Gene Trapping Uncovers Sex-Specific Mechanisms for Upstream Stimulatory Factors 1 and 2 in Angiotensinogen Expression

Sungmi Park, Xuebo Liu, Deborah R. Davis, Curt D. Sigmund

Abstract—A single-nucleotide polymorphism (C/A) located within an E-box at the −20 position of the human angiotensinogen (AGT) promoter may regulate transcriptional activation through differential recruitment of the transcription factors upstream stimulatory factor (USF) 1 and 2. To study the contribution of USF1 on AGT gene expression, mice carrying a (−20C) human AGT (hAGT) transgene were bred with mice harboring a USF1 gene trap allele designed to knock down USF1 expression. USF1 mRNA was reduced relative to controls in liver (9 ± 1%), perigenital adipose (16 ± 3%), kidney (17 ± 1%), and brain (34 ± 2%) in double-transgenic mice. This decrease was confirmed by electrophoretic mobility shift assay. Chromatin immunoprecipitation analyses revealed a decrease in USF1, with retention of USF2 binding at the hAGT promoter in the liver of male mice. hAGT expression was reduced in the liver and other tissues of female but not male mice. The decrease in endogenous AGT expression was insufficient to alter systolic blood pressure at baseline but caused reduced systolic blood pressure in female USF1 gene trap mice fed a high-fat diet. Treatment of USF1 knockdown males with intravenous adenoviral short hairpin RNA targeting USF2 resulted in reduced expression of USF1, USF2, and hAGT protein. Our data from chromatin immunoprecipitation assays suggests that this decrease in hAGT is attributed to decreased USF2 binding to the hAGT promoter. In conclusion, both USF1 and USF2 are essential for AGT transcriptional regulation, and distinct sex-specific and tissue-specific mechanisms are involved in the activities of these transcription factors in vivo. (Hypertension. 2012;59:1212-1219.) ● Online Data Supplement

Key Words: polymorphism ■ transcription ■ adenovirus ■ short hairpin RNA ■ hypertension

The angiotensinogen (AGT) gene has long been studied as a candidate gene in hypertension, and many studies have illustrated a direct correlation between AGT levels and blood pressure (BP). Increasing the copy number of the AGT gene increases BP by ~7 mm Hg per copy, delivery of anti-AGT antibodies into the blood reduces BP, and injection of AGT causes increased BP.1-3 However, the precise mechanisms by which genetic polymorphisms in the AGT gene or its promoter region could influence BP remain unclear.

It is widely accepted that hypertension is heritable and, consequently, has a genetic component. Single nucleotide polymorphisms (SNPs) in the human AGT (hAGT) promoter have been implicated in the pathogenesis of high BP, including those at nucleotides −6, −20, −217, −517, and −792 (reviewed in Reference 4). Some of the polymorphisms in the hAGT promoter have been reported to affect transcriptional regulation of the gene, thus providing a potential mechanism for dysregulation.5 For example, we reported that a genetic variant in the hAGT promoter (G-6A) might have physiological relevance under high-salt diet conditions.6 In that study, we examined BP in 4 triple-transgenic mouse models expressing either the −6G or −6A haplotypes of hAGT, combined with 2 different human renin transgenes (one well regulated and the other poorly regulated), all on an endogenous murine AGT null genetic background. We found increased salt sensitivity of BP in mice carrying the −6A hAGT haplotype when on a genetic background of poorly controlled human renin expression.

The A-20C variant in hAGT is independently associated with increased BP, cardiac hypertrophy, cerebral small vessel disease, and a blunted aldosterone response to angiotensin II.7-10 We demonstrated that the −20 position lies within an “E-box (CANNTG)” sequence, which binds the transcription factors upstream stimulatory factor (USF) 1 and USF2, and differential binding of USF1 and USF2 to the −20C or −20A alleles of the AGT promoter may lead to differential expression of AGT in AGT-expressing cell lines.11 Moreover, clinical studies show that AGT expression is higher in human subjects carrying the −20C SNP compared with those carrying the −20A SNP.12 However, the molecular mechanism...
regulating hAGT expression by transcription factors that bind to physiologically relevant promoter sequences is unclear in vivo.

USF1 and USF2 are ubiquitously expressed transcription factors controlling several critical genes in lipid and glucose metabolism.\(^1^3\) They are recruited to the transcriptional complex to modulate the state of chromatin.\(^1^4^,1^5\) USF1-null mice were viable and fertile with elevated levels of USF2, which may compensate for the absence of USF1.\(^1^6^,1^7\) In contrast, USF2-null mice exhibited reduced levels of USF1 and displayed an obvious growth defect.\(^1^6\) Although USF1 or USF2 single knockouts survive, the double null is lethal, further supporting a functional redundancy in USF1 and USF2. Herein, we determined the in vivo importance of the single knockouts survive, the double null is lethal, further supporting a functional redundancy in USF1 and USF2.

**Materials and Methods**

Please see the online-only Data Supplement for a more detailed Materials and Methods section.

**Animals**

We used a USF1 gene trap (USF1\(^{GT}\)) allele (USF1\(^{GT}\) [XG830] Byg) derived from embryonic stem cells obtained from the Mutant Mouse Regional Resource Center. We also used hAGT transgenic mice as reported.\(^1^8\) All of the mice were fed standard mouse chow and had access to water ad libitum. All of the procedures were approved by the University of Iowa University Animal Care and Use Committee and conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Molecular Analysis**

Gene expression, Western blotting, gel shift and supershift assays (electrophoretic mobility-shift assay [EMSA]), and chromatin immunoprecipitation (ChIP) were performed as detailed in the Methods section of the online-only Data Supplement.

**Intravenous Adenoviral Delivery In Vivo**

Adenoviral delivery of short hairpin RNA targeting USF2 (AdshUSF2.3) was prepared and administered as described.\(^1^9\)

**BP Measurements**

Systolic BP (SBP) was measured using the Visitech Systems BP-2000 tail-cuff BP monitoring system, as described previously.\(^1^9\)

**Statistical Analysis**

All of the comparisons were performed between USF1\(^{GT}\) and wild-type USF1 (USF1\(^{WT}\)) littermates expressing the hAGT transgene. Data were analyzed by \(t\) test or ANOVA as appropriate and were expressed as mean±SEM. Statistical significance was defined at a value of \(P<0.05\).

**Results**

**Mice Carrying −20C SNP of hAGT Transgene and USF1\(^{GT}\)**

To identify the role of USF1 on hAGT mRNA expression in vivo, we bred transgenic mice carrying a high expressing allele (−20C) of hAGT onto the USF1\(^{GT}\) genetic background and then examined human and mouse AGT expressions in hAGT/USF1\(^{WT}\) and hAGT/USF1\(^{GT}\) mice. Body weights between hAGT/USF1\(^{WT}\) and hAGT/USF1\(^{GT}\) littermates at 3 months of age were not different (26.9±0.7 versus 25.9±0.9 in male and 19.8±0.4 versus 19.5±0.7 g in female; \(n=7\)). No differences in the weights of liver, kidney, and reproductive adipose tissue or glucose levels were found between hAGT/USF1\(^{WT}\) and hAGT/USF1\(^{GT}\) mice.

**Regulation of USF1 and USF2 mRNA**

Expression of USF1 mRNA in USF1\(^{GT}\) mice was significantly decreased in male and female liver (11±1% and 8±1% of WT, respectively; Figure 1A and 1B). Variable decreases relative to WT were observed in other tissues, including kidney and brain, and reproductive, mesenteric, perirenal, and brown adipose tissues indicating that the gene...
trap is not a complete null but result in an effective knockdown of USF1 mRNA (Figure 2A and 2B). Although the expression of USF2 mRNA, which possess 2 potential E-box motifs, was not changed in USF1GT male liver (Figure 1A), it was significantly decreased in the USF1GT female liver (Figure 1B). Modest reductions of USF2 mRNA were found in USF1GT female reproductive and brown adipose tissues (Figure 2D). The levels of USF1 but not USF2 protein in USF1GT male and female liver were reduced significantly (Figure 1C through 1E).

Regulation of hAGT as a USF Target Gene In Vivo

No changes in the expression of hAGT mRNA were found in male USF1GT liver and reproductive adipose, which are known as major sites of AGT production, whereas hAGT mRNA expressions in male USF1GT kidney, brain, and mesenteric adipose tissue were decreased significantly (Figure 3A). In females, the expression of hAGT in most tissues, including liver, kidney, brain, and reproductive adipose tissue, was decreased significantly (Figure 3B). The level of hAGT was not affected in the USF1GT mice compared to the wild-type (WT) mice.
hAGT protein in USF1<sup>GT</sup> male plasma was unchanged (1.13±0.30-fold versus WT) but was decreased significantly in plasma from female USF1<sup>GT</sup> mice (0.65±0.04-fold versus WT). In males, the expression of endogenous mouse AGT in USF1<sup>GT</sup> was not changed in liver, kidney, and reproductive adipose tissue, but a significant reduction of mouse AGT mRNA in brain from USF1<sup>GT</sup> mice was found (Figure 3C). In female USF1<sup>GT</sup> mice, mouse AGT mRNA expression was significantly decreased in liver, kidney, brain, reproductive, and brown adipose tissue, consistent with hAGT expression patterns in USF1<sup>GT</sup> females (Figure 3D). The expression of p53, Fasn, and peroxisome proliferator-activated receptor-γ mRNA, known USF target genes, were not altered in liver from USF1<sup>GT</sup> males (Figure 4A) but were significantly lower in liver from USF1<sup>GT</sup> females (Figure 4B).

**BP in USF1<sup>GT</sup> Mice**

We next determined whether the reduction in mouse AGT expression was sufficient to lower BP in female USF1<sup>GT</sup> mice. We measured SBP in USF1<sup>GT</sup> mice lacking the hAGT transgene because of the known species specificity of the renin-AGT enzymatic reaction (and the lack of human renin in the model).<sup>21</sup> There was no significant difference in SBP in male or female USF1<sup>GT</sup> mice fed a low-fat diet (Figure 5A). In females, high-fat diet feeding increased body weight in both USF1<sup>WT</sup> and USF1<sup>GT</sup> mice (Table S1), but SBP was blunted in USF1<sup>GT</sup> mice (Figure 5B). There was no difference in plasma aldosterone in female mice fed a high-fat diet (Table S1). Other parameters, including food and water intake, plasma fasting glucose, and metabolic rate, are presented in Table S1.

**Evidence for USF1 and USF2 Binding on hAGT Promoter**

To explore molecular mechanisms underlying the effects of USF1 knockdown in the USF1<sup>GT</sup> liver, we first performed EMSA using nuclear extracts from liver and labeled hAGT promoter oligo probes (containing A or C at position –20; Figure S1A, online-only Data Supplement). One strong major shift product (closed arrowhead) along with 1 weak shift product (open arrowhead) was observed in nuclear extracts from liver derived from USF1<sup>WT</sup> mice. Both shift products were competed by an excess cold WT but not mutant probe. Consistent with our previous observation in cultured cells,<sup>11</sup> the gel shift signal using the –20C probe was stronger than the signal obtained with the –20A probe. It was initially interesting to note that the same pattern of gel shift products was observed in nuclear extracts from USF1<sup>WT</sup> and USF1<sup>GT</sup> livers. Therefore, we performed EMSA using antisera directed against USF1 (α1) and USF2 (α2) to identify the shifted products (Figure S1B). αUSF1 caused a weak supershift and strongly inhibited the formation of the major shift product from WT extracts, suggesting that this complex must contain USF1. This complex also likely contains USF2, because it is retained in the USF1<sup>GT</sup> nuclear extract and supershifts with αUSF2. Consequently, these data suggest that the major product likely contains USF1/USF2 heterodimers but may also contain USF2/USF2 homodimers, particularly when USF1 is depleted. This is consistent with the very close localization of the USF1/USF2 and USF2/USF2 products in nuclear extracts from a variety of AGT-expressing cells<sup>11</sup> and the observation that the αUSF1 antisera does not reduce or supershift the major product in extracts from USF1<sup>GT</sup> mice. The minor shifted product does not shift with αUSF1 and is retained in USF1<sup>GT</sup>, suggesting that it does not contain USF1. However, this product is effectively supershifted by αUSF2, suggesting that it contains USF2. It remains unclear whether it is in complex with some other protein. A similar pattern of shifted and supershifted products was observed when the E-box from the p53 promoter, a known USF target gene, was used (Figure S1C).
Physiological Binding Between USF1 and the Promoters In Vivo

Quantitative ChIP was performed to assess the binding of USF1 and USF2 in chromatin surrounding the hAGT promoter from the liver of male and female mice. In males, a 60% reduction in USF1 binding to the −20 region was observed in USF1GT mice. USF2, which was more abundant than USF1 at the hAGT promoter, was unchanged (Figure 6A). In female liver, the level of USF1 binding was much closer to the IgG background, and, thus, a difference between USF1WT and USF1GT was not clearly evident (Figure 6B). USF2 binding was similarly unaffected (Figure 6B). Because of the low level of USF1 binding in the female mice, where the effect on hAGT was the largest, we repeated the quantitative PCR to query USF1 and USF2 binding to the p53 promoter. USF1 and USF2 have been reported previously to strongly bind to the E-box in the p53 promoter. In this experiment, a significant decrease in USF1 binding to the p53 promoter was observed in the liver of male and female mice. Moreover, a decrease in USF2 binding was detected in females (Figure 6D). This correlated with a decrease in p53 expression in the liver of female mice (Figure 4B). There was no change in the level of RNA polymerase II binding consistent with studies showing that the level of poised RNA polymerase II does not directly correlate with the level of transcription.

USF2 Knockdown by AdshUSF2 In Vivo

To dissect the mechanisms of USF-dependent regulation on hAGT in vivo, we used AdshUSF2, validated previously to efficiently infect the liver. We asked if the preserved expression of hAGT in the liver of male USF1GT mice was attributed to retention of USF2 binding, as suggested by the EMSA and ChIP studies. USF1WT and USF1GT male mice were treated intravenously with either a targeting AdshUSF2 or a control adenoviral delivery of short hairpin green fluorescent protein–expressing virus. USF1 protein levels were reduced by 55% by shUSF2 in USF1WT mice but were largely ablated in USF1GT mice (Figure 7A and 7B). USF2 protein levels in liver were significantly decreased in both USF1WT and USF1GT mice by AdshUSF2 delivery compared with mice infected with adenoviral delivery of short hairpin targeting green fluorescent protein (Figure 7A and 7C). A significant reduction in hAGT protein was observed in both USF1WT and USF1GT mice by AdshUSF2, but the reduction was greater in mice lacking both USF1 and USF2 (Figure 7D and 7E). Quantitative ChIP using USF2 antisera confirmed the loss of USF2 binding to the hAGT promoter in USF1GT mice infected with AdshUSF2 virus (Figure 7F).

Discussion

Studies presented herein examined the relationship between USF1 and USF2 binding to the E-box sequence (CANNTG) overlying the −20 position in the promoter region of the hAGT gene using a transgenic mouse expressing hAGT, gene trap mice disrupting USF1 expression, and adenoviruses expressing short hairpin RNAs targeting USF2. We conclude from these studies that USF1 and USF2 binding are essential for normal AGT transcriptional regulation and that USF2 might be able to functionally compensate for the loss of USF1, especially in males. Loss of both USF1 and USF2 leads to a significant decline in hepatic hAGT expression.

USF1 or USF2 null mutants have been reported previously. USF1-null mice are viable and fertile and exhibit elevated levels of USF2, which may potentially compensate for the absence of USF1. USF1 and USF2 can homodimerize, but they preferentially heterodimerize in vivo and have similar transactivating capacities. Moreover, different cell types have distinct cell-specific ratios of USF homodimers and heterodimers. Consequently, it is possible that USF2 homodimer formation became favored in mice lacking USF1. This is supported by the EMSA studies using antisera directed against USF2 and by the preservation of USF2 binding to the hAGT and p53 promoters in chromatin from the liver in USF1GT mice. Our data are consistent with the
hypothesis that USF1-knockdown mice exhibit markedly lower levels of AGT mRNA expression in tissues only if both USF1 and USF2 are decreased simultaneously, such as was observed in females or in males after short hairpin RNA mediates ablation of USF2. Thus, USF2 may functionally compensate for decreased USF1 in males. This situation is similar to the regulation of \( L \)-type pyruvate kinase and Spot14 by glucose, where residual USF2 homodimers can compensate for decreased USF1/2 heterodimers in USF1 knockout mice.\(^{25,26}\)

The effects of USF1 deficiency were much more pronounced in females than in males. Sexual dimorphism in the phenotype of USF1- and USF2-deficient mice has been reported previously. Abnormal brain development and epileptic seizures were observed primarily in female USF1 knockout mice.\(^{25,26}\) Spot14 by glucose, where residual USF2 homodimers can compensate for decreased USF1/2 heterodimers in USF1 knockout mice.\(^{25,26}\)

Interestingly, USF1 polymorphisms were reported to be associated with the level of AGT expression in human fat biopsies,\(^{34}\) and we observed that knockdown of USF1 leads to decreased expression of AGT in several adipose tissue depots. Thus, polymorphisms that affect the activity of USF1 or USF2 may modulate the level of AGT in adipose tissues. Interestingly, transgenic studies suggest that adipose AGT can contribute to the circulating pool of AGT and, thus, can regulate arterial pressure.\(^{35}\) The status of the renin-angiotensin system can also have a profound effect on energy homeostasis and adiposity. Global knockout or pharmacological interference with renin, AGT, angiotensin-converting enzyme, and angiotensin II type 1 receptor, all result in lower body mass, altered body composition, and/or abnormal adipose development.\(^ {36-38} \) Interestingly, although there was no difference in SBP under baseline conditions, female USF1-deficient mice exhibited a decrease in SBP after high-fat diet feeding. This is consistent with the observation that females exhibited larger decreases in AGT expression than male. It remains unclear whether this is because of decreased AGT expression in the liver (ie, circulation) or in adipose tissue.

USF proteins bind close to the transcription start site of many genes and may be recruited to the transcriptional

![Figure 7](https://example.com/figure7.png)

**Figure 7.** Effect of intravenous adenoviral delivery of short hairpin RNA targeting USF2 (AdshUSF2) on upstream stimulatory factor (USF) 1, USF2, and human (h) AGT protein in vivo. AdshUSF2 or adenoviral delivery of short hairpin targeting green fluorescent protein (AdshGFP) was injected to age-matched USF1 wild-type (WT) and USF gene trap (GT) male mice (each n=3). A, Western blots showing USF1, USF2, and \( \beta \)-actin. Quantification of USF1 (B) and USF2 (C) protein expression. D, Western blots showing hAGT and \( \beta \)-actin. E, Quantitation of hAGT protein expression. F, Quantitative chromatin immunoprecipitation (ChIP) for USF2 binding on hAGT promoter. *\( \sim \)0.05 vs USF1WT. All of the data are presented as mean±SEM.
complex to modulate the state of chromatin. A USF1-dependent increase in transcriptional activity at the β-globin locus involves a complex among USF1, USF2, and chromatin-modifying proteins and is accompanied by localized increases in methylated histone 4 and acetylated histone 3 and histone 4. Recent advances in microarray and ChIP sequencing technologies provide relevant information of transcriptional control, associated with common metabolic disorders. Profiles of USF binding in a liver cell line by ChIP-ChIP demonstrated that there is a positive correlation between binding and target gene expression levels. Proximal binding to the transcription start site was critical for the assembly of a transcription complex including transcriptional coactivators, such as p300/CREB-binding protein and enzymes involved in chromatin modification, such as histone acetyltransferases P300/CBP associated factor and steroid receptor coactivator-1 and arginine methyltransferases such as protein arginine N-methyltransferase-1. In our study, loss of USF1 and USF2 binding to the hAGT promoter in chromatin led to a 90% reduction in hAGT expression, suggesting that a similar recruitment of chromatin-modifying enzymes may be required to activate hAGT transcription in response to USF binding. However, we did not observe any difference in the binding (using quantitative ChIP) of acetylated (164.8±11.7-fold versus 168.12±1.8-fold of IgG) or methylated (1.3±0.1-fold versus 1.3±0.1-fold of IgG) histone 3 (lysine 27; H3K27) to the hAGT promoter in AdshUSF2-treated USF1GT compared with liver from normal mice. Therefore, we must consider the possibility that the effects of USF1 and USF2 depletion on AGT expression are indirect. For example, genes potentially regulated by USF (eg, transcription factors hepatic nuclear factor 4 and peroxisome proliferator-activated receptor-γ) are involved in energy homeostasis, which, in turn, can directly or indirectly regulate hAGT gene expression.

In conclusion, by examining the transcriptional regulation of the hAGT gene, we have uncovered novel mechanisms regulating hAGT expression by transcription factors that bind to physiologically relevant promoter sequences (A-20C SNP). This sequence is significant because it overlies polymorphisms that are genetically associated with hypertension and other cardiovascular and metabolic phenotypes. A decrease in USF1 but not USF2 binding was observed in USF1GT mice, suggesting that USF2 might be able to functionally compensate for loss of USF1, especially in males. We provide evidence that the retention of USF2 was critical for the regulation of the USF target gene.

**Perspectives**

Essential hypertension is influenced by both genetic and environmental factors. The AGT gene has long been studied as a candidate gene in hypertension, but numerous studies have failed to elucidate the mechanisms by which polymorphisms in AGT cause hypertension. In our study, both USF1 and USF2 play an important role in hAGT regulation, and distinct sex-specific mechanisms are involved in the regulation of their expression in vivo. We hypothesize that the −20C polymorphism results in increased USF recruitment to the AGT promoter, which results in increased AGT expression. This elevation in AGT expression may cause increases in BP and may have metabolic effects.

**Acknowledgments**

We thank Drs Justin Grobe, Henry Keen, and Eric Weatherford for generous discussion and review of the article. Transgenic mice were generated at the University of Iowa Transgenic Animal Facility supported in part by grants from the National Institutes of Health and from the Roy J. and Lucille A. Carver College of Medicine. We thank Norma Sinclair, JoAnne Schwarting, and Patricia Yarolem for genotyping mice.

**Sources of Funding**

This work was supported through research grants from the National Institutes of Health (to C.D.S.; HL084207, HL048058, and HL061446). We also gratefully acknowledge the generous research support of the Roy J. Carver Trust.

**Disclosures**

None.

**References**


Gene Trapping Uncovers Sex-Specific Mechanisms for Upstream Stimulatory Factors 1 and 2 in Angiotensinogen Expression

Sungmi Park, Xuebo Liu, Deborah R. Davis and Curt D. Sigmund

Hypertension. 2012;59:1212-1219; originally published online April 30, 2012; doi: 10.1161/HYPERTENSIONAHA.112.192971

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/59/6/1212

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2012/04/30/HYPERTENSIONAHA.112.192971.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension 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 Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
ONLINE SUPPLEMENTAL MATERIAL

Gene Trapping Uncovers Gender-Specific Mechanisms for Upstream Stimulatory Factors 1 and 2 in Angiotensinogen Expression

Sungmi Park, Xuebo Liu, Deborah R. Davis, and Curt D. Sigmund
Departments of Pharmacology, Roy J. and Lucille A. Carver College of Medicine,
University of Iowa, Iowa City, IA 52242
Supplemental Methods:

Animals. Due to an inability to obtain USF1 null mice we employed a USF1 gene trap (USF1\textsuperscript{GT}) allele (USF1\textsuperscript{GT (XG830) Byg}) obtained from BayGenomics (now the Mutant Mouse Regional Resource Center, MMRRC, affiliated with the KnockOut Mouse Project, KOMP). XG830 ES cells were injected into the blastocysts in the University of Iowa Gene Targeting Facility and the chimeras were bred to C57BL/6 to confirm germline transmission. Mice carrying a hAGT transgene\textsuperscript{1} were bred with USF1\textsuperscript{GT} mice. Double transgenic mice carrying hAGT and USF1\textsuperscript{GT} were confirmed by PCR based genotyping in the transgenic facility at the University of Iowa.

Gene expression. Animals were sacrificed by CO\textsubscript{2} asphyxiation between 9-12 am. Tissues were collected by blunt dissection, weighed, snap frozen at -80°C immediately, and total RNA was extracted using TRIzol\textsuperscript{R} Reagent according to the protocol provided by the manufacturer (Invitrogen) and QIAGEN RNaseasy Mini kit's manual. The expression levels of Usf1, Usf2, mAgt and hAGT genes were assessed by real-time RT-PCR using TaqMan primer/probe sets from Applied Biosystems (Usf1, Mm01290210_g1; Usf2, Mm00495959_m1; Agt, Mm00599662_m1; hAGT, Hs01586213_m1). The quantitative expression of p53, Fasn, and PPAR\textgamma were performed by SYBR green mix using iCycler (Biorad). Primer sequences follows: 1) p53 5’-AAAGGATGCCCATGCTACAGAGGA-3’, and 5’-AGTAGACTGCCCCTTCTTGTCTT-3’; 2) Fasn 5’-TGCTCCCAGCTGCGCC-3’, and 5’-GCCCGGTAGCCTGGTGTA-3’; 3) PPAR\textgamma 5’-GATGGAAGACCACCACCGCATT-3’, and 5’-AACCATTGGGGTCAGCTTTT-3’. All data were normalized by beta actin probe and quantified by the 2\textsuperscript{-ΔΔCT} method.\textsuperscript{2,3}

Western blot. Antibodies against USF1 (1:10000 sc-229X, Santa Cruz Biotechnology), USF2 (1:10000 sc-861X, Santa Cruz Biotechnology), hAGT (1:10000, gift from Duane Tewksbury, Marshfield Medical Research Foundation, Marshfield, Wis), β-actin (1:5000 ab8227, Abcam) and albumin primary antibody (1:10000 sc46293, Santa Cruz Biotechnology) were used for western blotting. Quantitative Western analysis was performed using imaging software (ImageJ, NIH) after normalization by β-actin for tissues or albumin for plasma.

Gel Shift and Super shift Assays. Nuclear extracts were prepared from liver samples and 7ug per lane were used for electrophoretic mobility shift assay (EMSA). Double-stranded DNA oligos were labeled by \textsuperscript{32}P ATP. The sequences were as follows; 5’-GAT CTAAATAGGGC\textunderscore X TCGTGACCAGCCGCAAGG-3’ where \textit{X}=A or C for the -20 SNP in hAGT. Gel shifts were carried out using 5% polyacrylamide gel and USF1 (sc-229X), USF2 (sc-861X) (1 μg/reaction) were added for super shift under the same condition. A 2-50 excess of unlabelled double stranded DNA (C or mutant G at -20 region of hAGT promoter sequence) were used as competitors to confirm specific DNA-protein interactions.

Chromatin Immunoprecipitation (ChIP). Mouse liver tissues were harvested and frozen. Tissues were minced on ice and incubated in PBS containing 1% (v/v) formaldehyde at
room temperature (15 min). The reaction was stopped by addition of glycine (0.125M, 5 min at room temperature), homogenized on ice, and then rinsed with ice-cold PBS including proteinase inhibitors three times. The pellet was resuspended in lysis buffer with proteinase inhibitors (EZ-CHIP, Millipore) and sonicated on ice (Amplitude 30%, time 12 seconds, cooling 30 seconds, 10-15 times using a Sonic Dismembrator Model 500, Fisher Scientific). The size of the sonicated chromatin was verified as between 200-1000 bp by electrophoresis. Immunoprecipitation was performed with the EZ-CHIP kit using 5ug antibody against USF1 (sc-229X), USF2 (sc-861X), or non specific IgG as a negative control, RNA polymerase II provided by the kit as a positive control as described by us.4 Real-time PCR reactions utilized SYBR-Green mix (Applied Biosystems). The sequence for amplification of the proximal region of the hAGT promoter (-129+76) was 5'- AGTGTCGTTCTGGCATCTGT -3' and 5'-AGAGACAA GACCGAGAAGGAG-3'.

Intravenous Adenoviral Delivery of shRNA against USF2 (AdshUSF2) in vivo. AdshUSF2.3 expressing the 21-nt core sequence (5'-GGATCGTCCAGCTTTCGAAAA-3’ for acute Usf2 inhibition in vivo was prepared as described.4 Intravenous jugular cannulation, adenoviral delivery, and extraction of proteins from tissues were performed as described.4 We injected 2X10^{10} pfu of AdshGFP as a control or 1X10^{10} pfu of AdshUSF2.3 and tissues were harvested after 5 days.

Plasma Aldosterone. Plasma aldosterone was determined using an ELISA kit from Cayman Chemical (Cat # 1004377) after 1:2 dilution of plasma.

Metabolic rate: Individual Mice were placed into a water-jacketed 2 L beaker (Ace Glass, Vineland, NJ) maintained at 30°C, and room air was drawn through the chamber at 300 mL/min (R2 flow control, AEI) described as in 5.

Food and fluid Intake: Mice were acclimated to Nalgene (Rochester, NY) single-mouse-sized metabolism cages for at least 2 days before the measurement for water intake, food intake, and urine volume averaged over 24 hour collections for a 3 day period.
References


### Supplemental Table S1. Physiologic and Metabolic Parameters

<table>
<thead>
<tr>
<th></th>
<th>10% Fat Diet</th>
<th>60% Fat Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>USF1&lt;sup&gt;WT&lt;/sup&gt;</td>
<td>USF1&lt;sup&gt;GT&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(n≥6)</td>
<td>(n≥6)</td>
</tr>
<tr>
<td><strong>Female</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma Aldo</td>
<td>pg/mL</td>
<td>1158 ± 48</td>
</tr>
<tr>
<td>Body Weight</td>
<td>g</td>
<td>20.9 ± 0.7</td>
</tr>
<tr>
<td>Food Intake</td>
<td>Kcal/day</td>
<td>13.6 ± 0.3</td>
</tr>
<tr>
<td>Glucose</td>
<td>mg/dL</td>
<td>146 ± 26</td>
</tr>
<tr>
<td>Water Intake</td>
<td>mL/day</td>
<td>2.8 ± 0.1</td>
</tr>
<tr>
<td>Urine Output</td>
<td>mL/day</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>VO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>mL/100g/min</td>
<td>3.18 ± 0.02</td>
</tr>
<tr>
<td>RQ</td>
<td></td>
<td>0.92 ± 0.02</td>
</tr>
<tr>
<td>Heat</td>
<td>Kcal/hr</td>
<td>0.20 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Male</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma Aldo</td>
<td>pg/mL</td>
<td>N.D</td>
</tr>
<tr>
<td>Body Weight</td>
<td>g</td>
<td>26.7 ± 1.1</td>
</tr>
<tr>
<td>Food Intake</td>
<td>Kcal/day</td>
<td>13.2 ± 0.6</td>
</tr>
<tr>
<td>Glucose</td>
<td>mg/dL</td>
<td>140 ± 13</td>
</tr>
<tr>
<td>Water Intake</td>
<td>mL/day</td>
<td>2.7 ± 0.3</td>
</tr>
<tr>
<td>Urine Output</td>
<td>mL/day</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>VO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>mL/100g/min</td>
<td>2.83 ± 0.05</td>
</tr>
<tr>
<td>RQ</td>
<td></td>
<td>0.89 ± 0.01</td>
</tr>
<tr>
<td>Heat</td>
<td>Kcal/hr</td>
<td>0.23 ± 0.01</td>
</tr>
</tbody>
</table>

N.D., not determined  
<sup>*</sup>, P<0.05 vs. USF1<sup>WT</sup>  
<sup>†</sup>, P<0.05 vs. 10% fat diet
Supplemental Figures

Figure S1. EMSA of liver nuclear extract from USF1\(^{WT}\) and USF1\(^{GT}\) male mice.

A. Competition EMSA using labeled -20C probe. Ramped increases in cold wildtype (WT) and mutant (MUT) probes are indicated by the closed triangles. The source of the extracts (USF1\(^{WT}\) and USF1\(^{GT}\)) is indicated. The mutant probe substitutes -20G for -20C. B. EMSA and supershift using -20A (A) or -20C (C) probes and antisera directed against USF1 (\(\alpha_1\)) and USF2 (\(\alpha_2\)). C. EMSA and supershift using p53 probes and antisera directed against USF1 (\(\alpha_1\)) and USF2 (\(\alpha_2\)). Closed arrowhead, major shift product; open arrowhead, minor shift product; S-arrows, supershift products.