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Chapter 4

Intestinal bile salt absorption in Atp8b1 deficient mice

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Abstract

Background/Aims: Mutations in the ATP8B1 gene can cause Progressive Familial Intrahepatic Cholestasis type 1. We have previously reported that Atp8b1<sup>G308V/G308V</sup> mice, a model for PFIC1, have slightly, but significantly, higher baseline serum bile salt (BS) concentrations compared to wt mice. Upon BS feeding, serum BS concentrations strongly increased in Atp8b1-deficient mice. Despite these findings, we observed only mildly impaired canalicular BS transport. In the present report we tested the hypothesis that Atp8b1<sup>G308V/G308V</sup> mice hyperabsorb BS in the intestine during BS feeding.

Methods: Intestinal BS absorption was measured in intestinal perfusion and in intestinal explants. In addition, we measured BS concentrations in portal blood. Ileal expression of the Fxr-targets Asbt, Ilbp and Shp was assessed.

Results: In wt and Atp8b1<sup>G308V/G308V</sup> mice, intestinal taurocholate absorption is primarily mediated by the ileal bile salt transporter Asbt. Neither of the experimental systems revealed enhanced absorption of BS in Atp8b1<sup>G308V/G308V</sup> mice compared to wt mice. In line with these observations, we found no difference in the ileal protein expression of Asbt. Induction of Shp expression during BS feeding also demonstrated that Fxr signalling is intact in Atp8b1<sup>G308V/G308V</sup> mice.

Conclusions: The accumulation of BS in plasma of Atp8b1<sup>G308V/G308V</sup> mice during BS feeding is not caused by increased intestinal BS absorption.
Introduction

The enterohepatic cycle of bile salts (BS) involves various transporters in liver and intestine. The bile salt export pump (ABCB11) transports BS from the hepatocytes into bile\textsuperscript{1,2}. After release in the gut, BS are reabsorbed in the terminal ileum by the apical sodium-dependent bile salt transporter, ASBT (SLC10A2)\textsuperscript{3-5}. In ileal enterocytes, the ileal lipid binding protein, ILBP (FABP6), is thought to mediate transcellular BS transport\textsuperscript{6,7}. The heterodimer Osta/p facilitates the transport of BS from the enterocytes into the blood\textsuperscript{8}. Via the portal vein, BS reach the liver and are taken up by hepatocytes via the Na+–taurocholate co-transporting protein (SLC10A1)\textsuperscript{9,10}, which completes the enterohepatic cycle.

Impaired bile formation leads to cholestasis. Mutations in ATP8B1 can cause progressive familial intrahepatic cholestasis type 1 (PFIC1)\textsuperscript{11-13}. Cholestatic symptoms in patients with PFIC1 include high serum and low biliary BS levels\textsuperscript{14}. Many PFIC1 patients exhibit extrahepatic symptoms, like diarrhea, hearing defects, and pancreatitis\textsuperscript{14-17}. Liver transplantation in these patients resolves the cholestatic symptoms but the extrahepatic symptoms remain\textsuperscript{14}. This suggests that ATP8B1 is of physiological importance in many different tissues.

ATP8B1 is a type 4 P-type ATPase and is considered to function as a lipid flippase, translocating phospholipids from the outer to the inner leaflet of the membrane bilayer\textsuperscript{18-20}. The protein is expressed in apical membranes of epithelial cells including hepatocytes, cholangiocytes, and enterocytes\textsuperscript{21}. Ujhazi et al.\textsuperscript{22} reported enhanced translocation of a fluorescently labelled phosphatidylserine (PS) analog in cells transfected with ATP8B1 cDNA, suggesting a role for ATP8B1 in the flipping of aminophospholipids. We have recently shown in mice that absence of Atp8b1 decreases the resistance of the canalicular membrane to hydrophobic BS. This leads to enhanced extraction of PS and cholesterol from this membrane domain; consequently, this may impair hepatobiliary BS transport\textsuperscript{23}.

Chen et al.\textsuperscript{24} showed that ASBT expression in ileal tissue from three PFIC1 patients was increased. They also observed a strong decrease in ileal FXR (NR1H4) expression, the nuclear receptor involved in regulation of several key steps in BS metabolism\textsuperscript{25}. The authors have suggested a role for ATP8B1 in cytosol-to-nuclear signalling, which would be impaired in PFIC1 patients and would lead to (amongst other phenotypic characteristics) enhanced intestinal uptake of BS.

A common mutation in PFIC1 patients is a glycine-to-valine amino acid substitution at position 308 (G308V)\textsuperscript{26}. Pawlikowska et al.\textsuperscript{27} produced mutant mice in which this mutation was introduced (Atp8b1\textsuperscript{G308V/G308V}), and showed that it leads to near absence of the protein. These animals displayed mildly, but significantly, increased serum BS levels. Upon feeding a
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diet containing 0.5% cholate (CA), the serum BS levels of the Atp8b1<sup>G308V/G308V</sup> mice increased up to 35 times the control values, while these were unaffected in wt mice. The dramatic rise in serum BS concentrations upon CA feeding might suggest that mutant mice hyper-absorb BS in the small intestine.

In the present report we measured intestinal BS absorption in wt and Atp8b1<sup>G308V/G308V</sup> mice in intestinal perfusions and in Ussing chambers. We also measured portal and systemic BS concentrations upon feeding of a CA-supplemented diet. In addition, we analyzed the contribution of Fxr signalling by determining expression levels of the Fxr targets Asbt and the short heterodimer Shp-1.

Methods

In vivo experiments

All experiments were performed with age-matched male mice at 26 months of age. The bile salt (BS) retention experiments were performed with age-matched female mice aged 4-12 months. Wild-type and Atp8b1<sup>G308V/G308V</sup> mice were bred against identical genetic backgrounds (129/Sv background) in a pathogen free environment at the animal facility of the Academic Medical Center.

Mice were fed standard rodent chow or a commercial purified diet (K4068.02, Arie Blok Diervoeders, Woerden, The Netherlands), the latter diet either or not supplemented with 0.5% w/w sodium cholate (Merck, Darmstadt, Germany). Food and water were supplied ad libitum.

Bile salt uptake and retention experiment with and without Asbt-inhibitor HMR 1453

All mice were conventionally housed with 2 animals or more and weighed daily. From day 1 to 4, all mice were fed the purified diet and received the vehicle (PBS + 0.5% methylcellulose + 2% ethanol) by gavage twice a day. The Asbt-inhibitor HMR 1453 (Aventis Pharma Deutschland, Germany) was dissolved in 100% ethanol and was diluted with the vehicle. On days 5-14, mice received either vehicle or Asbt-inhibitor HMR 1453 (5 mg/kg per day), twice a day. On day 13, all animals received 2 µCi/25 g mouse of [3H]TC (Firm New England Nuclear, NET322) in PBS. On day 15, the mice were sacrificed, blood was collected, organs (gallbladder, total intestine and liver) were isolated and solubilized with 100% Soluene (Packard). Radioactivity was determined by liquid scintillation counting.

Ileal and jejunal perfusions

The method used was adapted from Athman et al.<sup>29</sup>. After feeding a purified or cholate-supplemented diet for one week, wt and Atp8b1<sup>G308V/G308V</sup> mice were anesthetized by intraperitoneal injection of Hypnorm/Diazepam<sup>30</sup>. The gallbladder was cannulated and mice were depleted from endogenous BS for 120 min. Bile samples were collected every 15 min. The last three segments of blood supply before the cecum (about 10 cm intestine) were externalized. The inflow cannula was inserted at the proximal end and the outflow cannula was inserted at the ileocecal junction. The cannulated part of the intestine was rinsed with warm saline. The tissue was perfused for 2 h with Krebs/bicarbonate, 5 mM taurocholate (TC) (Sigma, T-4009), and 1 µCi [3H]TC (Firm New England Nuclear), at a rate of 3 ml/h. Perfusate and bile samples were collected every 15 min. Radioactivity in all fractions was determined by liquid scintillation counting.
For the jejunal perfusion, the perfused part contained three segments in the middle of the small intestine. Jejunal perfusions were performed with 2mM cholate (Calbiochem), spiked with 0.16 µCi [3H]CA.

**Ussing chamber experiments**

Ussing chamber experiments were performed as described previously with explants from jejunum, ileum, and colon of wt and Atp8b1G308V/G308V mice. A Krebs/bicarbonate buffer supplemented with 2 mM L-Glutamine and 10 mM Hepes/NaOH, pH 7.4, was applied to both compartments of the Ussing chamber. The buffer and chambers were kept at 37 °C and were gassed continuously with carbogen (95%O2, 5%CO2). Mice were sacrificed by cervical dislocation and the intestine was externalized. Pieces of 1 cm² intestinal tissue were excised and installed on a support, which was placed in the Ussing chamber. The tissue was equilibrated for 10 min in the Krebs/bicarbonate buffer. Cholate (Calbiochem) was administered to one compartment of the Ussing chamber at a final concentration of 3 mM. At different time points, 100 µl samples were taken from the opposite compartment, followed by addition of 100 µl Krebs to adjust the volume. BS concentrations were determined enzymatically.

**Portal blood sampling**

After feeding a purified or cholate-supplemented diet for 1 day, wt and Atp8b1G308V/G308V mice were anesthetized. Peripheral blood was collected by orbital puncture. The vena cava caudalis was injected with 50 µl heparin (1000 U/ml). The portal vein was cannulated and blood was collected during 5 min. Serum BS levels were determined by HPLC/MS.

**Assays**

Biliary BS concentrations were determined enzymatically. Serum BS levels were determined by HPLC/MS.

**Western blotting**

Protein extraction, SDS-PAGE, and Western blotting were performed as previously described. The last 10 cm of the small intestine was cut into 1 cm pieces and was extracted. Total ileum homogenates were also prepared. Ilbp was detected using rabbit anti-serum raised against recombinant murine Ilbp. Anti-Asbt was a gift from B. Stieger. Anti-Atp1a1 (Na+/K+-ATPase) was used as loading control. The blots shown are representative blots from one mouse per condition.

**Quantitative PCR analysis of Shp gene expression**

Quantitative PCR was performed as described previously; Shp expression was quantified by means of the Assays-on-Demand primer/probe set (Applied Biosystems, Foster City, CA, USA) ABI Mm00442278.

**Statistics**

All data are given as means ± SD. Significance was tested by use of the One-Way ANOVA with Bonferroni’s correction for multiple testing.

**Results**

**Asbt-dependent bile salt uptake**

We determined whether intestinal BS absorption is similarly dependent on Asbt activity in both Atp8b1G308V/G308V and wt mice. Mice orally received either vehicle or the Asbt-inhibitor HMR 1453. Forty-eight hours after [3H]TC administration, gallbladder, liver, and the intestine were collected, and radioactivity was measured. The results were expressed as percentage of the administered dose. Fig. 1 shows that the total body retention of [3H]TC was 1.5-fold higher in
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Atp8b1<sup>G308V/G308V</sup> compared to wt mice. The higher retention of [<sup>3</sup>H]TC in Atp8b1<sup>G308V/G308V</sup> mice is most likely caused by the mild cholestasis<sup>23</sup> and by the enlarged bile salt pool<sup>27</sup> observed in these mice. However, BS retention was equally reduced by the Asbt-inhibitor HMR 1453.

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**Asbt and Ilbp protein levels**

Western blotting of the last 10 centimeters of the small intestine, which includes the terminal ileum, revealed no increased Asbt expression levels in Atp8b1<sup>G308V/G308V</sup> mice compared to wt mice (Fig. 2). By contrast, the intestinal Asbt protein levels in Atp8b1<sup>G308V/G308V</sup> mice were somewhat lower than in wt mice. We also observed this for Ilbp protein (Fig. 3). In both genotypes, BS feeding induced Ilbp expression.
Quantitative determination of Shp and Fgf15 gene expression

In wt mice CA feeding did not influence serum BS concentrations. In contrast CA feeding led to very high BS concentrations in Atp8b1<sup>G308V/G308V</sup> mice. When fed a control diet, both hepatic and intestinal Shp expression levels were not significantly different in Atp8b1<sup>G308V/G308V</sup> mice compared to wt mice, although expression tended to be lower in the Atp8b1<sup>G308V/G308V</sup> mice (Fig. 4). However, on a CA-supplemented diet, Shp expression was induced only in Atp8b1<sup>G308V/G308V</sup> mice in both liver and ileum demonstrating that Fxr signalling is intact in Atp8b1<sup>G308V/G308V</sup> mice. Fgf15 mRNA expression shows no difference between wt and Atp8b1<sup>G308V/G308V</sup> mice on a control diet. The BS diet induces the expression two- to threefold, in both wt and Atp8b1<sup>G308V/G308V</sup> mice (data not shown).

Intestinal bile salt perfusion

To investigate BS uptake in vivo, we performed in situ ileal and jejunal perfusions. Five millimolar TC (spiked with [3H]TC) was perfused. BS absorption was assessed by measuring the
amount of radioactivity in bile. In the ileal perfusions, Atp8b1<sup>G308V/G308V</sup> mice fed a control diet displayed a similar hepatobiliary TC transport rate as wt mice (Fig. 5a). In Atp8b1<sup>G308V/G308V</sup> mice on a CA-supplemented diet there was a tendency towards a lower biliary TC excretion than in wild types but this was only significant after 195 min (Fig. 5b). To assess whether the elevated serum BS levels in Atp8b1<sup>G308V/G308V</sup> mice were caused by non-specific uptake of CA in the intestine, we also performed perfusions of the jejunum with this unconjugated BS. These experiments were performed with mice fed a CA-supplemented diet for 1 week. The jejunum was perfused with 2 mM CA spiked with [3H]CA. In wt mice, the hepatobiliary excretion rates of radioactive bile salt were approximately fourfold lower than in the ileal perfusion (Figs. 6 vs. 5). This was expected since non-specific uptake of BS in the jejunum is a much slower process than active, Asbt-mediated, uptake in the ileum. Strikingly, Atp8b1<sup>G308V/G308V</sup> animals displayed a three-to fourfold reduced hepatobiliary CA excretion compared to wt mice (Fig. 6). The reduced hepatobiliary excretion in CA-fed Atp8b1<sup>G308V/G308V</sup> animals possibly was caused by dilution of the radioactive BS in the larger BS pool of Atp8b1<sup>G308V/G308V</sup> mice compared to wt animals (see discussion) rather than by reduced intestinal uptake. At any rate these data indicate that Atp8b1<sup>G308V/G308V</sup> animals have neither enhanced Asbt-mediated nor enhanced non-specific BS uptake in the intestine.

![Fig. 5. Taurocholate absorption during ileal perfusion. Ileal perfusions were performed with WT and Atp8b1<sup>G308V/G308V</sup> mice on a control diet (a) or a 0.5% CA-supplemented diet for 7 days (b). The ileum was in situ luminally perfused with saline containing 5 mM taurocholate (TC) and 1 µCi [3H]TC. The amount of radioactive TC excreted into bile (expressed as dpm [3H]-TC/min*100gr) was used as a measure of ileal absorption. ■, WT n = 5; O, Atp8b1<sup>G308V/G308V</sup> n = 4. *p < 0.05.](image-url)
Bile salt levels in portal blood

To get a more direct estimation of the net in vivo BS uptake by the entire gut, we measured BS concentrations in peripheral and portal blood from animals that were fed a control or a 0.5% CA-supplemented diet for 1 day. This short time-span was chosen to rule out secondary effects due to the BS feeding as much as possible. Subtraction of peripheral from portal BS concentrations yielded an estimation of the amount of BS taken up in the gut. In accordance with the diet and perfusion experiments, this experiment showed that intestinal BS uptake in Atp8b1<sup>G308V/G308V</sup> mice was not increased compared to wt mice (Fig. 7).

**Fig. 6.** Cholate absorption during jejunal perfusion. Jejunal perfusions were performed with WT and mutant mice fed a 0.5% CA-supplemented diet for 7 days. The jejunum was in situ luminaul perfused with saline containing 2 mM cholate (CA) and 0.16 μCi [³H]CA per ml. The amount of radioactivity recovered in bile was used as a measure for intestinal absorption.

- **WT** n = 5; O, Atp8b1<sup>G308V/G308V</sup> n = 3. *p < 0.05.

**Fig. 7.** Bile salt concentrations in peripheral and portal blood. Peripheral (orbita) and portal blood was sampled and bile salts were analyzed by HPLC-MS. The difference between peripheral and portal blood concentration was calculated and taken as an estimation of bile salt absorption in the gut at the time of blood sampling. Average serum bile salt levels in peripheral (a), portal (b) and difference between portal and peripheral serum (c) after one-day control or 0.5% CA-supplemented diet. Black bars - WT, control diet; white bars - Atp8b1<sup>G308V/G308V</sup>, control diet; dark grey bars - WT, 0.5% CA diet; light grey bars - Atp8b1<sup>G308V/G308V</sup>, 0.5% cholate diet. All groups n = 3. *p < 0.05.
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Fig. 8. Intestinal taurocholate transport in Ussing chambers. Explants from jejunum, ileum, and colon from control or 0.5% cholate fed mice were mounted in an Ussing chamber set-up. After equilibration of the tissue in Krebs/bicarbonate, 3 mM cholate (CA) was administered apically. Samples were taken at the indicated time points from the opposite basolateral compartment and were subsequently analyzed for bile salt concentration. As a control, cholate was administered basolaterally, and samples were taken from the apical compartment. (a) Time-dependent appearance of cholate in the basolateral compartment after apical administration. ♦, WT; □, Atp8b1\(^{G308V/G308V}\) ileal explants. (b) Transport rate of cholate in wildtype jejunal, ileal, and colon explants from the apical to the basolateral compartment or from the basolateral to the apical compartment. The ileal apical to basolateral transport was inhibited by HMR 1453 (100 µg/ml). All \( n = 3-11 \). Cholate transport rate in ileal (c), jejunal (d), and colon (e) explants from WT and Atp8b1\(^{G308V/G308V}\) mice fed a control or a 0.5% CA diet. Black bars - WT, control diet; white bars - Atp8b1\(^{G308V/G308V}\), control diet; dark grey bars - WT, 0.5% CA diet; light grey bars - Atp8b1\(^{G308V/G308V}\), 0.5% cholate diet. All groups \( n = 3-11 \). *\( p < 0.05 \)
Bile salt transport in intestinal explants

Transport of CA in explants of ileum, jejunum, and colon was studied in Ussing chambers. Three millimolar CA was administered to the apical compartment (representing the intestinal lumen) and samples were taken from the basolateral compartment at the indicated time points (Fig. 8). Fig. 8a shows that there was a linear increase in the basolateral CA concentration when CA was applied to the apical compartment, so vectorial BS transport could be properly measured in ileal explants. Basolateral-to-apical transport of CA was fivefold lower compared to apical-to-basolateral transport (Fig. 8b). When CA was administered apically together with the Asbt-inhibitor HMR 1453 (100 µg/ml), apical-to-basolateral CA transport was also dramatically reduced (Fig. 8b). These data demonstrate that this system is suitable to measure Asbt-mediated BS transport. There was no difference in the ileal transport rate of CA between wt and Atp8b1\(^{G308V/G308V}\) mice on either diet (Fig. 8c). In the jejunal and colon explants, apical-to-basolateral CA transport was much lower (but not absent), which is consistent with the expression of Asbt (Figs. 8b,d, and 8e). In jejunal explants from wt animals fed a CA-supplemented diet, CA transport was slightly but significantly lower than that seen in the other groups (Fig. 8d).

Discussion

The enterohepatic circulation of BS is highly efficient. This, combined with the fact that hepatic BS synthesis is tightly regulated, makes it rather unlikely that increased BS absorption leads to accumulation of endogenous BS in the enterohepatic cycle. However, we have previously observed that Atp8b1\(^{G308V/G308V}\) mice accumulate high amounts of BS in the circulation if they are challenged with a diet containing 0.5% cholate. Under such experimental conditions of exogenous BS overload intestinal hyperabsorption may cause accumulation of BS in the circulation. We therefore investigated intestinal BS absorption in the present study.

First, we demonstrated that Asbt mediates BS uptake in the intestine of Atp8b1\(^{G308V/G308V}\) mice to the same extent as in wt mice (Fig. 1). Second, we showed that Asbt and Ilbp protein expression is comparable in Atp8b1\(^{G308V/G308V}\) mice and wt mice (Fig. 2 and 3). Third, we demonstrated that BS transport rates can be adequately determined in ileal explants in Ussing chambers and we observed no differences between the rates in Atp8b1\(^{G308V/G308V}\) and wt mice (Fig. 8). Fourth, we performed ileal and jejunal perfusions with radioactive BS and showed that there is no increase in uptake of radioactivity between Atp8b1\(^{G308V/G308V}\) and wt mice as measured by the appearance of radioactive BS in bile. Instead, we found a decrease in radioactivity recovered in bile in cholate-fed Atp8b1\(^{G308V/G308V}\) mice (Fig. 5 and 6). We have demonstrated in
the past that Atp8b1<sup>G308V/G308V</sup> mice have a fourfold increased BS pool size under this condition compared to wt mice<sup>27</sup>. Most likely, the decreased recovery of radioactive BS is caused by dilution of the label in the larger BS pool of Atp8b1<sup>G308V/G308V</sup> mice. The partial cholestasis that we recently described in Atp8b1<sup>G308V/G308V</sup> mice may also contribute to this phenomenon<sup>23</sup>. Finally, we sampled portal blood under control and cholate-fed conditions and, again, found no increase in portal BS concentrations in Atp8b1<sup>G308V/G308V</sup> mice as compared to wt mice (Fig. 7). We therefore conclude that there is no increase in the absorption of BS in the intestine of Atp8b1<sup>G308V/G308V</sup> mice.

Our findings in mice are at variance with those reported in human tissue samples<sup>24,36</sup>. Chen et al.<sup>24</sup> reported an upregulation of ASBT mRNA levels in ileal tissue from PFIC1 patients as compared to control ileal tissue. In in vitro experiments with Caco-2 cells they observed a similar phenomenon; transfection with ATP8B1 antisense RNA caused an upregulation of ASBT expression that was accompanied by a downregulation of FXR, SHP and ILBP expression. On the basis of these observations they hypothesized that FXR signalling is dependent on proper ATP8B1 function. This would also explain the reduction of SHP and ILBP expression, which are downstream targets of FXR. It remains unclear, however, how functioning of ATP8B1 in the plasma membrane relates to that of FXR in the nucleus. Our earlier results with the Atp8b1<sup>G308V/G308V</sup> mice also do not suggest any aberration of Fxr signalling in these mice<sup>27</sup>. We found that Fxr and Ilbp expression in the ileum of these mice is not different from that in wt animals. Furthermore, impaired Fxr signalling would lead to enhanced expression of Cyp7a1 in the liver<sup>37</sup> and we have not observed this in Atp8b1<sup>G308V/G308V</sup> mice<sup>27</sup>. In addition, we showed that Cyp7a1 and Cyp8b1 expression in livers of Atp8b1<sup>G308V/G308V</sup> mice was strongly downregulated during BS administration. Since these two genes are also Fxr targets, it appears that Fxr signalling is intact in these animals. Finally, we now report that Shp and Fgf15 expression is sensitive to BS feeding in Atp8b1<sup>G308V/G308V</sup> mice (Fig. 4), again indicative for intact Fxr signalling.

Strikingly, we observed that Shp expression in Atp8b1<sup>G308V/G308V</sup> mice on a control diet tended to be lower than in wt mice, both in liver and ileum. In contrast, induction of Shp in Atp8b1<sup>G308V/G308V</sup> mice by BS feeding was significantly stronger than in wt mice. This may correlate with the difference in BS composition in these two genotypes; the mutant mice have a higher amount of tauromuricholate and less TC<sup>27</sup>. We speculate that tauromuricholate is a weaker Fxr agonist, but this has never been tested.

It should be stressed here that in the study of Chen et al.<sup>24</sup> the ileal tissue was obtained during ileal exclusion at which time the patients were strongly cholestatic. The high circulating BS
levels may have influenced the expression of FXR per se and, as a consequence, its downstream targets. Indeed, it was recently reported that liver specimens of patients with other cholestatic syndromes also have low FXR expression levels. In line with this report we have observed reduced Fxr expression in Atp8b1G308V/G308V mice fed a cholate-containing diet, which causes major accumulation of BS in their circulation. Hence, a specific relation between the absence of ATP8B1 function and downregulation of FXR expression remains to be confirmed and elucidated.

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