Quantitative Lipid Droplet Proteome Analysis Identifies Annexin A3 as a Cofactor for HCV Particle Production

Graphical Abstract

Highlights
- Quantitative lipid droplet proteome analysis of HCV-infected cells is presented
- ANXA3 is recruited to lipid-rich fractions in HCV-infected cells
- ANXA3 functions as a host factor required for efficient HCV particle production
- ANXA3 promotes virion maturation by facilitating incorporation of apolipoprotein E

In Brief
Cytoplasmic lipid droplets are vital to hepatitis C virus particle production. Rösch et al. report a quantitative lipid droplet proteome analysis and identify annexin A3 as a regulator of HCV particle maturation and egress.

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Quantitative Lipid Droplet Proteome Analysis Identifies Annexin A3 as a Cofactor for HCV Particle Production

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SUMMARY

Lipid droplets are vital to hepatitis C virus (HCV) infection as the putative sites of virion assembly, but morphogenesis and egress of virions remain ill defined. We performed quantitative lipid droplet proteome analysis of HCV-infected cells to identify co-factors of that process. Our results demonstrate that HCV disconnects lipid droplets from their metabolic function. Annexin A3 (ANXA3), a protein enriched in lipid droplet fractions, strongly impacted HCV replication and was characterized further: ANXA3 is recruited to lipid-rich fractions in HCV-infected cells by the viral core and NS5A proteins. ANXA3 knockdown does not affect HCV RNA replication but severely impairs virion production with lower specific infectivity and higher density of secreted virions. ANXA3 is essential for the interaction of viral envelope E2 with apolipoprotein E (ApoE) and for trafficking, but not lipidation, of ApoE in HCV-infected cells. Thus, we identified ANXA3 as a regulator of HCV maturation and egress.

INTRODUCTION

Hepatitis C virus (HCV) infection is one of the leading causes of liver-related morbidity and mortality worldwide, accounting for approximately 0.5 million deaths every year (Wedemeyer et al., 2015). The true number of HCV infections is unknown, but recent estimates suggest 80 (64–103) million viraemic HCV infections, and the disease burden will likely continue to rise in most countries (Gower et al., 2014). No vaccine exists, but the recently developed direct-acting antivirals dramatically increase therapeutic responses compared to the standard interferon-based therapy. However, worldwide, the treatment of patients will likely be restricted due to the extremely high costs of the new therapeutics.

HCV belongs to the family of Flaviviridae. The enveloped viral particles contain a single positive-stranded RNA genome of 9.6 kb in length and are associated with lipoproteins and neutral lipids and accordingly named lipoviroparticles. After receptor-mediated endocytosis, fusion, and uncoating, the viral genome is translated into one polyprotein precursor (reviewed in Lindenbach and Rice, 2005). Host and viral proteases process the viral polyprotein, releasing the three structural proteins (nucleocapsid core, E1, and E2), the viroporin p7, and six non-structural (NS) proteins (NS2, 3, 4A, 4B, 5A, and 5B). Multi-protein RNA replication complexes containing minimally NS3–5B proteins replicate the viral RNA within ER (endoplasmic reticulum)-derived structures termed the membranous web. The membranous web contains single-, double-, and multi-membrane vesicles (Romero-Brey et al., 2012), as well as cytosolic lipid droplets that may serve as viral assembly sites (reviewed in Lindenbach and Rice, 2013). After encapsidation of newly synthesized viral RNA at ER membranes near lipid droplets, the virus is thought to exit the cell via the secretory pathway, thereby maturing to low-density lipoviroparticles (reviewed in Lindenbach and Rice, 2013).

In the absence of full viral replication, only two viral proteins localize to lipid droplets, core and NS5A (Barba et al., 1997; Shi et al., 2002). All other viral proteins are found in close proximity to lipid droplets in infected cells (Miyanari et al., 2007), but they lack intrinsic lipid-droplet-targeting features, as they fail to localize to lipid droplets when expressed individually (Camus et al., 2013). Translocation of both core and NS5A to lipid droplets requires triglyceride biosynthesis as inhibitors of diacylglycerol acyltransferase-1 (DGAT1) impair trafficking to lipid droplets and subsequent HCV assembly (Camus et al., 2013; Herker et al., 2010). Proper processing of core is a prerequisite for core’s ability to traffic to lipid droplets (Targett-Adams et al., 2008), and mutations in either core or NS5A that disrupt lipid droplet binding suppress HCV assembly (Boulant et al., 2007; Miyanari et al., 2007). Trafficking of core to lipid droplets additionally requires cytosolic phospholipase A2 activity (Menzel et al., 2012), and lipid-droplet-binding proteins act as host factors with, e.g., PLIN3/TIP47 required for both HCV RNA replication and release of virions (Ploen et al., 2013a, 2013b; Vogt...
Despite recent advances, the mechanistic details of the late stages of HCV replication are still ill defined.

Here, we performed an extensive quantitative lipid-droplet proteome analysis of HCV-infected cells to identify host factors for HCV particle production. One of the highly enriched proteins was annexin A3 (ANXA3), a member of the annexin family of calcium-dependent, phospholipid-binding proteins. All annexins show cytosolic and membrane localizations, and some including ANXA3 are also secreted. They are involved in endo- and exocytosis and trafficking, and they serve as membrane scaffolds organizing specific membrane microdomains (reviewed in Gerke et al., 2005). Membrane recruitment is most likely regulated by calcium influx, and, depending on the phospholipid-binding specificity, annexins may target different cellular membranes (reviewed in Gerke et al., 2005; Gerke and Moss, 2002). We found that ANXA3 is specifically recruited to lipid-rich fractions in cells infected with HCV and that it participates in HCV maturation and release. Thus, by performing quantitative lipid droplet proteomics, we revealed that HCV disconnects lipid droplets from their metabolic function and identified ANXA3 as a host factor for HCV replication.

**RESULTS**

**HCV Infection Profoundly Changes the Lipid Droplet Proteome**

To identify regulators of HCV replication, we performed quantitative lipid droplet proteome analysis of HCV-infected versus uninfected control cells, using stable isotope labeling by amino acids in cell culture (SILAC) (Figure 1A). We labeled HCV-permissive Huh7.5 cells with heavy amino acids for at least six passages until near-complete (>95%) incorporation of the heavy amino acids into the cellular proteins was achieved. Cells cultured in media containing the normal light amino acids were transfected with lentiviral stocks expressing the indicated shRNAs, followed by infection with HCV Jc1 NS5AB-EGFP. 6 days post-infection, cells were fixed and analyzed by flow cytometry of EGFP to measure HCV infection rates. Shown is the relative infection normalized to the non-targeting shRNA (shNT) control (mean ± SD, n = 2). Red indicates proteins that are enriched at lipid droplets.

See also Figure S1 and Tables S1, S2, S3, S4, S5, S6, and S7.

Figure 1. Quantitative Lipid Droplet Proteome Analysis of HCV-Infected versus Uninfected Control Cells

(A) Huh7.5 cells cultured in media containing either heavy or light amino acids were infected with an HCV reporter virus. Lipid droplets were isolated by two sequential sucrose gradient centrifugations, washed, and subjected to LC-ESI-MS/MS.

(B) Western blot of the lipid droplet (LD) fraction shows enrichment of the lipid-droplet-binding proteins PLIN2/ADRP and PLIN3/TIP47. Other subcellular compartment markers as calreticulin (ER), MnSOD (mitochondria), or β-tubulin were undetectable in lipid droplet fractions.

(C) Coomassie-stained SDS-PAGE of a purified lipid droplet (LD) fraction (M, molecular weight marker).

(D) Heatmap depicting enriched or depleted proteins (1.5-fold cutoff). "p < 0.05; **p < 0.01; ***p < 0.001.

(F) Protein interaction networks of dysregulated lipid-droplet-associated proteins. Shown is the interaction network of significantly enriched (red) or depleted (blue) proteins visualized using the cisPath package in R.

(G) Huh7.5 cells were transduced with lentiviral stocks expressing the indicated shRNAs, followed by infection with HCV Jc1 NS5AB-EGFP. 6 days post-infection, cells were fixed and analyzed by flow cytometry of EGFP to measure HCV infection rates. Shown is the relative infection normalized to the non-targeting shRNA (shNT) control (mean ± SD, n = 2). Red indicates proteins that are enriched at lipid droplets.

See also Figure S1 and Tables S1, S2, S3, S4, S5, S6, and S7.
followed by a blasticidin resistance gene (blasticidin S deaminase [BSD]) in between a duplicated NS5A-NS5B cleavage site described previously (Webster et al., 2013). After 2 to 3 weeks, HCV-infected cells that displayed infection rates higher than 95% were used for the experiments. Of note, we used selected HCV-infected cells in half of the experiments. One day prior to lipid droplet isolation, HCV-infected and heavy-amino-acid-labeled control cells were plated at equal cell densities. For lipid droplet isolation, 5 × 10^7 cells were mixed and lysed in hypotonic lysis buffer with a Dounce homogenizer. Lipid droplets were isolated by two sequential sucrose density centrifugations of post-nuclear supernatant, followed by three washing steps in isotonic buffer. The isolated lipid droplet fractions were subsequently analyzed by western blotting to confirm enrichment of lipid-droplet-binding proteins (PLIN2/ADRP and PLIN3/TIP47) (Bickel et al., 2009) and depletion of markers of other cellular compartments (Figure 1B). SDS-PAGE followed by Coomassie staining was used to separate the lipid droplet fractions (Figure 1C), and after trypptic digest, peptides were analyzed by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). As a control, we switched labeling conditions in one experiment, thus culturing the HCV-infected cells with heavy amino acids to exclude isotope-specific effects.

In four independent experiments (on two different MS platforms), we identified around 1,500 proteins, of which 316 were detected through multiple peptides in each experiment and selected for further analysis (datasets are listed in Tables S1, S2, S3, and S4).

To correct for different cell numbers or numbers of lipid droplets, we next centered the detection ratios of light over heavy peptides (L/H) or vice versa in swapped labeling conditions (h/L) by dividing through the median of the identified proteins (Table S5). Correlation analysis over all identified proteins revealed reproducible protein quantification independent of the labeling conditions, blasticidin selection, or MS platform used, with a mean Pearson correlation coefficient of r = 0.54 for all experiments (Figure S1). Detection ratios of the identified proteins were then ranked according to their mean enrichment at lipid droplets in the four independent experiments. For further analysis, we used cutoff ratios of greater than 1.5-fold enrichment or depletion and selected the proteins with significantly altered abundance (Figure S1). This generated a list of 16 proteins that are enriched significantly in lipid droplet fractions of HCV-infected cells and 21 proteins that are depleted; these proteins showed similar ratios of lipid droplet localization in all four independent experiments (Figure 1D).

Next, we conducted gene ontology (GO) enrichment analysis and further characterized the lipid droplet proteome of HCV infection (Figure 1E; Tables S6 and S7). Recruited proteins are enriched for proteins usually not associated with lipid droplet function, such as RNA-binding proteins and proteins associated with cytoplasmic stress granules and vesicles. Intriguingly, the depleted proteins are enriched for lipid metabolic annotations such as lipid particle as the cellular compartment and fatty acid and triglyceride metabolism for biological processes; thus, proteins generally associated with lipid droplets are depleted from lipid droplets in HCV-infected cells. Protein network analysis highlights the protein clusters identified both for enriched and for depleted proteins (Figure 1F).

**ANXA3 Is Required for Efficient HCV Spreading Infection**

To validate our approach of identifying regulators of HCV replication, we used short hairpin RNA (shRNA)-mediated knockdown of a subset of proteins detected at lipid droplets, some of which were enriched in HCV-infected cells. We transduced Huh7.5 cells with lentiviral particles encoding the shRNAs and simultaneously expressing mCherry to monitor transduction efficacy. Subsequently, cells were infected with a fluorescently labeled HCV reporter virus (Jc1NS5AB-EGFP) to identify proteins that impact HCV replication. Among them, knockdown of ANXA3 and ARF4 (ADP-ribosylation factor 4) showed the strongest effect on HCV replication (Figure 1G). As ARF4 is a brefeldin A (BFA)-sensitive ARF, and previous studies had investigated the effect of BFA on HCV replication, we focused on ANXA3 in the present study. In infection experiments, Jc1NS5AB-EGFP efficiently spread in mock- and control shRNA-transduced cells, while knockdown of ANXA3 severely impaired viral spreading (Figures 2A–2D). Knockdown of ANXA3 was confirmed by qRT-PCR and western blotting (Figures 2B and 2C). Previous studies had identified a different annexin, ANXA2, as a host factor for HCV RNA replication or virion assembly (Backes et al., 2010; Saxena et al., 2012), while others only found a minor effect (Deux et al., 2012). We detected ANXA2, -3, -4, and -5 in lipid droplet fractions, but only ANXA3 was enriched in HCV-infected cells (Table S5). We wanted to clarify the impact of knocking down each of the ANXA proteins on viral replication and found that, in our experimental setup, only ANXA3 knockdown efficiently blocked spreading of Jc1NS5AB-EGFP reporter viruses (Figures 2E and 2F).

To independently confirm a role for ANXA3 in HCV replication, we created clonal knockout cell lines using the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 system (Figure 2G). Huh7.5 cells were transiently transfected with a Cas9 expression plasmid encoding single guide RNA (sgRNA) targeting the ANXA3 locus right after the translation start codon. After single-cell cloning, indels were verified by sequencing (Figure S2), and ANXA3 knockout was confirmed by western blotting (Figure 2H). We generated three cell lines harboring ANXA3 deletions and one cell line that transiently expressed the Cas9 construct but still harbored the wild-type alleles. When we infected these cell lines with Jc1NS5AB-mKO2 reporter viruses, spreading infection was nearly completely blocked in the ANXA3-knockout cell lines as compared to the wild-type control clone or the parental Huh7.5 cells (Figure 2I). We also analyzed this phenotype by fluorescence microscopy, and, indeed, Jc1NS5AB-mKO2 was impaired from spreading in ΔANXA3 cells, as indicated by the very low number of infected cells per foci of infection as compared to the control cells (Figure 2J).

**ANXA3 Is Recruited to Lipid-Rich Fractions in HCV-Infected Cells**

In order to confirm the re-localization of ANXA3 to lipid droplet fractions in HCV infection, we isolated lipid droplets of naive and HCV-infected Huh7.5 cells by sucrose gradient centrifugation and analyzed ANXA3 levels via western blotting. Despite
Figure 2. ANXA3 Controls HCV Replication

(A) Scheme of the experimental design using ANXA3-knockdown cells.

(B) Knockdown efficacy was verified by qRT-PCR.

(C) Western blot analysis of shANXA3 (shRNA targeting ANXA3)-transduced and control cells. Asterisk indicates an unspecific band recognized by the ANXA3 antibody.

(D) Huh7.5 cells were transduced with lentiviral stocks expressing shRNAs targeting ANXA3 (shANXA3) or non-targeting (NT) control (shNT) and infected with Jc1\(^{NS5AB-EGFP}\). 2, 4, and 6 days post-infection, cells were fixed and analyzed by flow cytometry of EGFP to measure spreading infection (mean ± SEM, n = 3). *p < 0.05.

(E) HCV spreading infection in ANXA2–5 knockdown cells. Shown is one experiment performed in triplicate (mean ± SD).

(F) Knockdown efficacies were verified by qRT-PCR.

(G) Scheme of the experimental design using ANXA3-knockout cells.

(H) Western blot of ANXA3-knockout and wild-type clones as well as parental Huh7.5 cell lines. Asterisk marks an unspecific band.

(I) ANXA3-knockout (clone #3), wild-type (clone #4), and parental Huh7.5 cells were infected with Jc1\(^{NS5AB-mKO2}\) viral stocks and analyzed by flow cytometry of mKO2 to measure spreading infection (mean ± SEM, n = 3). *p < 0.05; **p < 0.01.

(J) Confluent ANXA3-knockout (clone #3), wild-type (clone #4), and parental Huh7.5 cells were infected with a very low MOI of Jc1\(^{NS5AB-mKO2}\) and fixed 3 days post-infection for microscopic analysis. Cells were stained with NS5A antibodies and counterstained with Hoechst (scale bar, 10 μm). The number of infected cells per infection focus was counted in two independent experiments in two to three different wells (mean ± SEM). **p < 0.01.

See also Figure S2.
loading the same amount of protein as indicated by similar PLIN2/ADRP levels, ANXA3 was nearly undetectable in lipid droplet fractions from control cells but readily detectable in lipid droplet fractions from HCV-infected cells (Figure 3A). Therefore, ANXA3 is localized to lipid droplet fractions in cells that are infected with HCV. This increase in ANXA3 levels could either reflect an overall increased expression or a specific recruitment of ANXA3 to lipid droplets. Therefore, we analyzed ANXA3 expression following HCV infection by western blotting. Overall, ANXA3 protein expression was unchanged in infected cells, as compared to control cells, pointing to enhanced recruitment of ANXA3 to lipid-rich fractions following infection with HCV (Figure 3B).

Next, we investigated ANXA3 localization in cells transfected with a bicistronic Con1 subgenomic replicon (Con1 SGR) that lacks all structural proteins, including core, as well as p7 and NS2, and only expresses NS3 to NS5B, as well as a neomycin resistance gene (Choi et al., 2004). After selection for cells that replicate HCV RNA, we isolated lipid droplets of replicon and control cells and analyzed them by western blotting. As observed before, ANXA3 was barely detectable in the lipid droplet fraction of control cells (Figure 3C). In contrast, in cells selected for expression of the subgenomic HCV replicon and expressing the NS3-5B RNA replicase, ANXA3 was recruited to the lipid droplet fraction (Figure 3C). The HCV RNA is replicated within the membranous web. PLIN3/TIP47 is a lipid-droplet-binding protein that acts as a host factor for HCV RNA replication and is re-localized to low-density lipid-rich membranes in membrane floatation assays (Vogt et al., 2013). Hence, we analyzed ANXA3 localization in HCV RNA—replicating cells by...
membrane floatation assay. Post-nuclear cellular lysates were separated by a discontinuous iodixanol gradient and analyzed by western blotting. ANXA3 localized to high-density membranes but additionally co-fractionated with NS3A and PLIN2/ADRP in low-density lipid-rich membranes in cells actively replicating HCV RNA (Figure 3D). Thus, expression of NS3A, NS5B and active viral RNA replication are sufficient to re-localize ANXA3 to lipid-rich membranes.

To analyze which viral protein re-localizes ANXA3, we ectopically expressed the two viral proteins that traffic to lipid droplets independently of viral replication, core and NS5A, and performed lipid droplet isolations. In control cells, ANXA3 was barely detectable in lipid droplet fractions. In contrast, ANXA3 traffics to lipid-rich fractions in cells expressing either core or NS5A (Figure 3E). We next assessed co-localization of both proteins together with hemagglutinin (HA)-tagged ANXA3 in immunofluorescence microscopy. We could not analyze endogenous ANXA3, as all antibodies tested failed to specifically detect endogenous ANXA3 in immunofluorescence staining. Overexpressed ANXA3-HA mainly showed cytoplasmic staining pattern. Co-localization analysis through quantifying co-localization according to Manders and Pearson revealed that ANXA3-HA partially co-localized both with NS5A and with core protein (Figure 3F) but did not display clear ring-like lipid droplet staining patterns. Taken together, the data indicate that ANXA3 is specifically recruited to lipid-rich membrane fractions by core and NS5A during HCV infection.

HCV RNA Replication Is Independent of ANXA3
Next, we dissected which step of viral replication depends on ANXA3 expression. As ANXA3 is recruited to lipid-rich membrane fractions in cells replicating a subgenomic replicon RNA, we first used the Con1 SGR system to analyze viral RNA replication in cells harboring ANXA3 shRNA (Figure 4A). Con1 SGR RNA was transfected into Huh7.5 cells transduced with the different shRNAs. After 3 weeks in selection media, growing cell colonies were visualized by crystal violet staining. We observed slightly fewer colonies in ANXA3-knockdown cells as compared to control cells (Figure 4A). In order to verify this result, we used a monocistronic genotype 2a replicon system containing a firefly luciferase in between a duplicated NS5A-NS5B cleavage site and control cells (Figure 4B). In contrast to the subgenomic replicon, this system is very sensitive and allows the examination of viral RNA replication in short-term experiments. In-vitro-transcribed Jc1 and partially deleted in E1 and E2 to prevent viral spreading Jc1ΔE1E2NS5AB-Fluc RNA was transfected into ANXA3-knockdown or control cells and subjected to luciferase assays at different time points post-electroporation. To normalize to the transfection efficacy, all values are expressed as fold increase of luciferase activity per microgram of protein over a 4-hr time point before viral RNA replication occurs. Compared to the control, we could not detect any differences in viral RNA replication rates in ANXA3-knockdown cells (Figure 4B). In addition, we analyzed the viral protein expression in ANXA3-knockdown and control cells after electroporation of wild-type JFH1 RNA by western blotting. ANXA3-knockdown and control cells showed comparable levels of core and NS5A, indicating that viral RNA replication and translation are not affected by knockdown of ANXA3 (Figure 4C).

ANXA3 Is Required for Efficient HCV Particle Production
We next asked whether ANXA3 influences the later stages of HCV replication. We transfected full-length HCV Jc1ΔE1E2NS5AB-EGFP genomes into ANXA3-knockdown or control cells and confirmed equal transfection efficacy by flow cytometry of EGFP. We then isolated total cellular RNA and viral RNA from the culture supernatant and measured HCV copy numbers by qRT-PCR at day 3 and day 6 post-transfection. In line with the previous experiments, intracellular HCV copy numbers did not differ significantly between ANXA3-knockdown and control cells, indicating equal viral RNA replication rates (Figure 4D). In contrast, ANXA3 knockdown significantly decreased HCV RNA copy numbers as well as core protein released in the culture supernatant both on day 3 and day 6 post-transfection (Figures 4D and 4E). As HCV RNA copy numbers only poorly reflect the amount of infectious particles, we determined infectious viral titers by measuring the 50% tissue culture infective dose (TCID50) (Lindenbach et al., 2005), using the same experimental setup as described earlier. Viral titers were severely reduced at day 3 and day 6 post-transfection in ANXA3-knockdown cells in comparison to control cells (Figure 4F), supporting the model that ANXA3 influences HCV particle production.

Core Translocation to Lipid Droplets and Capsid Envelopment Is Independent of ANXA3
No accumulation of infectious viral particles was observed inside ANXA3-knockdown cells; instead, we detected slightly reduced intracellular viral titers pointing to HCV viral assembly or maturation as the steps in the viral life cycle requiring proper ANXA3 function (Figure 5A). A prerequisite for HCV assembly is the trafficking of the capsid protein core and NS5A to lipid droplets. Therefore, we first analyzed the subcellular localization of the two viral proteins by immunofluorescence microscopy and lipid droplet isolations. Core and NS5A strongly localized to lipid droplets in both control and ANXA3-knockdown cells, excluding that disturbed trafficking of these proteins was responsible for the defect in virus production observed in ANXA3-knockdown cells (Figures 5B and 5C). Next, we examined the formation of high-molecular-mass (HMM) core complexes and envelopment of the capsids as described previously (Gentzsch et al., 2013). Cell lysates were analyzed by 2D blue native SDS-PAGE, and results indicated that formation of HMM core complexes is independent of ANXA3 expression for Jc1 as well as JFH1 strains and independent on the ANXA3-inactivation method (Figure 5D). For envelopment assays, cell lysates were treated with proteinase K to determine the amount of core that is enveloped and, thus, protected from proteinase K digestion. As a control, lysates were incubated with Triton X-100, which disrupts all membranes. The amount of core protein that was protected was similar in control and in ANXA3-knockdown cells, demonstrating that the envelopment of core was not dependent on ANXA3 expression (Figure 5E). As control, the membrane was probed with NS5A antibodies, and, as expected, NS5A is not protected against proteinase K digestion, similar to core in envelope-deleted viral strains (Figure 5E). As annexins had been previously described...
to modulate phospholipase A2 (PLA2) activity (Gerke and Moss, 2002) and cPLA2 is required for HCV virion production (Menzel et al., 2012), we probed cPLA2 activity in HCV-infected control and ANXA3-knockdown cells and did not detect any difference (Figure S3).

Knockdown of ANXA3 Affects the Density and Specific Infectivity of HCV Particles

To address whether ANXA3 influences the specific infectivity of the particles released, we divided the amount of infectious particles (focus-forming units; FFUs) by the amount of the viral capsid protein core or the number of HCV genomes. Intriguingly, particles released by cells lacking ANXA3 had a lower specific infectivity than the particles released by control cells (Figure 6A). To analyze whether ANXA3 directly influences particle infectivity, we analyzed whether ANXA3 is incorporated into the virions. Therefore, we purified E2-FLAG tagged viral particles from culture supernatant by subsequent precipitation, ultracentrifugation, and affinity purification, but we did not detect ANXA3 attached to the purified HCV particles (Figure S3). In addition, antibodies directed against ANXA3 failed to neutralize HCV (Figure S3).

One major determinant of the specific infectivity of HCV particles is the density of the lipoviroparticles. When we compared the densities by density gradient centrifugation, we observed the peak of infectivity and secreted core protein at lower densities in culture supernatant from cells expressing ANXA3 (Figure 6B); secreted HCV RNA displayed only a slight shift (indicated by arrows). Of note, the higher plateau at lower densities most likely reflects that there is less viral RNA in ANXA3-knockdown cell supernatant; thus, this plateau seems higher than in the control cells when shown as percentage of total RNA. The change in density during HCV maturation critically depends on

Figure 4. ANXA3 Regulates HCV Progeny Virion Production

(A) Huh7.5 cells transduced with lentiviral shRNAs were electroporated with Con1 SGR and kept under selective pressure for 3 weeks, and surviving colonies were stained with crystal violet. Shown is one representative experiment. shNT, non-targeting shRNA; shANXA3, shRNA targeting ANXA3.

(B) Luciferase activity of Jc1ΔE1E2NS5AB-Fluc-transfected cells was measured 4, 24, 48, and 72 hr post-electroporation. Shown is the luciferase activity (RLU, relative light units) per microgram of protein standardized to the 4-hr time point (mean ± SEM, n = 3).

(C) JFH1-electroporated cells were lysed and analyzed by western blotting.

(D–F) Huh7.5 cells transduced with lentiviral shRNAs were electroporated with full-length HCV Jc1NS5AB-EGFP RNA. Equal transfection rates were verified by flow cytometry of EGFP 3 days post-electroporation. (D) Intra- and extracellular HCV RNA copy numbers were determined by qRT-PCR. Shown is the absolute quantification of the HCV copy number (GE, genome equivalents) per microgram of total RNA (intracellular) or milliliter of culture supernatant (extracellular) (mean ± SEM, n = 3). **p < 0.01. (E) Quantification of HCV core protein released in the supernatants (mean ± SEM, n = 3) **p < 0.01. (F) The infectious titers of the culture supernatants were determined by TCID₅₀ titration on naive Huh7.5 cells (mean ± SEM, n = 3–5). *p < 0.05; **p < 0.01.
very low-density lipoprotein secretion (Gastaminza et al., 2008; Huang et al., 2007; Jiang and Luo, 2009). Therefore, we investigated the activity of the microsomal transfer protein (MTP) and could not detect dependence on ANXA3 in the presence or absence of HCV replication (Figure 6C). Secretion of apolipoproteins ApoE and ApoB into the culture supernatant of ANXA3-knockdown and control cells was similar in uninfected cells (Figure 6D; Figure S4). In contrast, ApoE, but not ApoB, secretion was significantly reduced in ANXA3-knockdown cells that replicated either subgenomic or genomic HCV RNA (Figure 6D; Figure S4). Of note, even in HCV-infected cells, the density of total cellular and secreted ApoE was not affected by ANXA3 knockdown (Figure 6B; Figure S4). Thus, in uninfected cells, lipoprotein secretion functions independently from ANXA3, but in HCV-infected cells, ANXA3 contributes to ApoE secretion but not lipidation.

ANXA3 Promotes Viral Particle Maturation by Facilitating Incorporation of ApoE

During HCV maturation, ApoE likely attaches to HCV particles via its interaction with the envelope protein E2 (Lee et al., 2014). We probed this interaction using a fully infectious HCV construct expressing FLAG-tagged E2. In cells expressing ANXA3, we were able to detect the ApoE-E2 interaction. In contrast, in ANXA3-knockdown or -knockout cells, this interaction was nearly abolished (Figures 7A and 7B). ANXA3 was additionally required for the interaction between E2 and the capsid core, implicating that incorporation of the envelope proteins, as well as ApoE, requires ANXA3 (Figures 7A and 7B). Importantly, the interactions between E2 and ApoE, as well as core, were only observed in the context of full viral replication, as co-expressed core and E1/E2 expression constructs containing the signal peptide of core to ensure correct
subcellular localization did not interact with each other or with endogenous ApoE (Figure 7C). Prior to interaction with E2, ApoE interacts with NS5A, an interaction that is thought to recruit ApoE to virion assembly sites. We used a viral strain that encodes an HA-tagged NS5A to study this interaction. Intriguingly, we detected no change in the level of ApoE interacting with NS5A in ANXA3-knockdown cells (Figure 7D). Therefore, ANXA3 is required for virion maturation steps in the ER. To verify our immunoprecipitation results, we analyzed the subcellular localization of E2/core and ApoE. We observed that, in uninfected cells, ApoE mainly localizes to the Golgi compartment, while in HCV-infected cells, ApoE is scattered in a punctuate pattern throughout the cell co-localizing with E2 (Figure 7E). This pattern is reverted in ANXA3-knockdown cells where ApoE is, again, in the Golgi co-localizing with Golgi matrix protein GM130 (Figure 7F). We observed a similar pattern of less co-localization in ANXA3-knockdown cells when we probed for E2 and core (Figure 7E).

In summary, our results suggest that ANXA3 acts as a host factor for HCV particle maturation, with subsequent effects on the number and infectivity of particles released (Figure 7G).

**DISCUSSION**

Here, we performed an extensive quantitative lipid droplet proteome analysis of HCV-infected versus uninfected control cells to reveal the perturbations caused by HCV infection and to identify regulators of HCV replication. We compiled a list of 316 proteins that were reliably identified in the lipid droplet fractions, with 16 proteins being recruited to lipid droplets and 21 proteins being displaced from lipid droplets in HCV-infected cells. Strikingly, some of the recruited proteins are annotated for RNA-binding proteins, which fits with the present model that part of the membranous web, where the HCV RNA replicates, as well as viral translation centers, are found in close proximity of lipid droplets. Two of our top hits were DEAD box proteins 1 and 3 (DDX1 and DDX3), which both have been described to interact with the 3’UTR of the HCV RNA activating a non-canonical IκB kinase (IKK)-alpha pathway that, in turn, induces lipogenic genes, resulting in more lipid droplets, enhanced core-lipid droplet interaction, and virion assembly (Ariumi et al., 2007; Li et al., 2013).
Proteins depleted from lipid droplets were mainly annotated for lipid metabolic processes, indicating that HCV perturbs the protein composition to disconnect the lipid droplets from their normal metabolic function. This phenomenon might contribute to the development of steatosis in patients and is line with our previous data that lipid droplets are stabilized in cells expressing the capsid protein core (Harris et al., 2011).

One of the proteins highly enriched in lipid droplet fractions was the phospholipid-binding protein ANXA3 that was studied in greater molecular detail. This enrichment was not due to an ANXA3-dependent mechanism, as knock-down of ANXA3 did not relieve the lipid droplet association of ApoE or E2 (Figure 7A, B, C). shRNA-transduced cells were infected with Jc1 Flag-E2, fixed, and stained with ApoE, FLAG, core, and GM130 (Golgi) antibodies. Samples were analyzed by confocal microscopy; single channels are shown in black and white, and the merged images are pseudocolored as indicated (scale bars, 10 μm).

Co-localization was analyzed using coloc2 in Fiji of individual cells of two to three independent experiments by calculating the degree of co-localization using the Manders’ colocalization coefficient (mean ± SEM). *p < 0.05; **p < 0.01; ***p < 0.001. (G) Model: HCV infection causes re-localization of ANXA3 to lipid droplet fractions to ensure efficient HCV maturation and egress.
overall increased expression but due to a specific re-localization to lipid-rich fractions in HCV-infected cells. As overexpressed ANXA3 failed to localize to ring-like structures, ANXA3 is likely trafficked to lipid-rich membrane structures and not to the lipid droplet surface. We found that expression of single viral proteins, either core or NS5A, is sufficient to re-localize ANXA3. However, we were unable to detect a direct interaction by co-immunoprecipitation experiments (data not shown) indicating that ANXA3 is recruited by interaction partners or by changes in the membrane composition that enable binding of ANXA3. Alternatively, HCV infection could also cause an efflux of calcium from the ER at ER-lipid droplet contact sites that, in turn, could enable the binding of ANXA3 to membranes. Interestingly both core and NS5A can affect calcium homeostasis in the ER (Dionisio et al., 2009). Thus, locally elevated calcium levels could trigger enhanced ANXA3 binding.

We observed that ANXA3 is required for a step in HCV particle production during envelopment of the viral capsids. Viral particles released from cells lacking ANXA3 showed a higher density and less specific infectivity pointing to a defect in proper virion assembly or maturation. ANXA3 expression was required for ApoE secretion, but not ApoB secretion or MTP activity, in cells infected with HCV but not in uninfected cells. Interestingly, ApoE subcellular localization changes in response to HCV infection, a phenotype reverted by ANXA3 knockdown. Through interaction studies, we show that the recruitment of ApoE via NS5A to virion assembly sites is independent of ANXA3, while ApoE/NS5A interaction requires ANXA3. Thus, ANXA3 mediates ApoE trafficking (but not lipidation) and HCV maturation steps within the ER. Interestingly, it was recently reported that ANXA3 is secreted and that it functions in a positive-feedback loop that promotes cancer stem cell self-renewal and tumor growth in hepatocellular carcinoma (Tong et al., 2015). ANXA3 expression is approximately 10-fold higher in cancer cell lines as Huh7 cells, thus, ANXA3 effect on HCV replication might contribute to the higher replication rates in Huh7 cells. However, further studies have to explore in greater detail the mechanistic role of ANXA3 in the late stages of the HCV life cycle.

**EXPERIMENTAL PROCEDURES**

For detailed experimental procedures, see the Supplemental Information.

**Cell Lines and Culture Conditions**

HEK293T cells obtained from the American Type Culture Collection and Huh7.5 cells obtained from C.M. Rice were grown under standard cell culture conditions.

**HCV Infection and Replicon Assays**

HCV Jc1 reporter constructs encoding fluorescent proteins, selection markers, or the firefly luciferase between a duplicated NS5A-NS5B cleavage site and JctTAG-EG2 were described previously (Eggert et al., 2014; Webster et al., 2013). For HCV infection and transfection experiments, in-vitro-transcribed HCV RNA was electroporated into Huh7.5 cells. Culture supernatant was harvested, filtered, and concentrated by polyethylene glycol 8000 precipitations. For subgenomic replicon assays, replicon RNA was electroporated into Huh7.5 cells and viral RNA replication was measured by either survival under G418 treatment or luciferase activity as described previously (Vogt et al., 2013).

**Knockdown and Knockout of ANXA3**

To knock down ANXA3, we used lentiviral shRNA constructs (pSicoR-MS1) as described previously (Herker et al., 2010; Wissing et al., 2011). We created clonal ANXA3-knockout cell lines using the CRISPR/Cas9 system as described previously (Ran et al., 2013).

**SILAC Labeling, HCV Infection, and Lipid Droplet Isolation for Mass Spectrometry**

For isotope metabolic protein labeling, we used the SILAC Protein Quantitation Kit according to the manufacturer’s instructions (Thermo Scientific). After the incorporation was confirmed, light or heavy Huh7.5 cells were infected with Jc1 reporter strains and cultured for 2 to 3 weeks. Lipid droplets were isolated by two sequential sucrose density centrifugations of post-nuclear supernatant, followed by three washing steps in isotonic buffer. The lipid-droplet-associated proteins were separated by SDS-PAGE. After tryptic in-gel digestion, LC-ESI-MS/MS analyses were performed on a quadrupole-time-of-flight (Q-TOF) mass spectrometer (Q-TOF Premier, Micromas/Waters) or on a linear trap quadrupole (LTQ) orbitrap mass spectrometer (Orbitrap Fusion, Thermo Scientific). Both instruments were coupled with an ESI source to a nano-UPLC (ultra-performance liquid chromatography) system (nanoACQUITY, Waters; Dionex UltiMate 3000 RSLCnano, Thermo Fisher Scientific). Data from LC-ESI-Q-TOF-MS/MS analysis were analyzed using the open-source software framework OpenMS (Sturm et al., 2008) and the OpenMS Proteomic Pipeline (TOPPAS) (Kohlbacher et al., 2007). For peptide and protein identification, LC-MS/MS raw data were processed as described previously (Kwiatkowski et al., 2015). SILAC pairs were detected and quantified using SILACAnalyzer (Nilse et al., 2010). The LC-MS/MS data from orbitrap analysis were processed with MaxQuant version 1.5.2.8 (Cox and Mann, 2008). The MS proteomics data have been deposited into the ProteomeXchange Consortium (http://proteomcentral.proteomexchange.org) via the PRIDE partner repository (Vizcaíno et al., 2013) with the dataset identifier PXD004707.

**Biochemical and Cell Biological Methods**

Lipid droplets were isolated by sucrose gradient centrifugation (Herker et al., 2010). Iodixanol gradient centrifugations for membrane flotation and to determine HCV particle density were performed as described previously (Cataneese et al., 2013; Vogt et al., 2013). 2D blue native SDS-PAGE and proteasome K digestion protection assay were performed as described (Gentzsch et al., 2013). Co-immunoprecipitation, determination of ApoE/B secretion, and immunofluorescence microscopy were performed as described previously (Herker et al., 2010).

**Bioinformatics and Statistical Analysis**

For bioinformatics and statistical analysis, we used R (R Core Team, 2015), RStudio (RStudio Team, 2015), and GraphPadPrism (GraphPad Software). Statistical analysis was performed using unpaired two-tailed Student’s t test and, in the case of normalized data, one-sample t test.

**ACCESSION NUMBERS**

The accession number for the data reported in this paper is PRIDE: PXD004707.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, four figures, and seven tables and can be found with this article online at [http://dx.doi.org/10.1016/j.celrep.2016.08.052](http://dx.doi.org/10.1016/j.celrep.2016.08.052).

**AUTHOR CONTRIBUTIONS**

K.R. designed, conducted, and analyzed the experiments. M.K., M.W., and H.S. performed the MS experiments for proteome analysis and analyzed the data. S.H., A.S., and C.G. provided reagents and performed some experiments. E.H.
designed and analyzed the experiments and supervised the project. The manuscript was written by E.H. and K.R., with input from all authors.

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