Wnt/β-Catenin Signaling Regulates the Expression of the Ammonium Permease Gene RHBG in Human Cancer Cells

Ahmad Merhi, Christelle De Mees, Rami Abdo, Jennifer Victoria Alberola, Anna Maria Marini*

Biology of Membrane Transport Laboratory, IBMM, Université Libre de Bruxelles, Gosselies, Belgium

* amarini@ulb.ac.be

Abstract

Ammonium is a metabolic waste product mainly detoxified by the liver. Hepatic dysfunction can lead to cytotoxic accumulation of circulating ammonium and to subsequent encephalopathy. Transmembrane ammonium transport is a widely spread process ensured by the highly conserved proteins of the Mep-Amt-Rh superfamily, including the mammalian Rheus (Rh) factors. The regulatory mechanisms involved in the control of RH genes expression remain poorly studied. Here we addressed the expression regulation of one of these factors, RHBG. We identify HepG2 hepatocellular carcinoma cells and SW480 colon adenocarcinoma cells as expressing RHBG and show that its expression relies on β-catenin signaling. siRNA-mediated β-catenin knockdown resulted in significant reduction of RHBG mRNA in both cell lines. Pharmaceutical inhibition of the TCF4/β-catenin interaction or knockdown of the transcription factor TCF4 also downregulated RHBG expression. We identify a minimal RHBG regulatory sequence displaying a promoter activity and show that β-catenin and TCF4 bind to this fragment in vivo. We finally characterize the role of potential TCF4 binding sites in RHBG regulation. Taken together, our results indicate RHBG expression as a direct target of β-catenin regulation, a pathway frequently deregulated in many cancers and associated with tumorigenesis.

Introduction

Ammonium, hereafter referring to the sum of the NH3 and NH4+ molecular species, serves as principal nitrogen source for micro-organisms and plants [1]. It is however mostly described for the cytotoxic consequences of its accumulation in animals [2]. Hepatic metabolism of ammonium towards urea and glutamine synthesis is critical to maintain a low plasmatic level of the ammonium emerging from the catabolism of proteins and the activity of the intestinal flora. The impairment of ammonium detoxification occurring in case of liver dysfunction can lead to the development of hepatic encephalopathy and, in acute cases, to lethal cerebral paralysis. In parallel to these toxic effects, renal ammonium production from glutaminolysis and its subsequent urinary excretion is a crucial process to ensure blood pH homeostasis [3]. The view...
that the transmembrane fluxes of ammonium are the sole consequence of NH₃ free diffusion was held for decades, till the first genes encoding specific ammonium permeases were identified [4–6]. Sequence analysis enabled to define a new and widely conserved family of proteins termed Mep-Amt-Rh, represented in vertebrates by the well known human Rhesus blood group factors [7]. Non-erythroid Rh factors, RhBG and RhCG, were subsequently discovered and notably found to be expressed in specific epithelial cells of several organs including mouse and human liver and kidney [8–14]. Mice knockout studies revealed the role of Rhbg and Rhcg in renal urinary ammonium excretion while their potential involvement in the liver physiology remains unsolved [15–17]. Of note, RHBG appears overexpressed in human hepatocellular carcinoma bearing activating mutations in β-catenin [18], suggesting a correlation between Wnt/β-catenin signaling and human RHBG regulation. This correlation appears to hold true in a normal mouse liver context as transgenic models enabling targeted inactivation or activation of β-catenin signaling show downregulation or upregulation of RHBG expression, respectively [19,20]. The Wnt/β-catenin pathway is highly conserved across metazoans and regulates cell proliferation, differentiation, and survival [21–23]. The secreted proteins of the WNT family are able to bind specific Frizzled/Lrp receptors and activate signal transduction via different mechanisms. In the canonical Wnt/β-catenin mechanism, absence of Wnt signaling is accompanied by a low cytosolic β-catenin level. The β-catenin stability is regulated by a destruction complex, formed by axin, adenomatous polyposis complex (APC), casein kinase 1 and glycogen synthase kinase-3β (GSK-3β) that phosphorylates β-catenin at its N-terminus and leads to its ubiquitylation and subsequent proteasomal degradation [21,22]. Wnt signaling triggers the dissociation of the β-catenin/destruction complex [23–25]. The resulting inhibition of phosphorylation leads to cytosolic β-catenin accumulation and translocation into the nucleus. Beta-catenin can then activate the transcription of various target genes as a cofactor bound to members of the T-cell factor (TCF)/lymphoid enhancer factor (LEF) transcription factor family and drive Wnt-specific transcriptional programs [23]. Abnormal activation of Wnt/β-catenin signaling, due to loss-of-function mutations in APC or activating mutations in β-catenin has been linked to tumorigenesis in many settings including melanoma, breast, colon and hepatocellular carcinomas [21,23].

The regulatory mechanisms involved in the control of human RH genes expression and the signaling pathways potentially implicated are so far poorly documented. Here, we sought to identify human cancer cell lines expressing the RHBG gene to study its expression regulation and address the potential direct influence of the Wnt/β-catenin signaling. We show that RHBG is highly expressed in HepG2 hepatoma cells and relies on β-catenin signaling. Similarly, the RHBG expression revealed in SW480 colon cancer cells is dependent on β-catenin, further supporting the role of β-catenin signaling in RHBG regulation. Promoter analysis and chromatin immunoprecipitation assays are consistent with a direct involvement of TCF4/β-catenin in RHBG up-regulation in HepG2 cells.

Materials and Methods

Cell culture and reagents

The human hepatocellular carcinoma (HepG2 and Hep3B) and human embryonic kidney (HEK293T) cell lines were kindly provided by Professor Claude Szpirer, Université Libre de Bruxelles, Belgium. The human colon adenocarcinoma (SW480) cell line was purchased from CLS (Germany). HepG2, Hep3B, SW480 and HEK293T cells were cultured in advanced DMEM medium (Invitrogen) supplemented with 10% fetal bovine serum (Biowest), 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. Cells were maintained in an
incubator with humidified air (5% CO2) at 37°C. PFK118-310 (#K4394) was purchased from Sigma and used at a 0.2 or 0.4 μM concentration from a DMSO solution.

**Plasmids construction**

DNA fragment (2492 bp) corresponding to the potential RHBG promoter was amplified using polymerase chain reaction with the F-Pr- BG- and R-Pr-BG (Table 1) primers and the human genomic DNA of HEK293T cells as a template. The PCR product was digested with SacI and BglII restriction enzymes and cloned into pGL3-Basic (Promega) that has been linearized with the same restriction enzymes. Deletion mutants were then constructed using the pGL3-RHBG plasmid as a template and the indicated primers (Table 1). The PCR products were digested with SacI and BglII restriction enzymes and cloned into pGL3-Basic. All constructs were verified by sequencing.

**RNA extraction and qRT-PCR**

Total cellular RNA was extracted using TRIzol reagent (Invitrogen) according to manufacturer instructions. DNase treatment was done using a DNA Removal Kit (Invitrogen, #AM1906). One μg of total RNA was reverse-transcribed to cDNA using the SuperScriptIII First-Strand Synthesis SuperMix (Invitrogen) according to manufacturer instructions. Realtime RT-PCR were performed on a StepOnePlus Real-Time PCR System (Applied Biosystems) using GoTaq qPCR Master Mix (Promega) using the indicated primers (Table 2) and normalized to β-actin mRNA level measured in parallel.

**Immunofluorescence staining**

Cells were cultured in Millicell EZ 8 chamber slide (Millipore). Cells were fixed with 4% paraformaldehyde (PFA) for 15 min and subsequently permeabilized with 0.1% TritonX-100 for 3 minutes and were next blocked with goat serum (5%) for 30 minutes. Cells were incubated with β-catenin antibody (Cell signaling #8480, 1:100 in 5% goat serum) overnight at 4°C. Slides were washed and incubated with anti-rabbit IgG (H+L), F(ab’)2 Fragment (Alexa Fluor 594 Conjugate, Cell signaling, 1:250 in 5% goat serum) for 1 hour at room temperature. After washing with PBS, the slides were mounted with ProLong Gold antifade reagent with DAPI (Invitrogen).

---

**Table 1. Primer sequences used for cloning.**

|          |       |                                  |                                    |
|----------|-------|----------------------------------|-----------------------------------|
| RHBG     | Forward| CCCGAGCTCCAGTCCTGATTGGACAGCC    |                                   |
|          | Reverse| CCCAGATCTGGCGACAAAGACAGCAAAGA   |                                   |
| Mut-A    | Forward| CCCGAGCTCACTGAGTCTGGTAACACAC    |                                   |
| Mut-B    | Forward| CCCGAGCTACAGAGACGTGGTTCTGGGA    |                                   |
| Mut-C    | Forward| CCCGAGCTCTGAGACGACTATTATCTTC    |                                   |
| Mut-D    | Forward| CCCGAGCTCAGATCATCTGTATGAGGGTG    |                                   |
| Mut-E    | Forward| CCCGAGCTCTACAGGGCAGAACATAGG     |                                   |
| Mut-F    | Forward| CCCGAGCTCTTTCCAGGGTCGCGTCCAA    |                                   |
| Mut-G    | Forward| CCCGAGCTCCGCCCGCCCTCTG          |                                   |
| Mut-H    | Forward| CCCGAGCTCAGAGGCCCTGTTG          |                                   |
| Mut-I    | Forward| CCCGAGCTCCGCGCCGGAATTGTG        |                                   |
| ΔTCF3    | Reverse| CCCGATCTACAGAAAAGAGACGCCAGTG    |                                   |
| ΔTCF2, 3 | Reverse| CCCGATCTCGGAGCTGGCCGCTGGAG      |                                   |

doi:10.1371/journal.pone.0128683.t001
Western Blot analysis

Total proteins were extracted using RIPA (25mM Tris-HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) lysis buffer supplemented with cocktail of phosphatase and protease inhibitors (Roche). After centrifugation, proteins were quantified using Pierce Microplate BCA Protein Assay Kit—Reducing Agent Compatible assay (Thermo Scientific). Equal amounts (~20μg) of proteins were then separated by Mini-PROTEAN TGX gels (Bio-Rad). Proteins were transferred to nitrocellulose membrane (Protran, VWR). Membranes were blocked with 5% milk and incubated with anti-β-catenin (Cell signaling, #8480, 1:1000), anti-TCF4 (#2569, 1:1000), anti-Glutamine synthetase (Abcam, #ab73593, 1:1000) or anti-β-Actin (Sigma, #A2228, 1:2000). Primary antibodies were detected with horseradish-peroxidase-conjugated anti-rabbit or anti-mouse-IgG secondary antibodies (GE Healthcare) followed by measurement of chemoluminescence (Lumi-LightPLUS, Roche).

RNA interference

Cells were reverse transfected with pre-designed silencer select non-targeting control (Invitrogen, #4390843) or siRNAs targeting β-catenin (Invitrogen, #s438) or TCF4 (Invitrogen, #s13880) with Lipofectamine siRNA-MAX (Invitrogen). Cells were incubated at 37°C for 72 or 96 hours and the indicated mRNA and proteins level were examined by qRT-PCR and western blotting, respectively.

Transfection and luciferase assay

Cells were transiently co-transfected with the pTK-Renilla luciferase reporter vector (Promega) and empty plasmid (pGL3-Basic, Promega) or the plasmid containing the indicated RHBG promoter constructs for 48h using the Viafect transfection reagent (Promega). Luciferase activity in total cell lysates was measured using the Dual-Glo luciferase reporter assay (Promega).
according to manufacturer instructions. Luminescence was measured using GloMax-96 Microplate Luminometer (Promega).

**Chromatin immunoprecipitation (CHIP) assay**

CHIP assay was done using SimpleChIP Enzymatic Chromatin IP Kit (Magnetic Beads, Cellsignaling) according to manufacturer instructions. Briefly, cells were fixed with 1% formaldehyde solution to cross-link histone and non-histone proteins to DNA. Nuclear chromatin was digested with Micrococcal Nuclease for 20 min at 37°C and then incubated overnight at 4°C with either anti-β-catenin (Cell signaling, #8480, 1:50), anti-TCF4 (#2569, 1:50) Normal Rabbit IgG (Cell signaling, #2729). Following washing with low and high salt ChIP buffers, the protein-DNA complexes were eluted and cross-links were then reversed. After proteinase K digestion, DNA is purified and quantified by Real time-PCR as described earlier using primers listed in Table 2 and designed to amplify the indicated promoter regions of the target genes.

**Statistical analysis**

Data are expressed as means ± S.E.M. Statistical comparisons were assessed by Student’s t-tests using Graph Pad Prism version 5.00 software (Graph Pad Software). Differences were considered significant when the p value is below 0.05 ( * p < 0.05, ** p < 0.01, *** p < 0.001), n = 3, except for Chip experiments where n = 2.

**Results**

*RHBG is highly expressed in HepG2 hepatoma cells*

The human RHBG gene was found to be overexpressed in a subset of hepatocellular carcinoma [18], however, the regulatory mechanisms involved remain unknown. In order to identify a cancer cell line that could be used to address the regulation of RHBG, we evaluated its expression levels in the HepG2 and Hep3B hepatoma cells. HepG2 cells bear a heterozygous deletion in the exon3 of the β-catenin CTNNB-1 gene, producing a truncated β-catenin protein lacking key residues for its phosphorylation by the destruction complex and thus resulting in its cellular accumulation [26–28]. Hep3B cells are derived from a hepatitis B- infected liver tumor, and do not contain mutations in the β-catenin gene [29,30].

The RHBG gene appeared highly expressed in HepG2 compared to Hep3B cells, the latter showing slightly higher expression level compared to that of HEK293T cells taken as a normal cell model (Fig 1A). Expression level of GLUL, the gene of glutamine synthetase (GS) was also upregulated in HepG2 cells compared to Hep3B (Fig 1B). As the GS gene is a reported target of β-catenin [31,32], this could be consistent with a more effective β-catenin signaling in HepG2 cells compared to Hep3B. The GLUL expression levels were corroborated by the resulting GS protein levels which appeared higher in HepG2 compared to Hep3B cells (Fig 1C). Upon checking the expression of the second non-erythroid RH gene, RHCG, very low levels were found in HepG2, Hep3B and HEK293T cells (Fig 1D). These data thus point to HepG2 cells as suitable for the study of RHBG expression.

We next checked the β-catenin protein levels in HepG2, Hep3B and HEK293T cells. Consistent with previous reports [33,34], HepG2 cells harbored two β-catenin species likely corresponding to the wild-type and the truncated forms (Fig 1E). A β-catenin protein with the size of the wild-type form was detected in both HEK293T and Hep3B, with no obvious accumulation in the latter cell line. It was previously shown that β-catenin is present in the nucleus of HepG2 cells in contrast to Hep3B cells [34]. These data suggest a correlation between RHBG expression in HepG2 cells and nuclear localization of β-catenin.
Silencing of β-catenin correlates with RHBG down-regulation in HepG2 cells

We next used β-catenin siRNA to test whether the RHBG gene expression observed in HepG2 cells is related to β-catenin function. Transfection of HepG2 cells with β-catenin siRNA for 72 hours led to a decrease in β-catenin mRNA level compared to cells transfected with non-targeting siRNA (Fig 2A). The reduction of β-catenin mRNA resulted in a reduction of β-catenin protein levels (Fig 2B). Of note, the RHBG gene expression was largely reduced upon β-catenin silencing (Fig 2C). Similarly, expression levels of Axin2 and Cyclin D1, two targets of β-catenin regulation [35–38], were also decreased with β-catenin silencing (Fig 2D and 2E). In contrast, the low expression level of RHCG observed in HepG2 cells was not affected by β-catenin.
silencing (Fig 2F). Hence, inhibition of β-catenin is accompanied by the down-regulation of RHBG gene expression in HepG2 cells.

Beta-catenin drives RHBG expression in SW480 colon cancer cells

Mutations inducing β-catenin activation have been identified in various types of tumors, including melanoma, prostate, breast, and colon cancers [21,33,39,40]. We next tested whether the β-catenin signaling could be correlated with RHBG expression in another cancer cell line harboring β-catenin signaling activating mutations. The SW480 colon cancer cells bear a truncating mutation in the APC gene, resulting in stabilization and nuclear accumulation of β-catenin and leading to constitutive activation of β-catenin signaling [41–44]. Consistently, immunofluorescence experiments revealed a strong nuclear localization of β-catenin in these cells (Fig 3A). Transfection of SW480 cells with β-catenin siRNA for 72 hours led to a decrease in both mRNA and protein levels of β-catenin (Fig 3B and 3C). Similarly to HepG2 cells, β-catenin silencing was accompanied by a large decrease in RHBG expression in SW480 cells.
The expression levels of Axin2, and CyclinD1 (Fig 3E and 3F) were also decreased upon β-catenin silencing, consistent with β-catenin signaling inhibition. RHCG expression was not significantly affected upon β-catenin silencing (Fig 3G).

These results indicate that the correlation between β-catenin signaling and RHBG expression can be extended to SW480 colon cancer cells.

**Activation of β-catenin correlates with RHBG gene induction in HEK293T cells**

We next addressed whether activation of β-catenin in a cell line with no major nuclear activity of β-catenin could be sufficient to induce RHBG expression. LiCl is reported to inhibit GSK3 kinase [45,46], leading to β-catenin stabilization and nuclear accumulation [47,48]. We therefore treated HEK293T cells with LiCl (10 and 20 mM) to activate the Wnt/β-catenin pathway. In keeping with previous observations, immunofluorescence and western blot experiments revealed that treatment of HEK293T cells with LiCl for 24 hours induced a nuclear accumulation and stabilization of β-catenin (Fig 4A and 4B). Of note, LiCl treatment concomitantly induced an increase in the mRNA level of RHBG in a dose-dependent manner (Fig 4C). This result indicates that RHBG expression can be upregulated by artificial activation of β-catenin signaling.
RHBG expression in HepG2 cells is dependent on TCF4

The Wnt/β-catenin pathway drives Wnt-specific transcriptional programs via the interaction with DNA-binding factors of the TCF/LEF family [21,22]. However, it is reported that β-catenin can also activate gene expression in a TCF4-independent manner [49–51]. To address a role of the TCF4/β-catenin complex in RHBG expression, we evaluated the effect of inhibiting the β-catenin activity in HepG2 cells by using an antagonist of the TCF4/β-catenin complex, PKF118-310. This compound disrupts the TCF4/β-catenin complex and inhibits expression of TCF4-dependent genes [52]. Treatment of HepG2 cells with PKF118-310 was accompanied by a decrease in RHBG expression (Fig 5A). The GLUL expression level was also decreased by the treatment, consistent with a likely reduction of TCF4/β-catenin mediated transcription in these conditions (Fig 5B).

To further assert a role of TCF4 in RHBG expression, we tested the impact of TCF4 knockdown in HepG2 cells. Transfection of the latter cells with TCF4 siRNA for 72 hours decreased both the mRNA and protein levels of TCF4 compared to cells transfected with non-targeting siRNA (Fig 5C and 5D). Importantly, the RHBG mRNA level was reduced upon TCF4 silencing (Fig 5E). Similarly, the Axin2 and Cyclin D1 mRNA levels were also decreased (Fig 5F and 5G), in keeping with previous observations describing the corresponding genes as targets of TCF4 [38,40]. Moreover, similar TCF4 silencing experiments performed in SW480 colon adenocarcinoma cells also decreased RHBG, Axin2 and Cyclin D1 mRNA levels (Fig 6A–6E).
Fig 5. **RHBG expression is dependent on TCF4.** A-B) HepG2 cells were treated with PKF118-310 (0.2 or 0.4 μM) for 24 hours. The RHBG (A) and GLUL (B) mRNA levels were determined by qRT-PCR. C-G) HepG2 cells were reverse transfected with TCF4 or control (scramble) siRNAs. 72 hours after transfection, levels of TCF4 mRNA (C), TCF4 protein (D), RHBG mRNA (E), Axin2 mRNA (F), and Cyclin D1 mRNA (G), were determined.

doi:10.1371/journal.pone.0128683.g005
These results together indicate that β-catenin-mediated expression of RHBG is at least partially TCF4-dependent in both HepG2 and SW480 cells.

The RHBG promoter is activated by β-catenin/TCF4

To further study RHBG expression and identify potential regulators, a genomic fragment (Fig 7) containing 2349 bp upstream and 142 bp downstream of the RHBG predicted transcriptional start site (TSS) was directionally subcloned into the pGL3-basic firefly luciferase reporter vector. To test whether this fragment possesses a promoter activity, the RHBG promoter construct (pGL3-RHBG) and the native pGL3-basic vector were used for transient co-transfection
Fig 7. Promoter region of RHBG gene. The potential human RHBG promoter sequence was obtained from eukaryotic promoter database (http://epd.vital-it.ch/). Black arrow (A) indicates the predicted transcription start site (TSS) which is designated nucleotide 0. The GC boxes are shadowed. A selection of potential binding sites (with 0 or less than 5% dissimilarity) of transcription factors identified using PROMO [54,55] is underlined. Potential TCF4 binding sites are indicated with empty boxes. Horizontal arrows (---) indicate the starting residue position of each promoter construct analyzed in Fig 8.
of HepG2 cells together with the pTK-Renilla luciferase reporter vector as transfection control. 48 hours after transfection, the luciferase activity in pGL3-RHBG transfected cells was about 30 fold higher than with the pGL3-basic plasmid indicating that the cloned RHBG sequence contains an active promoter (Fig 8A).

Sequence analysis of the RHBG regulatory sequence did not reveal a potential TATA box, while the region proximal to the predicted TSS was enriched in G/C content, indicating that the RHBG promoter likely corresponds to a TATA-less GC-rich promoter. Two potential GC boxes were depicted, embedded in potential Sp-1 binding sites (Fig 7). To further dissect the regions important for the activity of the cloned RHBG promoter, a series of constructs were generated bearing progressive deletions in this DNA fragment (Figs 7 and 8).

Though fluctuations were noted according to the considered fragment, all the constructs containing the -60/+142 region produced a luciferase activity very close or higher than the
full-length pGL3-RHBG construct (Fig 8B). Of note the -60/+142 region of fragment H, comprising only one of both GC boxes, retained high luciferase activity. The constructs bearing further 5’ truncation into this region led to a major decrease of the RHBG promoter function, the -22/+142 region showing a very low luciferase activity (Fig 8B). This underlines the importance of the DNA segment between fragment H and I, and indicates that the expression impairment is most likely due to the loss of the second GC box. Additionally, analysis of the -60/+142 functional segment revealed the presence of three CTTTG/CAAAG motifs which could serve as TCF4 binding sites (Fig 7). These motifs are either juxtaposed to or downstream of the putative TSS. To evaluate a potential contribution of these motifs to the regulation of RHBG gene expression, promoter constructs bearing the -60/+142 region of fragment H with deletion of potential TCF4 binding motif 2, or motifs 2 and 3, were generated. Both constructs showed a promoter activity (Fig 8C). However, deletion of motif 2 reduced the promoter activity to the half of fragment H, and simultaneous deletion of motifs 2 and 3 further decreased the promoter activity, suggesting a contribution of these motifs to the functionality of the RHBG promoter.

We finally performed chromatin immunoprecipitation (ChIP) assays to determine whether TCF4 and β-catenin are capable of binding the -60/+142 segment of RHBG promoter in vivo. Nuclear extracts obtained from the HepG2 cells were subjected to protein/DNA complex cross-linking and immunoprecipitation was performed using antibodies targeting either β-catenin, TCF4 or IgG, as a control. qPCR using primers within the H region reveal that TCF4 and β-catenin bind to this fragment of the RHBG promoter (Fig 9). Consistently, TCF4 and β-catenin did also bind to the Axin2 promoter, taken as control, as previously reported [37,53].

These results indicate that TCF4/β-catenin specifically binds to the -60/+142 region of the RHBG promoter, and likely enhances RHBG expression in HepG2 cells.

**Discussion**

This study identifies the hepatoma HepG2 cells as expressing the RHBG gene encoding an ammonium transport protein. We show that in these cells RHBG expression is largely dependent on β-catenin function. We perform a functional analysis of the RHBG upstream regulatory sequence, revealing a minimal region bearing a promoter activity. Our data indicate that the RHBG regulatory sequence is a TATA-less GC-rich promoter. We show that β-catenin and TCF4 are both able to bind the minimal promoter region in vivo and characterize potential TCF4 binding motifs important for the promoter activity. Our data support a direct role of β-catenin/TCF4 in the regulation of RHBG expression in this cell line, and further indicate that RHBG could serve as a direct reporter of the Wnt/β-catenin pathway in specific cancer cell contexts.

Hepatocellular carcinoma is the most common adult liver malignancy and many lines of evidence associate hyperactivation of the Wnt/β-catenin pathway to its initiation and development [56]. Abnormal activation of Wnt/β-catenin signaling, due to loss-of-function mutations in APC or activating mutations in β-catenin has been linked to various human malignancies including melanoma, breast, and colon carcinomas [22]. For instance, more than 80% of colon cancers bear truncations in APC, resulting in active β-catenin accumulation in the nucleus, the initial stage of transformation [56–58]. We show that RHBG is expressed in the colon cancer SW480 cells bearing an APC mutation and that its expression is also dependent on TCF4/β-catenin. In contrast, the expression of the gene encoding the second non erythroid ammonium transport protein, RhCG, is independent of β-catenin signaling in either HepG2 or SW480 cells.

Our data are consistent with the RHBG overexpression observed in hepatocarcinoma obtained from surgical resections and bearing activating mutations in the β-catenin gene.
CTNNB-1 [18]. RHBG was up-regulated in 9 over 10 HCC of the latter resections compared to normal liver, while it was slightly overexpressed in only one over 15 HCC showing wild-type CTNNB-1. RHBG overexpression was strongly correlated with upregulation of GLUL, but also of SLC13-A3 encoding a sodium-dicarboxylate (including α-ketoglutarate and succinate) transporter and GPR49, also known as LGR5, a coreceptor of Wnt signaling. A correlation between HCC with activated β-catenin pathway and upregulation of GS, of ornithin amino transferase (involved in glutamate synthesis), and of the Glt1 glutamate transporter, were also reported [31]. The upregulation of GS suggests that HepG2 cells, and possibly specific HCC, could be able to adapt their metabolism to favor glutamine synthesis from glutamate and ammonium, a function restricted to perivenous hepatocytes in normal liver. For instance, in mouse, the Wnt/β-catenin pathway has been shown to play a key role in liver zonation [19,20]. This process ensures a functional specialization of hepatocytes along the porto-central axis of the liver lobule and determines the fate of periportal hepatocytes, active in urea synthesis, or perivenous hepatocytes, active in glutamine synthesis for instance.

Fig 9. TCF4/β-catenin binds to the RHBG promoter. HepG2 cells were cross-linked with formaldehyde followed by chromatin digestion. Chromatin immunoprecipitations were performed using antibodies targeting either β-catenin, TCF4 or IgG, as a control. Purified DNA was analyzed by qPCR using the indicated primers. The amount of immunoprecipitated DNA with each antibody is represented as signal relative to IgG (equivalent to 1) (n = 2).

doi:10.1371/journal.pone.0128683.g009
Mouse Rhbg is specifically present at the cell surface of the latter hepatocytes and co-localizes with GS [59]. HepG2 cells were shown to have a reduced activity of the urea cycle [60]. However, it should be kept in mind that these cells show important plasticity of the metabolic networks according to the availability of key metabolite in the surrounding medium as glucose and insulin [61]. Of note, it was recently shown that HepG2 cells have a glutamine-addiction phenotype [34]. Addiction to glutamine is a metabolic particularity of many cancer cells showing concomitant high rates of glutamine transport and metabolism [62]. Proliferation of HepG2 was importantly reduced upon withdrawal of exogenous glutamine, and simultaneous drug-mediated inhibition of GS activity further hampered proliferation [34]. In conditions where glutamine synthesis would be favourable, it is tempting to hypothesize that correlated upregulation of RhBG could help to scavenge ammonium, providing one of the substrates of GS. Whether RhBG actively participates to cancer cell metabolism will require further investigation. Rh factors were shown to act as bidirectional ammonium transport proteins [8,63]. Rhcg is expressed at the apical membrane of specific epithelial kidney cells, together with the H+ V-ATPase which is supposed to drive NH3 efflux by favouring urinary trapping of NH4+ [12,13,17]. The GS activity could serve as a trapping system, driving ammonium influx via co-expressed Rh factors such as RhBG by consuming ammonium for glutamine generation. Interestingly, a corresponding mechanism exists in E. coli where the GS activity is strictly required to drive substrate uptake via the Rh orthologue AmtB [64]. However, to date, a role of Rhbg in the process of ammonium detoxification via glutamine synthesis has not been highlighted in vivo, as plasma levels of glutamine and urea appear normal in mice lacking Rhbg [15]. GS is the sole enzyme catalyzing glutamine synthesis. In addition to its presence in perivenous hepatocytes, a detailed analysis in mouse revealed that it is also highly expressed and active in the epididymis epithelial cells and in Leydig cells, the testosterone-producing cells in the testis [65]. Although the physiological role of GS in these cells is unknown, it should be noted that Rh factors are also co-expressed [17,66].

Ammonium was recently proposed to play a particular role in a tumoral context [67]. Up-regulated glutaminolysis in glutamine-addicted cancer cells results in NH3 production. The latter molecule was shown to act as an autocrine and paracrine diffusible signal that triggers a specific autophagic program, in turn enabling survival and proliferation of cancer cells deep in a tumour mass [67,68]. Whether Rh factors could play a role in these processes by participating to trans-cellular ammonium movements remains to be evaluated.

Acknowledgments

We thank Fadi Abdel-Sater, Mélanie Boeckstaens, Jérôme Kucharczak, Claude Szpirer and all members of the lab for fruitful discussions and support.

Author Contributions

Conceived and designed the experiments: AM AMM. Performed the experiments: AM CD RA JVA. Analyzed the data: AM CD RA JVA AMM. Wrote the paper: AM AMM.

References

1. Von Wirén N, Merrick M. Regulation and function of ammonium carriers in bacteria, fungi, and plants. 2004; 1–26. doi:10.1007/b95775
2. Auron A, Brophy PD. Hyperammonemia in review: pathophysiology, diagnosis, and treatment. Pediatr Nephrol. 2012; 27: 207–22. doi:10.1007/s00467-011-1838-5 PMID: 21431427
3. Weiner ID, Verlander JW. Renal ammonia metabolism and transport. Compr Physiol. 2013; 3: 201–20. doi:10.1002/cphy.c120010 PMID: 23720285
1. Ninnemann O, Jauniaux JC, Frommer WB. Identification of a high affinity NH4+ transporter from plants. EMBO J. 1994; 13: 3464–3471. PMID: 8062823

2. Marini AM, Vissers S, Urrestarazu A, Andre B. Cloning and expression of the MEP1 gene encoding an ammonium transporter in Saccharomyces cerevisiae. EMBO J. AD—Laboratoire de Physiologie Cellulaire et de Genetique des Levures, Universite Libre de Bruxelles, Belgium; 1994; 13: 3456–3463. PMID: 8062822

3. Marini AM, Soussi-Boudekou S, Vissers S, Andre B. A family of ammonium transporters in Saccharomyces cerevisiae. Mol Cell Biol. 1997; 17: 4282–93. PMID: 9234685

4. Marini AM, Urrestarazu A, Beauwens R, Andre B. The Rh (rhesus) blood group polypeptides are related to NH4+ transporters. Trends Biochem Sci. 1997; 22: 460–1. PMID: 9433124

5. Marini AM, Matassi G, Raynal V, Andre B, Cartron JP, Cherif-Zahar B. The human Rhesus-associated RhAG protein and a kidney homologue promote ammonium transport in yeast. Nat Genet. AD—Laboratoire de Physiologie Cellulaire, Universite Libre de Bruxelles, Institut de Biologie et de Medecine Moleculaires, Gosselies, Belgium; 2000; 26: 341–344. PMID: 11062476

6. Liu Z, Chen Y, Mo R, Hui C, Cheng JF, Mohandas N, et al. Characterization of human RhCG and mouse RhCG as novel nonerythroid Rh glycoprotein homologues predominantly expressed in kidney and tests. J Biol Chem. 2000; 275: 25641–25651. doi: 10.1074/jbc.M003533200 PMID: 10852913

7. Liu Z, Peng J, Mo R, Hui C, Huang CH. Rh type B glycoprotein is a new member of the Rh superfamily and a putative ammonia transporter in mammals. J Biol Chem. 2001; 276: 14247–33. doi: 10.1074/jbc.M007528200 PMID: 11204028

8. Nakhoul NL, Lee Hamm L. Characteristics of mammalian Rh glycoproteins (SLC42 transporters) and their role in acid-base transport. Molecular Aspects of Medicine. 2013. pp. 629–637. doi: 10.1016/j.mam.2012.05.013 PMID: 23506896

9. Quentin F, Eladari D, Cheval L, Lopez C, Goossens D, Colin Y, et al. RhBG and RhCG, the putative ammonium transporters, are expressed in the same cells in the distal nephron. J Am Soc Nephrol. 2003; 14: 545–554. doi: 10.1097/01.ASN.0000050413.43662.55 PMID: 12595489

10. Verlander JW, Miller RT, Frank AE, Royaux IE, Kim Y-H, Weiner ID. Localization of the ammonium transporter proteins RhBG and RhCG in mouse kidney. Am J Physiol Renal Physiol. 2003; 284: F323–F337. doi: 10.1152/ajprenal.00050.2002 PMID: 12388412

11. Weinier ID, Miller RT, Verlander JW. Localization of the ammonium transporters, Rh B glycoprotein and Rh C glycoprotein, in the mouse liver. Gastroenterology. 2003; 124: 1432–40. PMID: 12730882

12. Chambrey R, Goossens D, Bourgeois S, Picard N, Bloch-Faure M, Levieil F, et al. Genetic ablation of Rhb in the mouse does not impair renal ammonium excretion. Am J Physiol Renal Physiol. 2005; 289: F1281–F1290. doi: 10.1152/ajprenal.00172.2005 PMID: 16077082

13. Bishop JM, Verlander JW, Lee H-W, Nelson RD, Weiner AJ, Handlogten ME, et al. Role of the Rhesus glycoprotein, Rh B glycoprotein, in renal ammonia excretion. Am J Physiol Renal Physiol. 2010; 299: F1065–F1077. doi: 10.1152/ajprenal.00277.2010 PMID: 20719974

14. Biver S, Belge H, Bourgeois S, Van Voren P, Nowik M, Scoby S, et al. A role for Rhesus factor Rhcg in renal ammonia excretion and male fertility. Nature. 2008; 456: 339–343. doi: 10.1038/nature07518 PMID: 19020163

15. Stahl S, Ittrich C, Marx-Stoelting P, Köhle C, Altug-Teber O, Riess O, et al. Genotype-phenotype relationships in hepatocellular tumors from mice and man. Hepatology. 2005; 42: 353–61. doi: 10.1002/hep.20768 PMID: 15965925

16. MacDonald BT, Tamaki K, He X. Wnt/β-Catenin Signaling: Components, Mechanisms, and Diseases. Developmental Cell. 2009. pp. 9–26. doi: 10.1016/j.devcel.2009.06.016 PMID: 19619488

17. Cong F, Schweizer L, Varmus H. Wnt signals across the plasma membrane to activate the beta-catenin pathway by forming oligomers containing its receptors, Frizzled and LRP. Development. 2004; 131: 5103–15. doi: 10.1242/dev.01318 PMID: 15459103
25. Kim S-E, Huang H, Zhao M, Zhang X, Zhang A, Semenov MV, et al. Wnt stabilization of β-catenin reveals principles for morphogen receptor-scaffold assemblies. Science. 2013; 340: 867–70. doi: 10.1126/science.1233839 PMID: 23579495

26. Amit S, Hatzubai A, Birman Y, Andersen JS, Ben-Shushan E, Mann M, et al. Axin-mediated CKI phosphorylation of beta-catenin at Ser 45: a molecular switch for the Wnt pathway. Genes Dev. 2002; 16: 1066–76. doi: 10.1101/gad.230302 PMID: 1200790

27. Wu G, Huang H, Garcia Abreu J, Jin D-Y, editor. PLoS One. Public Library of Science; 2009; 4: e4926. doi:10.1371/journal.pone.0004926 PMID: 19293931

28. Liu X, Rubin JS, Kimmel AR. Rapid, Wnt-induced changes in GSK3beta associations that regulate phosphorylation of beta-catenin via phosphorylated PPPSPXS motifs of Wnt coreceptor LR6P. J Biol Chem. 2002; 277: 20056–63. doi:10.1074/jbc.M200139200 PMID: 12000790

29. Cha M-Y, Kim C-M, Park Y-M, Ryu W-S. Hepatitis B virus X protein is essential for the activation of Wnt/beta-catenin signaling in hepatoma cells. Hepatology. 2004; 39: 1683–93. doi: 10.1002/hep.20245 PMID: 15185310

30. Desbois-Mouthon C, Blivet-Van Eggelpoël M-J, Beurel E, Boissan M, Delélo R, Cadoret A, et al. Dysregulation of glycogen synthase kinase-3beta signaling in hepatocellular carcinoma cells. Hepatology. 2002; 36: 1528–36. doi: 10.1053/hep.2002.37192 PMID: 12447879

31. Cadoret A, Ovejero C, Terris B, Souil E, Lévy L, Lamers WH, et al. New targets of beta-catenin signaling in colon cancer. Oncogene. 2002; 21: 8293–301. doi: 10.1038/sj.onc.1206118 PMID: 12447692

32. Zeng G, Apte U, Cleypie B, Singh S, Monga SPS. siRNA-Mediated B-Catenin Knockdown in Human Hepatoma Cells Results in Decreased Growth and Survival 1. 2007; 9: 951–959. doi:10.1593/neo.07469 PMID: 18030363

33. Coste A de La, Romagnolo B, Billuart P, Renard C-A, Buendia M-A, Soubrane O, et al. Mutations of the β-catenin gene are frequent in mouse and human hepatocellular carcinomas. Proc Natl Acad Sci U S A. 1998; 95: 8847–8851. doi: 10.1073/pnas.95.15.8847 PMID: 9671767

34. Tardito S, Chiu M, Uggeri J, Zerbini A, Da Ros F, Dall’Asta V, et al. L-Asparaginase and inhibitors of glutamine synthetase disclose glutamine addiction of β-catenin-mutated human hepatocellular carcinoma cells. Curr Cancer Drug Targets. 2011; 11: 929–43. PMID: 21834755

35. Tetsu O, McCormick F. Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature. 1999; 398: 422–6. doi: 10.1038/18884 PMID: 10201372

36. Shitujman M, Zhurinsky J, Simcha I, Albanese C, D’Amico M, Pestell R, et al. The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. Proc Natl Acad Sci U S A. 1999; 96: 5522–7. PMID: 10318916

37. Jho E, Zhang T, Domon C, Joo C-K, Freund J-N, Costantini F. Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. Mol Cell Biol. 2002; 22: 1172–83. PMID: 11809808

38. Leung JY, Kolligs FT, Wu R, Zhai Y, Kuick R, Hanash S, et al. Activation of AXIN2 expression by beta-catenin-T cell factor. A feedback repressor pathway regulating Wnt signaling. J Biol Chem. 2002; 277: 21657–65. doi:10.1074/jbc.M200139200 PMID: 11940574

39. Rubinfield B, Robbins P, El-Gamil M, Albert I, Polakis P. Stabilization of beta-catenin by genetic defects in melanoma cell lines. Science. 1997; 275: 1790–2. PMID: 9065403

40. Lin SY, Xia W, Wang JC, Kwong KY, Spohn B, Wen Y, et al. Beta-catenin, a novel prognostic marker for breast cancer: its roles in cyclin D1 expression and cancer progression. Proc Natl Acad Sci U S A. 2000; 97: 4262–6. doi:10.1073/pnas.060025397 PMID: 10759547

41. Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B, et al. Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. Science. 1997; 275: 1787–1790. doi: 10.1126/science.275.5307.1787 PMID: 9065402

42. Munemitsu S, Albert I, Souza B, Rubinfield B, Polakis P. Regulation of intracellular beta-catenin levels by the adenomatous polyposis coli (APC) tumor-suppressor protein. Proc Natl Acad Sci U S A. 1995; 92: 3046–50. PMID: 7708772

43. Rubinfield B, Albert I, Porfiri E, Munemitsu S, Polakis P. Loss of beta-catenin regulation by the APC tumor suppressor protein correlates with loss of structure due to somatic mutations of the gene. Cancer Res. 1997; 57: 4624–4630. PMID: 9377578

44. Rubinfield B, Souza B, Albert I, Munemitsu S, Polakis P. The APC protein and E-cadherin form similar but independent complexes with alpha-catenin, beta-catenin, and plakoglobin. J Biol Chem. 1995; 270: 5549–55. PMID: 7890674
45. Klein PS, Melton DA. A molecular mechanism for the effect of lithium on development. Proc Natl Acad Sci U S A. 1996; 93: 8455–9. PMID: 8710892
46. Stambolic V, Ruel L, Woodgett JR. Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells. Curr Biol. 1996; 6: 1664–8. PMID: 8994831
47. Najafov A, Seker T, Even I, Hoxhaj G, Selvi O, Ozell DE, et al. MENA is a transcriptional target of the Wnt/beta-catenin pathway. PLoS One. 2012; 7: e37013. doi: 10.1371/journal.pone.0037013 PMID: 22615875
48. Yamashita T, Budhu A, Forgues M, Wang XW. Activation of hepatic stem cell marker EpCAM by Wnt-beta-catenin signaling in hepatocellular carcinoma. Cancer Res. 2007; 67: 10831–9. doi: 10.1158/0008-5472.CAN-07-0908 PMID: 18006828
49. Soloran G, Porta-de-la-Riva M, Agustí C, Casagoida D, Sánchez-Aguilera F, Larriba MJ, et al. E-cadherin controls beta-catenin and NF-kappaB transcriptional activity in mesenchymal gene expression. J Cell Sci. 2008; 121: 2224–34. doi: 10.1242/jcs.021667 PMID: 18565826
50. Saegusa M, Hashimura M, Kuwata T, Hamano M, Okayasu I. Induction of p16INK4A mediated by beta-catenin in a TCF4-independent manner: implications for alterations in p16INK4A and pRb expression during trans-differentiation of endometrial carcinoma cells. Int J Cancer. 2006; 119: 2294–303. doi: 10.1002/ijc.22112 PMID: 16858682
51. Xu L, Corcoran RB, Welsh JW, Pennica D, Levine AJ. WISP-1 is a Wnt-1- and beta-catenin-responsive gene. Genes Dev. 2000; 14: 585–95. PMID: 10716946
52. Lepourcetel M, Chen Y-NP, France DS, Wang H, Crews P, Petersen F, et al. Small-molecule antagonists of the oncogenic Tcf/beta-catenin protein complex. Cancer Cell. 2004; 5: 91–102. PMID: 14749129
53. Bottomly D, Kyler SL, McWeeney SK, Yochum GS. Identification of (beta)-catenin binding regions in colon cancer cells using ChIP-Seq. Nucleic Acids Res. 2010; 38: 5735–45. doi: 10.1093/nar/gkq363 PMID: 20460455
54. Farré D, Roset R, Huerta M, Aduaure JE, Roselló L, Albà MM, et al. Identification of patterns in biological sequences at the ALGGEN server: PROMO and MALGEN. Nucleic Acids Res. 2003; 31: 3651–3. PMID: 12824386
55. Messeguer X, Escudero R, Farré D, Núñez O, Martínez J, Albà MM. PROMO: detection of known transcription regulatory elements using species-tailored searches. Bioinformatics. 2002; 18: 333–4. PMID: 11847087
56. Ma L, Wei W, Chua M-S, So S. WNT / beta-catenin pathway activation in hepatocellular carcinoma: a clinical perspective. Gastrointest Cancer Targets Ther. 2014; 49–63.
57. Rowan AJ, Llamul H, Ilyas M, Wheeler J, Straub J, Papadopoulou A, et al. APC mutations in sporadic colorectal tumors: A mutational “hotspot” and interdependence of the “two hits”. Proc Natl Acad Sci U S A. 2000; 97: 3352–7. PMID: 10737795
58. Jones S, Chen W-D, Parmigiani G, Diehl F, Beerenwinkel N, Antal T, et al. Comparative lesion sequencing provides insights into tumor evolution. Proc Natl Acad Sci U S A. 2008; 105: 4283–8. doi: 10.1073/pnas.0712345105 PMID: 18337506
59. Weiner ID, Verlander JW. Renal and hepatic expression of the ammonium transporter proteins, Rh B Glycoprotein and Rh C Glycoprotein. Acta Physiologica Scandinavica. 2003. pp. 331–338. doi: 10.1046/j.1600-6772.2003.01210.x PMID: 14656370
60. Mavri-Damelin D, Eaton S, Damelin LH, Rees M, Hodgson HJF, Selden C. Ornithine transcarbamylase and arginase I deficiency are responsible for diminished urea cycle function in the human hepatoblastoma cell line HepG2. Int J Biochem Cell Biol. 2007; 39: 555–64. doi: 10.1016/j.biocel.2006.10.007 PMID: 17098461
61. Iyer V V, Yang H, Ierapetritou MG, Roth CM. Effects of glucose and insulin on HepG2-C3A cell metabolism. Biotechnol Bioeng. 2010; 107: 347–56. doi: 10.1002/bit.22799 PMID: 20506178
62. Wise DR, Thompson CB. Glutamine addiction: a new therapeutic target in cancer. Trends Biochem Sci. 2010; 35: 427–33. doi: 10.1016/j.tibs.2010.05.003 PMID: 20570523
63. Zidi-Yahiaoui N, Mouro-Chanteloup I, D’Ambrosio A-M, Lopez C, Gane P, Le van Kim C, et al. Human Rhesus B and Rhesus C glycoproteins: properties of facilitated ammonium transport in recombinant kidney cells. Biochem J. 2005; 391: 33–40. doi: 10.1042/BJ20050657 PMID: 15929723
64. Javelle A, Thomas G, Marin A-M, Krämer R, Merrick M. In vivo functional characterization of the Escherichia coli ammonium channel AmtB: evidence for metabolic coupling of AmtB to glutamine synthetase. Biochem J. 2005; 390: 215–22. doi: 10.1042/BJ20042094 PMID: 15876187
65. Van Straten HWM, He Y, van Duist MM, Labruyère WT, Vermeulen JLM, van Dijk PJ, et al. Cellular concentrations of glutamine synthetase in murine organs. Biochem Cell Biol. 2006; 84: 215–31. doi: 10.1139/o05-170 PMID: 16609703
66. Lee H-W, Verlander JW, Handlogten ME, Han K-H, Cooke PS, Weiner ID. Expression of the rhesus glycoproteins, ammonia transporter family members, RHCG and RHBG in male reproductive organs. Reproduction. 2013; 146: 283–96. doi:10.1530/REP-13-0154 PMID: 23904565

67. Eng CH, Yu K, Lucas J, White E, Abraham RT. Ammonia derived from glutaminolysis is a diffusible regulator of autophagy. Sci Signal. 2010; 3: ra31. doi: 10.1126/scisignal.2000911 PMID: 20424262

68. Harder LM, Bunkenborg J, Andersen JS. A comparative phosphoproteomic study of the cellular response to ammonia and rapamycin Inducing autophagy. 2014; 1–17. doi:10.4161/auto.26863 PMID: 24300666