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E4BP4 regulates hepatic SLC2A9 and uric acid disposition in mice

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Non-standard abbreviations:

BCRP, breast cancer resistance protein; bZIP, basic leucine zipper; CES2, carboxylesterase 2; ChIP, Chromatin immunoprecipitation; CYP3A11, cytochrome P450 3A11; DMEM, Dulbecco's modified Eagle's medium; DMEs, drug-metabolizing enzymes; E4BP4, E4 promoter-binding protein 4; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; HBSS, Hanks' balanced salt solution; MRP4, multidrug resistance-associated protein 4; OAT, organic anion transporter; *Ppib*, peptidylprolyl isomerase B; ROR γ , RAR-related orphan receptor γ ; SLC2A9, the member 9 of solute carrier family 2; URAT1, urate anion transporter 1.

Abstract

SLC2A9 is a voltage-driven transporter that mediates cellular uptake and efflux of various substrates such as uric acid. Here, we investigate the role of E4BP4, a transcription factor, in regulating hepatic SLC2A9 in mice. Effects of E4BP4 on hepatic SLC2A9 and other transporters were examined using *E4bp4* knockout (*E4bp4*^{-/-}) mice. Transporting activity of SLC2A9 was assessed using uric acid as a prototypical substrate. We found that three SLC genes (i.e., *Slc2a9*, *Slc17a1*, and *Slc22a7*) were up-regulated in the liver in *E4bp4*^{-/-} mice with *Slc2a9* altered the most. *E4bp4* ablation in mice dampened the daily rhythm in hepatic SLC2A9, in addition to increasing its expression. Furthermore, *E4bp4*^{-/-} mice showed increased hepatic uric acid but reduced uric acid in the plasma and urine. Consistently, allantoin, a metabolite of uric acid generated in the liver, was increased in the liver of *E4bp4*^{-/-} mice. *E4bp4* ablation also protected mice from potassium oxonate-induced hyperuricemia. Moreover, negative effects of E4BP4 on SLC2A9 were validated in Hepa-1c1c7 and primary mouse hepatocytes. Additionally, according to luciferase reporter and ChIP assays, E4BP4 repressed *Slc2a9* transcription and expression via direct binding to a D-box (-531 bp to -524 bp) on the P2 promoter. In conclusion, E4BP4 was identified as a novel regulator of SLC2A9 and uric acid homeostasis, which might facilitate new therapies for reducing uric acid in various conditions related to hyperuricemia.

Keywords: SLC2A9; E4BP4; hepatic uptake; uric acid

Significance statement

Our findings identify E4BP4 as a novel regulator of SLC2A9 and uric acid homeostasis, which might facilitate new therapies for reducing uric acid in various conditions related to hyperuricemia.

Introduction

SLC2A9 (the member 9 of solute carrier family 2, also known as GLUT9) is a voltage-driven transporter that mediates cellular uptake and efflux of various substrates such as uric acid, glucose and fructose (Vitart et al., 2008; Wright et al., 2010; Lu et al., 2019). SLC2A9 is highly expressed in the liver and kidney, consistent with its role in elimination of endobiotics and xenobiotics (Lu et al., 2019; Ruiz et al., 2018). Two functional splice variants have been identified for SLC2A9 and they differ only in the length of amino terminus (Prestin et al., 2014; Augustin et al., 2004). Compared to the long form, the short form has a shorter amino terminus (19 versus 34 amino acids) (Prestin et al., 2014; Augustin et al., 2004). Both forms can be found in the liver and kidney (Keembiyehetty et al., 2006). The long form (isoform 1) is distributed in the basolateral side and the short form (isoform 2) in the apical side of polarized renal epithelial cells (Kimura et al., 2014; Augustin et al., 2004), whereas both are distributed in the basolateral membrane of hepatocytes (Keembiyehetty et al., 2006). In addition to its role in transporting substances, SLC2A9 has been implicated in cancer development and Parkinson's disease (Gao et al., 2013; Han et al., 2019).

Uric acid is a main product of purine metabolism that mainly occurs in the liver. Since uric acid has an anti-oxidant activity, high circulating levels are beneficial against oxidative stress (Sautin et al., 2008). However, circulating uric acid level above the normal range (i.e., hyperuricemia) is frequently linked to poor health, including hypertension, gout, metabolic

syndrome, nephropathy and cardiovascular diseases (Sharaf El et al., 2017). The kidney plays a critical role in maintaining circulating uric acid concentration through both secretion and reabsorption by a network of uric acid transporters such as SLC2A9, URAT1, OAT1, OAT3, OAT4, BCRP, and MRP4 (Xu et al., 2017). In addition, the liver also has a role in uric acid homeostasis through hepatocyte uptake from the circulation that is mediated by SLC2A9 (Preitner et al., 2009; Lu et al., 2019). This is evidenced by the fact that liver-specific loss of *Slc2a9* in mice causes severe hyperuricemia and hyperuricosuria (Preitner et al., 2009). Uric acid in the liver can be either excreted to the bile (by the exporters such as BCRP) for clearance or degraded to allantoin (a pathway varies from species to species) (Ristic et al., 2020).

E4BP4 (NFIL3) is a basic leucine zipper (bZIP) transcription factor. E4BP4 negatively regulates the transcription of target genes by competing for D-box binding with the proline-alanine rich (PAR) family of bZIP transcription factors and recruiting histone deacetylase 2 and histone methyltransferase G9a via a repression domain consisting amino acids 299-363 (Zhao et al., 2021; Tong et al., 2013). The role of E4BP4 in immune system has been recognized. For instance, it is a critical regulator of IgE class switching, IL-3-mediated cell survival, NK and Th17 cell development (Motomura et al., 2011; Keniry et al., 2014). Additionally, E4BP4 is implicated in regulating circadian rhythms via repression of clock genes such as *Periods* (Ohno et al., 2007). Interestingly, E4BP4 integrates circadian clock and development of immune cells through inhibiting ROR γ , a Th17-determining factor (Yu et al., 2013).

Pharmacokinetics plays a critical role in determining drug concentrations in the plasma and

tissues, thereby profoundly affecting drug efficacy and side effects. Pharmacokinetic processes consist of absorption, distribution, metabolism and excretion which for most drugs depend on DMEs (drug-metabolizing enzymes) and drug transporters (Li et al. 2019). Studies in recent years have revealed E4BP4 as a key regulator of DMEs such as carboxylesterase 2 (CES2) and cytochrome P450 3A11 (CYP3A11) (Tong et al., 2019; Zhao et al., 2018). E4BP4 positively regulates CES2 enzymes through inhibiting their transcriptional repressor REV-ERB α , whereas it represses *Cyp3a11* transcription by binding to a D-box in gene promoter (Tong et al., 2019; Zhao et al., 2018). Therefore, it is of no surprise that E4BP4 is a determinant of metabolism and pharmacokinetics for some drugs including irinotecan and midazolam (Tong et al., 2019; Zhao et al., 2018). However, whether E4BP4 regulates drug transporters and chemical disposition remains largely unknown.

In this study, we investigate the role of E4BP4 in regulation of hepatic SLC2A9 in mice. Effects of E4BP4 on hepatic transporters were examined using *E4bp4* knockout (*E4bp4*^{-/-}) mice. Transporting activity of SLC2A9 was evaluated using uric acid as a prototypical substrate. We demonstrated that E4BP4 inhibits hepatic SLC2A9 expression in mice through a direct transrepression mechanism.

Materials and Methods

Materials

Assay kit for uric acid was obtained from Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). Uric acid was purchased from Yuanye Biotech (Shanghai, China). ELISA kit for allantoin was obtained from Jianglai Biotech (Shanghai, China). Anti-SLC2A9, anti-OAT2 and anti-GAPDH antibodies were obtained from Proteintech (Chicago, IL). TRIzol reagent was purchased from Accurate Biotech (Hunan, China). *E4bp4*-containing pcDNA3.1 plasmid (or *E4bp4* plasmid), siE4bp4 (siRNA targeting *E4bp4*) and a negative control, as well as *Slc2a9-P1* (-2000/+100 bp) and *Slc2a9-P2* (-2000/+100 bp) luciferase reporters (cloned into pGL4.10 vector) (sequences are shown in Table 1 & supplemental Table 1) were obtained from TranSheep Biotech (Shanghai, China).

Mice

E4bp4-deficient mice were obtained from Dr. Masato Kubo (Motomura et al., 2011). Wildtype mice (C57BL/6) were obtained from HFK Biotech (Beijing, China). All mice were kept on a 12-hour light, 12-hour dark cycle (lights on at 6:00 AM or ZT0 and lights off at 6:00 PM or ZT12), and food and water were available *ad libitum*. ZT stands for zeitgeber time which is conventionally used to describe the time in a 12 h light/dark cycle in which lights are turned on at ZT0 and off at ZT12. Male mice (8-12 weeks old) were used for experimentation.

PCR genotyping

PCR genotyping (primers shown in Table 1) was performed as described (Chen et al., 2021).

Hyperuricemia

E4bp4^{-/-} and wildtype mice ($n = 5$ per group) were intragastrically treated with potassium oxonate (PO, 250 mg/kg) at ZT2 for seven consecutive days. Urine samples were collected over a 0-24 h period after last drug dosing. On day 8, mice were sacrificed and plasma samples and livers were collected. Uric acid and allantoin levels were determined with the commercial kits.

Isolation of primary mouse hepatocytes

Mouse hepatocytes were isolated from wildtype and *E4bp4*^{-/-} mice using a collagenase perfusion method (Zhang et al., 2018).

Cell culture and treatment

Hepa-1c1c7 cells were cultured in DMEM containing 10% FBS and primary mouse hepatocytes in DMEM containing 10% FBS plus 1% penicillin/streptomycin. Cells were seeded in a six-well plate and grown to a confluence of about 70%, and then treated with *E4bp4* plasmid (2 μ g) or si*E4bp4* (50 nM) or control. After 1 or 2 days, cells were harvested for expression analyses.

Cellular uptake experiments

Cellular uptake of uric acid was carried out as described previously (Yu et al., 2018). Briefly, cells (Hepa-1c1c7 and primary mouse hepatocytes) with or without transfection were incubated

with uric acid (25, 50 and/or 100 μ M) in HBSS buffer. At specific time point (15, 30 or 60 min), the buffer was aspirated. Cells were washed using ice-cold PBS, and then solubilized in 50% methanol. After centrifugation, the supernatant was collected and uric acid was assayed.

qPCR assay

Total RNA was extracted with TRIzol reagent, and used for cDNA synthesis. qPCR reactions and amplification were performed as previously described (Wang et al., 2020; Wang et al., 2018). *Ppib* (*peptidylprolyl isomerase B*) was used as an internal control. Primers are listed in Table 1. Relative mRNA level was calculated using the $2^{-\Delta\Delta C_t}$ approach.

Western blotting and luciferase reporter assay

Western blotting and luciferase reporter assay were performed as described (Chen et al., 2021). In Western blotting, protein expression was normalized to GAPDH (as an internal control).

ChIP assay

ChIP assays were performed as previously described (Wang et al., 2020). In brief, mouse livers were fixed in formaldehyde. After sample lysis, chromatin was sheared and immunoprecipitated using anti-E4BP4 or normal IgG. DNA fragments were collected and purified, followed by qPCR quantification with specific primers (Table 1).

Data analyses

Data are shown as mean $\pm$ SD. Statistical analyses were performed with Student's t-test (for two group comparisons), and with ANOVA (one-way or two-way) and Bonferroni post hoc test (for

multiple group comparisons). $p < 0.05$ (*) was considered as a statistically significant difference.

Results

Validation of *E4bp4*-deficient mice

PCR genotyping of *E4bp4* was performed with mouse tails using a specific primer set (Table 1). *E4bp4*^{-/-} mice generated a 350 bp fragment, whereas the wildtype allele produced a 2.5 kb fragment (Figure 1A). By using primers targeting the deleted sequence, we demonstrated with qPCR assays the loss of *E4bp4* transcript (wildtype) in the livers of *E4bp4*^{-/-} mice (Figure 1B).

Altered SLC2A9 expression and uric acid levels in *E4bp4*-deficient mice

To assess potential effects of E4BP4 on SLC transporters, we determined hepatic expression of a number of SLC genes, many of which encode drug transporters, in *E4bp4*^{-/-} mice. The sample-collecting time point was set at ZT2 when the E4BP4 protein is highly expressed (Narumi et al., 2016). According to qPCR assays, three SLC genes (i.e., *Slc2a9*, *Slc17a1*, and *Slc22a7/Oat2*) were up-regulated in the liver in *E4bp4*^{-/-} mice with *Slc2a9* altered the most (Figure 2). To test if alterations in *Slc2a9* expression depend on the time of the day, we extended our examinations to six time points around the clock considering that *E4bp4* is a circadian gene (Zhao et al., 2012). We found that *Slc2a9* oscillated in a circadian time-dependent manner with lowest value at ZT18 in wildtype mice (Figure 3A). *E4bp4* ablation in mice increased the hepatic expression of *Slc2a9* mRNA throughout the whole day, and blunted its diurnal rhythm (Figure 3A). Renal expression of *Slc2a9* also varied according to the daily time, but was unaffected in *E4bp4*^{-/-} mice (Figure 3B). In line with the changes in mRNA, SLC2A9 protein was elevated in

the live of *E4bp4*^{-/-} mice and its diurnal rhythm was dampened (Figure 3C). By contrast, hepatic OAT2 protein was unaffected in *E4bp4*^{-/-} mice (Figure 3D) despite a slight change in the mRNA level (Figure 2). Because SLC2A9 is a uric acid transporter (Lu et al., 2019), we examined whether uric acid homeostasis is disrupted in *E4bp4*^{-/-} mice. *E4bp4*^{-/-} mice showed reduced plasma and urine uric acid but increased hepatic uric acid (Figure 4A & B). We also found allantoin, a metabolite of uric acid generated in the liver, was increased in the liver of *E4bp4*^{-/-} mice (Figure 4B). These changes can be attributed to up-regulation of hepatic SLC2A9 which mediates the uptake of uric acid to the liver from the blood circulation. Supporting the *in vivo* findings, *E4bp4*-deficient hepatocytes showed increased SLC2A9 expression and enhanced uptake of uric acid according to *in vitro* uptake experiments (Figure 5). Altogether, we identified E4BP4 as a key regulator of hepatic SLC2A9 and uric acid homeostasis.

***E4bp4* ablation desensitizes mice to hyperuricemia**

Because uric acid homeostasis was disrupted in *E4bp4*^{-/-} mice, we further attempted to examine whether the mice have an altered susceptibility to hyperuricemia. To this end, we established hyperuricemia models by treating mice intragastrically with 250 mg/kg PO for 7 days. Administration of PO induced hyperuricemia in both *E4bp4*^{-/-} and control mice. However, hyperuricemia was less severe in *E4bp4*^{-/-} mice than in control mice, as evidenced by significantly lower plasma and urine uric acid in the knockout mice (Figure 6A). The fold increase in circulating uric acid after PO treatment was greater in *E4bp4*^{-/-} mice compared to wildtype controls (3.4- versus 1.6-fold). Consistently, hepatic uric acid and allantoin were higher

in *E4bp4*^{-/-} mice compared to wildtype controls (Figure 6B). Altogether, *E4bp4* ablation protects mice from PO-induced hyperuricemia.

E4BP4 negatively regulates SLC2A9 expression in Hepa-1c1c7 and primary mouse hepatocytes

Next, regulation of SLC2A9 expression by E4BP4 were evaluated in both Hepa-1c1c7 (a mouse hepatoma cell line) cells and primary mouse hepatocytes by performing gain and loss-of-function experiments. It was validated that the overexpression plasmid can increase *E4bp4* expression, and that the specific siRNA can reduce the expression of *E4bp4* in Hepa-1c1c7 cells (Figure 7A & D). Overexpression of E4BP4 in Hepa-1c1c7 cells significantly reduced the SLC2A9 mRNA and protein, and also reduced the cellular uptake of uric acid (Figure 7A-C). Conversely, *E4bp4* knockdown enhanced the cellular expression of SLC2A9 and uptake of uric acid (Figure 7C-F). Similar effects of E4BP4 on SLC2A9 expression were observed in primary mouse hepatocytes (Figure 8A-F). Taken together, E4BP4 negatively regulates SLC2A9 in Hepa-1c1c7 cells and in primary mouse hepatocytes, congruent with the animal findings (Figure 3).

E4BP4 represses *Slc2a9* transcription

E4BP4 is a critical component of the auxiliary loop of molecular clock, functioning as a transcriptional repressor to inhibit the expression of clock-controlled genes through binding to D-box elements in their promoters (Zhao et al., 2021). Given that E4BP4 is a negative regulator of SLC2A9, we reasoned that E4BP4 may regulate SLC2A9 expression through a direct

transcriptional mechanism. Of note, two major *Slc2a9* transcripts with E4BP4 significantly enriched at their promoters were identified (Figure 9A). These two isoforms are driven by their own promoters, named P1 and P2, respectively. Intriguingly, E4BP4 dose-dependently inhibited the activity of a *Slc2a9*-P2-driven luciferase reporter in Hepa-1c1c7 cells (Figure 9B). In contrast, E4BP4 did not affect the activity of a luciferase reporter driven by *Slc2a9*-P1 (Figure 9B). *In silico* analysis revealed a D-box element (-531/-524 bp) in *Slc2a9*-P2 (Figure 9C), whereas, no D-box element was found in *Slc2a9*-P1. Also, *Slc2a9* transcript driven only by P1 promoter remained unchanged in the liver of *E4bp4*^{-/-} mice (Figure 9D). Furthermore, the inhibitory effects of E4BP4 on the activity of *Slc2a9*-P2 luciferase reporter were abrogated when the D-box sequence was mutated (Figure 9E). Moreover, ChIP assay demonstrated that E4BP4 protein was enriched at *Slc2a9*-P2 in wildtype liver, however, this enrichment was no longer available in the liver of *E4bp4*^{-/-} mice (Figure 9F). As expected, E4BP4 did not bind to *Slc2a9*-P1 or a non-specific region (NR) in the wildtype and *E4bp4*-deficient livers (Figure 9F). Taken together, E4BP4 negatively regulates *Slc2a9* transcription via binding to a D-box (-531/-524 bp) within *Slc2a9*-P2 promoter.

Discussion

We have unraveled that multiple transporters including *Slc2a9*, *Slc17a1*, and *Slc22a7* are regulated by E4BP4 in mouse liver (Figure 2). Importantly, we showed that increased expression of SLC2A9 led to enhanced hepatocyte uptake of its endogenous substrate uric acid, and thus to a lower level of circulating uric acid as well as attenuated chemical-induced hyperuricemia in *E4bp4*^{-/-} mice (Figures 4 and 6). Negative regulatory effects of E4BP4 on SLC2A9 were further validated in primary mouse hepatocytes and in Hepa-1c1c7 cells (Figures 7 and 8). Based on combined assays of luciferase reporter and ChIP, E4BP4 represses *Slc2a9* transcription via direct binding to a D-box (-531/-524 bp) in the P2 promoter (Figure 9). Taken together, E4BP4 inhibits hepatic SLC2A9 expression and activity in mice through a direct transrepression mechanism, thereby impacting transporting and elimination of substrate molecules. Identification of E4BP4 as a novel modulator of SLC2A9 and uric acid homeostasis might facilitate new therapies for reducing uric acid in various conditions related to hyperuricemia.

It is a novel finding that *Slc2a9* is cyclically expressed and its level oscillates according to time of the day (Figure 3A). *Slc2a9* mRNA peaks at ZT6, whereas its protein peaks at ZT10 with a 4-h phase delay (Figure 3). This is normal because certain amount of time is required for the translation of mRNA to protein (Narumi et al., 2016). The circadian expression of *Slc2a9* may be translated to circadian rhythm in liver uptake of uric acid, thereby contributing to daily oscillations in circulating level of uric acid as noted in rodents and humans (Kanemitsu et al., 2017). The *Slc2a9* rhythmicity was dampened in *E4bp4*-deficient mice, indicating E4BP4 as a

circadian regulator of *Slc2a9* (Figure 3). However, *E4bp4*^{-/-} mice retain a rhythmicity in SLC2A9 expression, suggesting that other factors play a role in regulating circadian expression of this transporter. Promoter analysis of *Slc2a9* gene using JASPAR algorithm identified two potential E-box elements (located at -1221/-1216 bp and -716/-711 bp) on which the core circadian oscillators BMAL1 and CLOCK may act to generate oscillations in gene expression (Wang et al., 2019; Zhao et al., 2019). Thus, it is likely that BMAL1 and CLOCK are involved in regulating circadian expression of *Slc2a9*. However, this requires further validations.

Uric acid homeostasis is dependent on the rates of its production and elimination. Uric acid is mainly synthesized from purine by xanthine oxidase (XO) in the liver. *Uox* gene encodes uricase that metabolizes uric acid to allantoin in the liver. We found that hepatic *XO* and *Uox* was unaffected in *E4bp4*^{-/-} mice (Figure 10A). Thus, the contribution of uric acid production to an altered plasma level of uric acid in *E4bp4*^{-/-} mice can be excluded. Uric acid is primarily eliminated in the kidney (about two-thirds) through filtration, reabsorption and secretion (Bobulescu et al., 2012). In addition, the liver is an organ where SLC2A9 is highly expressed and has a role in urate homeostasis through hepatocyte uptake from the circulation for hepatobiliary elimination (Lu et al., 2019). In the study of Preitner *et al*, liver-specific loss of *Slc2a9* in mice causes severe hyperuricemia and hyperuricosuria (Preitner et al., 2009). In line with this, circulating uric acid level was reduced in *E4bp4*^{-/-} mice with an elevated expression of hepatic SLC2A9 (Figure 3 & 4). Therefore, this study strongly supports the notion that uptake of uric acid by the liver is a contributing factor to uric acid homeostasis besides the disposal processes in

the kidney.

We focused primarily on assessment of the effects of E4BP4 on SLC2A9 and uric acid disposition in the liver. This was because neither SLC2A9 nor other uric acid transporters (including URAT1/SLC22A12, SLC22A6/ OAT1, SLC22A8/OAT3 and ABCG2/BCRP) were affected in the kidney of *E4bp4*^{-/-} mice (Figure 10B). The exact reason as to why E4BP4 has no effect on renal SLC2A9 remains unknown. However, transcriptional mechanisms for gene regulation are tissue-dependent because the transcription factors and coregulators vary across tissues (Jones et al., 2013; Xu et al., 2009). Tissue-specific regulatory effects for E4BP4 were also noted in previous reports in which E4BP4 positively regulates *Ces3* genes in the liver, whereas it may repress the expression of renal *Ces3* (Zhao et al., 2018; Gachon et al., 2006). Combined *in vivo* and *in vitro* evidence have been provided here that E4BP4 regulates SLC2A9 in mouse liver to alter uric acid homeostasis. However, whether E4BP4 regulates SLC2A9 and uric acid disposition in humans requires further studies.

E4BP4 in general competes for binding to D-box with the PAR bZIP proteins to regulate gene transcription and expression. In general, the PAR bZIP proteins activate, whereas E4BP4 represses, gene transcription. Their opposing roles in gene regulation are also true for DMEs and transporters such as *Fmo5*, *Mdr1a*, and *Mrp2*, and are critical for maintaining circadian rhythmicity in target genes (Yu et al., 2019; Zhou et al., 2019; Chen et al., 2019). Thus, there is a possibility that PAR bZIP factors activate *Slc2a9* transcription, and contribute to its circadian rhythm in expression. E4BP4 plays a role in immune system and in inflammatory responses. In

turn, E4BP4 expression can be modified by inflammatory factors including IL-3, -4, -6, and -15 (Kashiwada et al., 2010). Therefore, it is possible that E4BP4 expression is altered in immune and inflammatory diseases, and uric acid homeostasis is disrupted in these diseases due to changes in SLC2A9 expression.

It is noteworthy that *Slc2a9* gene has two major transcripts (splice variants), encoding two protein isoforms that differ in the length of amino terminus but do not differ in their functions (Keembiyehetty et al., 2006). Compared to the long form, the short form has a shorter amino terminus (19 versus 34 amino acids) (Keembiyehetty et al., 2006). Since the qPCR primers for *Slc2a9* analysis (Figures 2, 3, 7 & 8) cannot distinguish the two transcripts, the reported level of *Slc2a9* mRNA was the sum of both transcripts. Also, anti-SLC2A9 antibody used here reacts with both forms of SLC2A9 proteins, thus the western blot data measured the total level of SLC2A9 protein. We revealed that E4BP4 regulates transcription of *Slc2a9* isoform 2 (P2, short form) via binding to a D-box within its promoter region (Figure 9), but has no effect on *Slc2a9* isoform 1 (P1, lacking in a D-box). Additionally, we showed that *E4bp4* has no effect on *Slc2a9* transcript driven by P1 promoter by designing a specific primer set. Therefore, the isoform 2 was responsible for up-regulated *Slc2a9* expression in *E4bp4*^{-/-} mice.

In summary, our study reveals E4BP4 as a negative regulator of SLC2A9 in mice, affecting the uptake of uric acid by the liver and circulating uric acid level. Importantly, E4BP4 represses *Slc2a9* transcription via direct binding to a D-box (-531/-524 bp) in the P2 promoter. Future

investigations are suggested to study the role of human E4BP4 in regulating SLC2A9 and uric acid homeostasis.

Author contributions

ZW, SW, and BW participated in research design: ZW, LG and SR conducted experiments; GS and YL contributed new reagents or analytic tools: ZW and LG performed data analysis: ZW, SW, and BW wrote or contributed to the writing of the manuscript

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Footnotes

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ZW and LG contributed equally to this work.

Legends for Figures

- Figure 1 Characterization of *E4bp4*-deficient mice.** (A) PCR genotyping of wildtype (WT), heterozygous (*E4bp4*^{+/-}) and homozygous (*E4bp4*^{-/-}) mice. (B) qPCR assays of hepatic *E4bp4* in *E4bp4*^{-/-} and WT mice. Data are mean ± SD (*n* = 5). **p* < 0.05 (t test). ND, not detected. ZT, Zeitgeber time.
- Figure 2 mRNA expression of various SLC transporters in the liver of *E4bp4*-deficient and WT mice.** Data are mean ± SD (*n* = 5). **p* < 0.05 (t test).
- Figure 3 Hepatic SLC2A9 is increased in *E4bp4*-deficient mice.** (A) Diurnal *Slc2a9* in the liver of *E4bp4*^{-/-} and WT mice. (B) Diurnal *Slc2a9* in the kidney of *E4bp4*^{-/-} and WT mice. (C) Diurnal SLC2A9 protein in the liver of *E4bp4*^{-/-} and WT mice. (D) Effects of *E4bp4* knockout on OAT2 protein in mouse liver. In panels A-C, data are mean ± SD (*n* = 5). **p* < 0.05 at individual time points (two-way ANOVA and Bonferroni post hoc test). In panel D, data are mean ± SD (*n* = 3).
- Figure 4 Altered uric acid levels in *E4bp4*-deficient mice.** (A) Uric acid in the plasma and urine of *E4bp4*^{-/-} and WT mice. (B) Hepatic uric acid and allantoin in *E4bp4*^{-/-} and WT mice. Data are mean ± SD (*n* = 5). **p* < 0.05 (t test).
- Figure 5 Increased uptake of uric acid in *E4bp4*-deficient primary hepatocytes.** (A) protein expression of SLC2A9 in the hepatocytes of *E4bp4*^{-/-} and WT mice. **p* < 0.05 (t test). (B) Uric acid uptake in hepatocytes of *E4bp4*^{-/-} and WT mice at indicated time points (left panel, dosed at 50 μM) and concentrations (right panel, sampled at 30 min). **p* < 0.05 (two-way ANOVA and Bonferroni post hoc test). Data are mean ± SD (*n* = 3 or 5).
- Figure 6 *E4bp4* ablation protects mice from chemical-induced hyperuricemia.** (A) Uric acid in the plasma and urine of *E4bp4*^{-/-} and WT mice treated with PO. (B) Uric acid and allantoin in the liver of *E4bp4*^{-/-} and WT mice treated with PO. Data are mean ±

SD ($n = 5$). $*p < 0.05$ (t test)

Figure 7 E4BP4 regulates SLC2A9 expression in Hepa-1c1c7 cells. (A) Effects of *E4bp4* overexpression on *E4bp4* and *Slc2a9* mRNAs. (B) Effects of *E4bp4* overexpression on SLC2A9 protein. (C) Effects of *E4bp4* overexpression on cellular uptake of uric acid (50 μ M) at 30 min after incubation. (D) Effects of *E4bp4* knockdown on *E4bp4* and *Slc2a9* mRNAs. (E) Effects of *E4bp4* knockdown on SLC2A9 protein. (F) Effects of *E4bp4* knockdown on cellular uptake of uric acid (50 μ M) at 30 min after incubation. Data are mean $\pm$ SD ($n = 3$). $*p < 0.05$ (t test).

Figure 8 E4BP4 regulates SLC2A9 expression in primary mouse hepatocytes. (A) Effects of *E4bp4* overexpression on *E4bp4* and *Slc2a9* mRNAs. (B) Effects of *E4bp4* overexpression on SLC2A9 protein. (C) Effects of *E4bp4* overexpression on cellular uptake of uric acid (50 μ M) at 30 min after incubation. (D) Effects of *E4bp4* knockdown on *E4bp4* and *Slc2a9* mRNAs. (E) Effects of *E4bp4* knockdown on SLC2A9 protein. (F) Effects of *E4bp4* knockdown on cellular uptake of uric acid (50 μ M) at 30 min after incubation. Data are mean $\pm$ SD ($n = 3$). $*p < 0.05$ (t test).

Figure 9 E4BP4 regulates *Slc2a9* transcription. (A) Schematic diagram for ChIP-sequencing of liver at ZT22, showing the signals of E4BP4 on the P1 and P2 promoters of *Slc2a9*. Data were obtained from GSM1437733 of NCBI GEO. (B) Effects of *E4bp4* on the activities of *Slc2a9* P2-Luc and *Slc2a9* P1-Luc reporters. (C) Schematic diagram of D-box on the *Slc2a9* P2 promoter. (D) Effects of *E4bp4* on *Slc2a9* transcript driven by P1 promoter only. (E) Effects of *E4bp4* (200 ng) on a mutated version of *Slc2a9* P2-Luc reporter activity. (F) Enrichment of E4BP4 at P1 and P2 promoters of *Slc2a9* in liver of *E4bp4*^{-/-} and control mice at ZT2. A non-specific region (NR) was used as a negative control. Data are mean $\pm$ SD ($n = 3$ or 5). Percentage of input (% input) is defined as the amount of DNA pulled down by using anti-E4BP4 antibody, relative to the amount of starting material (input sample).

In panel B, $*p < 0.05$ (one-way ANOVA and Bonferroni post hoc test). In panel E, $*p < 0.05$ (two-way ANOVA and Bonferroni post hoc test). RLU, relative luciferase unit.

Figure 10 mRNA levels of xanthine oxidase (XO), *urate oxidase (Uox)* and *Bcrp* in the liver (A) and of three SLC transporters and *Bcrp* in the kidney (B) in *E4bp4*-deficient and WT mice at ZT2. Data are mean $\pm$ SD ($n=5$).

Table

Table 1. Oligonucleotides used for PCR genotyping, qPCR and ChIP

	Forward (5'-3' sequence)	Reverse (5'-3' sequence)
PCR genotyping		
<i>E4bp4</i>	GATTGCATGGGTCCAAGTCT	CACAAGGACACCCAGACAGA
qPCR		
<i>Bcrp</i>	CCTCACCTTACTGGCTTCCG	ATCCGCAGGGTTGTTGTAGG
<i>E4bp4</i>	CTTTCAGGACTACCAGACATCCAA	GATGCAACTTCCGGCTACCA
<i>Slc2a9</i>	GAAGCAAAGTACCACAGAGT	GATGTCGGTGGCCCTCTCT
<i>Slc2a9-P1</i>	TGTGGCTGGGAATGTGATCC	TGCCTCGTAACTGCAAGCTC
<i>Slc1a4</i>	GGCATCGCTGTTGCTTACTTC	CGAGGAAAGAGTCCACTGTCT
<i>Slc2a2</i>	TCAGAAGACAAGATCACCGGA	GCTGGTGTGACTGTAAGTGGG
<i>Slc4a4</i>	GATGCCACCGACAACATGC	TCAAGATGGTAAGCGGTTGAC
<i>Slc4a7</i>	GCTGGCTACCACCTTGACTTG	ACTTATAGACAACACTGTCGCAG
<i>Slc6a6</i>	GCACACGGCCTGAAGATGA	ATTTTGTAGCAGAGGTACGGG
<i>Slc6a8</i>	GCAGGGTGTGCATATCTCCAA	TACCCCCACTCACATCAGTCA
<i>Slc6a9</i>	AAAAGGTGCCAAAGGGATGTT	GTCAGTACAACTCGATCTGGTT
<i>Slc6a12</i>	GGTCCCTGAGGAAGGAGAGAT	GGGGATGAAGAAAGCTCCACC
<i>Slc6a13</i>	CAGTACACCAACCAGGGAGG	GCCAGGACAACGATGTAGTAGA
<i>Slc16a10</i>	GAGGTGGAGCTGACGAGGT	CATGGACACGAAGAGCACCC
<i>Slc16a11</i>	GCTTGGTCTTCTCGGCTTTC	GCGAGGATGCCCCATTACC
<i>Slc17a1</i>	TCTGTTCCCTCCGGTATGGAC	AGAACTGAGAATAAGCCCTTGGA
<i>Slc17a3</i>	GTAGAGATGAGCCCTACAAGTGA	CCATAGCGCATGGAACATAAACT
<i>Slc22a1</i>	GACGCCTGGAAAGTGGACC	GCAACATGGATGTATAGTCTGGG
<i>Slc22a3</i>	CAGCCCGACTACTATTGGTGT	TGAGCTGGTATTAGTGGCTTCC

<i>Slc22a4</i>	TGGTATGTCAGTCGTGTTTCCT	AGCCCCATCGCAGAGAAGT
<i>Slc22a5</i>	ACTGTGCCAGGGGTGCTAT	GCAACTGAGGCTTCGTAGAAT
<i>Slc22a6</i>	GCAAGGACGACCCGAATCTT	GAGCACACAAGGTTCCACTCA
<i>Slc22a7</i>	CAACTGCGGAATCTGGTGCT	ATCAGGCAGGGCACAATGATG
<i>Slc22a8</i>	AGAAGTGTCTCCGCTTCGTG	CGCATACCAAGTCCCCTCT
<i>Slc22a12</i>	TACGACCACAGCACCTTCAG	TTCTGCGCCCAAACCTATCT
<i>Slc22a15</i>	TTGTGTGCAGCTTGGTGACT	GCAGAGAGGGTAGGATGGAATCT
<i>Slc22a18</i>	CACTGGGCCTAGATTCTGTCT	CGCCAGGAAGGAGAGTGAGA
<i>Slc22a28</i>	TTAAGGGCAACAGGTATGGGA	GGGGCCAGGAATAATCCACT
<i>Slc22a30</i>	GTGGATGTCAGAGTCGGCTC	CCGCTTTCGTAAGATGGGTGT
<i>Slc25a15</i>	GCTGCCTCAAGACCTACTCC	CCGTAACACATGAACAGCACC
<i>Slc30a10</i>	TGTGGTCATCACGGCTATCAT	ATGTAGCACTGCCAGTTACAC
<i>Slc34a1</i>	TGCCTCTGATGCTGGCTTTC	GATAGGATGGCATTGTCCTTGAA
<i>Slc38a4</i>	GCGGGGACAGTATTCAGGAC	GGAACTTCTGACTTTCGGCAT
<i>Slc39a8</i>	GCCAAGCTCATGTACCTGTCT	AAGATGCCCCAATCGCCAA
<i>Slc39a13</i>	AGTGGCTATCTCAACCTGCTT	GCCCGATCTTTTTGCTCACAA
<i>Slc40a1</i>	ACCAAGGCAAGAGATCAAACC	AGACACTGCAAAGTGCCACAT
<i>Slco2a1</i>	TGAAGCGTTTTGTTTTCCCTCT	CGGGTGTGGAACATCCCATAA
<i>Slco2b1</i>	CTCAGGACTCACATCAGGATGC	CTCTTGAGGTAGCCAGAGATCA
<i>Slco3a1</i>	AGGTGTCCTGCTTCTCCAAC	GTCAACACGCTCACCAGGTAG
<i>Uox</i>	AAGAGGTCCCCTGGAAACGA	TGAATGACGGGAGGTCCGTT
<i>XO</i>	CCTTAGAAGAAAGTTGGGGCTG	CTGAAGGCGGTCATACTTGGA
<i>Ppib</i>	TCCACACCCTTTTCCGGTCC	CAAAAGGAAGACGACGGAGC
ChIP		
<i>Slc2a9-P1</i>	TAAGGGCTTGGGCATCTTGACA	GGGCTGTGCACCTGTCTTGT
<i>Slc2a9-P2</i>	TCCCCTCCCACCCTATCAGAA	CAACGACCTTGTATGCCCC
<i>NR</i>	TGCTTTTTTCACACCTTCAGCTC	TGCAAGGGACTGGGTAGGTA

siRNA

siE4BP4	GCACAAGCUUCGAUUAAT	UUUAAUCCGAAGCUUGCTT
Control	CGAUUAGUCUAUACGUUCUCCUG	CUCAGGAGAACGUAUAGACUAAUC

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10