ROS are toxic to cellular functions through having deleterious effects on proteins, lipids, and nucleic acids. To protect cellular functions from such oxidative stress, cells are equipped with antioxidants such as glutathione (GSH), which is oxidized by glutathione peroxidase to eliminate H_2O_2 (30). The oxidized form produced by the peroxidase reaction (GSSG) is then reduced again by glutathione reductase under consumption of NADPH. Overall, it is reasonable to assume that Ad4BP/SF-1 cooperates with two systems, one of which results in the increase of ROS through steroidogenesis and the other of which reduces ROS through NADPH production.

NADPH is consumed in the biosynthesis of building blocks for cellular macromolecules, such as fatty acids, nucleotides, and cholesterol. In proliferating cells, an impaired NADPH level will constrain the production of building blocks required for generating daughter cells. The role of Ad4BP/SF-1 in NADPH

production may thus explain the affected development of the steroidogenic organs in homozygous and heterozygous gene-disrupted mice (31-33), as well as the presence of ectopically enlarged steroidogenic organs in Ad4BP/SF-1 overexpressed mice (34).

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ACKNOWLEDGEMENTS

Hereby I would like to express my deepest respect to Prof. Morohashi Ken-ichirou and Dr. Baba Takashi for their guidance in the research ideas and experimental technics as well as for the assistances in writing the dissertation. I am sincerely gratitude for Dr. Shima-Miyabayashi Kanako for her carefully technical instructions and patient communications to solving obstacles during my study. I appreciate Dr. Shima Yuichi and Dr. Otake Hiroyuki for their valuable instructions and advices in many experimental procedures. I thank Drs. Shishido Yurina, Inoue Miki, Matuszaki Sawako for their discussions, encouragement and help in both of my research project and private life. I am grateful for all the lab members' (present and past) friendly help during my life as an international student in Japan.

Besides, I wish to thank Dr. Miura Daisuke and Dr. Ichinose Tomomi (Innovation Center for Medical Redox Navigation, Kyushu University, Japan) for their efforts in determining NADPH levels in the cultured cells. I appreciate Dr. Ohkawa Yasuyuki (Research Center for Transomics Medicine, Medical Institute of Bioregulation, Kyushu University, Japan) for deep sequencing of the ChIP-seq and mRNA-seq libraries. I also appreciate Dr. Sato Tetsuya and Dr. Suyama Mikita (Division of Bioinformatics, Medical Institute of Bioregulation, Kyushu University, Japan) for computational analyses for mRNA-seq and ChIP-seq.

Last but not least, I am grateful for China Scholarship Council for providing living expenses during my Ph.D. study at Kyushu University. I appreciate the supports of my devoted family.

FIGURES LIST

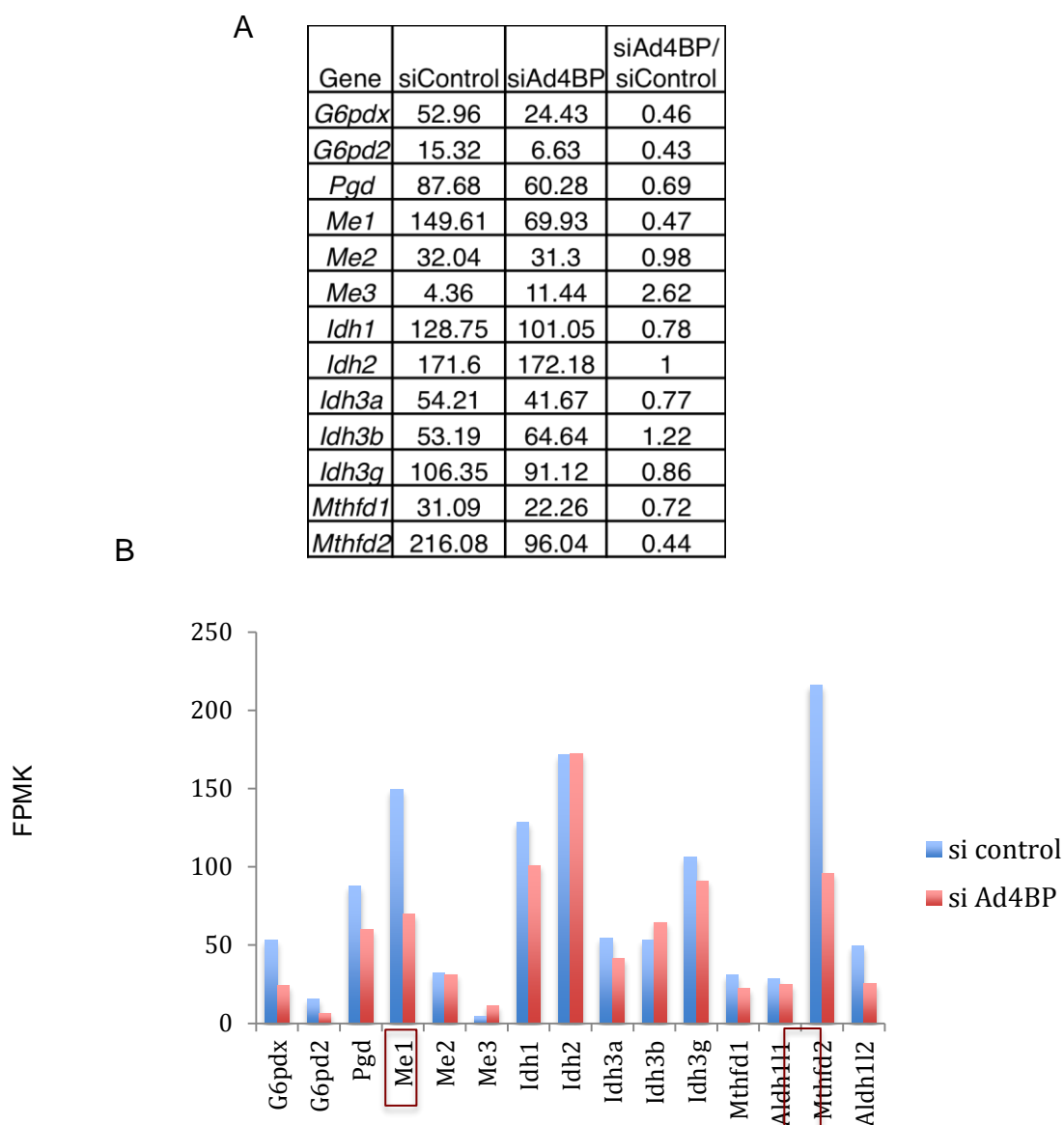


Fig. 1. Expression of genes involved in NADPH production are decreased by *Ad4BP/SF-1* knockdown in Y-1 cells revealed by mRNA-seq

(A) Previously published mRNA-seq data sets were investigated (15). Genes involved in NADPH production are listed. Levels of gene expression in Y-1 cells treated with *siControl* and *siAd4BP/SF-1* are shown as fragments per kilobase of transcript per million mapped fragments (FPKM) values. Ratios of gene expression between *siControl* and *siAd4BP/SF-1* treated cells are indicated on the right (*siAd4BP/siControl*). (B) Bar graph of the results shown in (A), genes *Me1* and *Mthfd2* displayed dominant expression level and were decreased severely by *siAd4BP/SF-1*.

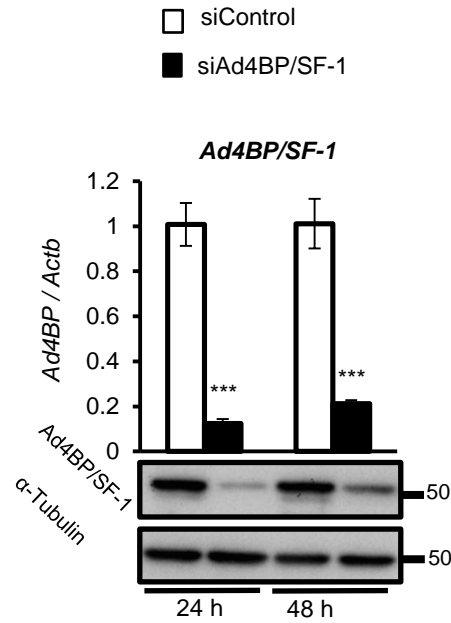


Fig. 2. *Ad4BP/SF-1* was successfully knocked down in Y-1 cells in both mRNA and protein levels

After Y-1 cells were treated with *siControl* or *siAd4BP/SF-1* for 24 or 48 h, whole-cell lysates and total RNAs were prepared and subjected to western blot (panels below) and qRT-PCR (column chart above) for *Ad4BP/SF-1*. α -Tubulin was used as a control for western blot. Open and closed bars indicate *siControl* and *siAd4BP/SF-1* treated cells, respectively. Averages of *siControl* treated cells for each time point were normalized to 1. Differences were tested for statistical significance using a two-tailed Student's t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 3$.

□ siControl ■ siAd4BP/SF-1

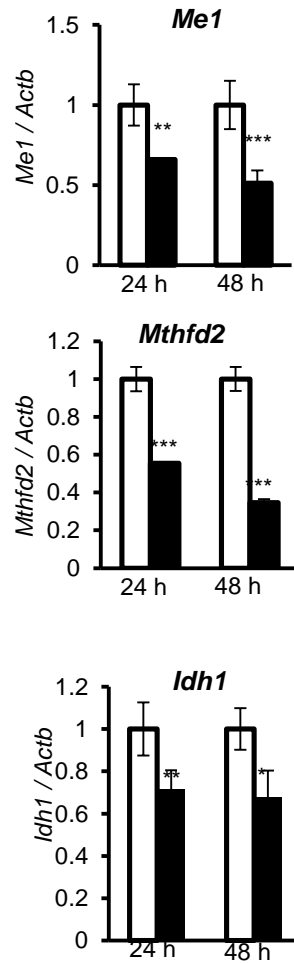


Fig. 3. Down-regulation of NADPH producing genes *Me1*, *Idh1*, *Mthfd2* were confirmed in siAd4BP/SF-1 treated Y-1 cells

Total RNAs were prepared from Y-1 cells treated with siControl (open bars) or siAd4BP/SF-1 (closed bars) and subjected to qRT-PCR for *Me1*, *Idh1*, and *Mthfd2*. Averages of siControl treated cells for each time point were normalized to 1. Differences were tested for statistical significance using a two-tailed student's t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 3$.

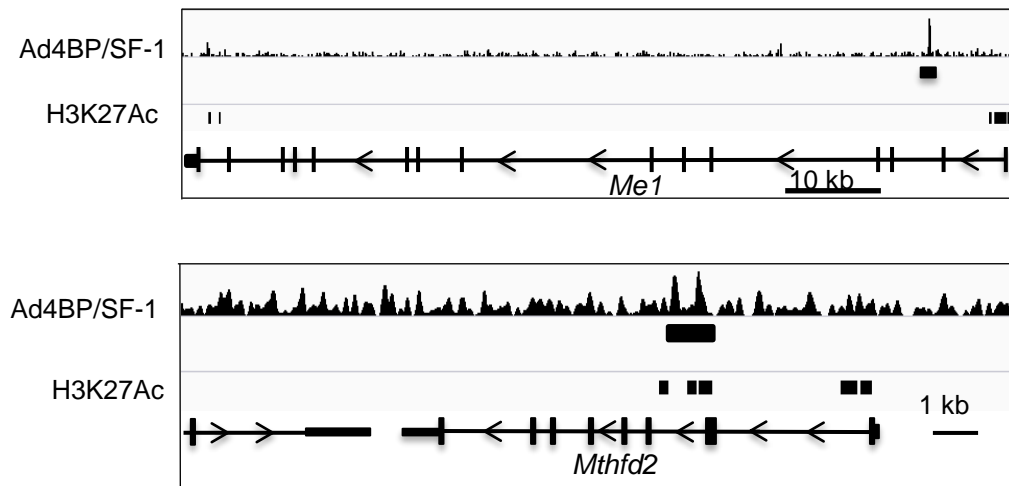


Fig. 4. Accumulation of Ad4BP/SF-1 in *Me1* and *Mthfd2* gene loci revealed by ChIP-seq results

Previously published ChIP-seq data sets for Ad4BP/SF-1 and H3K27Ac were investigated (15). Accumulation of Ad4BP/SF-1 and H3K27Ac in Y-1 cells are shown for *Me1* (upper) and *Mthfd2* (lower) gene loci. Exon/intron organization of *Me1* and *Mthfd2* together with surrounding genes is indicated at the bottom of each panel.

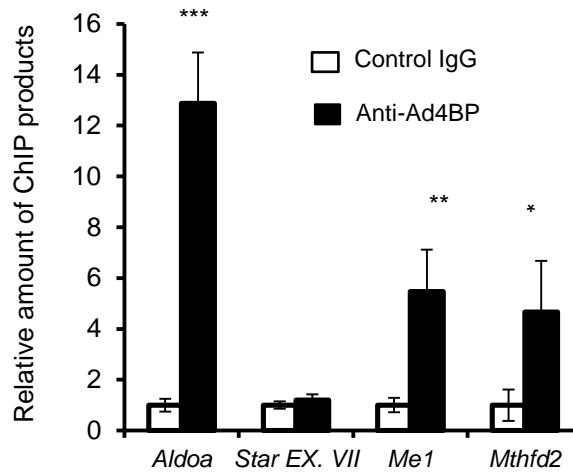


Fig. 5. ChIP-qPCR Confirmed the Accumulation of Ad4BP/SF-1 in *Me1* and *Mthfd2* gene loci

Accumulation of Ad4BP/SF-1 in *Me1* and *Mthfd2* gene loci was validated by ChIP-qPCR. The regions used for the ChIP-qPCR assays are indicated by boxes in A. ChIP-qPCR was performed with anti-Ad4BP/SF-1 (closed bars) and control IgG (open bars) antibodies. Regions in *aldolase A* (*Aldoa*) and *Star* (exon VII) gene loci were examined as positive and negative controls, respectively (14). Differences were tested for statistical significance using a two-tailed student's t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 3$.

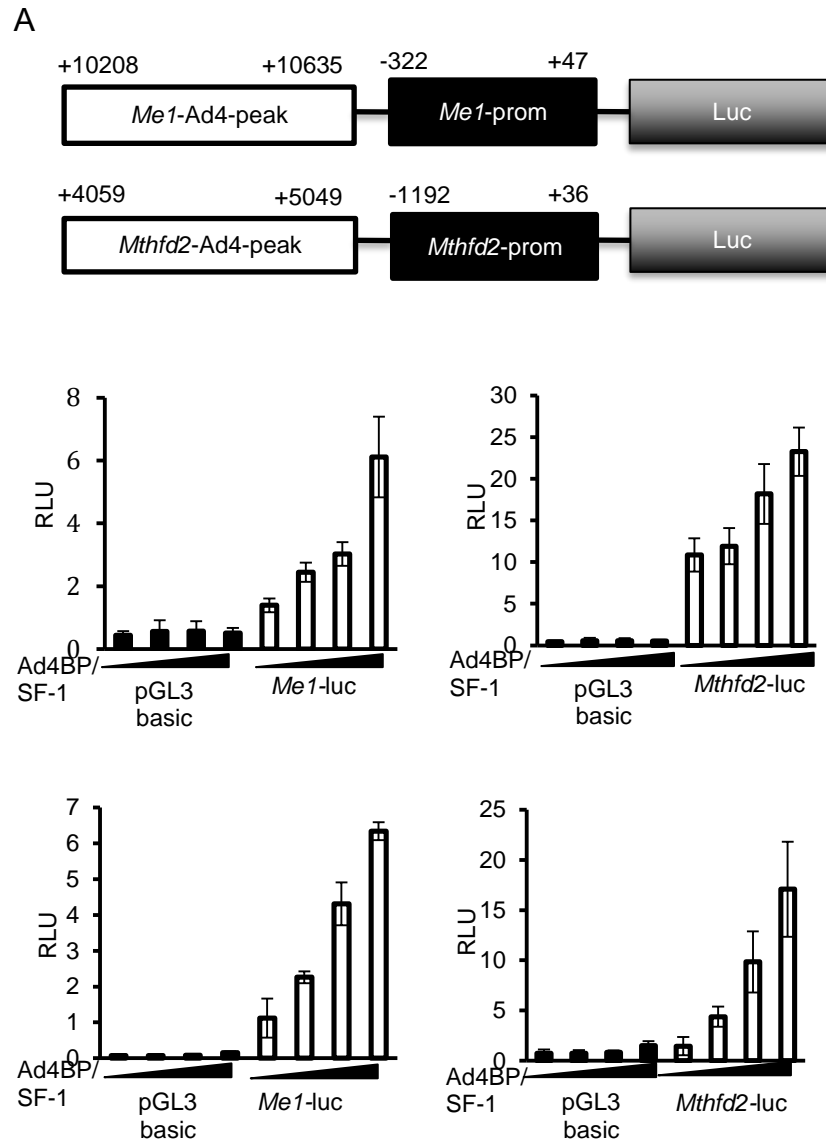


Fig. 6. Transcription activities of the Ad4BP/SF-1 accumulated (ChIP-peak) region in *Me1* and *Mthfd2* genes shown by wild-type reporter gene assay.

(A) Luciferase reporter gene constructs *Me1-luc* and *Mthfd2-luc* are shown. ChIP-peak regions (*Me1*-Ad4-peak and *Mthfd2*-Ad4-peak) and promoter regions (*Me1*-prom and *Mthfd2*-prom) were used in their construction. Their locations from the transcription start site are indicated. (B) *Me1-luc* or *Mthfd2-luc* (white) with increasing amounts of expression vector for Ad4BP/SF-1 (0, 10, 50, 250 ng) were transfected into non-steroidogenic HEK293 and HeLa cells. pGL3-basic (black) was used as the negative control. Relative luciferase activity (RLU) is shown.

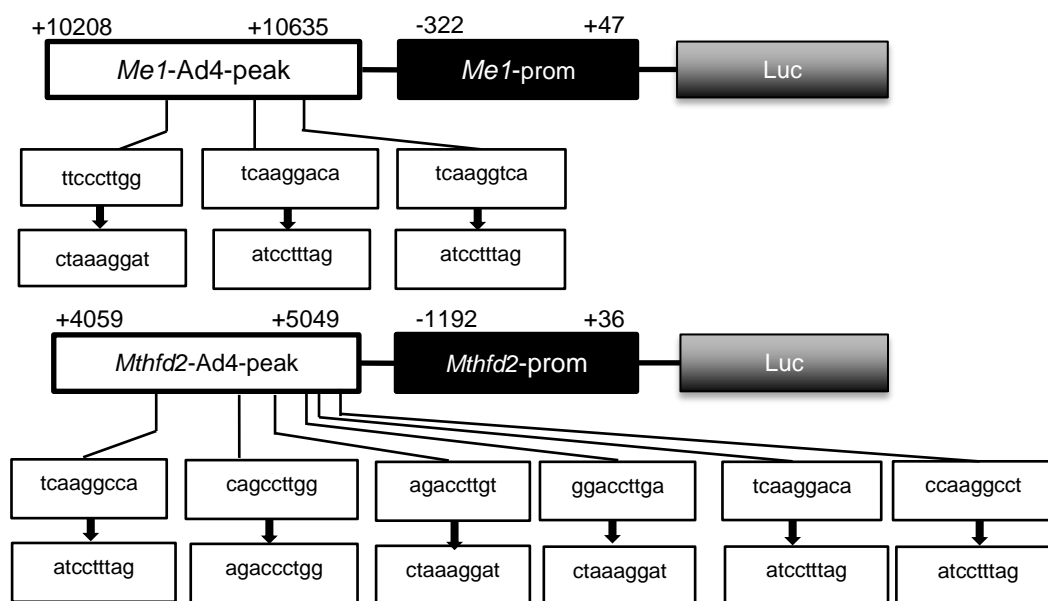


Fig. 7. Illustration of enhancer-mutated reporter constructs

All potential Ad4BP/SF-1 binding sequences localized in the ChIP-peak regions were mutated to produce the mutated reporter constructs *Me1*-mut-luc and *Mthfd2*-mut-luc. The mutated sequences were tested not able to be bound by Ad4BP/SF-1 (13,15). The DNA inserts including mutation sequences were ordered and synthesized by company (See “Materials and Methods”).

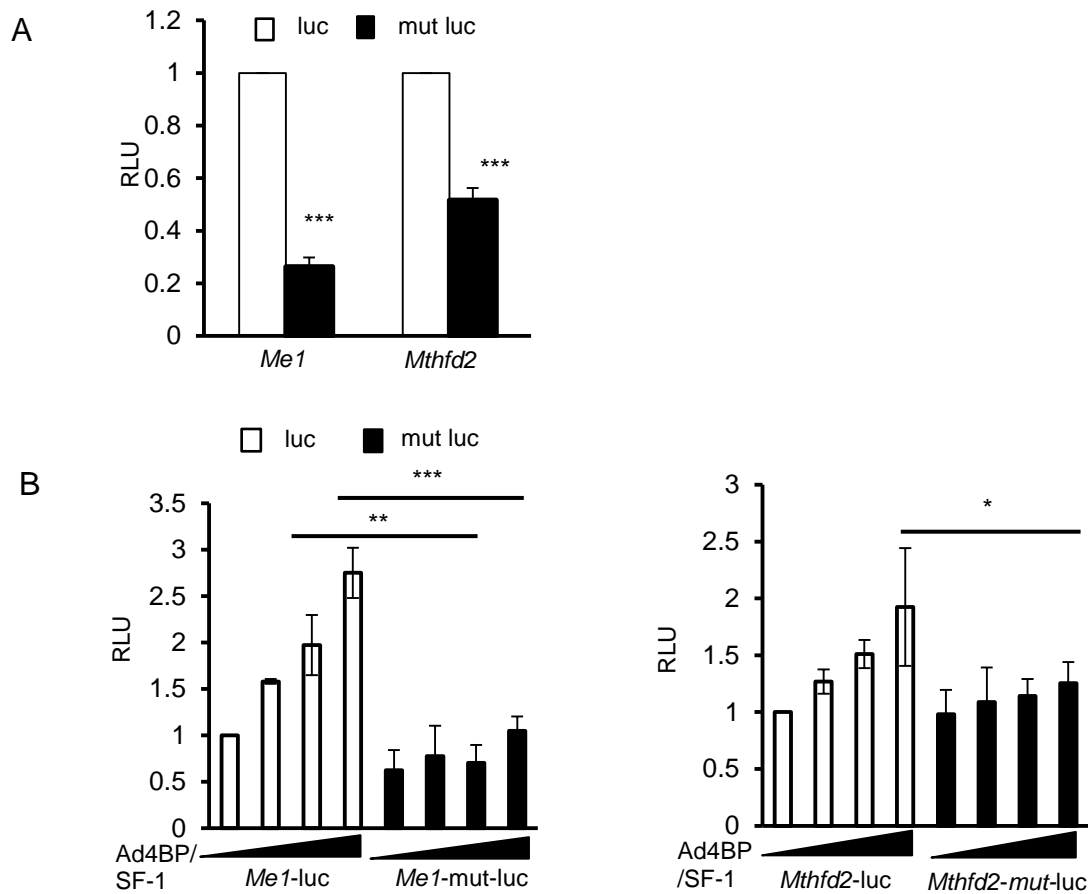


Fig. 8. Both mutated reporters displayed blunted transcriptional activity in luciferase assay.

(A) Wild type constructs, *Me1*-luc and *Mthfd2*-luc (white), and mutated constructs, *Me1*-mut-luc and *Mthfd2*-mut-luc (black), were transfected into Y-1 cells. Relative luciferase activity values are shown. (B) Wild type constructs, *Me1*-luc and *Mthfd2*-luc (white), and mutated constructs, *Me1*-mut-luc and *Mthfd2*-mut-luc (black), together with expression vector for Ad4BP/SF-1 (0, 10, 50, 250 ng) were transfected into non-steroidogenic HEK293 cells. Relative luciferase activity is shown. Average RLU of wild type reporter gene with 0 ng of Ad4BP/SF-1 expression plasmids was normalized to 1. Differences were tested for statistical significance using a two-tailed student's t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, $n = 3$. (A-B).

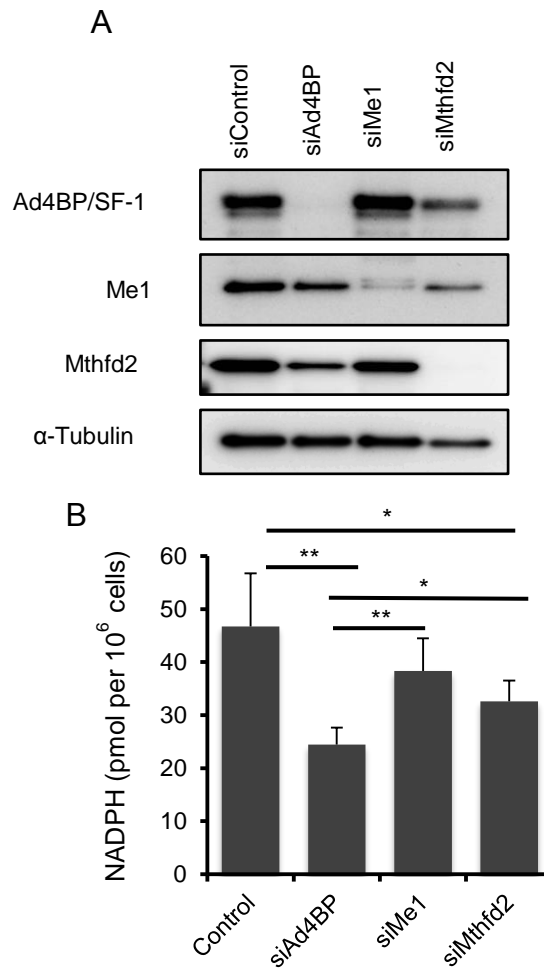


Fig. 9. NADPH concentration in Y-1 cells.

(A) Y-1 cells were treated with siControl, siAd4BP/SF-1, siMe1, or siMthfd2 for 48 h. Whole-cell lysates were prepared from the cells and subjected to western blotting using antibodies to Ad4BP/SF-1, ME1, and MTHFD2. α -Tubulin was used as a control. (B) The amount of NADPH per 10^6 cells was examined in Y-1 cells treated with siControl, siAd4BP/SF-1, siMe1, or siMthfd2 for 48 h. Differences were tested for statistical significance using a two-tailed student's t-test. $*p < 0.05$, $**p < 0.01$, $n = 5$.