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ORIGINAL ARTICLE

Tip60 might be a candidate for the acetylation of hepatic carbonic anhydrase I and III in mice

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Abstract

Background Carbonic anhydrases (CAs) play a significant role in maintaining pH balance by catalyzing the conversion of carbon dioxide to bicarbonate. The regulation of pH is critical for all living organisms. Although there are many studies in the literature on the biochemical, functional, and structural features of CAs, there is not sufficient information about the epigenetic regulation of CAs.

Methods and results The lysine acetyltransferase TIP60 (60 kDa Tat-interactive protein) was knocked out specifically in mouse liver using the Cre/loxP system, and knockout rate was shown as 83–88% by Southern blot analysis. The impact of *Tip60* on the expression of *Ca1*, *Ca3*, and *Ca7* was investigated at six Zeitgeber time (ZT) points in the control and liver-specific *Tip60* knockout mice (mutant) groups by real-time PCR. In the control group, while *Ca1* showed the highest expression at ZT8 and ZT12, the lowest expression profile was observed at ZT0 and ZT20. Hepatic *Ca1* displayed robust circadian expression. However, hepatic *Ca3* exhibited almost the same level of expression at all ZT points. The highest expression of *Ca7* was observed at ZT12, and the lowest expression was determined at ZT4. Furthermore, hepatic *Ca7* also showed robust circadian expression. The expression of *Ca1* and *Ca3* significantly decreased in mutant mice at all time periods, but the expression of *Ca7* used as a negative control was not affected.

Conclusions It was suggested for the first time that *Tip60* might be considered a candidate protein in the regulation of the *Ca1* and *Ca3* genes, possibly by acetylation.

Keywords Carbonic anhydrases · *Tip60* · Acetylation · Circadian rhythm · Mice

Introduction

Carbonic anhydrases (CAs), zinc-containing metalloenzymes that are common in living organisms, catalyze the reversible hydration of CO_2 to bicarbonate (HCO^-_3) and proton (H^+) [1–3]. Although these enzymes were first discovered in hemolyzed blood, nowadays, they are known to have activity in many tissues such as the kidney, liver, brain, muscle, and bone tissues [4–7]. CAs in mice are composed of 16 different isoenzymes and play a role in various functions such as respiration, acid–base homeostasis, ion transport, bone resorption, taste preferences, ureagenesis, and gluconeogenesis [3, 8, 9]. Eight of these zinc-containing metalloenzymes 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 the mitochondria (CAVA and VB), and one (CAVI) is secreted [10]. Although there are many studies in the literature on the biochemical, functional, and structural properties of the CA family, there is insufficient information on the regulation of CAs at the gene and protein levels, especially their epigenetic regulation [11].

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 [12, 13]. Protein acetylation, which refers to the covalent binding of an acetyl group to an amino

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acid residue of a protein, is the most well-known along with phosphorylation [14]. 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 the subcellular localization of the protein [15, 16]. A recent study has reported that the 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. However, the proteins involved in this acetylation are still unknown [11].

The lysine acetyltransferase TIP60 (60 kDa Tat-interactive protein), a member of the histone acetyltransferases (HATs) protein family, has important and vital functions such as transcriptional regulation, DNA repair, cell cycle, apoptosis mechanism, cancer, the circadian system and the generation of cellular signals, both directly and indirectly [17–20]. Studies have demonstrated 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 [21–23]. The study performed by Chen et al. showed that the c-Myb transcription factor increased *Ca1* expression by binding to its promoter in mouse erythroleukemia cells [24]. However, c-Myb inactivation is required to inhibit the *Ca1* gene when the division of the cell is achieved. The cell becomes cancer without this suppression. It is also known that *Tip60* is a regulating factor for c-Myb [21]. This study aimed to determine whether *Tip60* plays a role in the regulation of hepatic *Ca1* and *Ca3* predominantly expressed in the liver. To this end, liver-specific *Tip60* knockout mice were generated using Cre/loxP recombination. The quantitative expression of the *Ca1* and *Ca3* genes at different Zeitgeber time (ZT) points were determined for both the control and knockout groups and then compared to each other. *Ca7*, not regulated by acetylation, was used as a negative control [11].

Materials and methods

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 [20] were crossed to a SACre driver mouse line resulting in the Cre-mediated deletion of *Tip60* in the liver [25]. Mice had been previously backcrossed to a C57BL/6N background for at least ten generations. To delete *Tip60*, 10–12-week-old male mice ($Tip60^{fl/-}$; $SA^{+/Cre-ERT2}$) were injected daily with tamoxifen (10 mg/ml stock solution, Sigma, St. Louis, MO, USA) in corn oil for five consecutive days. The control group ($Tip60^{fl/fl}$;$SA^{+/+}$) was injected with corn oil. Genotyping was performed with gene-specific primers [20, 25]. Liver and kidney tissues were collected 5 days after the last injection. Genomic DNA was isolated from both tissues and analyzed by Southern blot analysis. Liver-specific *Tip60* knockout mice were generated at the Max Planck Institute for Biophysical Chemistry (MPIBPC), Germany.

Southern blot analysis

Genomic DNA from liver and kidney tissues was isolated using the DNeasy tissue kit (Qiagen Inc., Valencia, CA, USA), and fifteen micrograms of the genomic DNA were digested with BamHI overnight (NEB, Ipswich, MA, USA). DNA was separated on a 0.6% agarose gel and transferred onto a Hybond-XL positively charged nylon membrane (GE Healthcare/Amersham Biosciences, Sweden). The membranes were hybridized with a 32^{P}-dCTP labeled radioactive double-stranded DNA probe prepared by random priming using an appropriate commercial kit according to the manufacturer's instructions (Amersham Rediprime™ II DNA Labeling System, GE Healthcare) and purified with the illustra ProbeQuant™ G-50 Micro Columns (GE Healthcare). The hybridization of the radioactive probe (100 µl) to the membrane was performed at 65 °C overnight in the presence of a hybridization buffer. The membranes were washed with 2× SSC/0.1% SDS, 1× SSC/0.1% SDS, and 0.1× SSC/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 and 1000J Processors). Southern blot experiments were performed at the MPIBPC, Germany.

Experimental animals, feeding, and zeitgeber time

At least 3 weeks prior to any experiment, all mice were singly housed with food and water ad libitum under a 12-h-light/12-h-dark cycle (350 lx). During this study, time is indicated using Zeitgeber time (ZT) as the indicator for the phase of the rhythm, where ZT0 refers to the time when the lights are turned on (06:00), and ZT12 refers to the time when the lights are turned off (18:00). ZT4, ZT8, ZT16, and ZT20 in this study are equivalent to 10:00, 14:00, 22:00, and 02:00 respectively [26]. Artificial light was provided daily from ZT0 (06:00), with temperature (24 ± 1) °C, and humidity (55 ± 5%) kept constant [27]. In the first set of experiments, 10–12-week-old male C57BL/6N mice were used and divided into six groups corresponding to the six chosen time points (ZT0, ZT4, ZT8, ZT12, ZT16, and ZT20). In the second set of experiments, we used 10–12-week-old male

mutant mice (*Tip60*$^{fl/-}$; SA$^{+/Cre-ERT2}$) and their respective control littermates [28].

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. The concentrations and purities of RNA were measured by a 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 the SuperScript III First-Strand cDNA kit system (Invitrogen, California, USA), by utilizing random hexamers, according to the manufacturer's protocol. The resulting cDNA was diluted to 100 ng/µl with nuclease-free water and stored at − 20 °C [29].

Primer and probe design

Primer3 software (v. 0.4.0) (http://bioinfo.ut.ee/primer3-0.4.0/) was used for the design of gene-specific primers and probes meeting 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 the Blast tool (NCBI) to confirm their specificity for the desired target. Then they were synthesized and purchased from Methabion International (Martinsried, Germany). The gene symbols and GenBank ID numbers are as follows: *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 the treatments, *β-actin* was used as reference gene. The sequences of the specific primers of all genes are shown in Table 1.

Quantitative real-time PCR

To determine the expression levels of the *Ca1, Ca3,* and *Ca7* genes at different ZT points, real-time PCR (qPCR) was carried out on the Rotor-Gene Q instrument (QIAGEN, Inc., Hilden, Germany). Beta-actin was selected as a reference control gene. The qPCR reactions were carried out with 2 µl of cDNA (final concentration of 0.02 ng), 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 15 s, and annealing and extension at 60 °C for 1 min [30]. The expression results were analyzed using the ΔCT method [31].

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 comparison tests using the Prism software 7.0 (GraphPad Software, San Diego, CA). Statistically significant changes are displayed by a symbol (*). The symbol expressions are as follows: $p > 0.05$ (not significant, ns); $*p < 0.05$ (significant); $**p < 0.01$ (very significant); $***p < 0.001$ and $****p < 0.0001$ (extremely significant).

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

Primer name	Primer sequence (5′–3′)	Accession number	Product size (bp)	Tm (°C)
Ca1-forward	AGAGCCTGCAGTTCCAGTTC	XM_011248137.1	97	60
Ca1-reverse	CTCATTCCTTGCTGGGACTC			
Ca1-HYB Oligo	^{FAM}-TGAGCAACCACCGTCCACCC-$_{TAMRA}$			70
Ca3-forward	ACACACTTTGACCCATCATG	NM_007606.3	130	60
Ca3-reverse	GAGCTCACAGTCATGGGCTC			
Ca3-HYB Oligo	^{FAM}-TGTTCCCTGCTTGCCGGGAC-$_{TAMRA}$			70
Ca7-forward	TGGTTCACTGGAACGCCAAG	NM_053070.3	143	60
Ca7-reverse	AACCATGTAGAGGGCGTCTG			
Ca7-HYB Oligo	^{FAM}-TGGCCTGGCTGTGGTTGGTG-$_{TAMRA}$			70
β-actin-forward	AATCGTGCGTGACATCAAAG	BC138614.1	137	60
β-actin-reverse	CGTTGCCAATAGTGATGACCT			
β-actin-HYB Oligo	^{Cy5}-ATGGCCACTGCCGCATCCTC-$_{BQ2}$			70

Results

Liver-specific *Tip60* knockout mouse models

Tamoxifen-inducible Cre/lox recombination was used to create genetically modified mouse lines in C57BL/6N backgrounds for tissue-specific or conditional knockouts of *Tip60* due to the lethal effect of full knockout on mice [32]. The *Tip60* gene was specifically knocked out in the mouse liver using this system. The Cre-mediated deletion of *Tip60* in the mouse liver tissue was shown by Southern blot analysis. In Fig. 1, Cre-mediated deletion did not occur due to the absence of the SACre-driver in $Tip60^{fl/-}$;$SA^{+/+}$ mice.

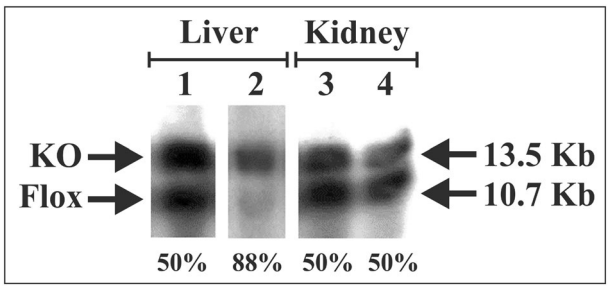

Fig. 1 Cre-mediated deletion of *Tip60* in the mouse liver tissue shown by Southern blot analysis. Line 1: $Tip60^{fl/-}$;$SA^{+/+}$, Line 2: $Tip60^{fl/-}$;$SA^{+/Cre-ERT2}$, Line 3: $Tip60^{fl/-}$;$SA^{+/+}$, Line 4: $Tip60^{fl/-}$;$SA^{+/Cre-ERT2}$. Densities and percentages of blot lines were measured quantitatively using the Image J 2.0 software (NIH, USA)

Therefore, the deletion efficiency of *Tip60* was 50% in the liver. Since $Tip60^{fl/-}$;$SA^{+/Cre-ERT2}$ mice contain the SACre driver, Cre-mediated deletion was achieved and the knockout rate was shown as 83–88%. The leakage of the Cre driver was also checked in the kidney tissues of both mouse lines, and any leakage was not observed within the kidney tissues (Fig. 1).

Expression levels of the *Ca1*, *Ca3*, and *Ca7* genes at different Zeitgeber time points in the control groups

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

Comparison of the gene expression levels of the control and mutant groups

It has been recently reported that while CAI and CAIII are acetylated by acetyltransferase enzymes, CAVII is not acetylated [11]. However, the proteins involved in this acetylation

Table 2 Comparison of the mRNA levels of *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 and ZT4	−0.00108 ± 0.00148	0.9928ns	0.0614 ± 1.423	0.9998ns	0.0003 ± 8.386e−005	0.1110ns
ZT0 and ZT8	−0.00736 ± 0.00162	0.0077**	0.1946 ± 0.148	0.9591ns	−0.0001867 ± 0.0001107	0.4711ns
ZT0 and ZT12	−0.00792 ± 0.00232	0.0037**	0.1757 ± 0.247	0.9735ns	−0.0004517 ± 4.686e−005	0.0017**
ZT0 and ZT16	−0.0009 ± 0.00124	0.9976ns	0.1901 ± 0.2412	0.9615ns	0.0001767 ± 5.496e−005	0.5305ns
ZT0 and ZT20	0.00018 ± 0.00073	> .9999ns	0.28 ± 0.184	0.8347ns	3.9e−005 ± 0.0001205	0.9991ns
ZT4 and ZT8	−0.00628 ± 0.00205	0.0306*	0.1332 ± 0.1786	0.9923ns	−0.0004617 ± 0.000128	0.0013**
ZT4 and ZT12	−0.00684 ± 0.00263	0.0153*	0.1144 ± 0.2662	0.9962ns	−0.0007267 ± 7.961e−005	< 0.0001****
ZT4 and ZT16	0.00018 ± 0.00186	> 0.9999ns	0.1287 ± 0.2609	0.9930ns	−9.833e−005 ± 8.463e−005	0.9276ns
ZT4 and ZT20	0.00126 ± 0.00145	0.9856ns	0.2186 ± 0.2092	0.9342ns	−0.000236 ± 0.0001399	0.2727ns
ZT8 and ZT12	−0.00055 ± 0.00272	0.9997ns	−0.0188 ± 0.2494	> 0.9999ns	−0.000265 ± 0.0001075	0.1348ns
ZT8 and ZT16	0.00646 ± 0.00201	0.0355*	−0.0045 ± 0.2439	> 0.9999ns	0.0003633 ± 0.0001113	0.0158*
ZT8 and ZT20	0.00754 ± 0.0016	0.0061**	0.0854 ± 0.1875	0.9991ns	−0.0002257 ± 0.0001611	0.3179ns
ZT12 and ZT16	0.00702 ± 0.00271	0.0185*	0.0144 ± 0.3137	> 0.9999ns	0.0006283 ± 4.824e−005	< 0.0001****
ZT12 and ZT20	0.0081 ± 0.0023	0.0029**	0.1043 ± 2722	0.9976ns	0.0004907 ± 0.0001169	0.0011**
ZT16 and ZT20	0.00108 ± 0.00121	0.9943ns	−0.090 ± 0.2671	0.9989ns	−0.0001377 ± 0.0001212	0.7944ns

Data are shown as mean ± SEM. The difference between the groups was evaluated by unpaired Student's t-test

Statistically significant differences are indicated as follows: $p > 0.05$ (not significant, ns); *$p < 0.05$ (significant); **$p < 0.01$ (very significant); ***$p < 0.001$ and ****$p < 0.0001$ (extremely significant)

are still unknown. Since *Tip60* is thought to be a candidate for the acetylation of *Ca1* and *Ca3* in the liver, the quantitative expression levels of *Ca1* and *Ca3* in the control and mutant groups were analyzed by qPCR. The analysis, determined that the expression amount in mutant tissues decreased significantly at all time points compared to the control groups. No change was observed in the expression of *Ca7* used as a negative control (Fig. 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 by 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 to HCO_3^- to regulate the pH balance of the blood [37–39].

Many studies have proven the significant role of CAs in physiological processes and shown that the 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 perform many functions, including digestion, glycogen synthesis, manufacturing triglycerides and cholesterol, bile production, storage of many essential vitamins and minerals [41]. It also metabolizes 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 the liver, there is very limited information about the regulation of CAs at the gene and protein levels, especially their transcriptional regulation [4, 42–44].

As a result of the analysis, the *Ca1*, *Ca3*, and *Ca7* genes were expressed at all ZT points in the liver tissues of the control group. It was observed that the expression of *Ca1* and *Ca7* reached the maximum level at ZT8 (the end of the light cycle) and ZT12 (the beginning of the dark cycle), and the *Ca3* gene was expressed in high amounts at all time

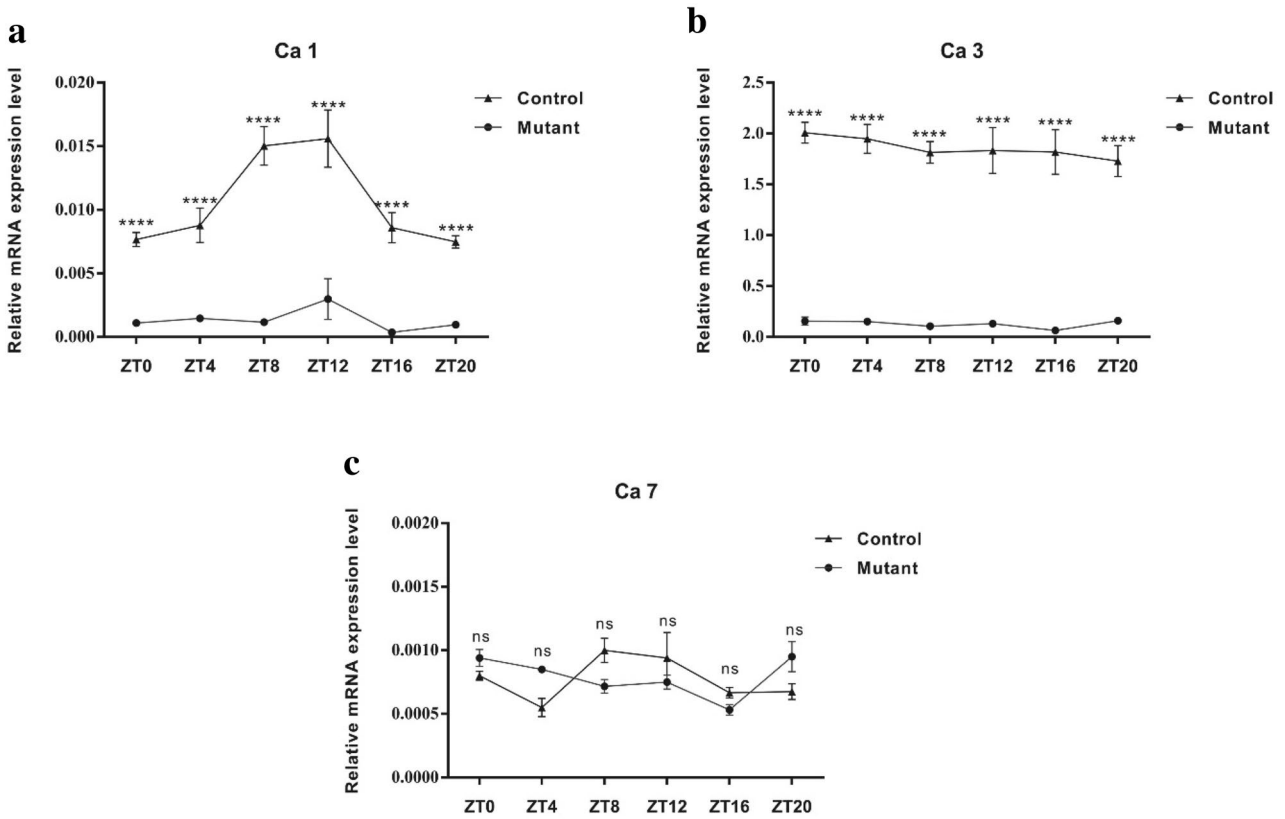

Fig. 2 Comparison of the gene expression levels in control and mutant tissues. *Ca1*, *Ca3* and *Ca7*. Changes in the gene expression levels of *Ca1* (**a**), *Ca3* (**b**), and *Ca7* (**c**) were detected by qPCR. *β-actin* was used as a housekeeping gene

points, except for ZT16. In the control group, upon comparing the 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].

The study performed by Chen et al. in 2006 on mouse erythroleukemia (MEL) cells revealed that the c-Myb transcription factor was bound to the promoter of the *Ca1* gene and increased the expression of *Ca1* for proliferation and differentiation [24]. Zhao et al. stated that TIP60, a histone acetyltransferase with activity in the cytoplasm and nucleus, belonged to the MYST (Moz-Ybf2/Sas3-Sas2-*Tip60*) family and was known to be responsible for acetylation in both mouse and human cells, was a regulating factor for c-Myb [21, 46–48]. Another study, investigated the relationship between hepatocellular carcinoma (HCC) and tumor development with the expression of CAs. The activity and protein expression of the CA family in tumor tissues were significantly lower than normal cells [49]. A recent study conducted by Di Fiore A, et al. has reported that CA I and CA III proteins are regulated by post-translational acetylation [11].

Many researchers have stated that knockout mice are widely used to better understand the biological role of specific genes, and molecular and cellular mechanisms [50–52]. The study carried out by Hu et al. showed that the homozygous disruption of the *Tip60* gene led to early embryonic death [32]. 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* at the gene level. Moreover, since TIP60 has been shown to play a role in the regulation of the circadian clock [20], it has been investigated whether this arrangement occurs at different ZT points. As shown in Fig. 1, the *Tip60* gene knockout rate was shown between 83 and 88% by the Southern blot technique in the liver.

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 [53, 54]. We also investigated the expression of the *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 at all time periods (Fig. 2a, b), the expression of *Ca7*, used as a negative control as mentioned above, was not affected (Fig. 2c). Potter and Harris stated that some cytoplasmic CAs were markers for human cancers [55]. Bekku et al. reported that *Ca1* expression decreased in colorectal tumors [56]. Chiang et al. observed that the expression of *Ca1* decreased in adenocarcinoma [57]. Kuo et al. revealed the reduced levels of CA I and III in human hepatocellular carcinoma (HCC). In contrast to this, a study conducted in 2008 showed that increased CA III expression accelerated HCC through the focal adhesion kinase signaling pathway [58]. It was hypothesized that CA III was re-expressed at the later stages of the metastatic progression of HCC, and it might have a significant impact on the development of metastasis in liver cancer [39]. According to the results obtained from these two studies, it was hypothesized that while the decreased expression of *Ca3* was important in the pathogenesis of HCC, increased *Ca3* expression was required for metastasis after cancer formation [39]. Based on the literature data, it is thought that decreased in *Ca1* and *Ca3* expression in mice due to the depletion of *Tip60* may lead to HCC.

In our preliminary study to show how total CA activity changes in the presence and absence of Tip60, total CA activity was measured by Wilbur and Anderson method in the control and mutant groups [59]. No statistically significant difference in total CA activity was observed in both groups (data not shown). There are 16 different isoforms of CAs in mice. It is not known which CAs activity can increase or decrease in the absence of TIP60. Although some compounds were synthesized for specific inhibition of CAs by Carta et al. [60], there are no specific inhibitors defined for CAI and CAIII. For this reason, no results could be obtained about how the enzyme activity of CAI and CAIII changes in the absence of Tip60.

In conclusion, TIP60 may be considered a candidate protein in the regulation of the *Ca1* and *Ca3* genes, possibly by acetylation. Moreover, our results showed 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.

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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.

Declarations

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.

Research involving human and animal rights 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).

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