Rab33B and its autophagic Atg5/12/16L1 effector assist in hepatitis B virus naked capsid formation and release

Tatjana Döring and Reinhold Prange*
Department of Medical Microbiology and Hygiene, University Medical Center of the Johannes Gutenberg University Mainz, Mainz D-55101, Germany

Summary
Hepatitis B virus morphogenesis is accompanied by the production and release of non-enveloped capsids/nucleocapsids. Capsid particles are formed inside the cell cytosol by multimerization of core protein subunits and ultimately exported in an uncommon coatless state. Here, we investigated potential roles of Rab GTPases in capsid formation and trafficking by using RNA interference and overexpression studies. Naked capsid release does not require functions of the endosome-associated Rab5, Rab7 and Rab27 proteins, but depends on functional Rab33B, a GTPase participating in autophagosome formation via interaction with the Atg5-Atg12/Atg16L1 complex. During capsid formation, Rab33B acts in conjunction with its effector, as silencing of Atg5, Atg12 and Atg16L1 also impaired capsid egress. Analysis of capsid maturation steps revealed that Rab33B and Atg5/12/16L1 are required for proper particle assembly and/or stability. In support, the capsid protein was found to interact with Atg5 and colocalize with Atg5/12/16L1, implicating that autophagy pathway functions are involved in capsid biogenesis. However, a complete and functional autophagy pathway is dispensable for capsid release, as judged by knockdown analysis of Atg8/LC3 family members and pharmaceutical ablation of canonical autophagy. Experiments aimed at analysing the capsid release-stimulating activity of the Alix protein provide further evidence for a link between capsid formation and autophagy.

Introduction
Hepatitis B virus (HBV) chronically infects about 350 million people worldwide and can ultimately lead to liver failure and liver cancer. Despite the availability of effective vaccines, HBV infection remains an important public health problem, as current therapeutics for chronic carriers cannot eradicate the virus completely. A better understanding of the HBV life cycle and the mechanisms by which it usurps established host cell pathways is thus essential towards the identification of novel antiviral targets.

HBV is an enveloped DNA virus that replicates by reverse transcription. With about 3 kb, the HBV genome is one of the smallest viral genomes known and encompasses four overlapping open reading frames (ORFs) encoding the polymerase/reverse transcriptase (RT), the capsid-forming core protein, three related envelope proteins (Env) and the regulatory X protein (Bruss, 2007; Nassal, 2008). The assembly of virions begins with the formation of icosahedral nucleocapsids within the cytoplasm that package the pregenomic RNA together with the viral polymerase. Inside the capsids, composed of 180 or 240 copies of the core protein, the progeny DNA genome is synthesized through reverse transcription of the pregenomic RNA. Mature nucleocapsids are then enclosed by the viral envelope concomitant with budding at intracellular membranes and subsequent release (Patient et al., 2009; Prange, 2012).

Beside infectious virions, HBV produces and releases subviral empty envelope particles and subviral non-enveloped capsid/nucleocapsid particles. The functional implication and pathway of release of naked capsids are less understood. While HBV-replicating cell lines export large quantities of naked capsids/nucleocapsids (Beterams and Nassal, 2001; Watanabe et al., 2007; Wittkop et al., 2010; Bardens et al., 2011), these particles are hardly found in the blood of infected patients (Possehl et al., 1992). Even so, high titres of anti-capsid antibodies are produced in virtually all patients who have been exposed to HBV (Milich and McLachlan, 1986). The potent immunogenicity of the (nucleo)capsid is related to its function as a T-cell-independent antigen that directly binds and activates B-cells to produce antibodies (Milich and McLachlan, 1986; Milich et al., 1997). Accordingly,
naked (nucleo)capsids appear to exist in vivo, but may be rapidly cleared from extracellular fluids by phagocytosis, endocytosis and/or degradation. Whether the release of naked (nucleo)capsids may be beneficial for the infected host or the virus or both also remains to be addressed. The release pathway of naked capsids is uncommon, as it proceeds in a non-lytic manner and renders extracellular particles devoid of a membrane coat (Bardens et al., 2011). This is in contrast to retroviral capsids or virus-like particles (VLPs) that are released in a lipid-coated state (Votteler and Sundquist, 2013). In further difference, HBV appears to exploit distinct cellular pathways to release its particle types, while retroviruses utilize equal host proteins and mechanisms for the egress of viral and subviral particles (Bardens et al., 2011; Prange, 2012; Votteler and Sundquist, 2013).

The budding and egress of HBV depend on scission functions provided by the endosomal sorting complex required for transport (ESCRT), including the ESCRT-II and ESCRT-III complexes together with the Vps4 ATPase (Kian Chua et al., 2006; Lambert et al., 2007; Watanabe et al., 2007; Steiler and Prange, 2014). By contrast, HBV naked capsid budding does not require the ESCRT machinery but can be stimulated by Alix [apoptosis-linked gene 2 (ALG-2)-interacting protein X, also known as PDCP6IP] (Bardens et al., 2011), a multifunctional adaptor protein with key roles in membrane biology, cell signalling and cytokinesis (Odorizzi, 2006). How Alix masters the release of coatless capsids is unknown, but may involve membrane structures as a topological mechanism to deliver cytoplasmic capsids to the extracellular milieu without lysing the cell. In support of this presumption, it had been reported that the HBV core protein can associate with membranes and that capsid particle formation requires intact membranes (Lingappa et al., 2005; Bardens et al., 2011).

In eukaryotic cells, Rab proteins are master regulators in the organization of membrane platforms. Rab proteins comprise a large group of small monomeric GTPases that control membrane identity and vesicle budding, trafficking and fusion. Rab proteins exert their functions through highly regulated GTP-GDP cycles, thereby enabling the GTP-bound, active forms to recruit specific effector proteins onto membranes (Stenmark, 2009; Bhuin and Roy, 2014). In recent years, it became evident that many viruses hijack Rab proteins to their advantage. Examples for Rab exploitation in the course of viral exit include Rab18 that facilitates membrane association of the hepatitis C virus during progeny production (Salloum et al., 2013), Rab27 that is co-opted by members of the herpes virus family during egress (Fraile-Ramos et al., 2010), and Rab11 that plays an important role in the assembly and release of negative-stranded RNA viruses (Bruce et al., 2012).

Some Rab proteins, like Rab5, Rab7, Rab11, Rab32 and Rab33, and their effectors also function in the formation and maturation of autophagic membrane platforms (Chua et al., 2011; Bento et al., 2013). Autophagy is a degradation pathway that delivers autophagic substrates to lysosomes via double-membrane vesicular structures called autophagosomes. It involves concerted action of more than 20 specific autophagy (Atg) proteins that mediate the formation and elongation of a double-membrane sack (the isolation membrane), cargo engulfment, autophagophore closure and autophagosome fusion with the lysosome (Geng and Klionsky, 2008; Shpilka et al., 2011; Weidberg et al., 2011). Although autophagy is an essential defence mechanism of the cell to confront viral invasion, many viruses evolved strategies to evade autophagy and even exploit the autophagy machinery for their own benefits (Dreux and Chisari, 2010; Dong and Levine, 2013). As exemplified by the poliovirus, a non-enveloped RNA virus, autophagic structures provide membranous surfaces for assembly of viral RNA replication complexes and serve as vehicles for virus exit without lysis (Jackson et al., 2005; Taylor et al., 2009). Similarly, other RNA viruses have been shown to induce double-membrane vesicles resembling small autophagosomes that support viral propagation (Blanchard and Roingeard, 2015). The autophagic machinery had been also implicated in HBV replication, as targeted knockdowns of essential Atg proteins impaired the release of viral particles (Sir et al., 2010; Li et al., 2011; Tian et al., 2011).

In this study, we investigated roles of candidate Rab proteins and their effectors in HBV naked capsid formation and release. We find that Rab33B along with its autophagic Atg5/12/16L1 effector is essential for naked capsid egress, while the entire autophagy machinery is dispensable. In addition, we provide evidence that Alix promotes capsid exocytosis via dysregulation of autophagic pathway functions.

**Results**

**Roles of Rab5, Rab7 and Rab27 GTPase in HBV naked capsid release**

To study HBV core/capsid maturation without an ongoing virus replication, the expression constructs pCore or pDPAF.Core were used. Both constructs carry the core ORF under the control of the human metallothionein IIA (hMTIIA) promoter but lack the HBV RT and Env ORFs. In difference to pCore, pDPAF.Core contains the core gene with an N-terminal insertion of a DPAF-epitope tag that did not interfere with the assembly and release of capsid particles (Bardens et al., 2011).

By using differentiated HepaRG liver cell lines that are permissive for HBV infection, it had been shown that HBV
entry depends on the endocytic Rab5 and Rab7 GTPases that likely facilitate transport of incoming virions and viral capsids from early to mature endosomes (Macovei et al., 2013). To analyse whether trafficking and export of newly synthesized naked capsids might have similar requirements, we perturbed Rab5 or Rab7 pathways in cells expressing DPAF.core. To impair Rab5A or Rab7A, ectopic overexpression of their GTP binding-deficient mutants was used that are known to act in a dominant-negative (DN) manner (Stenmark, 2009; Bhuin and Roy, 2014). Human hepatoma HuH-7 cells were cotransfected with the DPAF.Core construct plus expression vectors encoding enhanced green fluorescent (GFP)-tagged wild-type (WT) and DN forms of Rab5A and Rab7A. As shown by GFP-specific immunoblotting of cell lysates, all constructs were efficiently synthesized (Fig. 1A). Of note, Rab7.wt and Rab7.dn differ in their electrophoretic mobility due to differences in fusion to the GFP tag. Neither the WT nor DN forms of Rab5A and Rab7A grossly affected the levels of intracellular core or extracellular capsids, indicating that these two endocytic Rabs are dispensable for HBV naked capsid biogenesis and release (Fig. 1A).

Because the mechanism rendering the exocytosis of HBV capsids in a nude state is less clear, we next studied roles of Rab proteins involved in the regulation of exocytotic pathways, like Rab27 family members that control secretion of lysosomes-related organelles and exosomes (Stenmark, 2009; Ostrowski et al., 2010). For interference with Rab27 functions, Rab27A and Rab27B were depleted with specific siRNA pools consisting of four different duplexes prior to transfection with DPAF.Core. The knockdown of Rab27A was verified by immunoblotting with an isoform-specific antibody (Fig. 1B). Because this antibody did not recognize Rab27B, the degree of the knock-down was measured by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) of cellular RNAs that revealed silencing effects of about 70%. When culture media were analysed...
for capsids, the reduction of either Rab27 isoform surprisingly increased capsid export (Fig. 1B). Hence, the perturbations of Rab27-controlled exocytosis pathways appear to be beneficial for capsid egress.

**HBV naked capsid release requires functional Rab33B**

Next, we focused on Rab33 proteins, comprising the Rab33A and Rab33B isoforms. Unlike Rab33A that is preferentially expressed in the brain and cells of the immune system, the Rab33B isoform is ubiquitously expressed and was therefore studied here (Zheng et al., 1998). Rab33 was originally described as a Golgi-resident protein involved in Golgi-to-endoplasmic reticulum transport, but more recent studies documented a link between Rab33B and the autophagy machinery (Zheng et al., 1998; Itoh et al., 2008). Given the relationship between viruses and autophagy (Dreux and Chisari, 2010; Dong and Levine, 2013), a siRNA-mediated knockdown of Rab33B with a pool of four siRNA duplexes was performed in DPAF.Core-expressing cells. As shown by immunoblotting of lysates, Rab33B was effectively depleted (Fig. 2A). The inspection of the intra- and extracellular core/capsid levels revealed that the Rab33B knockdown reproducibly evoked a substantial decline in naked capsid export (Fig. 2A). To ascertain the role of Rab33B in capsid release, ectopic overexpression of WT and DN mutant proteins was used. Cells were cotransfected with DPAF.Core and GFP-tagged versions of Rab33B.wt or Rab33B.dn and processed as outlined above. As anticipated from the siRNA experiments, the up-regulation of the GTP binding-defective Rab33B.dn mutant impaired capsid release, while excess Rab33B.wt increased the amounts of extracellular capsids (Fig. 2B). Together, these data indicate that HBV capsid formation, trafficking and/or release require the assistance of catalytic active Rab33B.

Since retroviruses also release VLPs from infected cells, we next examined whether Rab33B might play a general role in subviral capsid release pathways. For reporters, we used a yellow fluorescent protein (YFP)-tagged Gag construct of the murine leukemia virus (MLV; Sherer et al., 2003), and GFP-tagged Gag constructs of the human immunodeficiency virus type 1 (HIV; Hermida-Matsumoto and Resh, 2000) and the equine infectious anaemia virus (EIAV; Fang et al., 2007). The release of the retroviral VLPs were inspected in Rab33B-depleted HuH-7 cells. Thereby, we observed that the knockdown of Rab33B had no impact on the export of MLV.Gag, HIV.Gag, and EIAV.Gag (Fig. 2C). Hence, Rab33B appears to play a specific role in HBV naked capsid egress.

Previously, we showed that the up-regulation of the cellular Alix protein accelerates HBV capsid export (Bardens et al., 2011). To analyse whether Alix maintained this ability upon inactivation of Rab33B, siControl- or siRab33B-treated cells were transfected with DPAF.Core both in the absence or presence of excess HA-tagged Alix. In agreement with published results, the ectopic Alix expression enhanced extracellular capsid levels in normal (i.e. control) cells (Fig. 2D). However, the capsid release-promoting activity of Alix was no longer evident in Rab33B-depleted HuH-7 cells (Fig. 2D), indicating that Alix might act either upstream or downstream of Rab33B.

**HBV naked capsid release requires Atg5, Atg12 and Atg16L1**

Recent works suggested an involvement of Rab33B in autophagy processes, as it binds to Atg16L1 in a GTP-dependent manner (Itoh et al., 2008). Atg16L1 comprises one factor that is essential for the formation of the isolation membrane and hence the autophagosome (Geng and Klionsky, 2008; Weidberg et al., 2011). Atg16L1 is part of an ubiquitin-like conjugation system and interacts with Atg5 that is in turn constitutively conjugated with Atg12. This ternary Atg5/12/16L1 complex initiates autophagophore formation and enables the lipidation of LC3 (Atg8) family members and their subsequent association with the autophagic membrane. LC3/Atg8 proteins are then responsible for expansion of the autophagophore (Shpilka et al., 2011; Romanov et al., 2012; Walczak and Martens, 2013; Kaufmann et al., 2014). To study whether Rab33B might act in concert with Atg16L1 and its complex partners during HBV capsid egress, the relevant autophagy factors were genetically ablated. HuH-7 cells were treated with control siRNA or siRNA pools against Atg5, Atg12 or Atg16L1 prior to transfection with DPAF.Core. To exclude possible off-target effects of the pools, single siRNAs targeting Atg5 and Atg12 were applied in addition. Anti-core and anti-β-actin Western blot analyses of lysates demonstrated that neither knockdown affected the intracellular core level or overall cell viabilities, indicating that the depletions did not interfere with the rate of DNA transfections and cell fitness (Fig. 3). In contrast, either knockdown reproducibly reduced the amounts of extracellular capsids in the magnitude Atg5 > Atg12 > Atg16L1 (Fig. 3). Since the Atg5/12/16L1 complex is essential for LC3 lipidation and hence for the conversion of the LC3-I to the LC3-II form (Shpilka et al., 2011; Romanov et al., 2012; Walczak and Martens, 2013), cell extracts were examined by anti-LC3-specific immunoblotting. The depletion of either complex member almost completely reduced the levels of the lipidated LC3-II isoform, indicative of a high siRNA transfection rate and of functional ablation of the complex (Fig. 3). For further verification, we monitored for depletion/cross-depletion efficacies. Anti-Atg5-specific immunoblotting of

© 2014 John Wiley & Sons Ltd, *Cellular Microbiology*, 17, 747–764
Fig. 2. HBV naked capsid release requires catalytic active Rab33B.

A. Rab33B depletion decreases capsid release. HuH-7 cells were transfected with control siRNA or siRNAs against Rab33B. Two days later, cells were retransfected with DPAF.Core and harvested after additional 2 days as outlined in the legend of Fig. 1B. Lysates were probed by Rab33B-specific WB to monitor depletion. Uniformity of sample loading was probed by anti-β-actin WB. Lysates and concentrated supernatants were analysed by anti-DPAF immunoblotting to detect core/capsids. Experiments were done in triplicate and the degree of capsid release is demonstrated in percent amount relative to control cells (± SD).

B. Effects of overexpressing Rab33B.wt or Rab33B.dn on capsid release. DPAF.Core was cotransfected with control DNA or GFP-tagged WT or DN forms of Rab33B at a 1:3 ratio respectively. NP-40 lysates and concentrated supernatants were subjected to GFP-specific and DPAF-specific WB. Uniformity of sample loading is shown by a band cross-reacting with the DPAF antibody (Load). Quantification data of extracellular capsids are shown below (n = 3, ± SD).

C. Rab33B depletion does not inhibit retroviral VLP release. Cells were treated with control or Rab33B-specific siRNA duplexes as in A and retransfected with YFP-tagged MLV.Gag or GFP-tagged HIV.Gag or EIAV.Gag constructs. Extracellular VLPs were analysed by GFP-specific WB, quantified by densitometric analysis and demonstrated in percent amount relative to control cells (n = 2).

D. Overexpressed Alix does not enhance HBV naked capsid release in Rab33B-depleted cells. HuH-7 cells were transfected with control siRNA or the siRNA pool targeting Rab33B. Cells were retransfected with DPAF.Core in the absence or presence of HA-tagged Alix (1:3 ratio, respectively), as indicated. SDS lysates and cell supernatants were subjected to HA- and DPAF-specific WB.
lysates showed effective depletion of the Atg5/12 conjugate in siAtg5- and siAtg12-treated cells (Fig. 3). Notably, in siAtg12-treated cells, unconjugated Atg5 appeared that is absent in siControl-transfected cells. We deduced from this observation that the siRNAs against Atg12 were also operative. In contrast to the covalent and constitutive binding between Atg5 and Atg12, Atg16L1 is loosely associated with the complex (Walczak and Martens, 2013). Accordingly, we observed that the level of the Atg5/12 conjugate is diminished but not entirely reduced in siAtg16L1-treated cells (Fig. 3). In addition, the siRNAs against Atg16L1 proved to be effective as shown by anti-Atg16L1 immunoblotting. Here again, cross-depletion effects were evident in such that the siRNAs against Atg5 abrogated Atg16L1, while those against Atg12 diminished Atg16L1 levels (Fig. 3). Together, these data indicate that HBV naked capsids require the action of the Atg5/12 conjugate, likely in conjunction with Atg16L1, to exit cells.

Knockdowns of Rab33B, Atg5, Atg12 and Atg16L1 reduce the pool of intracellular HBV naked capsids

To gain insights into the mechanistic actions of Rab33B and the Atg5/12/16L1 complex, we inspected the core assembly reaction. During this process, monomeric core forms dimers that assemble into icosahedral capsids (Bruss, 2007; Selzer et al., 2014). HuH-7 cells were treated with control or Atg5-, Atg12-, Atg16L1- or Rab33B-specific siRNAs prior to transfection with core, and lysates were probed by core-specific WB (left). Uniformity of sample loading was assessed by anti-β-actin WB. The same samples were analysed for the ratio of LC3-I and LC3-II by immunoblotting with anti-LC3 antibodies (left). Extracellular capsid values were quantified by densitometric analysis and demonstrated in percent amount relative to control cells in the graph below. Error bars indicate the standard deviations from the mean of five experiments (left). To monitor depletion, the same lysates were examined by Atg5- and Atg16L1-specific immunoblotting (right).

**Fig. 3.** HBV naked capsule release requires Atg5, Atg12 and Atg16L1. HuH-7 cells were treated with control siRNA or siRNA pools targeting Atg5, Atg12 or Atg16L1 for 72 h. In cases where single siRNA duplexes were used, the target gene is designated with a star. Following transfection with core for 48 h, cells were lysed with SDS and supernatants were subjected to ultracentrifugation. Synthesis and release of core/capsids were probed by core-specific WB (left). Uniformity of sample loading was assessed by anti-β-actin WB. The same samples were analysed for the ratio of LC3-I and LC3-II by immunoblotting with anti-LC3 antibodies (left). Extracellular capsid values were quantified by densitometric analysis and demonstrated in percent amount relative to control cells in the graph below. Error bars indicate the standard deviations from the mean of five experiments (left). To monitor depletion, the same lysates were examined by Atg5- and Atg16L1-specific immunoblotting (right).
reduced the amounts of core in the particulate fractions, while the loss of Atg16L1 had weaker effects. The combined data indicate that Rab33B along with its putative Atg5/12/16L1 effector assist in the formation and/or stability of HBV capsids. In support, we also observed reduced levels of nucleocapsids containing the viral DNA genome in HBV-replicating HuH-7 cell lines depleted for Rab33B (Jens Stieler and Reinhild Prange, unpubl. obs.).

HBV core interacts and colocalizes with Atg5

To attain more clues how core engages Rab33B and its effector, deconvolution immunofluorescence microscopy studies were performed. Core-expressing cells were costained with specific antibody pairs to visualize the distribution of core and endogenous Rab33B, Atg5, Atg12 and Atg16L1. In either setting, core yielded its typical cytoplasmic staining with some nuclear labelling (Fig. 5A). Consistent with published data, Rab33B appeared in small vesicular structures, while the autophagic factors were found mostly dispersed throughout the cytoplasm (Fig. 5A; Itoh et al., 2008; Weidberg et al., 2011). Importantly, an overlay of the fluorescence patterns revealed partly colocalization between core and Atg5, Atg12 and Atg16L1. By contrast, no colocalization could be observed for core and Rab33B (Fig. 5A). This prompted us to analyse whether core might physically interact with the identified host factors critical for capsid maturation. Because the polyclonal antisera against the Atg proteins proved to be less suitable for immunoprecipitation, we used an expression vector encoding human Atg5 with a C-terminal HA-tag. This vector was cotransfected with control DNA or core, and lysates were subjected to immunoprecipitation with anti-core antibodies followed by HA-specific immunoblotting. Thereby, a specific interaction between core and Atg5 was clearly detectable (Fig. 5B). To unravel whether Atg5 binds to mono/dimeric core protein and/or capsids, we studied the core mutant Core.Y132A that reportedly forms dimers but no capsids (Bourne et al., 2009; Alexander et al., 2013). As shown in Fig. 5B, Core.Y132A bound to Atg5 with the same efficiency as wt core. By using a similar immunocapture analysis, we failed to observe a stable association between core and GFP-tagged Rab33B.wt (data not shown). Combining the imaging and biochemical data, we conclude that core dimers interact with Atg5 and presumably with the Atg5/12/16L1 complex, but do not firmly associate with Rab33B.

HBV naked capsid release does not require LC3

Because the Atg5/12/16L1 complex initiates autophagosomal biogenesis and is required for efficient

© 2014 John Wiley & Sons Ltd, *Cellular Microbiology*, 17, 747–764
LC3 lipidation, we next probed for a role of LC3 in HBV capsid formation. Mammalian cells contain three LC3 isoforms referred to A, B and C, with LC3B being the most abundant molecule in many cell types (Geng and Klionsky, 2008; Shpilka et al., 2011). Again, an RNA interference approach was applied using siRNA pools against LC3A and LC3C and a single siRNA targeting LC3B, either individually or in combination. As shown in Fig. 6A, neither of the treatments largely affected the intra- and extracellular core/capsid levels. Rather, LC3B silencing repeatedly enhanced capsid egress. To test for depletion, lysates were probed with an anti-LC3A/B antibody that

**Fig. 5.** Core partly colocalizes with Atg5, Atg12 and Atg16L1 and interacts with Atg5. A. Immunofluorescence analysis. HuH-7 cells were transfected with DPAF-Core and immunostained with antibodies reacting with endogenous Rab33B, Atg5, Atg12 or Atg16L1. To detect core, either rabbit anti-core (for Rab33B costaining) or mouse anti-DPAF (for Atg5/12/16L1 costaining) antibodies were applied. Following staining with secondary antibodies, cells were analysed by deconvolution fluorescence microscopy and representative images are shown. The staining pattern of the endogenous proteins is shown in red, and the fluorescent signal of core is in green. The overlays of the fluorescence patterns are shown in the right column with yellow color indicating colocalization. DNA staining is shown in blue. Bar: 10 μm. Outlined areas are shown at larger magnifications. B. Immunocapture analysis. HuH-7 cells were cotransfected with HA-tagged Atg5 together with control plasmid, wt core or the assembly-defective Core.Y132A mutant at a 1:1 DNA ratio. Synthesis (Input) of Atg5 and wt or mutant core is shown by WB of lysates with anti-HA and anti-core antibodies. Identical gel loading is shown by anti-α-tubulin staining. For co-immunoprecipitation (IP), lysates were incubated with anti-core antibodies followed by anti-HA WB.
particularly reacts with the LC3B isoform. Thereby, LC3B expression was clearly reduced upon individual and combinatorial RNA interference (Fig. 6A). The silencing effects of the LC3A and LC3C pools were assayed by qRT-PCR and calculated to 45% and 73% respectively (Fig. 6A). Together, we deduce from these results that HBV capsid egress does not require LC3. Consistent with the biochemical data, cell imaging analyses did not reveal colocalization between endogenous LC3 and core (Fig. 6B). Even after induction of autophagy with chemical compounds (i.e. rapamycin) that shifted the diffuse cytoplasmic pool of LC3 to punctate, autophagosome-reminiscent structures, the staining pattern of core did not coincide with LC3-positive puncta (Fig. 6B). The

Fig. 6. HBV naked capsid release does not require LC3 family members.
A. LC3-independent capsid release. HuH-7 cells were treated with control siRNA or siRNAs targeting LC3A, LCB or LC3C either alone or in combination for 72 h. If single siRNA duplexes were used, the target gene is designated with a star. Following transfection with core for 48 h, SDS lysates and concentrated supernatants were subjected to core- and LC3-specific WB. Identical gel loading is shown by anti-β-actin staining. In the case of LC3A and LC3C, the silencing efficacy was additionally examined by qRT-PCR and shown in the graph. Experiments were done in triplicate and the degree of capsid release is demonstrated in percent amount relative to control cells (± SD).
B. Core does not colocalize with LC3. DPAF.Core-transfected cells were treated without or with rapamycin (Rapa) for 3 h prior to fixation. Cells were stained with mouse anti-DPAF (green) and rabbit anti-LC3 (red) antibodies and processed as in the Fig. 5B legend. Outlined areas of these images are shown at larger magnification.
dispensability of LC3 together with the requirement of Atg5/12/16L1 implicates that HBV capsid formation needs only part of the autophagy machinery.

**HBV naked capsid release does not depend on canonical autophagy**

For corroboration, we examined the fate of core upon chemical manipulation of autophagy. Control- and core-expressing cells were left untreated or treated with rapamycin or 3-methyladenine (3-MA) that, respectively, induces or suppresses autophagy (Mizushima et al., 2010). Both drugs were applied in the absence or presence of lysosomal acidification inhibitors in order to monitor autophagic flux. For an autophagic marker, the amounts of the lipidated LC3-II form were traced. Untreated control- and core-expressing cells displayed equal LC3-II levels indicating that the synthesis of core did not affect autophagy (Fig. 7, lanes 1 and 2). Upon activation or inactivation of autophagy, the amounts of LC3-II increased or decreased, respectively, as expected. However, neither of the treatments led to a considerable change in intracellular core and extracellular capsid concentrations (Fig. 7). These results support the notion that the entire autophagy machinery is dispensable for capsid formation.

**The HBV naked capsid release-promoting Alix protein modulates autophagy**

As outlined above, overexpression of Alix promotes HBV naked capsid release. This stimulation, however, got lost upon functional ablations of Rab33B (see Fig. 2D) and Atg5/12/16L1 (data not shown). Because Alix had been shown to specifically act on capsids rather than on mono/dimeric core proteins (Bardens et al., 2011), the capsid assembly deficits in Rab33B- and Atg5/12/16L1-knockdown cells might account for the missing effect of Alix therein. Albeit plausible, we nonetheless reasoned to search for other possible interconnections between Alix and Rab33B along with its autophagic effector during HBV capsid maturation. To this aim, we examined autophagy activity in cells overexpressing Alix. For reporters, LC3 and the LC3-interacting p62 autophagy adaptor were used (Mizushima et al., 2010). Upon transient overexpression of HA-tagged Alix, the amount of lipiddated LC3-II substantially increased as compared with control cells (Fig. 8A). Concomitantly, the level of p62 was slightly elevated, implicating that excess Alix might either induce autophagy or suppress pathway steps downstream of autophagosome formation. To our great surprise, however, immunostaining of cells demonstrated that overexpressed Alix did not induce punctate LC3 structures, reminiscent for autophagosomes, but rather led to large LC3 clusters that poorly colocalized with Alix (Fig. 8B). Quantification analysis revealed a strict correlation between ectopic Alix expression and the appearance of LC3 clusters (Fig. 8B). Such Alix-induced LC3 structures were also detectable upon chemical induction and repression of autophagy (Fig. 8C and D), indicating that Alix appears to interfere with canonical autophagy by sequestration/aggregation of LC3. Similarly, the distribution of endogenous p62 shifted from small dots in normal cells (i.e. control cells) to large perinuclear clusters in Alix-overexpressing cells (Fig. 8E). Noteworthy, however, when cells were cotransfected with Alix plus DPAF. Core, core did not colocalize with the Alix-induced LC3 structures (Fig. 8F). Together, these results indicate that up-regulated Alix appears to entrap key players of the autophagy machinery.

---

**Fig. 7.** Modulation of canonical autophagy does not affect HBV naked capsid release. Cells were transfected with empty plasmid DNA (Con) or core and treated with or without the indicated chemical compounds for 7 h. SDS extracts were immunoblotted with anti-core- or anti-LC3 antibodies. A non-specific stained protein served as a gel loading control (left). Culture supernatants of accordingly treated cells were assayed by a core-specific ELISA and demonstrated in percent amount relative to control cells in the graph (n = 3, ± SD; right).
Fig. 8. Overexpressed Alix induces clustering of LC3- and p62-positive structures.

A. HuH-7 cells were transfected with HA-tagged Alix, and SDS lysates were probed by HA-, β-actin-, LC3- and p62-specific WB. The levels of LC3-II and p62 were quantified by densitometric analysis and demonstrated in percent amount relative to control cells.

B. Cells expressing HA-tagged Alix were left untreated and immunostained against HA (green) and endogenous LC3 (red). The epifluorescence of single and merged channel images are shown. For quantification, the correlation between ectopic Alix expression and LC3 clustering were inspected in about 150 cells from three independent experiments and plotted in the graph (right).

C, D, E. Cells expressing HA-tagged Alix were left untreated or treated with rapamycin or 3-MA for 3 h prior to processing for immunofluorescence microscopy. Cells were labelled with antibodies against HA (green) and endogenous LC3 or p62 (red). The ‘LC3 control’ and ‘p62 control’ images show the staining pattern of LC3 or p62 in non-transfected cells. E. Core does not colocalize with Alix-induced LC3-positive structures. Cells were cotransfected with HA-tagged Alix plus DPAF-Core and stained with anti-DPAF- and anti-LC3 antibodies. Cells carrying aberrant LC3 structures (red) were analysed for the distribution of core (green). A representative epifluorescence image is shown. DNA staining of the nuclei is in blue. Bar: 10 μm.
Congruence of Alix domains responsible for promoting HBV naked capsid release and modulating autophagy

Alix consists of three regions including the N-terminal Bro1 domain, the middle V domain and the C-terminal proline-rich region (Odorizzi, 2006). Our previous mapping analysis led to the identification of the Bro1 domain being responsible for the HBV capsid release-promoting activity (Bardens et al., 2011). Here, we re-examined the features of WT and mutant Alix proteins and concomitantly inspected the fate of LC3. Furthermore, the human Brox protein (Bro1 domain-only protein) was also analysed that shares high sequence and structure similarities to the Bro1 domain of Alix (Ichioka et al., 2008; Zhai et al., 2011). HuH-7 cells were cotransfected with DPAF.Core and HA-tagged Alix, AlixΔBro1, Alix.Bro1 or FLAG-tagged Brox. As shown in Fig. 9A, all constructs were expressed and could be identified by their predicted molecular weights. By inspecting the amounts of DPAF.Core in lysates and capsids in supernatants, both WT Alix and its sole Bro1 domain (Alix.Bro1) accelerated capsid release concomitant with a decline in intracellular core levels (Fig. 9A). Conversely, the lack of the Bro1 domain (AlixΔBro1) abrogated enhanced capsid export (Fig. 9A). Against expectation, overexpressed Brox also failed to stimulate capsid egress (Fig. 9A) despite its pronounced homology to Bro1. By analysing the endogenous LC3 pattern, WT Alix and Alix.Bro1 increased the pools of LC3-II, whereas AlixΔBro1 and Brox did not (Fig. 9A). An accompanying immunofluorescence study confirmed these phenotypes, as Alix and Alix.Bro1 induced the appearance of atypical LC3 clusters. Such LC3 aggre-
gates were hardly detectable in Alix△Bro1- and Brox-expressing cells (Fig. 9B). The exact correlation between the capsid release-stimulating activity of Alix mutants/relatives and their properties to alter normal LC3 ratios/structures and vice versa suggest that the Alix-induced dysregulation of autophagic factors favours HBV naked capsid egress.

Discussion

HBV is one of the smallest animal viruses yet one of the most successful human pathogens. Due to the tiny size of its genome, HBV likely depends on a close interplay between viral and host factors for the generation of new viral particles from infected cells. In this work, we investigated candidate Rab GTPases and their effector proteins for their involvement in HBV capsid trafficking and export. Among viral export routes, the exocytic pathway of HBV naked capsids is unconventional in that it renders extracellular particles devoid of a membrane coat. We found that HBV capsid formation depends on Rab33 and the autophagic Atg5/12/16L1 complex irrespective of canonical autophagy.

The endocytic Rab5 and Rab7 proteins were shown to be essential for HBV entry into susceptible cell lines and likely mediate trafficking events of incoming viral particles, including enveloped virions and non-enveloped nucleocapsids, along the endosomal pathway (Macovei et al., 2013). As shown herein, these Rabs are not required for transport events of newly synthesized naked capsids, implicating that they are seemingly able to discriminate between capsids and nucleocapsids and/or their degree of maturation. Both Rab proteins have been shown to be involved in autophagy in such that Rab5 controls autophagy formation and Rab7 is required for fusion between autophagosomes and lysosomes (Chua et al., 2011; Bento et al., 2013). We took the dispensability of Rab5 and Rab7 in HBV naked capsid formation as first indirect clues that this process does not rely on the entire canonical autophagy pathway.

Rab27A and Rab27B control the secretion of exosomes that correspond to intraluminal vesicles generated by ESCRT-driven inward budding reactions at multivesicular bodies (MVB). Both Rabs function in MVB docking at the plasma membrane concomitant with exosome release (Stenmark, 2009; Ostrowski et al., 2010). Hepatitis B virus naked capsids differ from exosomes in their ESCRT independence and their lack of a vesicular coat (Watanabe et al., 2007; Bardens et al., 2011). Hence, we did not surmise and discover a requirement of Rab27 in the egress of capsids, thereby adding auxiliary evidence for their non-exosomal release. Rather, we found that Rab27 knockdowns increased extracellular capsid levels. Although we do not know the underlying mechanism, we suspect an indirect role played by Alix. Alix is frequently identified in exosomes and regulates together with syndecan and syntenin the biogenesis of exosomes (Baietti et al., 2012). Because exosome secretion is inhibited in Rab27 knockdown cells (Ostrowski et al., 2010), more functional Alix might be available to promote HBV capsid export.

Conversely, we found that HBV capsid maturation and egress rely on catalytically active Rab33B, a Golgi-resident GTPase. Rab33B had been demonstrated to interact with Aut16L1 and as overexpression of a hyperactive Rab33B mutant attenuates autophagy (Zheng et al., 1998; Itoh et al., 2008). These findings were interpreted in such that Rab33B-containing vesicles derived from the Golgi bind to Aut16L1 and fuse with the elongating isolation membrane for supply of membranes (Itoh et al., 2008). Nonetheless, an essential role of Rab33B in canonical autophagy remained to be established, as siRNA-mediated silencing of Rab33B had little effect on autophagosome formation (Itoh et al., 2008). The brain-specific Rab33A isoform also interacts with Aut16L1 and appears to use the Aut5/12/16L1 complex as an effector (Ishibashi et al., 2012). In neuroendocrine PC12 cells, Aut16L1 is localized on hormone-containing dense-core vesicles by interaction with Rab33A. Knockdown of Aut16L1 in these cells caused marked inhibition of hormone secretion independently of autophagic activity (Ishibashi et al., 2012), implicating that Rab33 family members and their effectors are also able to regulate certain secretory events. In support, we showed herein that Rab33B is involved in the unconventional secretion of HBV naked capsids.

During this process, Rab33B likely acts in conjunction with its effector, the Aut5/12/16L1 complex, as the knockdowns of Aut5 or Aut12 also impaired capsid release. As expected from the covalent and constitutive conjugation of Aut12 to Aut5, either knockdown reduced the levels of the Aut5/12 conjugate. Upon Aut12 depletion, unconjugated Aut5 survived without enabling capsid release. Hence, the capsid maturation pathway likely depends on Aut12-conjugated Aut5. Despite the effective depletion of Aut16L1, its loss evoked only moderate reductions in intra- and extracellular capsid levels. To account for this, Aut16L1 might not contribute to capsid maturation per se; rather, the observed effects might be due to the partial codepletion of the Aut5/12 conjugate in Aut16L1-silenced cells. Alternatively, Aut5/12 might not pair with Aut16L1 during capsid maturation but rather with the Aut16L2 isoform that is not targeted by the siRNAs applied herein. Such an Aut5/12/16L2 complex had been demonstrated in vivo, but shown to be dispensable for canonical autophagy (Ishibashi et al., 2011). At first
glance, a possible dispensability of Atg16L1 challenges the linkage between autophagic factors and Rab33B in the HBV capsid pathway. However, a recent proteomic analysis of the autophagy interaction network using mass spectrometry revealed that not only Atg16L1 but also Atg5 interacts with Rab33B (Behrends et al., 2010).

The Atg5/12/16L1 complex initiates the formation of isolation membranes and facilitates the lipidation and membrane conjugation of Atg8 family members that in turn support membrane expansion and perpetuate the autophagic process (Geng and Klionsky, 2008; Romanov et al., 2012; Walczak and Martens, 2013). In humans, there are two families of Atg8 orthologues, the LC3s (LC3A, LC3B, LC3C) and GABARAPs (GABARAP, GABARAPL1, GABARAPL2/GATE-16), that operate at different steps whereupon LC3s mediate elongation of the autophagic membrane and GABARAPs participate in downstream steps concomitant with the dissociation of the Atg5/12/16L1 complex (Geng and Klionsky, 2008; Shpilka et al., 2011). Here, we found that none of the LC3s is required for capsid export implicating that neither Atg5/12/16L1-mediated LC3 membrane conjugation nor consecutive maturation steps of the autophagosomes are essential. Consistent with this, ablation of conventional autophagy with chemical compounds had no effect on capsid release. We thus deduce that the HBV core protein exploits only portions of the autophagy machinery, i.e. the Atg5/12/16L1 complex, possibly in conjunction with autophagy-related membranous structures, to exit cells.

By analysing the effects of deficit Atg5/12/16L1 and Rab33B on core/capsid formation, we found that they do not interfere with the expression and stability profile of core. Rather, they appear to direct steps involved in the assembly and/or disassembly reaction of capsids. Hepatitis B virus capsid assembly requires threshold levels of core proteins/dimers (Bruss, 2007; Selzer et al., 2014) and involves their multimerization in the crowded cytoplasm that is an unfavourable environment for homotypic interactions. Similar to other viruses, HBV appears to face this problem by directing capsid assembly to membranes in order to raise the local concentration of core dimers. When HBV capsid assembly was studied in wheat germ extracts, it was found to be detergent-sensitive, implying that an intact membrane surface is required for capsid formation in the cell-free system (Lingappa et al., 2005). Moreover, the core proteins of HBV and the related duck HBV have been shown to contain intrinsic membrane targeting signals that act before or during capsid assembly (Mabit and Schaller, 2000; Bardens et al., 2011). Given that the Atg5/12/16L1 complex as well as Rab33B efficiently bind to membranes (Itoh et al., 2008; Romanov et al., 2012; Kaufmann et al., 2014), it is thus tempting to speculate that they provide a scaffold for HBV capsid assembly and stable particle architecture. Thereby, the physical interaction between core and Atg5 might strengthen the pre-assembly complex. Reminiscent to this, positive-stranded RNA viruses, including poliovirus and corona viruses, use double-membrane vesicles, resembling autophagosomes, as viral replication centres along with the exploitation of some autophagy proteins in a non-degradative manner (Prentice et al., 2004; Jackson et al., 2005; Maier and Britton, 2012). Aside, the necessity of Atg5/12/16L1 and Rab33B in the egress of the HBV capsid might be related to its unconventional export in a naked state. Similar to a process previously described as autophagosome-mediated exit without lysis (AWOL) observed during replication of the non-enveloped poliovirus (Taylor et al., 2009), HBV capsid trafficking might involve an intracellular autophagosome-like vesicular intermediate in order to be delivered to the extracellular milieu.

Recent studies have shown that HBV enhances and uses autophagy for its replication (Sir et al., 2010; Li et al., 2011; Tian et al., 2011), but the underlying mechanisms are a matter of controversy. One set of experiments indicates that the viral regulatory X protein is essential for autophagy induction (Sir et al., 2010; Tian et al., 2011), while the other identified the small envelope protein as an inducer (Li et al., 2011). However, consistent with our results, neither study provided evidence for a role of the core protein in autophagy induction. The step(s) of HBV replication affected by autophagy are also a matter of debate. Upon pharmaceutical or genetic inhibition of autophagy, HBV reverse transcription within the viral capsid was found to be impaired in HBV transgenic mice (Tian et al., 2011), whereas in HBV-replicating cell lines, the envelopment of nucleocapsids was primarily inhibited (Li et al., 2011). To reconcile the proposed different roles of autophagy for the HBV replication cycle, further studies are needed. Our preliminary studies implicate that Rab33B together with its Atg5/12/16L1 effector is involved in HBV nucleocapsid formation (Jens Stieler and Reinhold Prange, unpubl. obs.).

Previously, we identified Alix as a regulator of HBV capsid budding, as it stimulates the release of capsids in a dose-dependent manner (Bardens et al., 2011). This stimulating activity got lost in Rab33B knockdown cells, implicating that Alix might act downstream of Rab33B. By probing for possible intersections between Rab33B, Atg5/12/16L1 and Alix, we surprisingly found that overexpressed Alix modulates autophagy proteins and autophagic structures with LC3 being a prominent target. Although one previous report failed to provide hints for a role of Alix in autophagy (Petiot et al., 2008), a more recent proteomic analysis showed that Alix (PDCD6IP) belongs to a cohort of 67 LC3-interacting proteins (Behrends et al., 2010). In vitro pull-down assays,
however, did not reveal a direct binding between LC3 and Alix, indicative for an indirect interaction mode. In agreement, our immunofluorescence analyses did not demonstrate a significant colocalization between Alix and its induced LC3-positive structures. Unlike typical LC3-positive autophagosomes (i.e. puncta), these structures had a clustered appearance and were triggered by excess Alix irrespective of basal, induced or repressed autophagy. To account for the paradox that core neither requires LC3 nor colocalizes with the Alix-induced LC3-positive structures, but is fostered by Alix, we speculate that excess Alix might dysregulate normal autophagy. Thereby, the availability of the Atg5/12/16L1 complex might increase that thence could accelerate capsid maturation and egress. As supporting evidence, we found a close correlation between the capsid release-promoting activity of Alix mutants/derivatives and their capacities to induce LC3-positive clusters.

Overall, the above findings identify Rab33B-Atg5/12/16L1 in conjunction with Alix as important regulators of the HBV capsid assembly pathway. Likewise, they provide further clues for viral exploitation of the autophagy machinery and for non-autophagic functions of autophagy proteins.

**Experimental procedures**

**Plasmids**

The vector pNL2.3 (pCore) contains the HBV core gene [nucleotide (nt) 1905 to 2454; numbering as referred to the plus strand of the HBV DNA genome, genotype D; GenBank™ accession number J02203] under the control of the hMTIIa promoter. At the 3′-end, the core gene is fused to nt 827 to 1986 of the HBV genome carrying the posttranscriptional regulatory element and the polyA signal. This region also contains the HBV X gene. However, as measured by qRT-PCR, pCore did not direct transcription and hence synthesis of X at detectable levels. However, as measured by qRT-PCR, pCore did not direct transcription and hence synthesis of X at detectable levels. pDPAF.Core is identical to pCore save for a tetrapeptide epitope (DPAF) fused to the N-terminus of core as described (Bardens et al., 2011). A cDNA clone encoding human Brox (CloneID 8322687) was purchased from Open Biosystems and the Brox gene was cloned into plasmid p3xFLAG.CMV-10 (Sigma-Aldrich) to generate an N-terminal FLAG-tagged version. Plasmid pCl-neo-hApg5-HA carries the human ATG5 gene with a C-terminal HA-tag and was provided by N. Mizushima (University of Tokyo, Japan) via Addgene (plasmid #22948; Mizushima et al., 1998). All constructs were verified by sequencing and cloning details are available on request.

**Antibodies**

Polyclonal antisera against recombinant native (K45) or denatured (K46) HBV core particles were raised in rabbits, as described (Rost et al., 2006). To detect DPAF-tagged core, the MA18/7 mouse antibody was used (a gift from K.-H. Heermann, University of Göttingen, Germany; herein termed DPAF antibody). Commercially available antibodies were as follows: mouse anti-p62 (Abcam); mouse anti-GFP (BD Biosciences); rabbit anti-Atg5, rabbit anti-Atg12, rabbit anti-LC3A/B (used for immunostaining; Cell Signaling); mouse anti-HA (Covance); mouse anti-β-actin, mouse anti-α-tubulin, mouse anti-FLAG, mouse anti-Rab27A, rabbit anti-LC3A/B (used for immunoblotting) and mouse anti-Rab33B (Sigma-Aldrich). Peroxidase-labelled, secondary antibodies were obtained from Dianova, and fluorophore-labelled antibodies were purchased from Molecular Probes.

**siRNAs, cell culture and transfection**

The human hepatocellular carcinoma cell line HuH-7 was obtained from the Japanese Collection of Research Bioresources. Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 5 μg ml⁻¹ ciprofloxacin, and 1 mM sodium pyruvate. Transfections of HuH-7 cells with plasmid DNAs were performed with Lipofectamine™ Plus (Invitrogen). The amounts of plasmid DNA used in cotransfection experiments are indicated in the figure legends. For transfection of HuH-7 cells with siRNAs plus plasmid DNA, the Lipofectamine™ RNAiMAX transfection reagent (Invitrogen) was used. Briefly, 5 x 10⁶ cells per well of a 6-well plate were transfected with 90 pmol siRNA according to the protocol of the supplier. After 48 to 72 h, cells were retransfected with plasmid DNA using Lipofectamine™ Plus and harvested after additional 48 h. siRNA oligo sequences are provided in Supporting Information Table S1. As a control, a nonsense siRNA with no known homology to mammalian genes was used (Qiagen). For drug treatment, cells were incubated with Earle’s Balanced Salt Solution (Life Technologies) supplemented with 20 mM NH4Cl, 10 mM 3-MA and/or 10 nM rapamycin (Sigma-Aldrich) for time periods indicated in the figure legends.
Cell lysis and VLP analysis

To probe for protein expression, cells were lysed with either the non-denaturing detergent Nonidet P-40 (NP-40) or the denaturing reagent sodium dodecylsulfate (SDS). NP-40 lysates were prepared by incubating the cells with Tris-buffered saline (TBS, 50 mM Tris-HCl pH 7.5/150 mM NaCl) containing 0.5% NP-40 for 20 min on ice. Thereafter, lysates were centrifuged for 5 min at 15 000×g and 4°C. For lysis with SDS, the cells were scraped from the plates using 1× Laemmli buffer, and cell suspensions were boiled for 10 min prior to centrifugation. To analyse the assembly and release of capsid particles from transfected cells, clarified culture medium was concentrated by ultracentrifugation through a 20% (w/v) sucrose cushion (4 h at 100 000×g and 4°C). Pellets were suspended in 1× Laemmli buffer. Unless otherwise indicated, 25% of cell lysates and 100% of supernatants were applied in Western blots using standard procedures. In addition, the amounts of extracellular capsids were measured by an HBV precore/core-specific ELISA (DiaSorin).

qRT-PCR analysis

Total mRNAs were isolated from cells using the TRIzol reagent (Life Technologies) according to the protocols of the supplier. The mRNA was treated with 5 U RNase-free, recombinant DNase I (Roche Diagnostics), and cDNA synthesis was performed by using the Transcripter Universal cDNA Master Kit (Roche Diagnostics). For RT-PCR, each reaction mixture (20 μl) contained 5 μl cDNA template, 1 μl forward primer (10 μM), 1 μl reverse primer (10 μM), 10 μl Fast Start Universal SYBR Green Probe Master (Roche Diagnostics) and 3 μl aqua bidest. Polymerase chain reaction analyses were performed with a 7300 Real-Time PCR System and Sequence Detection Software 4.0 (Applied Biosystems). For data analysis, the comparative cycle threshold method was used. Specific primer pairs were retrieved from PrimerBank (http://pga.mgh.harvard.edu/primerbank/), and sequences are available on request.

Polyethylene glycol (PEG) precipitation and Western blotting of cytoplasmic capsids

For concentration of intracellular capsids, they were precipitated with PEG as described (Bardens et al., 2011). Precipitates were then separated by native 1% (w/v) agarose-Tris-acetate-EDTA gel electrophoresis. The gel was blotted by capillary transfer using a nitrocellulose membrane and 10× SSC buffer (1.5 M NaCl/150 mM sodium citrate pH 7.0). The membrane was reacted with anti-capsid antibodies (K45) using standard techniques.

Preparation of particulate and soluble protein fractions

Cells were incubated with 0.1× TBS for 10 min on ice, and swollen cells were disrupted by dounce homogenization and adjusted to 1× TBS. After centrifugation for 20 min at 2500×g and 4°C, the postnuclear supernatant was layered on 250 mM sucrose/TBS and ultracentrifuged for 45 min at 160 000×g and 4°C in a SW60 rotor (Beckman) to separate particulate and soluble fractions. Proteins in the soluble fraction were precipitated with 10% trichloroacetic acid. The precipitates and pelleted particulate fraction were adjusted to the same volume sample buffer and analysed by SDS-PAGE.

Fluorescence microscopy

For immunostaining, cells grown on coverslips were fixed and permeabilized with ice-cold methanol containing 2 mM EGTA. Cells were blocked in phosphate-buffered saline (PBS) containing 2% animal serum, incubated with the indicated primary antibodies for 1 h at 37°C, rinsed with PBS, and then incubated with AlexaFluor-tagged secondary antibodies for 1 h at 37°C. DNA was stained with Hoechst 33342 (Sigma-Aldrich). Z-stack images were acquired separately for each channel using a Zeiss Axiosvert 200 M microscope equipped with a Plan-Apochromat 100× (1.4 NA) and a Zeiss Axiocam digital camera. Axiovision software 4.7.1 was used for merging pictures. Where indicated, Z-stack images were optically deconvoluted using the software supplied by Zeiss. Tiffs were assembled into figures using Photoshop CS2 (Adobe).

Co-immunoprecipitation assay

To probe for complex formation, cells were lysed with a 2% solution of the non-denaturating detergent CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate)-HBS (50 mM HEPES-KCl pH 7.5/200 mM NaCl), 20 mM N-ethylmaleimide, supplemented with 1× protease inhibitor mixture (Serva) for 20 min on ice. After centrifugation, lysates were immediately subjected to immunoprecipitation using tosyl-activated, superparamagnetic polystyrene beads (Dynabeads Sheep anti-rabbit IgG; DYNAL) that had been precocated with the anti-core antibody K46 as described (Rost et al., 2006). After incubation for 3 h at 4°C with agitation, the immune complexes were washed three times with 0.5% CHAPS/HBS, and once with PBS prior to SDS-PAGE and immunoblotting.

Acknowledgements

We thank M. Fukuda, S. Gould, K.-H. Heermann, N. Mizushima, W. Mothes, N. Sagaguchi and G. Spoden for generously providing plasmid DNA constructs and antibodies. We are grateful to J. Stieler and K. Gotthardt for experimental support and helpful discussion. This work was supported by grants to R.P. from the Deutsche Forschungsgemeinschaft (SFB 490-D1, PR 305/3-1).

References

Alexander, C.G., Jurgens, M.C., Shepherd, D.A., Freund, S.M., Ashcroft, A.E., and Ferguson, N. (2013) Thermodynamic origins of protein folding, allostery, and capsid formation in the human hepatitis B virus core protein. Proc Natl Acad Sci USA 110: E2782–E2791.

Baetle, M.F., Zhang, Z., Mortier, E., Melchior, A., Degeest, G., Geeraerts, A., et al. (2012) Syndecan-syntenin-ALIX regulates the biogenesis of exosomes. Nat Cell Biol 14: 677–685.

Bardens, A., Doring, T., Stieler, J., and Prange, R. (2011) Alix regulates egress of hepatitis B virus naked capsid particles in an ESCRT-independent manner. Cell Microbiol 13: 602–619.
HBV capsid biogenesis depends on Rab33B and Atg5/12/16L1

Behrends, C., Sowa, M.E., Gygi, S.P., and Harper, J.W. (2010) Network organization of the human autophagy system. Nature 466: 68–76.

Bento, C.F., Puri, C., Moreau, K., and Rubinsztein, D.C. (2013) The role of membrane-trafficking small GTPases in the regulation of autophagy. J Cell Sci 126: 1059–1069.

Beterams, G., and Nassal, M. (2001) Significant interference with hepatitis B virus replication by a core-nuclease fusion protein. J Biol Chem 276: 8875–8883.

Bhun, T., and Roy, J.K. (2014) Rab proteins: the key regulators of intracellular vesicle transport. Exp Cell Res 328: 1–19.

Blanchard, E., and Roingeard, P. (2015) Virus-induced double-membrane vesicles. Cell Microbiol 17: 45–50.

Bourne, C.R., Katen, S.P., Fulz, M.R., Packianathan, C., and Zlotnick, A. (2009) A mutant hepatitis B virus core protein mimics inhibitors of icosahedral capsid self-assembly. Biochemistry 48: 1736–1742.

Bruce, E.A., Stuart, A., McCaffrey, M.W., and Digard, P. (2012) Role of the Rab11 pathway in negative-strand virus assembly. Biochim Soc Trans 40: 1409–1415.

Bruss, V. (2007) Hepatitis B virus morphogenesis. World J Gastroenterol 13: 65–73.

Chua, C.E., Gan, B.Q., and Tang, B.L. (2011) Involvement of members of the Rab family and related small GTPases in autophagosome formation and maturation. Cell Mol Life Sci 68: 3349–3358.

Dong, X., and Levine, B. (2013) Autophagy and viruses: adversaries or allies? J Innate Immun 5: 480–493.

Dreux, M., and Chisari, F.V. (2010) Viruses and the autophagy machinery. Cell Cycle 9: 1295–1307.

Fang, Y., Wu, N., Gan, X., Yan, W., Morrell, J.C., and Gould, S.J. (2007) Higher-order oligomerization targets plasma membrane proteins and HIV gag to exosomes. PLoS Biol 5: e158.

Fraile-Ramos, A., Cepeda, V., Elstak, E., and van der Sluijs, P. (2010) Rab27a is required for human cytomegalovirus assembly. PLoS ONE 5: e15318.

Geng, J., and Klionsky, D.J. (2008) The Atg8 and Atg12 ubiquitin-like conjugation systems in macroautophagy. ‘Protein modifications: beyond the usual suspects’ review series. EMBO Rep 9: 859–864.

Hermida-Matsumoto, L., and Resh, M.D. (2000) Localization of human immunodeficiency virus type 1 Gag and Env at the plasma membrane by confocal imaging. J Virol 74: 8670–8679.

Ichikawa, K., Kobayashi, R., Katoh, K., Shibata, H., and Maki, M. (2008) Brox, a novel farnesylated Bro1 domain-containing protein that associates with charged multivesicular body protein 4 (CHMP4). FEBS J 275: 682–692.

Ishibashi, K., Fujita, N., Kanno, E., Omori, H., Yoshimori, T., Itoh, T., and Fukuda, M. (2011) Atg16L2, a novel isoform of mammalian Atg16L that is not essential for canonical autophagy despite forming an Atg12-5-16L2 complex. Autophagy 7: 1500–1513.

Ishibashi, K., Uemura, T., Waguiri, S., and Fukuda, M. (2012) Atg16L1, an essential factor for canonical autophagy, participates in hormone secretion from PC12 cells independently of autophagic activity. Mol Biol Cell 23: 3193–3202.

Itoh, T., Fujita, N., Kanno, E., Yamamoto, A., Yoshimori, T., and Fukuda, M. (2008) Golgi-resident small GTPase Rab33B interacts with Atg16L and modulates autophagosome formation. Mol Biol Cell 19: 2916–2925.

Jackson, W.T., Giddings, T.H., Jr, Taylor, M.P., Mulinyawe, S., Rabinovitch, M., Kopito, R.R., and Kirkegaard, K. (2005) Subversion of cellular autophagosomal machinery by RNA viruses. PLoS Biol 3: e156.

Kauffmann, A., Beier, V., Franquielim, H.G., and Wollert, T. (2014) Molecular mechanism of autophage membrane-scaffold assembly and disassembly. Cell 156: 469–481.

Kian Chua, P., Lin, M.H., and Shih, C. (2006) Potent inhibition of human Hepatitis B virus replication by a host factor Vps4. Virology 354: 1–6.

Lambert, C., Doring, T., and Prange, R. (2007) Hepatitis B virus maturation is sensitive to functional inhibition of ESCRT-III, Vps4, and gamma 2-adaptin. J Virol 81: 9050–906.0.

Li, J., Liu, Y., Wang, Z., Liu, K., Wang, Y., Liu, J., et al. (2011) Subversion of cellular autophagy machinery by hepatitis B virus for viral envelopment. J Virol 85: 6319–6333.

Lingappa, J.R., Newman, M.A., Klein, K.C., and Dooher, J.E. (2005) Comparing capsid assembly of primate lentiviruses and hepatitis B virus using cell-free systems. Virology 333: 114–123.

Mabit, H., and Schaller, H. (2000) Intracellular hepadnavirus nucleocapsids are selected for secretion by envelope protein-independent membrane binding. J Virol 74: 11472–11478.

Macovei, A., Petreaanu, C., Lazar, C., Florian, P., and Branza-Nichita, N. (2013) Regulation of hepatitis B virus infection by Rab5, Rab7, and the endolysosomal compartment. J Virol 87: 6415–6427.

Maier, H.J., and Britton, P. (2012) Involvement of autophagy in coronavirus replication. Viruses 4: 3440–3451.

Milich, D.R., and McLachlan, A. (1986) The nucleocapsid of hepatitis B virus is both a T-cell-dependent and a T-cell-independent antigen. Science 234: 1398–1401.

Milich, D.R., Chen, M., Scho.del, F., Peterson, D.L., Jones, J.E., and Hughes, J.L. (1997) Role of B cells in antigen presentation of the hepatitis B core. Proc Natl Acad Sci USA 94: 14648–14653.

Mizushima, N., Sugita, H., Yoshimori, T., and Ohsumi, Y. (1998) A new protein conjugation system in human. The counterpart of the yeast Apg12p conjugation system essential for autophagy. J Biol Chem 273: 33889–33892.

Mizushima, N., Yoshimori, T., and Levine, B. (2010) Methods in mammalian autophagy research. Cell 140: 313–326.

Nassal, M. (2008) Hepatitis B viruses: reverse transcription a different way. Virus Res 134: 235–249.

Odoi.rizzi, G. (2006) The multiple personalities of Alix. J Cell Sci 119: 3025–3032.

Ostrowski, M., Carmo, N.B., Krumeich, S., Fangelt, I., Raposo, G., Savina, A., et al. (2010) Rab27a and Rab27b control different steps of the exosome secretion pathway. Nat Cell Biol 12 (Suppl.): 11–13.

Patient, R., Hourioux, C., and Roingeard, P. (2009) Morphogenesis of hepatitis B virus and its subviral envelope particles. Cell Microbiol 11: 1561–1570.

© 2014 John Wiley & Sons Ltd, Cellular Microbiology, 17, 747–764
Petiot, A., Strappazzon, F., Chatellard-Causse, C., Blot, B., Torch, S., Verna, J.M., and Sadoul, R. (2008) Alix differs from ESCRT proteins in the control of autophagy. *Biochem Biophys Res Commun* **375**: 63–68.

Possehl, C., Repp, R., Heermann, K.H., Korec, E., Uy, A., and Gerlich, W.H. (1992) Absence of free core antigen in anti-Hbc negative viremic hepatitis B carriers. *Arch Virol Suppl* **4**: 39–41.

Prange, R. (2012) Host factors involved in hepatitis B virus maturation, assembly, and egress. *Med Microbiol Immunol (Berl)* **201**: 449–461.

Prange, R. (2012) Host factors involved in hepatitis B virus maturation, assembly, and egress. *Med Microbiol Immunol (Berl)* **201**: 449–461.

Prentice, E., Jerome, W.G., Yoshimori, T., Mizushima, N., and Denison, M.R. (2004) Coronavirus replication complex formation utilizes components of cellular autophagy. *J Biol Chem* **279**: 10136–10141.

Romanov, J., Walczak, M., Ibiricu, I., Schuchner, S., Ogris, E., Kraft, C., and Martens, S. (2012) Mechanism and functions of membrane binding by the Atg5-Atg12/Atg16 complex during autophagosome formation. *EMBO J* **31**: 4304–4317.

Rost, M., Mann, S., Lambert, C., Doring, T., Thome, N., and Prange, R. (2006) Gamma-adaptin, a novel ubiquitin-interacting adaptor, and Nedd4 ubiquitin ligase control hepatitis B virus maturation. *J Biol Chem* **281**: 29297–29308.

Sakaguchi, T., Kato, A., Sugahara, F., Shimazu, Y., Inoue, M., Kiyotani, K., et al. (2005) AIP1/Alix is a binding partner of Sendai virus C protein and facilitates virus budding. *J Virol* **79**: 8933–8941.

Salloum, S., Wang, H., Ferguson, C., Parton, R.G., and Tai, A.W. (2013) Rab18 binds to hepatitis C virus NS5A and promotes interaction between sites of viral replication and lipid droplets. *PLoS Pathog* **9**: e1003513.

Selzer, L., Katen, S.P., and Zlotnick, A. (2014) The hepatitis B virus core protein intradimer interface modulates capsid assembly and stability. *Biochemistry* **53**: 5496–5504.

Sherrer, N.M., Lehmann, M.J., Jimenez-Soto, L.F., Ingmundson, A., Horner, S.M., Cicchetti, G., et al. (2003) Visualization of retroviral replication in living cells reveals budding into multivesicular bodies. *Traffic* **4**: 785–801.

Shplika, T., Weidberg, H., Pietrokovski, S., and Elazar, Z. (2011) Atg8: an autophagy-related ubiquitin-like protein family. *Genome Biol* **12**: 226 (27 July 2011).

Sir, D., Tian, Y., Chen, W.L., Ann, D.K., Yen, T.S., and Ou, J.H. (2010) The early autophagic pathway is activated by hepatitis B virus and required for viral DNA replication. *Proc Natl Acad Sci USA* **107**: 4383–4388.

Stenmark, H. (2009) Rab GTPases as coordinators of vesicle traffic. *Nat Rev Mol Cell Biol* **10**: 513–525.

Stieler, J.T., and Prange, R. (2014) Involvement of ESCRT-II in hepatitis B virus morphogenesis. *PLoS ONE* **9**: e91279.

Taylor, M.P., Burgon, T.B., Kirkegaard, K., and Jackson, W.T. (2009) Role of microtubules in extracellular release of poliovirus. *J Virol* **83**: 6599–6609.

Tian, Y., Sir, D., Kuo, C.F., Ann, D.K., and Ou, J.H. (2011) Autophagy required for hepatitis B virus replication in transgenic mice. *J Virol* **85**: 13453–13456.

Votteler, J., and Sundquist, W.I. (2013) Virus budding and the ESCRT pathway. *Cell Host Microbe* **14**: 232–241.

Walczak, M., and Martens, S. (2013) Dissecting the role of the Atg12-Atg5-Atg16 complex during autophagosome formation. *Autophagy* **9**: 424–425.

Watanabe, T., Sorensen, E.M., Naito, A., Schott, M., Kim, S., and Ahlquist, P. (2007) Involvement of host cellular multivesicular body functions in hepatitis B virus budding. *Proc Natl Acad Sci USA* **104**: 10205–10210.

Weidberg, H., Shvets, E., and Elazar, Z. (2011) Biogenesis and cargo selectivity of autophagosomes. *Annu Rev Biochem* **80**: 125–156.

Wittkop, L., Schwarz, A., Cassany, A., Grun-Bernhard, S., Delaleau, M., Rabe, B., et al. (2010) Inhibition of protein kinase C phosphorylation of hepatitis B virus capsids inhibits virion formation and causes intracellular capsid accumulation. *Cell Microbiol* **12**: 962–975.

Zheng, J.Y., Koda, T., Fujiwara, T., Kishi, M., Ikehara, Y., and Kakinuma, M. (1998) A novel Rab GTPase, Rab33B, is ubiquitously expressed and localized to the medial Golgi cisternae. *J Cell Sci* **111**: 1061–1069.

**Supporting information**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Table S1.** Sequences of siRNA constructs used in this study.