



HAL
open science

Autophagy determines efficiency of liver-directed gene therapy with adeno-associated viral vectors

M. Hosel, A. Huber, S. Bohlen, J. Lucifora, G. Ronzitti, F. Puzzo, F. Boisgerault, U. T. Hacker, W. J. Kwanten, N. Kloting, et al.

► To cite this version:

M. Hosel, A. Huber, S. Bohlen, J. Lucifora, G. Ronzitti, et al.. Autophagy determines efficiency of liver-directed gene therapy with adeno-associated viral vectors. *Hepatology*, 2017, 66 (1), pp.252-265. 10.1002/hep.29176 . hal-02880988

HAL Id: hal-02880988


<https://hal.science/hal-02880988v1>

Submitted on 5 Dec 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Autophagy Determines Efficiency of Liver-Directed Gene Therapy With Adeno-Associated Viral Vectors

Marianna Hösel,^{1,2} Anke Huber,^{1,2} Susanne Bohlen,³ Julie Lucifora,⁴ Giuseppe Ronzitti,⁵ Francesco Puzzo,⁵ Florence Boisgerault,⁵ Ulrich T. Hacker,⁶ Wilhelmus J. Kwanten ,⁷ Nora Klötting,⁸ Matthias Blüher,⁹ Alexander Gluschko,^{1,3} Michael Schramm,^{1,3} Olaf Utermöhlen,^{1,3} Wilhelm Bloch,¹⁰ Federico Mingozzi,^{5,11*} Oleg Krut,^{3*} and Hildegard Büning^{1,2,12*}

Use of adeno-associated viral (AAV) vectors for liver-directed gene therapy has shown considerable success, particularly in patients with severe hemophilia B. However, the high vector doses required to reach therapeutic levels of transgene expression caused liver inflammation in some patients that selectively destroyed transduced hepatocytes. We hypothesized that such detrimental immune responses can be avoided by enhancing the efficacy of AAV vectors in hepatocytes. Because autophagy is a key liver response to environmental stresses, we characterized the impact of hepatic autophagy on AAV infection. We found that AAV induced mammalian target of rapamycin (mTOR)-dependent autophagy in human hepatocytes. This cell response was critically required for efficient transduction because under conditions of impaired autophagy (pharmacological inhibition, small interfering RNA knock-down of autophagic proteins, or suppression by food intake), recombinant AAV-mediated transgene expression was markedly reduced, both *in vitro* and *in vivo*. Taking advantage of this dependence, we employed pharmacological inducers of autophagy to increase the level of autophagy. This resulted in greatly improved transduction efficiency of AAV vectors in human and mouse hepatocytes independent of the transgene, driving promoter, or AAV serotype and was subsequently confirmed *in vivo*. Specifically, short-term treatment with a single dose of torin 1 significantly increased vector-mediated hepatic expression of erythropoietin in C57BL/6 mice. Similarly, coadministration of rapamycin with AAV vectors resulted in markedly enhanced expression of human acid- α -glucosidase in nonhuman primates. **Conclusion:** We identified autophagy as a pivotal cell response determining the efficiency of AAVs intracellular processing in hepatocytes and thus the outcome of liver-directed gene therapy using AAV vectors and showed in a proof-of-principle study how this virus-host interaction can be employed to enhance efficacy of this vector system. (HEPATOLOGY 2017;66:252-265)

The liver is a main target in human gene therapy. It holds about one third of the total blood volume, enabling efficient distribution of proteins secreted by vector-transduced hepatocytes. In addition, more than 100 liver-related single-gene defects have been described that are potential candidates for a gene therapeutic intervention.⁽¹⁾

Recombinant adeno-associated virus (rAAV) vectors are considered as delivery vehicle of choice for liver-directed gene therapy due to their strong natural tropism for this organ and excellent safety profile.⁽²⁾ They are derived from adeno-associated viruses (AAVs), replication-deficient parvoviruses with a single-stranded DNA genome (4.7 kb) and a nonenveloped

Abbreviations: AAV, adeno-associated virus; Atg, autophagy-related gene; CD, cluster of differentiation; CMV, cytomegalovirus; CQ, chloroquine; DMSO, dimethyl sulfoxide; 4EBP, 4E binding protein; Epo, erythropoietin; GOI, genomic particle per cell ratio; hGAA, human acid- α -glucosidase; LC3, microtubule-associated protein light-chain 3; LSP, liver-specific promoter; 3-MA, 3-methyladenine; mTOR, mammalian target of rapamycin; NHP, nonhuman primate; p70S6K, p70 ribosomal S6 kinase; PHH, primary human hepatocyte; PI3KC3, class III phosphatidylinositol 3 kinase; PIK3R4, phosphoinositide-3-kinase regulatory subunit 4; rAAV, recombinant AAV; siRNA, small interfering RNA; TTR, transthyretin; ULK1/2, Unc-51-like kinase 1 and 2; UVRAG, ultraviolet radiation resistance-associated gene; *vg*, vector genome; *wt*, wild-type.

Received November 8, 2016; accepted March 16, 2017.

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.29176/supinfo.

*These authors share senior authorship.

Supported by the Collaborative Research Center 670 "Cell-Autonomous Immunity" of the German Research Foundation (TP8, to H.B.; TP5, to O.K.; and TP4 to O.U.), by the Center for Molecular Medicine Cologne (CI, to H.B.), by Köln Fortune (to O.K.), by CRC1052/2 (B01 to M.B. and B04 to N.K.), and by the European Research Council (617432, to F.M.).

protein capsid (~20 nm in diameter) that triggers cell infection and defines AAV postentry processing.⁽³⁾

Among gene therapy clinical trials for liver-related diseases, those for hemophilia B show the most promising results,^(2,4-6) with long-term transgene expression of factor IX from rAAV-transduced hepatocytes leading to restored blood coagulation. The high vector doses required to reach therapeutic expression levels, however, pose a continuous challenge.⁽³⁾ Besides making rAAV manufacturing elaborate and expensive, application of high vector doses carries the risk of inducing immune responses, limiting long-term transgene expression.⁽⁷⁾ Specifically, capsid-specific cluster of differentiation (CD) 8⁺ T cells eliminated rAAV-transduced hepatocytes in a high-dose cohort (2×10^{12} vector genomes [vgs]/kg of body weight), while this response was not observed in patients treated with lower doses of rAAV.⁽⁷⁾ In line, we recently reported on innate immune responses elicited by the viral capsid in nonparenchymal liver cells that strictly correlated with vector dose.⁽⁸⁾

Thus, liver-directed gene therapy will benefit from strategies allowing high therapeutic expression levels at lower vector doses. Our strategy to tackle this challenge is in-depth characterization of the AAV–hepatocyte interaction as efficiency of viral transduction depends on the availability of largely unknown host factors and is influenced by promoting or impeding cell responses.

A cell response of utmost importance for the liver is autophagy (Greek: “self-eating”) by which the liver removes and recycles excessive levels of damaged organelles and protein aggregates that have accumulated over time.⁽⁹⁻¹¹⁾ In addition, autophagy functions as a regulator of hepatic metabolism and contributes to innate immune responses.^(9,12) The liver has a basal level of autophagic activity that becomes significantly increased in response to stress, e.g., starvation, toxic injuries, deprivation of growth factors, or infection. This response is mainly controlled by mammalian target of rapamycin (mTOR). Specifically, inhibition of mTOR in response to stress results in a signal cascade that employs the Unc-51-like kinase 1 and 2 (ULK1/2) complex and subsequently activates the class III phosphatidylinositol 3 kinase (PI3KC3) complex. The PI3KC3 complex produces phosphatidylinositol 3-phosphate, which then functions as a signaling molecule for initiating nucleation and expansion of the phagophore, the double-membrane autophagosome precursor. Elongation and closure of the phagophore to the autophagosome, a process that requires lipidated microtubule-associated protein light chain 3 (LC3), results in sequestering cytoplasmic material to be disposed. Finally, autophagosomes fuse with late endosomes and lysosomes, creating autophagolysosomes for degradation and recycling of their contents and their membranes.^(10,12)

Copyright © 2017 The Authors. HEPATOLOGY published by Wiley Periodicals, Inc., on behalf of the American Association for the Study of Liver Diseases. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

View this article online at wileyonlinelibrary.com.

DOI 10.1002/hep.29176

Potential conflict of interest: Dr. Ronzitti received grants from Sanofi Genzyme. Dr. Mingozzi consults and received grants from Selecta Bioscience. He received grants from Bayer, Sanofi Genzyme, and BioMarin.

ARTICLE INFORMATION:

From the ¹Center for Molecular Medicine Cologne (CMMC), University of Cologne, Cologne, Germany; ²German Center for Infection Research (DZIF), Partner sites Bonn-Cologne and Hannover-Braunschweig, Germany; ³Institute for Medical Microbiology, Immunology and Hygiene, University of Cologne, Cologne, Germany; ⁴INSERM, U1052, Cancer Research Center of Lyon (CRCL), University of Lyon, Lyon, France; ⁵Genethon and INSERM U951, Evry, France; ⁶University Medicine Leipzig, University Cancer Center Leipzig (UCCL), Leipzig, Germany; ⁷Laboratory of Experimental Medicine and Pediatrics (LEMP), University of Antwerp, Antwerp, Belgium; ⁸IFB Adiposity Diseases and ⁹Department of Medicine, University of Leipzig, Leipzig, Germany; ¹⁰Department of Molecular and Cellular Sport Medicine, German Sport University Cologne, Cologne, Germany; ¹¹University Pierre and Marie Curie, Paris, France; ¹²Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany.

ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO:

Prof. Dr. Hildegard Büning
Laboratory for Infection Biology & Gene Transfer,
Institute of Experimental Hematology
Hannover Medical School

Carl-Neuberg-Str. 1
30625 Hannover, Germany
E-mail: buening.hildegard@mh-hannover.de
Tel: + 49-511-532-5106

Given the key role of autophagy in maintaining liver homeostasis, we were interested in determining whether this cell response impacts on rAAV-mediated transduction of hepatocytes. Here, we report that both wild type (wt) AAV2 and rAAV of different serotypes induce autophagy in human hepatocytes and that rAAV requires this cell response for efficient transduction as inhibition of autophagy by drugs or small interfering RNAs (siRNAs) or *in vivo* gene transfer in conditions of impaired autophagy resulted in significantly decreased levels of transgene expression. The latter finding reveals autophagy as a critical, patient-related parameter that determines the outcome of liver-directed human gene therapy. On the other hand, autophagy also represents an easy manipulation of the target for improving the efficacy of the vector system because drugs increasing the level of autophagy are already in clinical use. For the latter, we provide a proof-of-concept in mice and in nonhuman primates (NHPs).

Materials and Methods

REAGENTS

3-Methyladenine (3-MA) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO), rapamycin was obtained from LC Laboratories (Woburn, MA), and torin 1 was from Selleckchem (Houston, TX).

AUTOPHAGY ASSAYS

Autophagy was determined either by quantifying the LC3-II/actin ratio following western blotting ([Supporting Information](#)) or using the Cyto-ID Autophagy detection kit (Enzo Life Sciences, Farmingdale, NY) according to the manufacturer's instructions. Briefly, HepG2 cells were treated with wtAAV2 or drugs for specific time periods. After trypsinization and washing, 5×10^5 cells were resuspended in $1 \times$ assay buffer and incubated with the Cyto-ID dye at 37°C for 30 minutes in the dark, followed by flow-cytometric analysis. For fluorescence microscopy, cells were grown on coverslips, treated with wtAAV2 or drugs for specific time periods, washed, and incubated with the dual detection reagent (Cyto-ID dye and Hoechst 33342 nuclear stain) at 37°C for 30 minutes in the dark. After washing, cells were fixed in 4% paraformaldehyde and analyzed by fluorescence microscopy using the Olympus IX81 equipped with Cell Imaging

software (Tokyo, Japan). Images were analyzed by CellProfiler 2.4.0rc1 (rev29342ff). Mean fluorescence intensities of Cyto-ID per cell were quantified using at least 100 cells from each individual experiment. The autophagy activity factor was calculated in both flow-cytometric and fluorescence microscopic analyses as described in the [Supporting Information](#).

ANIMAL EXPERIMENTS

Autophagic stimulation in mice using torin 1 was performed according to the established protocol.⁽¹³⁾ Female mice (C57BL/6) between 8 and 10 weeks of age (Charles River, Boston, MA) were treated with vehicle solution or with torin 1 (20 mg/kg) by intraperitoneal injection. After 2 hours, 2×10^{10} vgs of rAAV8-TTR-Epo vector encoding the mouse erythropoietin (*Epo*) gene under the control of the liver-specific transthyretin promoter (TTR) was administered by tail vein injection (intravenously). Suppression of liver autophagy in mice was achieved through the starvation-refeeding protocol.⁽¹⁴⁾ Ten-week-old female C57BL/6 mice were starved for 24 hours and then refed (or not) for 1 hour before injection with 1×10^{10} vgs of rAAV8-TTR-Epo. After 4 hours postinfection, both groups of mice (refed and starved) were normally fed. In a genetic model, *ob/ob* homozygote and *ob/+* heterozygote (B6.V-Lep^{ob/ob} and B6.V-Lep^{ob/J}) 12-week-old female mice, obtained from Taconic Europe (Ry, Denmark), were injected (intravenously) with 2.5×10^{10} vgs of rAAV8-TTR-Epo. In all mouse models, levels of Epo were determined in serum samples collected at indicated time points by the Mouse Erythropoietin ELISA (R&D Systems, Minneapolis, MN).

For the NHP experiment, male *Macaca fascicularis* animals aged around 2 years were prescreened for the presence of anti-AAV8 neutralizing antibodies as described,⁽¹⁵⁾ and three animals were included in the study. They received 2×10^{12} vgs/kg of rAAV8-LSP-hGAA vector encoding the human acid- α -glucosidase (hGAA) under the control of the liver-specific (LSP) promoter intravenously. Prior to vector application, animals received rapamycin (3 mg/kg; $n = 2$) or vehicle solution ($n = 1$). The infusion procedure was done under sedation. hGAA was detected by western blot analysis of undiluted plasma samples.

All mouse studies were performed in accordance with the German and French animal protection law as approved by local government authorities. The NHP experiment was performed at the *in vivo* platform

Oniris (Nantes, France). Protocol APAFIS 2651 was approved by the local ethics committee (Boisbonne Center) and the Ministère de l'Éducation nationale, de l'Enseignement supérieur et de la Recherche.

STATISTICAL ANALYSIS

In cell culture experiments, statistical significance was determined by the Student unpaired two-tailed *t* test. Differences between experimental groups in mouse experiments were assessed using two-way analysis of variance with *post hoc* Sidak correction when appropriate. $P < 0.05$ was considered statistically significant.

Results

AAV INDUCES AUTOPHAGOSOME FORMATION IN HUMAN HEPATIC CELLS

To investigate whether autophagy is triggered by AAV infection in human liver cells, we infected human HepG2 cells and primary human hepatocytes (PHHs) with wtAAV2 or with rAAV and determined accumulation of LC3-II as a marker for autophagosome formation.⁽¹⁶⁾ Western blot analyses revealed a dose-dependent increase in LC3-II levels for wtAAV and for rAAV of different serotypes (Fig. 1A-C). Levels were comparable to those measured in cells treated with rapamycin, a pharmacological inducer of autophagy that acts by inhibiting mTOR activity.⁽¹⁷⁾

We further examined induction of autophagosome formation indicated as double-membrane vesicles by transmission electron microscopy. Double-membrane vesicles were rarely observed in mock-infected cells (data not shown) but were frequently detected upon AAV infection. Moreover, these vesicles contained particles resembling wtAAV2 in size and structure (Fig. 1D; Supporting Fig. S1). Such particles were not observed in mock-infected cells (Supporting Fig. S1).

To distinguish between AAV-mediated induction of autophagy and blockage of lysosomal degradation as the cause of the increased LC3-II levels, we treated AAV-infected PHHs and HepG2 cells (Fig. 2A,B) with chloroquine (CQ). CQ is a lysosomal inhibitor which increases the vacuolar pH, thereby preventing the formation and degradation of autophagolysosomes and causing accumulation of autophagosomes.^(18,19) Treatment with rapamycin and torin 1 was the positive control. Torin 1 also inhibits mTOR but induces

autophagy to a higher level than rapamycin.⁽²⁰⁾ Accumulation of LC3-II was markedly increased upon AAV and CQ cotreatment in comparison to treatment with either AAV or CQ alone, indicating that the increase in LC3-II level following wtAAV2 or rAAV infection represents an induction of autophagic flux (Fig. 2A,B).

Next, autophagic flux was quantified by flow cytometry and fluorescence microscopy using a recently established method, the Cyto-ID Autophagy detection reagent.^(19,21) The Cyto-ID green dye specifically labels autophagic compartments (autophagosomes, amphisomes, and autolysosomes) with minimal staining of lysosomes and endosomes.⁽¹⁹⁾ Consistent with the LC3-II western blotting data (Fig. 2A,B), the Cyto-ID fluorescent signal was significantly higher after wtAAV2 and CQ cotreatment in comparison to either wtAAV or CQ treatment alone (Fig. 2C,D). The values of the autophagy activity factor using mean fluorescence intensity from flow cytometry and fluorescence microscopy revealed a comparable increase in autophagy level (Supporting Table S2).

Because suppression of mTOR is one of the central pathways leading to induction of autophagy, we next examined the activity of mTOR and measured the phosphorylation status of two mTOR-specific substrates, p70 ribosomal S6 kinase (p70S6K) and eukaryotic translation initiation factor 4E binding protein 1 (p4EBP1).⁽²²⁾ In PHHs (Fig. 2E) and in HepG2 cells (Fig. 2F) infected with wtAAV2, levels of phosphorylated p70S6K and 4EBP were markedly reduced compared to noninfected cells. Thus, in AAV-infected cells mTOR activity becomes inhibited.

Taken together, the results indicate that infection with wtAAV2 as well as transduction with rAAV induce autophagy in human hepatocytes through inhibition of mTOR.

INHIBITION OF AUTOPHAGY SUPPRESSES rAAV TRANSDUCTION IN HUMAN HEPATOCYTES

Because autophagy was reported to be detrimental to some viruses but beneficial for others,^(23,24) we next assessed the consequences of impaired autophagy on rAAV transduction. To accomplish this, we used the most widely applied pharmacological inhibitor of autophagy, 3-MA, or performed siRNA-mediated knockdown of essential components of the two autophagy initiation complexes, ULK1/2 and

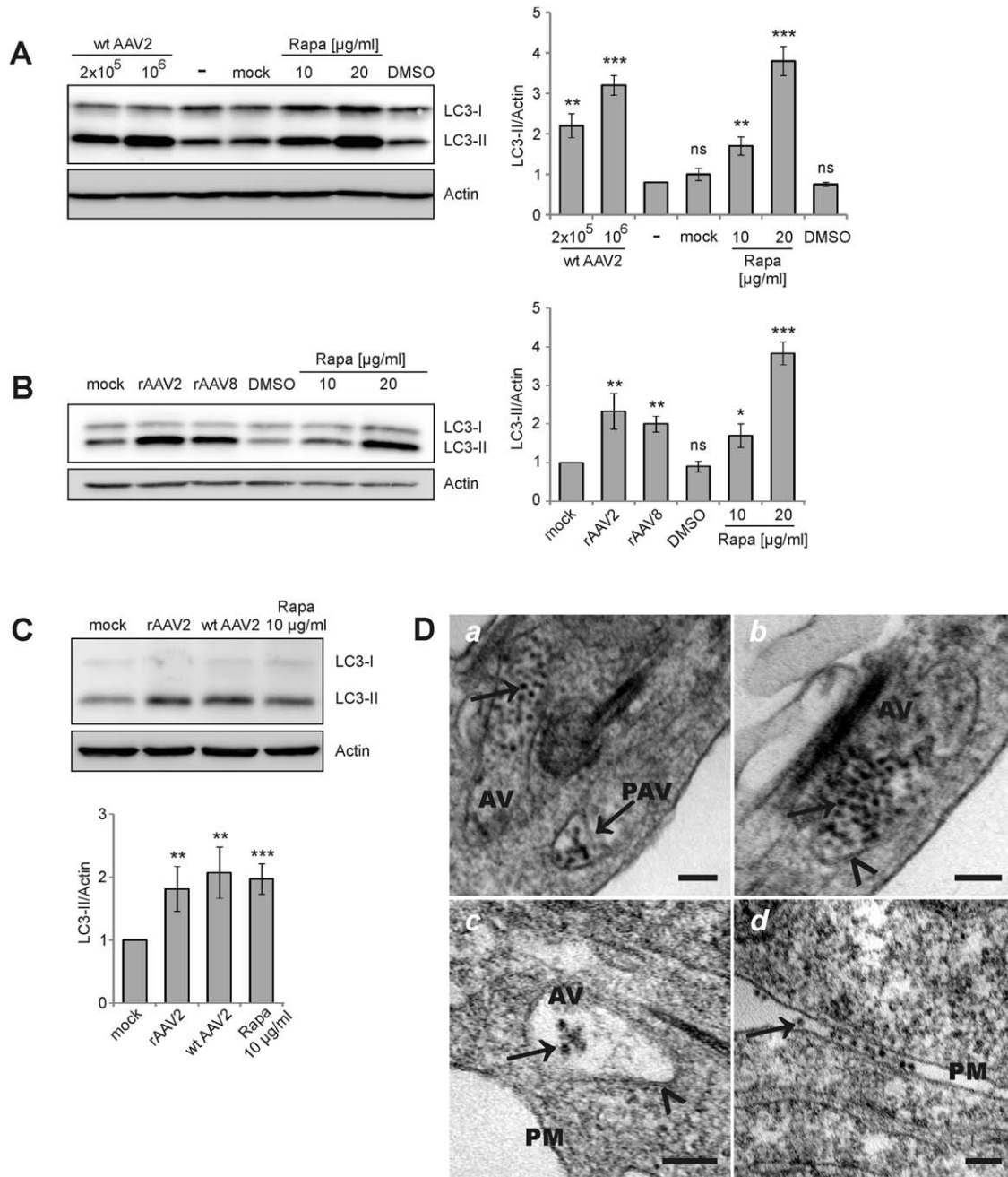


FIG. 1. AAV induces autophagosome formation in human hepatocytes. Representative immunoblottings and densitometric quantifications of LC3-II/actin ratio in HepG2 cells (A,B) and in PHHs (C) infected with wtAAV2 (A, genomic particles per cell [GOI] are indicated), with rAAV2-CMV-luc or rAAV8-CMV-luc (B, both at a GOI of 1×10^5), with wtAAV2 or rAAV2-CMV-eGFP (C, both at a GOI of 1×10^5) for 24 hours or after treatment with rapamycin at the indicated dose. Untreated cells (-, set to 1 in A), cells infected with the “mock” inoculum (set to 1 in B and C), and DMSO-treated cells were used as negative controls. Values are shown as mean \pm standard deviation ($n = 3$; $***P < 0.001$, $**P < 0.01$, $*P < 0.05$). (D) Transmission electron microscopic analysis of HepG2 cells infected with wtAAV2 (GOI of 1×10^5) at 3 hours p.i. Images a-c show preautophagic and autophagic vesicles that have engulfed several particles of the size and shape of AAV virions (dark arrows). Image d shows the particles in the extracellular space between cells. Dark arrowheads point to double-membrane of autophagic vesicles. Scale bars, 100 nm. Abbreviations: AV, autophagic vesicle; ns, not significant; PAV, preautophagic vesicle; p.i., postinfection; PM, plasma membrane; Rapa, rapamycin.

PI3KC3, followed by rAAV transduction. The vector rAAV2-CMV-luc used for this purpose encoded Renilla luciferase as transgene under control of the

cytomegalovirus (CMV) promoter, allowing us to measure the efficiency of cell transduction by luciferase assay.

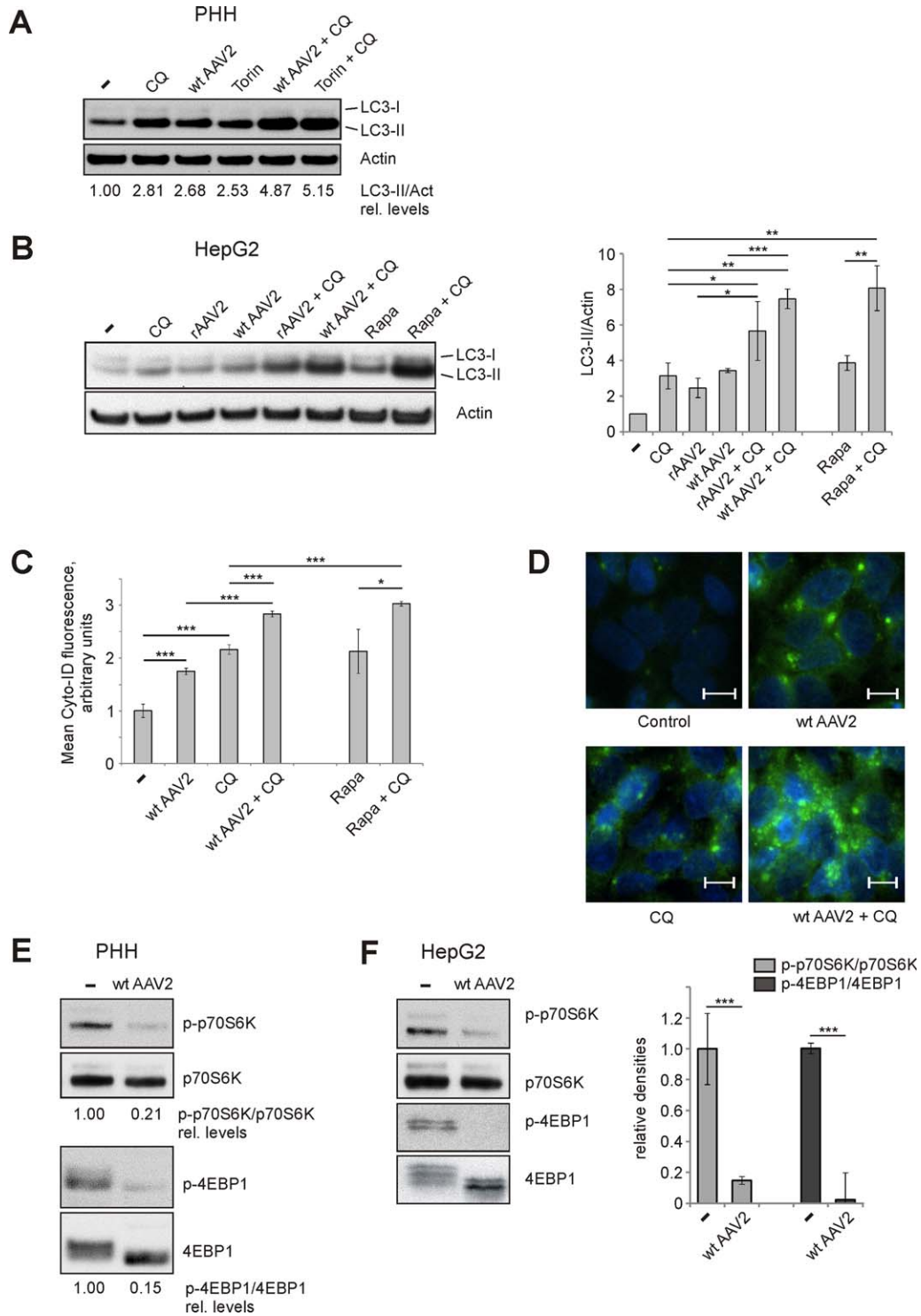


FIG. 2

Pretreatment of PHHs with 3-MA significantly reduced rAAV-mediated luciferase expression (Fig. 3A). Comparable results were obtained by siRNA-mediated knockdown that targeted phosphoinositide-3-kinase regulatory subunit 4 (PIK3R4), autophagy-related gene 13 (Atg13), Atg14, or ultraviolet radiation resistance-associated gene (UVRAG). Also in these cases, we found that luciferase activity was significantly reduced (Fig. 3B,C; Supporting Fig. S2). This indicates that autophagy is required for efficient rAAV-mediated transduction of hepatocytes.

INDUCTION OF AUTOPHAGY ENHANCES rAAV TRANSDUCTION OF LIVER CELLS

Because inhibition of autophagy resulted in strongly reduced rAAV-mediated transgene expression, we next examined the possibility of enhancing the efficacy of rAAV vectors by increasing the level of autophagy prior to rAAV transduction. HepG2 cells were therefore pretreated with pharmacological inducers of autophagy. In addition to rapamycin (Fig. 4), torin 1 was tested (Fig. 5). Both drugs increased the level of autophagy in HepG2 cells in a dose-dependent manner (Figs. 4A and 5A), with a positive correlation between the level of autophagy and the level of transgene expression (Figs. 4A,B and 5A-C). Pretreatment with torin 1 also significantly increased levels of transgene expression in mouse hepatocytes (Fig. 5D), in a set of further human hepatic cell lines (Supporting Fig. S3), in differentiated HepaRG cells (Fig. 5E), and in PHHs (Fig. 5F). Of further note, the promoting effect was observed independent of the serotype, the promoter, the transgene, or the vector genome conformation (Figs. 4C,D and 5B-F).

In contrast to our results, a recent study by Berry and Asokan⁽²⁵⁾ reported that neither the autophagy inducer nicardipine nor the autophagy inhibitor spautin-1 altered rAAV transduction in HeLa cells,

which are commonly used for the study of AAV infection biology. To explore the cell type-specific requirement for autophagy, we repeated our experiments with HeLa cells. In contrast to liver cells (Figs. 4 and 5) but confirming Berry and Asokan, torin 1 and rapamycin both failed to alter rAAV-mediated transgene expression in HeLa cells (Supporting Fig. S4A). Furthermore, although treatment with rapamycin led to increased accumulation of LC3-II, HeLa cells did not respond by autophagy induction when transduced with rAAV (Supporting Fig. S4B).

INDUCTION OF AUTOPHAGY IMPROVES INTRACELLULAR PROCESSING OF rAAV

To determine at which step(s) drug-induced autophagy improves AAV-hepatocyte interaction, we infected DMSO-pretreated or torin 1-pretreated HepG2 cells with rAAV2-CMV-luc and determined levels of total intracellular and intranuclear rAAV DNA as well as luciferase mRNA and protein expression. While the quantity of total intracellular rAAV vector genomes was not altered by autophagic stimulation, numbers of intranuclear genomes as well as levels of transgene expression and luciferase activity were increased by approximately 2-fold, 12-fold, and 14-fold, respectively (Fig. 6). Thus, pharmacological induction of autophagy enhances intracellular processing of rAAV.

LEVEL OF AUTOPHAGY DETERMINES EFFICIENCY OF LIVER-DIRECTED rAAV-MEDIATED TRANSGENE EXPRESSION *IN VIVO*

Given the dependence of rAAV on autophagy for efficient transduction of hepatocytes *in vitro* (Fig. 3)

FIG. 2. AAV induces autophagic flux and suppresses mTOR in human hepatocytes. (A,B) PHHs (A) and HepG2 cells (B) were infected with wtAAV2 or rAAV2-CMV-GFP (both at a GOI of 2×10^5) in the absence or presence of CQ (8 μ M) for 24 hours and subjected to immunoblot assay for LC3-II. For HepG2 cells, a representative blot out of three is shown. Noninfected cells (-), torin 1 (5 μ M), and rapamycin (10 μ g/mL) were used as controls. (A) LC3-II/actin ratios are indicated. (B, right panel) Densitometric quantification of LC3-II/actin ratios in HepG2 cells. Values are shown as mean \pm standard deviation ($n = 3$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$). (C,D) HepG2 cells were infected with wtAAV2 (GOI of 1×10^5) followed (or not) by treatment with CQ (8 μ M) for 4 hours (C) or for 16 hours (D) before labeling with Cyto-ID dye. Controls: noninfected cells (-), rapamycin (15 μ g/L). (C) Autophagic flux was assessed by flow cytometry. Values are shown as mean Cyto-ID green fluorescence intensities of treated cells \pm standard deviation relative to control ($n = 3$, *** $P < 0.001$, * $P < 0.05$). (D) Autophagic flux was analyzed by fluorescence microscopy. Nuclei were stained by Hoechst 33342 dye. Scale bars, 10 μ m. (E,F) Western blot analyses and relative densities for p-p70S6K/p70S6K and p4EBP1/4EBP1 in PHHs (E) or HepG2 cells (F) infected for 3 hours with wtAAV2 (GOI of 2×10^5). Protein levels in noninfected control (-) were set to 1. For HepG2, a representative blot out of three is shown. Densitometric data are expressed as mean \pm standard deviation ($n = 4$, *** $P < 0.001$). Abbreviation: Rapa, rapamycin.

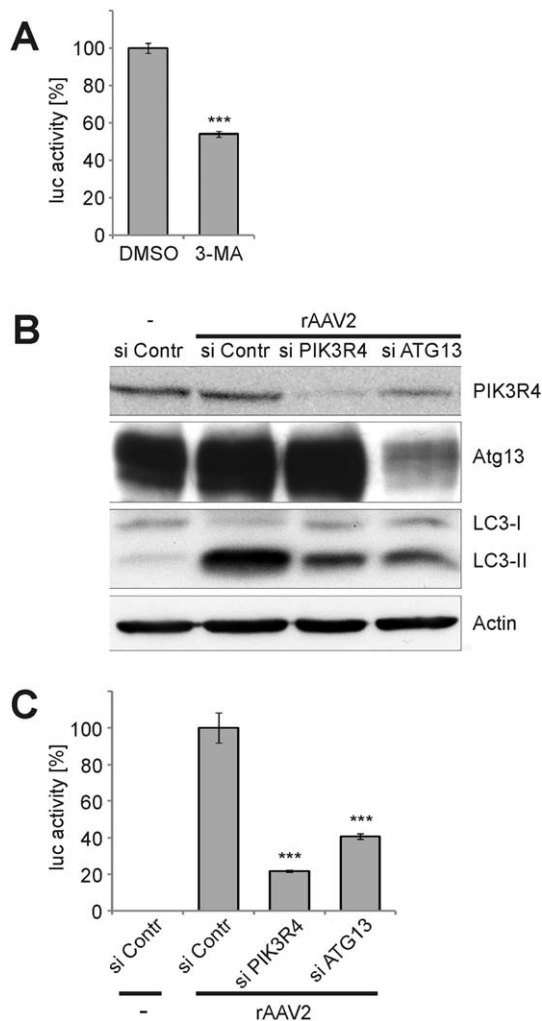


FIG. 3. Autophagy is required for efficient rAAV transduction of human hepatocytes. (A) PHHs were treated with DMSO or 5 mM of 3-MA for 1 hour, followed by infection with rAAV2-CMV-luc (GOI of 1×10^5). Luciferase activity was measured at 24 hours p.i. (DMSO-control, 100%). (B) HepG2 cells were transfected with control siRNA, with PIK3R4 siRNA or with ATG13 siRNA. At 48 hours after transfection, cells were infected with rAAV2-CMV-luc (GOI of 1×10^5) or left noninfected (-). Levels of PIK3R4, Atg13, LC3-II, and β -actin (loading control) were determined by immunoblotting 24 hours p.i. (C) Luciferase activity in knockdown cells infected with rAAV2-CMV-luc versus cells transfected with control siRNA and infected with rAAV2-CMV-luc (100%) was determined at 24 hours p.i. Noninfected cells transfected with control siRNA were used as negative controls. (A,C) Values are shown as mean \pm standard deviation ($n = 3$; *** $P < 0.001$). Abbreviation: p.i., postinfection.

and the positive correlation between the level of hepatic autophagy and rAAV-mediated transgene expression (Figs. 4 and 5), we hypothesized that autophagy may also represent a critical parameter determining the outcome of liver-directed gene therapy *in vivo*.

To assess this possibility, we first investigated whether a single, short-term induction of autophagy would be sufficient to enhance rAAV transduction in a healthy liver. C57BL/6 mice were pretreated with torin 1 for 2 hours, followed by injection of rAAV8 encoding the mouse *Epo* gene under the control of the liver-specific TTR promoter (rAAV8-TTR-Epo). This short autophagic stimulation was sufficient to increase serum levels of Epo up to 5-fold in comparison to animals that received vehicle prior to rAAV8-TTR-Epo (Fig. 7A).

Next, we assessed, whether under conditions of impaired autophagy, rAAV transduction efficiency is reduced. To this end, we employed a starvation-refeeding protocol developed by Mizushima and co-workers, which allows rapid suppression of liver autophagy in mice by food intake.⁽¹⁴⁾ C57BL/6 mice were starved for 24 hours and then refed for 1 hour, followed by intravenous injection of rAAV8-TTR-Epo. A starved group (control) was also injected with rAAV8-TTR-Epo but not refed. In agreement with published data,⁽¹⁴⁾ the amount of LC3-II was decreased in the liver after a 1-hour refeeding period (Fig. 7 B), confirming suppression of autophagy. Consistent with the markedly reduced luciferase expression level observed in liver cells under conditions of impaired autophagy (Fig. 3), refed mice showed significantly lower (~ 4 -fold) Epo levels in the serum compared to control mice (Fig. 7B).

In addition, we compared the transduction efficiency of rAAV8-TTR-Epo in leptin-deficient (*ob/ob*) mice, which have reduced hepatic autophagy as a consequence of a genetic defect (Supporting Fig. S5),^(26,27) with that in heterozygous (*ob/+*) mice, which mount normal autophagic activity. Again, under conditions of impaired autophagy (*ob/ob* mice), rAAV-mediated transgene expression was significantly reduced compared to control mice (Supporting Fig. S5).

To translate our findings to a clinically more relevant model, we assessed the effect of pharmacological autophagy induction in NHPs (*Macaca fascicularis*). We chose rapamycin, which induces autophagy in the liver,^(28,29) because this drug is already in clinical use.^(30,31) One NHP was infused with vehicle solution, and two NHPs were infused with 3 mg/kg of rapamycin. Immediately after rapamycin (or vehicle) administration, all three NHPs received a single injection of an rAAV8 dose equivalent to that used in human clinical trials.⁽⁷⁾ This rAAV8 vector encoded for hGAA under the control of the LSP (rAAV8-LSP-hGAA).⁽³²⁾ Autophagy induction also greatly improved rAAV-

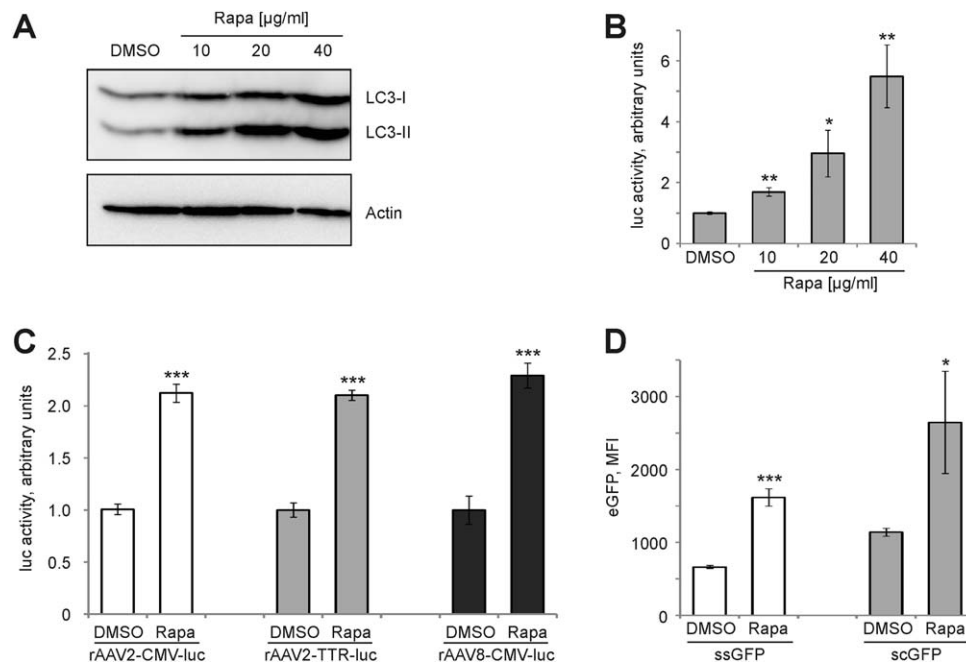


FIG. 4. Induction of autophagy by rapamycin enhances transduction efficiency of rAAV in human hepatic cells. (A,B) HepG2 cells were pretreated with DMSO or rapamycin for 1 hour, followed by infection with rAAV2-CMV-luc (GOI of 5×10^3). Accumulation of LC3-II (A) and luciferase activity (B) in rapamycin-treated versus DMSO-treated cells (set as 1) was determined at 24 hours p.i. (C,D) HepG2 cells were pretreated with DMSO or rapamycin (10 µg/mL) for 1 hour, followed by transduction (24 hours) with indicated rAAV encoding luciferase (C, all vectors at a GOI of 5×10^3) or enhanced green fluorescent protein (D, rAAV2-CMV-ssGFP and rAAV2-CMV-scGFP, both vectors at a GOI of 1×10^3). Luciferase activity and expression of eGFP (mean fluorescence intensity) were determined at 24 hours p.i. Values are shown as mean \pm standard deviation ($n = 3$; *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ relative to the corresponding DMSO-control). Abbreviations: eGFP, enhanced green fluorescent protein; MFI, mean fluorescence intensity; p.i., postinfection; Rapa, rapamycin; sc, self-complementary; ss, single stranded.

mediated liver transduction in NHPs (Fig. 7C). Specifically, monkeys that received rAAV8-LSP-hGAA together with rapamycin had more than 4-fold higher levels of transgene expression compared to the animal that received rAAV8-LSP-hGAA only. Overall, co-administration of rAAV8-LSP-hGAA together with rapamycin resulted in over 20-fold increased hGAA expression compared to the naive control (Fig. 7C).

Thus, our results show that the level of hepatic autophagy determines the efficiency of liver-directed rAAV-mediated gene transfer *in vivo*.

Discussion

In this study, we report that infection with wtAAV2 or transduction with rAAV induces autophagy in hepatocytes (Fig. 1). This cell response is centrally required for efficient transduction because under conditions of impaired autophagy (i.e., siRNA

knockdown, pharmacological inhibition, suppression by food intake), rAAV-mediated transgene expression was markedly reduced both *in vitro* and *in vivo* (Figs. 3 and 7B). Conversely, increasing the level of autophagy by short-term application of pharmacological autophagy inducers resulted in significantly improved transgene expression in mouse and human hepatocytes *in vitro* and in small and large animal models (Figs. 4–7).

Hepatocytes are in particular dependent on autophagy.⁽⁹⁾ As a long-lived cell type remaining in a quiescent state over prolonged periods of time, hepatocytes require autophagy to degrade damaged organelles and proteins. The constant basal level of hepatic autophagy is rapidly increased in response to a portfolio of environmental stressors to restore cellular homeostasis. For example, under starvation conditions, autophagy supplies hepatocytes with amino acids and substrates for energy production. In addition, autophagy contributes to hepatic glucose production by conversion of

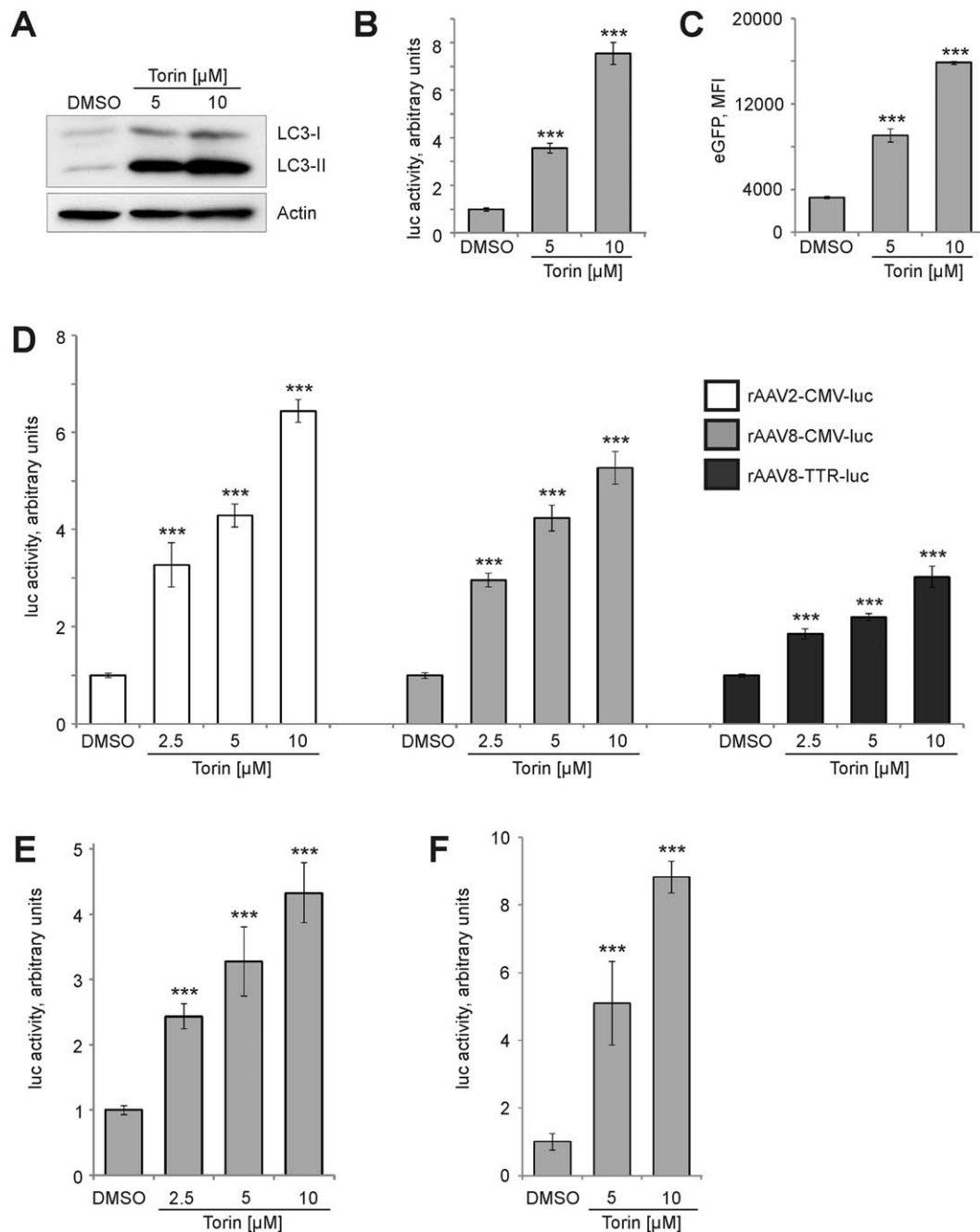


FIG. 5. Induction of autophagy by torin 1 promotes rAAV transduction in human and mouse hepatic cells. (A-C) HepG2 cells were pretreated with DMSO or torin 1 for 1 hour before infection with rAAV. Levels of LC3-II (A), luciferase activity (B, rAAV-CMV-luc, GOI of 1×10^3), and eGFP expression (C, rAAV2-CMV-sc GFP, GOI of 1×10^4) in torin 1-treated versus DMSO-treated cells were determined at 24 hours postinfection. (D) Luciferase activity in mouse hepatoma Hepa1-6 cells pretreated with DMSO or torin 1 for 1 hour before infection with rAAV2-CMV-luc (GOI of 1×10^3), with rAAV8-CMV-luc (GOI of 1×10^4), or with rAAV8-TTR-luc (GOI of 1×10^4) for 24 hours. (E,F) Luciferase activity in human HepaRG cells (E) or in PHHs (F) pretreated with DMSO or torin 1 for 1 hour before infection with rAAV2-CMV-luc (GOI of 1×10^3) for 24 hours. Values are shown as mean \pm standard deviation (B and C, $n = 3$; D-F, $n = 4$; *** $P < 0.001$ relative to control). Abbreviations: eGFP, enhanced green fluorescent protein; MFI, mean fluorescence intensity; sc, self-complementary.

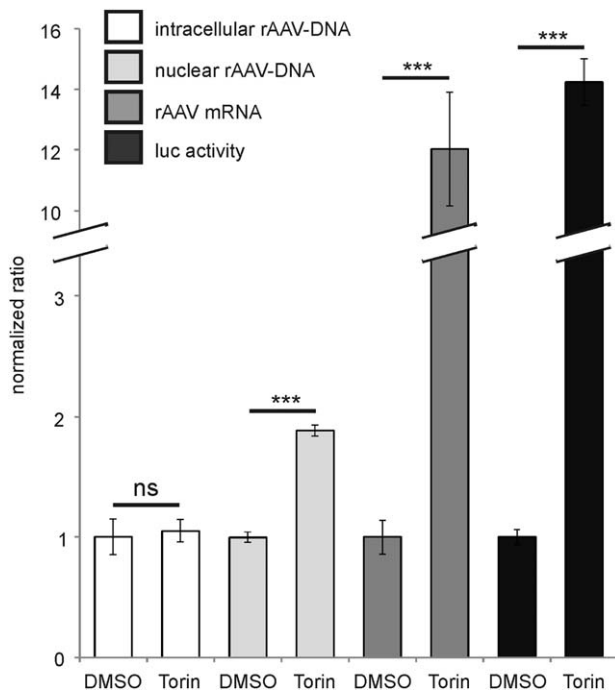


FIG. 6. Induction of autophagy promotes intracellular processing of rAAV. HepG2 cells were pretreated with DMSO or torin 1 (10 μ M) for 1 hour, followed by transduction with rAAV-CMV-luc (GOI of 1×10^3) for 24 hours. AAV genomes were determined in whole cellular extracts and in nuclear fraction by quantitative PCR. Luciferase expression was determined by reverse-transcriptase quantitative PCR at the mRNA level and by luciferase assay at the protein level. Relative levels in the corresponding DMSO-control were set to 1. Mean values \pm standard deviation are shown (n = 3; *** $P < 0.001$). Abbreviation: ns, not significant.

glucogenic amino acids through gluconeogenesis. Also, pathogen infection increases the level of autophagy to eliminate intracellular microorganisms.^(23,24)

In the case of rAAV, autophagy is not only induced upon cell infection but also required for efficient hepatocyte transduction, suggesting the existence of intracellular barriers sensitive to autophagy-based mechanisms. In fact, the 2-fold increase in the levels of intranuclear rAAV DNA in hepatocytes pretreated with torin 1 (Fig. 6) may indicate an enhanced transport of rAAV particles toward the nucleus. This is in line with results from a previous study in which we reported on inefficient intracellular transport as a barrier against rAAV transduction in human hepatocytes.⁽³³⁾ Vesicular acidification during autophagy, which occurs after fusion of autophagosomes with endosomes forming the so-called amphisomes and precedes delivery of cytoplasmic cargo to lysosomes,⁽³⁴⁾

may also contribute by providing a beneficial acidic environment for rAAV. The latter is required to prepare the viral capsid for endosomal/amphisomal escape and nuclear entry.⁽³⁵⁾ Given the high levels of transgene mRNA measured in torin 1-stimulated cells (Fig. 6), induction of autophagy may additionally improve transcription from rAAV vector genomes.

The observed strict correlation between the level of autophagy and rAAV-mediated hepatocyte transduction has a direct impact on the use of rAAV as a gene delivery tool in human clinical trials as exemplified by our preclinical models. Specifically, we found that hepatocellular transgene expression was significantly reduced upon food intake in a starvation-refeeding mouse model (Fig. 7B). In addition, reduced rAAV transduction efficiency correlated with impaired autophagy levels in the liver of *ob/ob* mice. Thus, our results strongly suggest that a competence to mount a valid autophagy response is a critical patient-related or disease-related parameter which needs to be considered when planning rAAV-based liver-directed gene therapy as decreased levels of autophagy have been described not only for steatosis—a condition found in *ob/ob* mice—but also for a number of other liver diseases like hepatocellular carcinoma, alcoholic liver disease, or α_1 -antitrypsin deficiency.^(9,36,37) On the other hand, our results also call for the development of strategies to use the dependence on autophagy to improve efficacy of rAAV. In our proof-of-concept experiments, we decided to use pharmacological inducers of autophagy such as mTOR inhibitors^(30,38) but also other already clinically used drugs such as metformin and carbamazepine,⁽³⁹⁾ or different strategies such as calorie restriction or caffeine intake could be considered.^(40,41) Of note, a single shot of the drug was sufficient to significantly improve the level of transgene products secreted from vector-transduced hepatocytes not only in mice (Fig. 7A) but also in NHPs (Fig. 7C). The latter is of importance as this animal model is very close to the human system and because rapamycin, the mTOR inhibitor explored in the NHPs, has been in clinical use for years. Assuming that results can be directly translated to the human situation, vector dose could be reduced 4-fold to 5-fold, which—based on the available data—should be sufficient to avoid induction of capsid-specific CD8+ T cells and immunotoxicity in humans. Additionally, as rapamycin also down-regulates effector T-cell responses to antigens including AAV capsids,⁽⁴²⁾ while sparing/enhancing survival of regulatory T-cell responses,⁽⁴³⁾ coadministration of rapamycin may represent not only a strategy

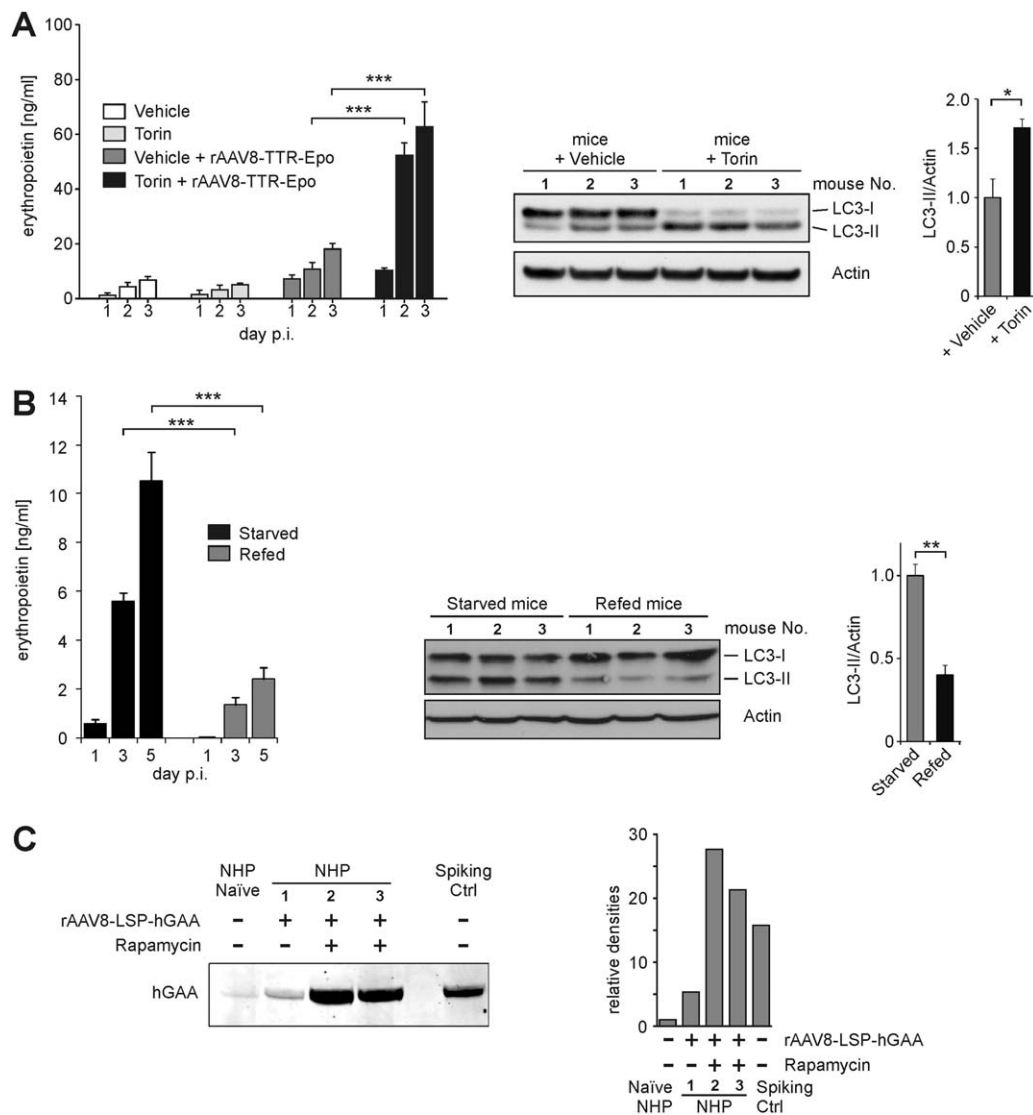


FIG. 7. *In vivo* effects of autophagy on rAAV transduction efficiency. (A) C57BL/6 mice were injected intraperitoneally either with vehicle or with torin 1. Induction of hepatic autophagy was assessed by immunoblot assay for LC3-II (three mice per group), and LC3-II/actin densitometric values are shown as mean \pm standard error of the mean. After 2 hours, mice were intravenously injected with 2×10^{10} vgs/animal of rAAV8-TTR-Epo. Levels of erythropoietin were determined on days 1, 2, and 3 p.i. Data are shown as mean \pm standard error of the mean ($n = 3$ for noninfected control mice treated with vehicle or with torin 1; $n = 5$ for rAAV-injected mice pretreated with vehicle or with torin-1). (B) C57BL/6 mice were starved for 24 hours and then fed (or not) for 1 hour. Inhibition of liver autophagy during a 1-hour refeeding period was examined by immunoblot assay for LC3-II (three mice per group), and LC3-II/actin densitometric values are shown as mean \pm standard error of the mean. Both groups were injected intravenously with 1×10^{10} vgs/animal of rAAV8-TTR-Epo. Levels of erythropoietin were determined on days 1, 3, and 5 p.i. Data are shown as mean \pm standard error of the mean; $n = 5$ for both groups. (A,B) $***P < 0.001$, $**P < 0.01$, $*P < 0.05$. (C) Three NHPs (1, 2, 3) received 2×10^{12} vgs/kg body weight of rAAV8-LSP-hGAA. Prior to vector administration, one NHP was infused with vehicle (NHP 1) and two with rapamycin (NHPs 2 and 3). Levels of hGAA were determined by western blot analysis of plasma proteins (1 mL of undiluted plasma per lane) at day 3 posttreatment. Plasma collected prior to treatment (Naive) was used as baseline, while spiking this plasma with 5 ng of recombinant hGAA (Spiking Ctrl) served as a positive control. The histogram plot shows the quantification of the bands in the western blot normalized to the naive control. Abbreviation: p.i., postinfection.

to enhance liver transduction by increasing autophagy (Figs. 4 and 7C) but also a potential advantage related to the immunomodulatory effect of the drug.

In summary, we identified autophagy as a cell response induced in hepatocytes upon rAAV transduction. This cell response determines the outcome of

rAAV-mediated liver-directed gene transfer because transgene expression was markedly reduced under conditions of impaired autophagy. By exploring pharmacological inducers of autophagy, we provided proof-of-concept for a simple, clinically feasible, and potent strategy to reduce viral vector doses and to significantly improve the safety and efficacy of rAAV-based liver-directed gene therapies.

Acknowledgment: We gratefully acknowledge Jude Samulski (University of North Carolina), Jim Wilson (University of Pennsylvania), and Sibille Quadt-Humme (University of Cologne) for providing us with pXX6, pXR8, and pscEpo, respectively. We thank Michel Rivoire (Centre Léon Bérard, Lyon) for providing human liver tissue and Peter Zentis (Imaging Facility, University of Cologne) for analysis of fluorescence microscopic data. We thank Michael Morgan for critical reading of the manuscript and Arlette Pailard, Hanna Janicki, Maureen Menning, Markus Loy, Ulrike Karow (all from University Hospital Cologne), and Mojgan Ghilav (German Sport University Cologne) for their excellent technical support. We thank Martin Krönke (University of Cologne) for helpful discussions and continuous support.

REFERENCES

- Aravalli RN, Steer CJ. Gene editing technology as an approach to the treatment of liver diseases. *Expert Opin Biol Ther* 2016;16:595-608.
- Brimble MA, Reiss UM, Nathwani AC, Davidoff AM. New and improved AAVenues: current status of hemophilia B gene therapy. *Expert Opin Biol Ther* 2016;16:79-92.
- Buning H, Huber A, Zhang L, Meumann N, Hacker U. Engineering the AAV capsid to optimize vector-host interactions. *Curr Opin Pharmacol* 2015;24:94-104.
- Mingozzi F, High KA. Immune responses to AAV vectors: overcoming barriers to successful gene therapy. *Blood* 2013;122:23-36.
- van der Laan LJ, Wang Y, Tilanus HW, Janssen HL, Pan Q. AAV-mediated gene therapy for liver diseases: the prime candidate for clinical application? *Expert Opin Biol Ther* 2011;11:315-327.
- Wang YG, Huang PP, Zhang R, Ma BY, Zhou XM, Sun YF. Targeting adeno-associated virus and adenoviral gene therapy for hepatocellular carcinoma. *World J Gastroenterol* 2016;22:326-337.
- Nathwani AC, Reiss UM, Tuddenham EG, Rosales C, Chowdary P, McIntosh J, et al. Long-term safety and efficacy of factor IX gene therapy in hemophilia B. *N Engl J Med* 2014;371:1994-2004.
- Hosel M, Broxtermann M, Janicki H, Esser K, Arzberger S, Hartmann P, et al. Toll-like receptor 2-mediated innate immune response in human nonparenchymal liver cells toward adeno-associated viral vectors. *HEPATOLOGY* 2012;55:287-297.
- Czaja MJ, Ding WX, Donohue TM Jr, Friedman SL, Kim JS, Komatsu M, et al. Functions of autophagy in normal and diseased liver. *Autophagy* 2013;9:1131-1158.
- Feng Y, He D, Yao Z, Klionsky DJ. The machinery of macroautophagy. *Cell Res* 2014;24:24-41.
- Flageul M, Aubert D, Pichard V, Nguyen TH, Nowrouzi A, Schmidt M, et al. Transient expression of genes delivered to newborn rat liver using recombinant adeno-associated virus 2/8 vectors. *J Gene Med* 2009;11:689-696.
- Dash S, Chava S, Chandra PK, Aydin Y, Balart LA, Wu T. Autophagy in hepatocellular carcinomas: from pathophysiology to therapeutic response. *Hepat Med* 2016;8:9-20.
- Liu Q, Chang JW, Wang J, Kang SA, Thoreen CC, Markhard A, et al. Discovery of 1-(4-(4-propionylpiperazin-1-yl)-3-(trifluoromethyl)phenyl)-9-(quinolin-3-yl)benzo[h][1,6]naphthyridin-2(1H)-one as a highly potent, selective mammalian target of rapamycin (mTOR) inhibitor for the treatment of cancer. *J Med Chem* 2010;53:7146-7155.
- Naito T, Kuma A, Mizushima N. Differential contribution of insulin and amino acids to the mTORC1-autophagy pathway in the liver and muscle. *J Biol Chem* 2013;288:21074-21081.
- Meliani A, Leborgne C, Triffault S, Jeanson-Leh L, Veron P, Mingozzi F. Determination of anti-adeno-associated virus vector neutralizing antibody titer with an *in vitro* reporter system. *Hum Gene Ther Methods* 2015;26:45-53.
- Kabaya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, et al. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J* 2000;19:5720-5728.
- Thomson AW, Turmquist HR, Raimondi G. Immunoregulatory functions of mTOR inhibition. *Nat Rev Immunol* 2009;9:324-337.
- Barth S, Glick D, Macleod KF. Autophagy: assays and artifacts. *J Pathol* 2010;221:117-124.
- Guo S, Liang Y, Murphy SF, Huang A, Shen H, Kelly DF, et al. A rapid and high content assay that measures cyto-ID-stained autophagic compartments and estimates autophagy flux with potential clinical applications. *Autophagy* 2015;11:560-572.
- Thoreen CC, Kang SA, Chang JW, Liu Q, Zhang J, Gao Y, et al. An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1. *J Biol Chem* 2009;284:8023-8032.
- Chan LL, Shen D, Wilkinson AR, Patton W, Lai N, Chan E, et al. A novel image-based cytometry method for autophagy detection in living cells. *Autophagy* 2012;8:1371-1382.
- Nojima H, Tokunaga C, Eguchi S, Oshiro N, Hidayat S, Yoshino K, et al. The mammalian target of rapamycin (mTOR) partner, raptor, binds the mTOR substrates p70 S6 kinase and 4E-BP1 through their TOR signaling (TOS) motif. *J Biol Chem* 2003;278:15461-15464.
- Chiramel AI, Brady NR, Bartenschlager R. Divergent roles of autophagy in virus infection. *Cells* 2013;2:83-104.
- Deretic V, Saitoh T, Akira S. Autophagy in infection, inflammation and immunity. *Nat Rev Immunol* 2013;13:722-737.
- Berry GE, Asokan A. Chemical modulation of endocytic sorting augments adeno-associated viral transduction. *J Biol Chem* 2016;291:939-947.
- Inami Y, Yamashina S, Izumi K, Ueno T, Tanida I, Ikejima K, et al. Hepatic steatosis inhibits autophagic proteolysis via impairment of autophagosomal acidification and cathepsin expression. *Biochem Biophys Res Commun* 2011;412:618-625.

- 27) **Yang L, Li P**, Fu S, Calay ES, Hotamisligil GS. Defective hepatic autophagy in obesity promotes ER stress and causes insulin resistance. *Cell Metab* 2010;11:467-478.
- 28) Farah BL, Landau DJ, Sinha RA, Brooks ED, Wu Y, Fung SY, et al. Induction of autophagy improves hepatic lipid metabolism in glucose-6-phosphatase deficiency. *J Hepatol* 2016;64:370-379.
- 29) **Lin CW, Zhang H**, Li M, Xiong X, Chen X, Chen X, et al. Pharmacological promotion of autophagy alleviates steatosis and injury in alcoholic and non-alcoholic fatty liver conditions in mice. *J Hepatol* 2013;58:993-999.
- 30) Waldner M, Fantus D, Solari M, Thomson AW. New perspectives on mTOR inhibitors (rapamycin, rapalogs and TORKinibs) in transplantation. *Br J Clin Pharmacol* 2016;82:1158-1170.
- 31) **Zoncu R, Efeyan A**, Sabatini DM. mTOR: from growth signal integration to cancer, diabetes and ageing. *Nat Rev Mol Cell Biol* 2011;12:21-35.
- 32) Nathwani AC, Cochrane M, McIntosh J, Ng CY, Zhou J, Gray JT, et al. Enhancing transduction of the liver by adeno-associated viral vectors. *Gene Ther* 2009;16:60-69.
- 33) **Hosel M, Lucifora J**, Michler T, Holz G, Gruffaz M, Stahnke S, et al. Hepatitis B virus infection enhances susceptibility toward adeno-associated viral vector transduction *in vitro* and *in vivo*. *HEPATOLOGY* 2014;59:2110-2120.
- 34) Eskelinen EL. Maturation of autophagic vacuoles in mammalian cells. *Autophagy* 2005;1:1-10.
- 35) Nonnenmacher M, Weber T. Intracellular transport of recombinant adeno-associated virus vectors. *Gene Ther* 2012;19:649-658.
- 36) Rautou PE, Mansouri A, Lebrec D, Durand F, Valla D, Moreau R. Autophagy in liver diseases. *J Hepatol* 2010;53:1123-1134.
- 37) Ueno T, Komatsu M. Autophagy in the liver: functions in health and disease. *Nat Rev Gastroenterol Hepatol* 2017;14:170-184.
- 38) Nelson V, Altman JK, Plataniotis LC. Next generation of mammalian target of rapamycin inhibitors for the treatment of cancer. *Expert Opin Investig Drugs* 2013;22:715-722.
- 39) Rubinsztein DC, Codogno P, Levine B. Autophagy modulation as a potential therapeutic target for diverse diseases. *Nat Rev Drug Discov* 2012;11:709-730.
- 40) Jia K, Levine B. Autophagy is required for dietary restriction-mediated life span extension in *C. elegans*. *Autophagy* 2007;3:597-599.
- 41) Sinha RA, Farah BL, Singh BK, Siddique MM, Li Y, Wu Y, et al. Caffeine stimulates hepatic lipid metabolism by the autophagy-lysosomal pathway in mice. *HEPATOLOGY* 2014;59:1366-1380.
- 42) **Mingozzi F, Maus MV**, Hui DJ, Sabatino DE, Murphy SL, Rasko JE, et al. CD8⁺ T-cell responses to adeno-associated virus capsid in humans. *Nat Med* 2007;13:419-422.
- 43) Biswas M, Sarkar D, Kumar SR, Nayak S, Rogers GL, Markusic DM, et al. Synergy between rapamycin and FLT3 ligand enhances plasmacytoid dendritic cell-dependent induction of CD4⁺CD25⁺FoxP3⁺ Treg. *Blood* 2015;125:2937-2947.

Author names in bold designate shared co-first authorship.

Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.29176/supinfo.