Tip60 Might Be a Candidate For The Acetylation of Hepatic Carbonic Anhydrase I and III in Mice

Nurdan GONUL BALTACI
Ataturk University: Ataturk Universitesi
Enver Fehim KOCPINAR
Muş Alparslan Üniversitesi: Mus Alparslan Universitesi
Harun Budak (hbudak@atauni.edu.tr)
Ataturk University https://orcid.org/0000-0002-7371-8959

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Abstract

Carbonic anhydrases (CAs) play an important role in maintaining pH balance by catalyzing the conversion of carbon dioxide to bicarbonate. Since this pH balance is critical to health, all organisms must develop mechanisms to control and regulate it. Although there is a great deal of literature on the biochemical, functional, and structural properties of the CA family, there is no enough knowledge on the regulation of CAs at gene and protein levels, especially their epigenetic regulation. In this study, impact of Tip60, a member of histone acetyltransferases family, on the expression of *Ca1* and *Ca3* genes in liver tissue was investigated at different zeitgeber time points in control and liver-specific Tip60 knockout mice (mutant) groups. First of all, Tip60 was specifically knocked out in mouse liver the using Cre/loxP system and knockout rate was shown as 83% - 88% by southern blot. Expression profiles of *Ca1* and *Ca3* genes in both groups were determined by Real-Time PCR at six different time points. While *Ca1* showed the highest expression at ZT8 and ZT12, the lowest expression profile was observed at ZT0 and ZT20. Hepatic *Ca1* showed a robust circadian expression. While hepatic *Ca3* showed almost the same level of expression at different time units. The expression of *Ca1* and *Ca3* significantly decreased in the absence of *Tip60* in mouse liver all time period. In conclusion, it was suggested for the first time that *Tip60* may be considered a candidate protein in the regulation of *Ca1* and *Ca3* genes, possibly by acetylation.

Introduction

Carbonic anhydrases (CAs), zinc-containing metalloenzymes that are commonly found in living organism, catalyze the reversible hydration of CO2 to bicarbonate (HCO-3) and proton (H+) [1-3]. Although these enzyme were first discovered in hemolyzed blood, today it is known to have activity in many tissues such as kidney, liver, brain, muscle, and bone tissues [4-7]. CAs found in vertebrates (α-CA) are composed of 16 different isoenzymes and play a role in a wide variety of functions such as respiration, acid-base homeostasis, ion transport, bone resorption, taste preferences, ureagenesis, and gluconeogenesis [8-10]. Eight of these proteins are in the cytosol (CAI, CAII, CAIII, CAVII, CA VIII, CA X, CA XI and CA XIII), five are transmembrane or membrane bound (CAIV, IX, XII, XIV and XV), two are in mitochondria (CAVA and VB), and one (CAVI) is secreted [11]. While there is a great deal of literature on the biochemical, functional, and structural properties of the CA family, there is no enough knowledge on the regulation of CAs at gene and protein levels, especially their epigenetic regulation [12]. Epigenetic mechanisms such as acetylation, phosphorylation, methylation, ubiquitination, sumoylation, and glycosylation are required to regulate gene expression and chromatin structure in mammalian cells without modulating the DNA sequence [13, 14]. Protein acetylation, which refers to the covalent binding of an acetyl group to an amino acid residue of a protein, is the most well-known along with phosphorylation [15]. The correlation between increased transcription and histone acetylation has been known for many years. Thus, acetylation regulates a number of metabolic and physiological processes by affecting protein functions, protein-DNA and protein-protein interactions, and subcellular localization of the protein [16, 17]. A recent study reported that members of the α-CA family, CAI (at the N-terminus), CAII (at the N-terminus, K18, K39, and K113), CAIII (at the N-terminus and K126), and CAXII (at K194), are acetylated by acetyltransferase enzymes. But

the proteins involved in this acetylation are still unknown [12]. TIP60 protein, a member of the histone acetyltransferases (HATs) protein family, has important and vital functions such as transcriptional regulations, DNA repair, cell cycle, apoptosis mechanism, cancer, circadian system and generation of cellular signals, both directly and indirectly. [18-21]. Studies have shown that the Tip60 protein is associated with many transcription factors and proto-oncogenes such as androgen receptor, c-Myb, c-Myc, STAT3, NF-Kb, E2F1, p53 and acts as a regulator / correlator [22-24]. The study was performed by Chen et al. showed that c-Myb transcription factor increases Ca1 expression by binding to its promoter in mouse erythroleukemia cells [25]. However, c-Myb inactivation is required to inhibit the Ca1 gene when the division of the cell is achieved. The cell goes to cancer without this suppression. It is also known that Tip60 is a regulating factor for c-Myb [22]. In this study, it is aimed that does Tip60 have a role in the regulation of hepatic Ca1 and Ca3 which are predominantly expressed in the liver? For this purpose, liver-specific Tip60 knockout mice was generated by using Cre/loxP recombination. Quantitative expression of Ca1 and Ca3 genes at different zeitgeber time (ZT) points were determined for both control and knockout groups and then compared each other. Ca7, which is not regulated by acetylation, was used as a negative control [12]

Materials And Methods

2.1. Liver-Specific Conditional Knockout Mouse Model

To generate liver-specific Tip60 knockout mice (mutant), Tip60 floxed mice (10-12 week old male) with loxP sites flanking exons 1 and 9 of the Tip60 gene [21] were crossed to a SACre driver mouse line resulting in Cre-mediated deletion of Tip60 in the liver [26]. Mice were previously backcrossed to a C57BL/6N background for at least 10 generations. To delete Tip60, 10-12 week-old male mice ($Tip60^{fl/-}$; $SA^{+/Cre\text{-}ERT2}$) were injected daily with tamoxifen (10mg/ml stock solution, Sigma, St. Louis, MO, USA) in corn oil for five consecutive days. The control group ($Tip60^{fl/fl}$;$SA^{+/+}$), in which corn oil were injected. Genotyping was performed with gene-specific primers [21, 26]. Liver and kidney tissues were collected five days after the last injection. Genomic DNA was isolated from both tissues and analyzed by southern blot.

2.2. Southern Blot Analysis

Genomic DNA from liver and kidney tissues was isolated with the DNeasy Tissue kit (Qiagen Inc., Valencia, CA, USA) and digested with BamHI (NEB, Ipswich, MA, USA). DNA was separated on a 0.6% agarose gel and transferred onto Hybond-XL positive charged nylon membrane (GE Healthcare/Amersham Biosciences, Sweden). The membranes were hybridized with a 32^{P}-dCTP labeled radioactive double-stranded DNA probe was prepared by random priming using an appropriate commercial kit according to manufacturer's instructions (Amersham Rediprime™ II DNA Labeling System, GE Healthcare) and purified with the illustra ProbeQuant™ G-50 Micro Columns (GE Healthcare). Hybridization of the radioactive probe (100 µl) to the membrane was performed at 65°C overnight in the presence of a hybridization buffer. Membranes were washed with 2xSSC/0.1% SDS, 1xSSC/0.1% SDS,

and 0.1xSSC/0.1% SDS, at 60°C until the excess label was removed and exposed to a sensitive X-ray film (Kodak X-Omat 1000,1000A ve 1000J Processors).

2.3. Experimental Animals, Feeding, and Zeitgeber Time

At least 3 weeks prior any experiment, all mice were singly housed with food and water ad libitum under a 12-hour-light/12-hour-dark cycle (350 lux). Throughout this study, time is indicated using zeitgeber time (ZT) as the indicator for the phase of the rhythm, wherein ZT0 refers to the time that lights went on (06:00), and ZT12 refers to the time that the lights went off (18:00). ZT4, ZT8, ZT16, and ZT20 in this study are equivalent to 10:00, 14:00, 22:00, and 02:00 respectively [27]. Artificial light was provided daily from ZT0 (06:00), with temperature (24 ± 1) °C, and humidity (55 ± 5%) kept constant [28]. In the first set of experiments, 10–12-week-old male C57BL/6N mice were used and split up into six groups corresponding to the six chosen timepoints (ZT0, ZT4, ZT8, ZT12, ZT16, and ZT20). For the second set of experiments, we used 10–12-week-old male mutant mice ($Tip60^{fl/-}$; $SA^{+/Cre\text{-}ERT2}$) and their respective control littermates [29].

2.4. RNA Extraction and First Strand cDNA Synthesis

Total RNA isolation from approximately 50 mg mice liver tissues was performed using the RNeasy Lipid Tissue Mini Kit (Qiagen-74804) following the manufacturer's instructions. Concentrations and purities of RNAs were measured by spectrophotometer. (Thermo Scientific, Multiskan GO, USA), RNA quality was checked by agarose gel electrophoresis and stored at −80°C until use. Total RNA was converted into first strand cDNA using SuperScript III First-Strand cDNA kit system (Invitrogen, California, USA), utilizing random hexamers, according to the manufacturer's protocol. The resulting cDNAs were diluted to 100 ng/µL with nuclease-free water and stored at −20 °C [30].

2.5. Primers and Probes Design

Primer3 software (v. 0.4.0) (http://bioinfo.ut.ee/primer3-0.4.0/) was used for gene-specific primers and probes design that met the following criteria; amplicon size 75-200 bp, ≤3 G or C repetitions, GC content 50-65%, ≤4 base repetitions, melting temperature (Tm) 60°C. Primers and probes were verified with Blast Tool (NCBI) to confirm its specificity for the desired target. Then they were synthetized and purchased from Methabion International (Martinsried, Germany). Gene symbols and GenBank ID numbers are; *Ca1* (Gene ID: 12346), *Ca3* (Gene ID: 12350), *Ca7* (Gene ID: 12354), and Actb (Gene ID: 11461). Since housekeeping genes were not affected by any of treatments, *β-actin* was used as reference gene. The sequences of specific primers of all genes were shown in Table 1.

2.6. Quantitative Real-Time PCR

To determine expression levels of *Ca 1, Ca 3,* and *Ca 7* genes in different circadien points, Real-time PCR (qPCR) was carried out on Rotor-Gene Q instrument (QIAGEN, Inc., Hilden, Germany). Beta-actin was selected as reference control gene. The qPCR reactions were carried out with 2 µL of cDNA (final

concentration is 0.02ng), 4 pmol of TaqMan probe, 8 pmol of forward and reverse primers and 10 µL FastStart TaqMan Probe Master Mix (Roche Diagnostics GmbHCorp, Mannheim, Germany) in a final volume of 20 µL. Optimal cycling conditions were 50 °C for 2 min, 95 °C for 10 min, 45 cycles of 95 °C for 15s, and annealing and extension at 60 °C for 1 min [31]. The expression results were analyzed using the ΔCT method [32].

2.7. Statistical Analysis

Each group contained three animals, and all measurements were triplicated for each animal. Statistical analysis was performed for each experiment using one-way and two way analysis of variance (ANOVA) with Tukey's and Bonferroni's multiple comparisons test using the Prism software 7.0 (GraphPad Software, San Diego, CA). A value of $P < .05$ was considered to indicate a statistically significant difference (*$P < .05$, **$P < .01$, ***$P < .001$, **** $P < .0001$).

Results

3.1. Liver Specific Tip60 Knockout Mice Models

Tamoxifen-inducible Cre/lox recombination was used to perform genetically modified mouse lines in C57BL/6N backgrounds tissue-specific or conditional knockouts of *Tip60* due to the lethal effect of full knockout on mice. *Tip60* gene was specifically knocked out in mouse liver the using this system and the knockout rate was shown as 83% - 88% by southern blot. The leakage of CRE driver also checked in kidney tissues and was not seen any leak within the kidney tissues (Fig. 1).

3.2. Expression levels of *Ca1*, *Ca3*, and *Ca7* genes at different zeitgeber time periods in the control groups

Circadian expression of *Ca1*, *Ca3*, and *Ca7*, which are expressed in the liver, was investigated in mice at six different ZT points in the contol group. While *Ca1* showed the highest expression at ZT8 and ZT12, the lowest expression profile was observed at ZT0 and ZT20. Hepatic *Ca1* showed a robust circadian expression. While hepatic *Ca3* showed almost the same level of expression at different ZT periods, it revealed no circadian expression. While the highest expression of *Ca7* was observed at ZT12, the lowest expression was seen at ZT4. Furthermore, hepatic *Ca7* was also showed a robust circadian expression (Table 2).

3.3. Comparison of gene expression levels of control and mutant groups

A recently reported that while CAI and CAIII are acetylated by acetyltransferase enzymes, CAVII is not acetylated [12]. However, the proteins involved in this acetylation are still unknown. Since Tip60 is thought to be a candidate for the acetylation of *Ca1* and *Ca3* in the liver, quantitative expression levels of *Ca1* and *Ca3* in control and mutant groups were analyzed by qPCR. As a result of the analysis, it was determined that the expression amount in mutant tissues decreased significantly in all time points compared to control groups. No change was observed in the expression of *Ca7* used as negative control. (Figure 2).

Discussion

CO_2 is one of the simplest molecules involved in important physiological processes for all life domains. It is produced as part of the metabolic process and is quickly transported in the body through the blood [33, 34]. The acidity of the blood increases due to the high solubility and rapid spread of CO_2. Since the pH of the human body is critical to health, all organisms must develop mechanisms to control it [35, 36]. CAs are an important family of enzymes that catalyze the conversion of CO_2 into HCO_3^- to regulate the pH balance of the blood. [37-39].

Many studies have proved the important role of CAs in physiological processes and showed that abnormal activity levels of these enzymes are associated with various human diseases such as glaucoma, erythroleukemia malignant brain tumors, and renal, gastric, and pancreatic carcinomas [35, 40]. Furthermore, HCO_3^- produced by carbonic anhydrases is essential for the function of metabolic liver enzymes that performs many functions, including digestion, glycogen synthesis, manufacturing triglycerides and cholesterol, bile production, storage for many essential vitamins and minerals [41]. It is also metabolizing many drugs, medications, chemicals, and natural substances. Although several CA isoforms, including CA I, CA II, CA III, CAVII and CA IX, have been described in liver, there is very limited information about regulation of CAs at the gene and protein levels, especially their transcriptional regulation [4, 42-44].

As a result of the analysis, *Ca1*, *Ca3*, and *Ca7* genes were expressed all ZT points in the liver tissues of control group. It was observed that the expression of Ca1 and Ca7 reached the maximum level in ZT8 (end of light cycle) and ZT12 (beginning of dark cycle) time unit and Ca3 gene was expressed high amounts in all time units except ZT16. In control group, comparing quantitative expression levels of *Ca1*, *Ca3*, and *Ca7* in liver tissues, genes with the highest expression were *Ca3*, *Ca1*, and *Ca7* respectively. In this respect, our findings are consistent with the "NCBI-Mouse ENCODE transcriptome data"[45].

In the study performed by Chen et al in 2006 in mouse erythroleukemia (MEL) cells that c-Myb transcription factor binds to the promoter of carbonic anhydrase 1 (*Ca1*) gene and increases the expression of *Ca1* for proliferation and differentiation [25]. Zhao et al. was stated that TIP60, a histone acetyl transferase with activity in cytoplasm and nucleus, belongs to MYST (Moz-Ybf2 / Sas3-Sas2-Tip60) family and is known to be responsible for acetylation in both mouse and human cells, is a regulating factor for c-Myb [22, 46-48]. In another study, the relationship between hepatocellular carcinoma (HCC) and tumor development with the expression of CAs was studied. The activity and protein expression of CA family in tumor tissues were observed to be significantly lower than normal cells [49]. Recent study reported by Di Fiore A, et al., CA I and CA III proteins are regulated by post-translational acetylation [12].

Many researchers have reported that knockout mice are widely used to better study the biological role of specific genes, as well as molecular and cellular mechanisms [50-52]. The study by Hu et al. showed that homozygous disruption of the Tip60 gene lead to early embryonic death [53]. Therefore, we generated

liver-specific Tip60 knockout mice using the tamoxifen-inducible Cre/ loxP system to investigate the role of *Tip60* in the regulation of *Ca1* and *Ca3* was investigated at the gene level. In addition, since TIP60 has been shown to have a role in the regulation of the circadian clock [21], it has been investigated whether this arrangement occurs in different ZT points. As shown in figure 1, Tip60 gene knockout rate was shown between 83% - 88% by southern blot technique in the liver. The leakage of CRE driver also checked in kidney tissues and was not seen any leak within the kidney tissues.

The knockout rate of *Tip60* in the liver tissue obtained from our study is efficient and useful for further studies as shown in the literature [54, 55]. And then, we investigated the expression of *Ca1* and *Ca3* genes at six different ZT points in the control and mutant groups. While the expression of *Ca1* and *Ca3* significantly decreased in the absence of *Tip60* in mouse liver all time period (Figure 2a and b), the expression of *Ca7*, which is a negative contol as we mentioned above, was not affected (Figure 2c). Potter and Harris stated that some cytoplasmic CAs are markers for human cancers [56]. Bekku et al., reported that *Ca1* expression decreased in colorectal tumors [57]. Chiang et al., observed that expression of *Ca1* decreased in adenocarcinoma [58]. Kuo et al. revealed that reduced levels of CA I and III in human hepatocellular carcinoma (HCC). In contrast to this, in 2008, a study showed that increased CA III expression accelerates HCC through the focal adhesion kinase signaling pathway [59]. It was hypothesized that CA III is re-expressed in later stages of metastatic progression of HCC, and it might have an important influence in the development of metastasis in liver cancer [39]. Following the results obtained from these two studies, It is hypothesized that while the decreased expression of *Ca3* is important in the pathogenesis of HCC, increased *Ca3* expression is required to metastasis after the process of cancer formation [39]. Based on the literature data, it is thought that decrease in *Ca1* and *Ca3* expression in mice due to the depletion of *Tip60* may lead to HCC.

In conclusion, TIP60 may be considered a candidate protein in the regulation of *Ca1* and *Ca3* genes, possibly by acetylation. Moreover, our results show that *Tip60* could be a new actor in explaining the molecular mechanism of hepatocellular carcinoma. However, it is clear that more studies including in vitro and in vivo tests are needed to support this hypothesis.

Declarations

Author contributions

Conceived and designed the experiments: HB (group leader). Performed the experiments: NGB, EFK, and HB. Analyzed the data: NGB, EFK, and HB. Contributed reagents/materials/analysis tools: HB. Wrote the paper: HB and NGB. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that there is no potential conflict of interest with respect to the research, authorship, and/or publication of this article. All authors read and approved the final manuscript.

Compliance with ethical standards

This article does not contain any studies with human participants. Animal experimentation: Mouse handling was carried out in accordance with the German Law on Animal Welfare and was ethically approved and licensed by the Office of Consumer Protection and Food Safety of the State of Lower Saxony (license numbers 33.11.42502-04/072/07 and 33.9-42502-04-12/0719).

References

1. Gilmour KM: **Perspectives on carbonic anhydrase**. *Comp Biochem Physiol A Mol Integr Physiol* 2010, **157**(3):193-197.
2. Ozensoy Guler O, Capasso C, Supuran CT: **A magnificent enzyme superfamily: carbonic anhydrases, their purification and characterization**. *J Enzyme Inhib Med Chem* 2016, **31**(5):689-694.
3. Li Z, Jiang L, Toyokuni S: **Role of carbonic anhydrases in ferroptosis-resistance**. *Arch Biochem Biophys* 2020, **689**:108440.
4. Cankaya M, Hernandez AM, Ciftci M, Beydemir S, Ozdemir H, Budak H, Gulcin I, Comakli V, Emircupani T, Ekinci D *et al*: **An analysis of expression patterns of genes encoding proteins with catalytic activities**. *Bmc Genomics* 2007, **8**.
5. Haapasalo J, Nordfors K, Haapasalo H, Parkkila S: **The Expression of Carbonic Anhydrases II, IX and XII in Brain Tumors**. *Cancers* 2020, **12**(7).
6. Shaikh AB, Fang HW, Li M, Chen SY, Shan P, Shang XL: **Reduced expression of carbonic anhydrase III in skeletal muscles could be linked to muscle fatigue: A rat muscle fatigue model**. *J Orthop Transl* 2020, **22**:116-123.
7. Chang XT, Zheng YB, Yang QR, Wang L, Pan JH, Xia YF, Yan XF, Han JX: **Carbonic anhydrase I (CA1) is involved in the process of bone formation and is susceptible to ankylosing spondylitis**. *Arthritis Res Ther* 2012, **14**(4).
8. Supuran CT: **Carbonic anhydrase activators**. *Future Med Chem* 2018, **10**(5).
9. Li Z, Jiang L, Toyokuni S: **Role of carbonic anhydrases in ferroptosis-resistance**. *Archives of Biochemistry and Biophysics* 2020, **689**.
10. Supuran CT: **Diuretics: From classical carbonic anhydrase inhibitors to novel applications of the sulfonamides**. *Curr Pharm Design* 2008, **14**(7):641-648.

11. Bayram E, Senturk M, Kufrevioglu OI, Supuran CT: **In vitro inhibition of salicylic acid derivatives on human cytosolic carbonic anhydrase isozymes I and II**. *Bioorgan Med Chem* 2008, **16**(20):9101-9105.
12. Di Fiore A, Supuran CT, Scaloni A, De Simone G: **Human carbonic anhydrases and post-translational modifications: a hidden world possibly affecting protein properties and functions**. *J Enzym Inhib Med Ch* 2020, **35**(1):1450-1461.
13. Chen JZ, Liu Q, Zeng LB, Huang XT: **Protein Acetylation/Deacetylation: A Potential Strategy for Fungal Infection Control**. *Front Microbiol* 2020, **11**.
14. Li XJ, Egervari G, Wang YG, Berger SL, Lu ZM: **Regulation of chromatin and gene expression by metabolic enzymes and metabolites**. *Nat Rev Mol Cell Bio* 2018, **19**(9):563-578.
15. Xia C, Tao Y, Li MS, Che TJ, Qu J: **Protein acetylation and deacetylation: An important regulatory modification in gene transcription (Review)**. *Exp Ther Med* 2020, **20**(4):2923-2940.
16. Kouzarides T: **Acetylation: a regulatory modification to rival phosphorylation?** *EMBO J* 2000, **19**(6):1176-1179.
17. Narita T, Weinert BT, Choudhary C: **Functions and mechanisms of non-histone protein acetylation**. *Nat Rev Mol Cell Biol* 2019, **20**(3):156-174.
18. Miyamoto N, Izumi H, Noguchi T, Nakajima Y, Ohmiya Y, Shiota M, Kidani A, Tawara A, Kohno K: **Tip60 is regulated by circadian transcription factor clock and is involved in cisplatin resistance**. *J Biol Chem* 2008, **283**(26):18218-18226.
19. Sun Y, Jiang X, Price BD: **Tip60: connecting chromatin to DNA damage signaling**. *Cell Cycle* 2010, **9**(5):930-936.
20. Sun Y, Jiang X, Chen S, Fernandes N, Price BD: **A role for the Tip60 histone acetyltransferase in the acetylation and activation of ATM**. *Proc Natl Acad Sci U S A* 2005, **102**(37):13182-13187.
21. Petkau N, Budak H, Zhou X, Oster H, Eichele G: **Acetylation of BMAL1 by TIP60 controls BRD4-P-TEFb recruitment to circadian promoters**. *Elife* 2019, **8**.
22. Zhao H, Jin S, Gewirtz AM: **The histone acetyltransferase TIP60 interacts with c-Myb and inactivates its transcriptional activity in human leukemia**. *J Biol Chem* 2012, **287**(2):925-934.
23. Frank SR, Parisi T, Taubert S, Fernandez P, Fuchs M, Chan HM, Livingston DM, Amati B: **MYC recruits the TIP60 histone acetyltransferase complex to chromatin**. *EMBO Rep* 2003, **4**(6):575-580.
24. Xiao H, Chung J, Kao HY, Yang YC: **Tip60 is a co-repressor for STAT3**. *J Biol Chem* 2003, **278**(13):11197-11204.
25. Chen J, Kremer CS, Bender TP: **The carbonic anhydrase I locus contains a c-Myb target promoter and modulates differentiation of murine erythroleukemia cells**. *Oncogene* 2006, **25**(19):2758-2772.
26. Schuler M, Dierich A, Chambon P, Metzger D: **Efficient temporally controlled targeted somatic mutagenesis in hepatocytes of the mouse**. *Genesis* 2004, **39**(3):167-172.
27. Jiao X, Wu M, Lu D, Gu J, Li Z: **Transcriptional Profiling of Daily Patterns of mRNA Expression in the C57BL/6J Mouse Cornea**. *Curr Eye Res* 2019, **44**(10):1054-1066.

28. Zhu X, Yang L, He Y, Sun Y, Shi W, Ou C: **Liver Function of Male Rats Exposed to Manganese at Different Time Points**. *Biol Trace Elem Res* 2020, **198**(1):224-230.
29. Perreau-Lenz S, Zghoul T, de Fonseca FR, Spanagel R, Bilbao A: **Circadian regulation of central ethanol sensitivity by the mPer2 gene**. *Addict Biol* 2009, **14**(3):253-259.
30. Budak H, Kocpinar EF, Gonul N, Ceylan H, Erol HS, Erdogan O: **Stimulation of gene expression and activity of antioxidant related enzyme in Sprague Dawley rat kidney induced by long-term iron toxicity**. *Comp Biochem Physiol C Toxicol Pharmacol* 2014, **166**:44-50.
31. Ceylan H, Budak H, Kocpinar EF, Baltaci NG, Erdogan O: **Examining the link between dose-dependent dietary iron intake and Alzheimer's disease through oxidative stress in the rat cortex**. *J Trace Elem Med Bio* 2019, **56**:198-206.
32. Pfaffl MW: **A new mathematical model for relative quantification in real-time RT-PCR**. *Nucleic Acids Res* 2001, **29**(9):e45.
33. Neri D, Supuran CT: **Interfering with pH regulation in tumours as a therapeutic strategy**. *Nat Rev Drug Discov* 2011, **10**(10):767-777.
34. Supuran CT: **Structure and function of carbonic anhydrases**. *Biochem J* 2016, **473**:2023-2032.
35. Alterio V, Di Fiore A, D'Ambrosio K, Supuran CT, De Simone G: **Multiple Binding Modes of Inhibitors to Carbonic Anhydrases: How to Design Specific Drugs Targeting 15 Different Isoforms?** *Chem Rev* 2012, **112**(8):4421-4468.
36. Capasso C, Supuran CT: **An overview of the alpha-, beta- and gamma-carbonic anhydrases from Bacteria: can bacterial carbonic anhydrases shed new light on evolution of bacteria?** *J Enzym Inhib Med Ch* 2015, **30**(2):325-332.
37. Blandina P, Provensi G, Passsani MB, Capasso C, Supuran CT: **Carbonic anhydrase modulation of emotional memory. Implications for the treatment of cognitive disorders**. *J Enzym Inhib Med Ch* 2020, **35**(1):1206-1214.
38. Lee D, Hong JH: **The Fundamental Role of Bicarbonate Transporters and Associated Carbonic Anhydrase Enzymes in Maintaining Ion and pH Homeostasis in Non-Secretory Organs**. *Int J Mol Sci* 2020, **21**(1).
39. Mboge MY, Mahon BP, McKenna R, Frost SC: **Carbonic Anhydrases: Role in pH Control and Cancer**. *Metabolites* 2018, **8**(1).
40. Clare BW, Supuran CT: **A perspective on quantitative structure-activity relationships and carbonic anhydrase inhibitors**. *Expert Opin Drug Metab Toxicol* 2006, **2**(1):113-137.
41. Diez-Fernandez C, Rufenacht V, Santra S, Lund AM, Santer R, Lindner M, Tangeraas T, Unsinn C, de Lonlay P, Burlina A *et al*: **Defective hepatic bicarbonate production due to carbonic anhydrase VA deficiency leads to early-onset life-threatening metabolic crisis**. *Genet Med* 2016, **18**(10):991-1000.
42. Alchera E, Tacchini L, Imarisio C, Dal Ponte C, De Ponti C, Gammella E, Cairo G, Albano E, Carini R: **Adenosine-dependent activation of hypoxia-inducible factor-1 induces late preconditioning in liver cells**. *Hepatology* 2008, **48**(1):230-239.

43. Bejaoui M, Pantazi E, De Luca V, Panisello A, Folch-Puy E, Hotter G, Capasso C, Supuran CT, Rosello-Catafau J: **Carbonic Anhydrase Protects Fatty Liver Grafts against Ischemic Reperfusion Damage (vol 10, e0134499, 2015)**. *Plos One* 2015, **10**(9).
44. Thiry A, Dogne JM, Masereel B, Supuran CT: **Targeting tumor-associated carbonic anhydrase IX in cancer therapy**. *Trends Pharmacol Sci* 2006, **27**(11):566-573.
45. Yue F, Cheng Y, Breschi A, Vierstra J, Wu WS, Ryba T, Sandstrom R, Ma ZH, Davis C, Pope BD *et al*: **A comparative encyclopedia of DNA elements in the mouse genome**. *Nature* 2014, **515**(7527):355-+.
46. Dong Y, Isono K, Ohbo K, Endo TA, Ohara O, Maekawa M, Toyama Y, Ito C, Toshimori K, Helin K *et al*: **EPC1/TIP60-Mediated Histone Acetylation Facilitates Spermiogenesis in Mice**. *Molecular and Cellular Biology* 2017, **37**(19).
47. Li TY, Song LT, Sun Y, Li JY, Yi C, Lam SM, Xu DJ, Zhou LK, Li XT, Yang Y *et al*: **Tip60-mediated lipin 1 acetylation and ER translocation determine triacylglycerol synthesis rate**. *Nat Commun* 2018, **9**.
48. Smith ER, Cayrou C, Huang R, Lane WS, Cote J, Lucchesi JC: **A human protein complex homologous to the Drosophila MSL complex is responsible for the majority of histone H4 acetylation at lysine 16**. *Molecular and Cellular Biology* 2005, **25**(21):9175-9188.
49. Kuo WH, Chiang WL, Yang SF, Yeh KT, Yeh CM, Hsieh YS, Chu SC: **The differential expression of cytosolic carbonic anhydrase in human hepatocellular carcinoma**. *Life Sci* 2003, **73**(17):2211-2223.
50. Dubois EL, Guitton-Sert L, Beliveau M, Parmar K, Chagraoui J, Vignard J, Pauty J, Caron MC, Coulombe Y, Buisson R *et al*: **A Fanci knockout mouse model reveals common and distinct functions for FANCI and FANCD2**. *Nucleic Acids Res* 2019, **47**(14):7532-7547.
51. Austin CP, Battey JF, Bradley A, Bucan M, Capecchi M, Collins FS, Dove WF, Duyk G, Dymecki S, Eppig JT *et al*: **The knockout mouse project**. *Nat Genet* 2004, **36**(9):921-924.
52. Skarnes WC, Rosen B, West AP, Koutsourakis M, Bushell W, Iyer V, Mujica AO, Thomas M, Harrow J, Cox T *et al*: **A conditional knockout resource for the genome-wide study of mouse gene function**. *Nature* 2011, **474**(7351):337-U361.
53. Hu Y, Fisher JB, Koprowski S, McAllister D, Kim MS, Lough J: **Homozygous disruption of the Tip60 gene causes early embryonic lethality**. *Dev Dyn* 2009, **238**(11):2912-2921.
54. Hanly PJ, Roberts D, Dobson K, Light RB: **Effect of indomethacin on arterial oxygenation in critically ill patients with severe bacterial pneumonia**. *Lancet* 1987, **1**(8529):351-354.
55. Tang Y, Tang LY, Xu X, Li C, Deng C, Zhang YE: **Generation of Smurf2 Conditional Knockout Mice**. *Int J Biol Sci* 2018, **14**(5):542-548.
56. Potter CP, Harris AL: **Diagnostic, prognostic and therapeutic implications of carbonic anhydrases in cancer**. *Br J Cancer* 2003, **89**(1):2-7.
57. Bekku S, Mochizuki H, Yamamoto T, Ueno H, Takayama E, Tadakuma T: **Expression of carbonic anhydrase I or II and correlation to clinical aspects of colorectal cancer**. *Hepatogastroenterology* 2000, **47**(34):998-1001.

58. Chiang WL, Chu SC, Yang SS, Li MC, Lai JC, Yang SF, Chiou HL, Hsieh YS: **The aberrant expression of cytosolic carbonic anhydrase and its clinical significance in human non-small cell lung cancer**. *Cancer Lett* 2002, **188**(1-2):199-205.

59. Dai HY, Hong CC, Liang SC, Yan MD, Lai GM, Cheng AL, Chuang SE: **Carbonic Anhydrase III Promotes Transformation and Invasion Capability in Hepatoma Cells Through FAK Signaling Pathway**. *Mol Carcinogen* 2008, **47**(12):956-963.

Tables

Table 1. Sequence of PCR primers and TaqMan probes for genes used in qPCR.

Primer name	Primer sequence (5'–3')	Accession number	Product Size	Tm (°C)
Ca1-Forward	AGAGCCTGCAGTTCCAGTTC	XM_011248137.1	97 bp	60
Ca1-Reverse	CTCATTCCTTGCTGGGACTC			
Ca1-HYB Oligo	FAM-TGAGCAACCACCGTCCACCC-TAMRA			70
Ca3-Forward	ACACACTTTGACCCATCATG	NM_007606.3	130 bp	60
Ca3-Reverse	GAGCTCACAGTCATGGGCTC			
Ca3-HYB Oligo	FAM- TGTTCCCTGCTTGCCGGGAC-TAMRA			70
Ca7-Forward	TGGTTCACTGGAACGCCAAG	NM_053070.3	143 bp	60
Ca7-Reverse	AACCATGTAGAGGGCGTCTG			
Ca7-HYB Oligo	FAM-TGGCCTGGCTGTGGTTGGTG-TAMRA			70
β-actin-Forward	AATCGTGCGTGACATCAAAG	BC138614.1	137 bp	60
β-actin-Reverse	CGTTGCCAATAGTGATGACCT			
β-actin HYB Oligo	Cy5- ATGGCCACTGCCGCATCCTC-BQ2			70

Table 2. Comparison of mRNA levels *Ca1*, *Ca3* and *Ca7* in liver tissues at different zeitgeber time points

Groups	*Ca1*		*Ca3*		*Ca7*	
	Difference between means	p values	Difference between means	p values	Difference between means	p values
ZT0 & ZT4	-0,00108 ± 0,00148	0,9928ns	0,0614 ± 1,423	0,9998 ns	0,0003 ± 8,386e-005	0,1110 ns
ZT0 & ZT8	-0,00736 ± 0,00162	0,0077**	0,1946 ± 0,148	0,9591 ns	-0,0001867 ± 0,0001107	0,4711 ns
ZT0 & ZT12	-0,00792 ± 0,00232	0,0037**	0,1757 ± 0,247	0,9735 ns	-0,0004517 ± 4,686e-005	0,0017**
ZT0 & ZT16	-0,0009 ± 0,00124	0,9976ns	0,1901 ± 0,2412	0,9615 ns	0,0001767 ± 5,496e-005	0,5305 ns
ZT0 & ZT20	0,00018 ± 0,00073	>0,9999ns	0,28 ± 0,184	0,8347 ns	3,9e-005 ± 0,0001205	0,9991 ns
ZT4 & ZT8	-0,00628 ± 0,00205	0,0306*	0,1332 ± 0,1786	0,9923 ns	-0,0004617 ± 0,000128	0,0013**
ZT4 & ZT12	-0,00684 ± 0,00263	0,0153*	0,1144 ± 0,2662	0,9962 ns	-0,0007267 ± 7,961e-005	<0,0001****
ZT4 & ZT16	0,00018 ± 0,00186	>0,9999ns	0,1287 ± 0,2609	0,9930 ns	-9,833e-005 ± 8,463e-005	0,9276 ns
ZT4 & ZT20	0,00126 ± 0,00145	0,9856ns	0,2186 ± 0,2092	0,9342 ns	-0,000236 ± 0,0001399	0,2727 ns
ZT8 & ZT12	-0,00055 ± 0,00272	0,9997ns	-0,0188 ± 0,2494	>0,9999 ns	-0,000265 ± 0,0001075	0,1348 ns
ZT8 & ZT16	0,00646 ± 0,00201	0,0355*	-0,0045 ± 0,2439	>0,9999 ns	0,0003633 ± 0,0001113	0,0158*
ZT8 & ZT20	0,00754 ± 0,0016	0,0061**	0,0854 ± 0,1875	0,9991 ns	-0,0002257 ± 0,0001611	0,3179 ns
ZT12 & ZT16	0,00702 ± 0,00271	0,0185*	0,0144 ± 0,3137	>0,9999 ns	0,0006283 ± 4,824e-005	<0,0001****
ZT12 & ZT20	0,0081 ± 0,0023	0,0029**	0,1043 ± 2722	0,9976 ns	0,0004907 ± 0,0001169	0,0011**
ZT16 & ZT20	0,00108 ± 0,00121	0,9943ns	-0,090 ± 0,2671	0,9989 ns	-0,0001377 ± 0,0001212	0,7944 ns

Data are shown as the mean ± SEM. Difference between groups was evaluated with the unpaired Student t-test. Statistically significant differ-ences are indicated as follows: p > 0.05 (not significant, ns); *p < 0.05 (significant); **p < 0.01 (very significant); ***p < 0.001 (extremely significant), ****p < 0.0001 (extremely significant).

Figures

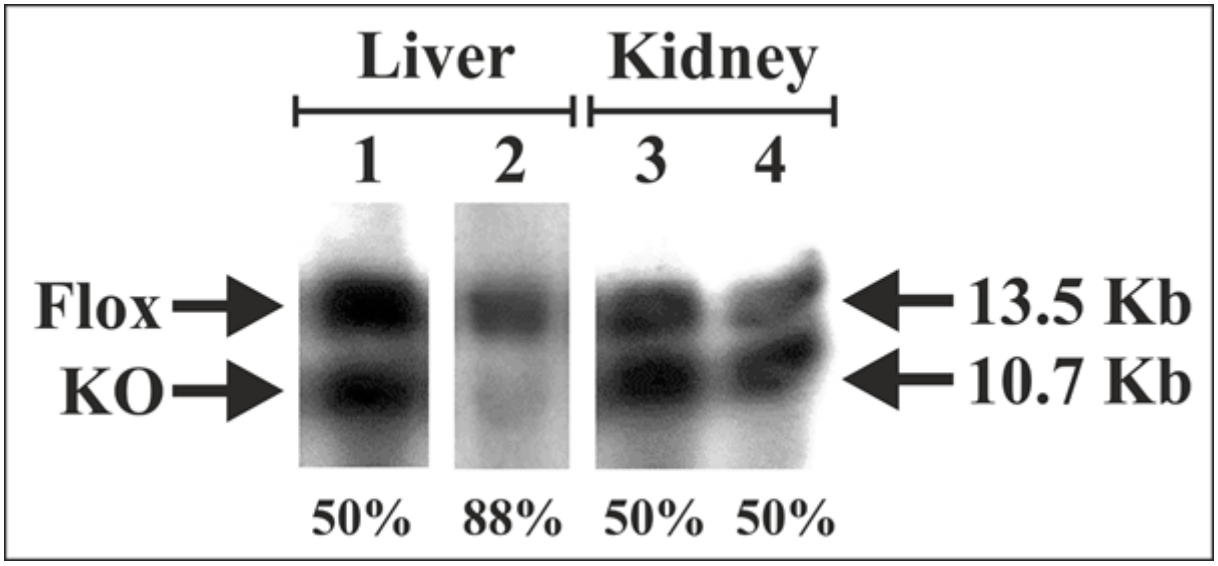

Figure 1

Cre-mediated deletion of Tip60 in the mouse liver tissue showed by southern blot technique. Line1: Tip60fl/-;SA+/+, Line2: Tip60fl/-;SA+/Cre-ERT2, Line3: Tip60fl/-;SA+/+, Line4: Tip60fl/-;SA+/Cre-ERT2 (mutant). Densities and percentages of blot lines were measured quantitatively using the Image J 2.0 software (NIH, USA) (E).

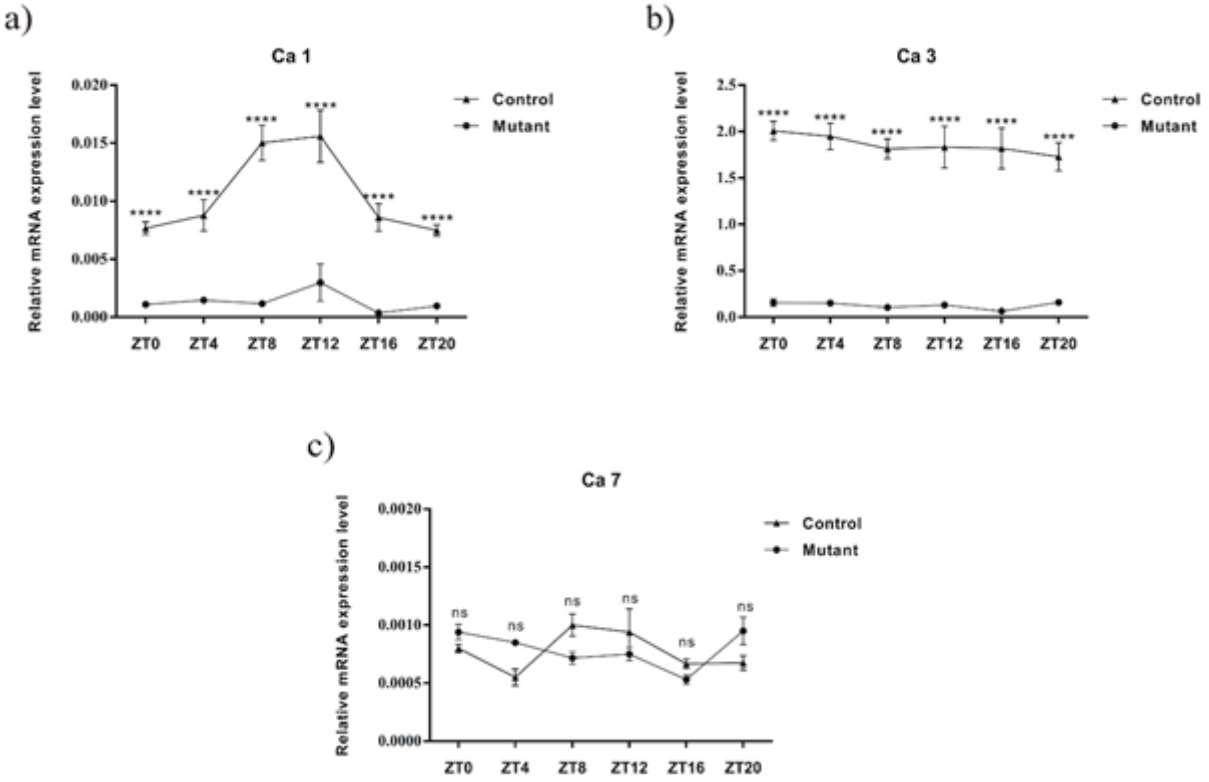

Figure 2

Comparison of genes expression levels in control and mutant tissues. Ca1, Ca3 and Ca7. Changes in the gene expression levels of Ca1 (a), Ca3 (b) and Ca7 (c) were detected by qPCR. β-actin was used as a housekeeping gene.