An HCMV mutant with an autofluorescent capsid reveals UL77 and UL93 as capsid constituents required for viral genome packaging

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HCMV capsid assembly is governed by a network of interacting essential viral proteins. Among them are the UL77 and UL93 proteins, which were suggested to participate in capsid maturation. However, data about UL77 is scarce and UL93 is uncharacterized. To study the role of these proteins, we constructed an HCMV mutant expressing a UL77-mGFP fusion protein, as well as HCMV genomes lacking UL77 or UL93. Both UL77 and UL93 were associated with nuclear capsids as well as with extracellular virions when assessed by immunoblotting. Using a specifically generated UL93 monoclonal antibody, we provide evidence that UL93 translation starts predominantly at the originally proposed ATG codon and not at putative downstream initiation sites, as suggested by a recent study using ribosome profiling. Immunogold labelling of nuclear capsids demonstrated the presence of UL77-mGFP on DNA-filled C capsids and on empty A and B capsids in comparable amounts. This is in contrast to the UL77 orthologue UL25 of HSV-1, which was reported to be preferentially attached to C capsids. When UL93 or UL77 was missing, only B capsids were produced and viral genome concatemers remained uncleaved, with the UL77 phenotype again being different to α-herpesvirus mutants. Moreover, HCMV BACs deficient in the expression of different capsid proteins or the β-herpesvirus-specific tegument protein pp150 allowed us to determine the components required for the correct subnuclear localization of UL77-mGFP and for its interaction with UL93.

These data for the first time describe an HCMV mutant with an autofluorescent capsid, ascribe an essential role to UL77 and UL93 in viral genome packaging, and provide insight into the interplay of HCMV proteins constituting the encapsidation network.

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Mapping the interaction between VASP and HTLV-1 p8

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The Human T-cell leukemia virus type 1 (HTLV-1)-encoded protein p8 induces cellular, actin-containing conduits facilitating virus transmission. Host factors interacting with p8 that mediate virus transmission remain still unknown. Here we searched for novel interaction partners of p8 using bioinformatics, co-immunoprecipitations (co-IP), peptide competition assays, and immunofluorescence. We identified the vasodilator-stimulated phosphoprotein (VASP), a promoter of actin-filament elongation, as a novel interaction partner of p8. VASP contains an N-terminal EVH1 domain that targets Ena/VASP proteins to focal adhesions. Bioinformatics identified p8 as a non-globular protein with putative linear motifs, amongst them a degenerated EVH1 binding motif, which suggests interactions of p8 with the EVH1 domain of VASP. Precipitation of VASP led to a specific co-precipitation of p8 in 293T cells and in Jurkat T cells. Mutational studies revealed that the EVH1-domain of VASP is necessary, but not sufficient for the interaction with p8. Deletion of the G- and F-actin binding domains within VASP strongly inhibited co-precipitation of p8. Using peptides mimicking different motifs in p8, we identified a short amino acid stretch in the intracellular part of p8 that is responsible for the interaction with VASP. Immunofluorescence analysis revealed that VASP and p8 partially co-localize at the plasma membrane and in protrusive structures. Thus, we identified VASP as a novel interaction partner of p8 and narrowed down the interaction interface in both proteins. Together, the p8:VASP interaction could contribute to formation of actin-containing conduits to promote HTLV-1 transmission.

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**Structure and Assembly**

OP118
ABHD5/CGI-58, the Causative Protein for the Chanarin-Dorfman Syndrome, Supports Hepatitis C Virus Assembly and Release

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**Introduction:** Hepatitis C virus (HCV) assembly depends on cytosolic lipid droplets (LDs) and on very low density lipoprotein (VLDL) synthesis, and virions are secreted as lipovirobparticles.

**Objectives:** Our study aims at identifying new cell factors involved in HCV assembly to better understand how HCV usurps the VLDL synthesis pathway and LD machinery.

**Material & Methods:** We conducted a rational siRNA-based screen by selecting host factors involved in LD biology and VLDL secretion and searching for genes regulating HCV assembly and release.

**Results:** The knockdown of nearly half of our candidates significantly inhibited HCV assembly or release. Among primary hits, ABHD5 knockdown repressed infectious HCV production similar to ApoE, a known HCV assembly factor. Importantly, this defect was rescued by expression of an RNAi-resistant ABHD5 variant. ABHD5 is a LD-associated protein responsible for the Chanarin-Dorfman syndrome (CDS), a rare inherited lipid storage disease associated with ichthyosis and liver steatosis. In our Huh-7-derived hepatoma cell line, ABHD5 was cytoplasmic and enriched at the LD surface and the Golgi. ABHD5 expression had no effect on the virion specific infectivity but regulated both the efficiencies of HCV assembly and release. Intriguingly, ABHD5 mutants causative of CDS did not support HCV production and showed an aberrant subcellular localization.

**Conclusion:** In conclusion, we identified a new host factor supporting HCV production. The reported role of ABHD5 in VLDL maturation and its dual function in HCV assembly and release shed new light both on the HCV egress pathway and on the poorly understood function of ABHD5 in the liver.

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Introduction: A key step in replication of human cytomegalovirus (HCMV) in the host cell is the generation and packaging of unit-length genomes into preformed capsids. Enzymes involved in this process are so-called terminases. These essential proteins are conserved throughout the herpesviruses and many double-stranded DNA bacteriophages. HCMV terminase consists of the two subunits, the ATPase pUL56 and the nuclease pUL89, and a potential third component pUL51.

Objectives: Even though the terminase subunit pUL89 has been shown to interact with pUL56 and is essential for DNA packaging, it is not known how pUL89 assumes this important role. In order to further characterize pUL89 structure-function studies were carried out.

Material & Methods: DNA binding studies were performed with dsDNA sepharose columns and extracts from transfected cells. In order to determine specific DNA binding ability of pUL89 to the packaging motifs, pac1 and pac2 as well as pac2a and pac2c, biotinylated oligonucleotides together with avidin sepharose resin were used. Recombinant baculovirus-pUL89-infected cell extracts were purified by cation-exchange and gel permeation chromatography. The fractions were subjected to nuclease activity assays. First structural analyses of pUL89 were performed using electron microscopy of negatively stained single particles in conjunction with image processing.

Results: These data indicates that the small terminase subunit is able to bind to dsDNA but did not recognize packaging motifs. Image analysis of purified, active pUL89 indicates that the molecule can exist as a dimer comprised of two ring-like structures positioned on top of each other.

Conclusion: The 3D data described, albeit preliminary, represent the first 3D structure of pUL89 and allow first insights into structure-function relationships of this important viral enzyme.
Introduction: HIV-1 is released from infected cells in an immature, non-infectious form. In immature particles the structural polyprotein Gag is arranged in a hexameric lattice, forming an incomplete spherical shell. Maturation is mediated by the viral protease that cleaves Gag at 5 sites, followed by a drastic structural rearrangement. Mature, infectious HIV-1 is characterised by a conical capsid encasing the condensed RNA genome. Structures of immature and mature particles suggest that maturation involves immature lattice disassembly followed by assembly of a conical capsid. However, the pathway of this process is currently not understood and it is unclear what initiates cone assembly.

Objectives: It was suggested that RNA, which represents an integral structural component of retroviruses, might nucleate mature capsid assembly. We addressed this hypothesis by studying the role of RNA in mature HIV-1 morphogenesis.

Methods: We replaced the RNA-binding NC domain of HIV-1 Gag by a protein-protein interaction domain (leucine zipper, LZ) in the viral context; this results in formation and release of virus-like particles devoid of RNA. Particles were characterised with respect to protein composition and polyprotein processing. Virion structure was analysed by cryo-electron tomography.

Results: GagLZ carrying virus assembled and viral polyproteins were proteolytically processed. Particles displayed lower density and lacked detectable RNA. Structural analyses revealed that a significant proportion of particles did contain mature capsids, while lacking a dense nucleoprotein core. Core morphologies showed a larger variability than wt viruses.

Conclusion: Our results show that RNA is dispensable as a nucleator for mature core assembly.
Structure and Assembly

OP121
Cellular microtubule scaffold promotes efficient assembly and genome packaging of the non-enveloped virus reovirus

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Introduction: Replication of RNA viruses occurs predominantly within viral-induced factories derived from host intracellular membranes. Some viruses such as reovirus and rotavirus, have developed strategies independent of cellular membranes. To date, very little is known how these membrane-independent viral factories are formed and how they regulate virus replication and assembly.

Objectives: Using the mammalian Reovirus as a model, our aim is to identify the molecular mechanisms by which these membrane-independent viral factories are formed, support virus replication and promote de-novo virus assembly and genome packaging.

Material and methods: Using a combination of confocal and structure illuminated microscopy and Electron tomography of high-pressure frozen-freeze substitution samples, we determined the ultrastructural organization of reovirus factories.

Results: Cells infected with the reovirus wt strain display filamentous viral factories due to their association with microtubules. Electron microscopic survey revealed both efficient virus assembly in paracrystalline arrays and efficient genome packaging. Electron tomography revealed the eccentric tiered organization of the progeny virions from the microtubule filaments. On the contrary, when cells are infected with the laboratory-adapted strain that can no longer associates with microtubule, virus replication was reduced and genome packaging was severely impaired. The importance of this cytoskeleton scaffold to promote genome packaging was further demonstrated by destabilizing microtubule.

Conclusion: Our results demonstrate that reovirus highjack the microtubule network to act as a scaffold promoting virus assembly and better genome packaging.

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The HIV-1 p6 Gag protein contains two late assembly (L-) domains that recruit components of the ESCRT pathway to mediate membrane fission between the nascent virion and the cell membrane. Also, the ubiquitin proteasome system (UPS) plays a certain, still enigmatic, role in those late processes of replication.

Mutational scanning analyses; membrane-association by density gradient flotation; polyubiquitination of Gag; electron microscopy; MHC-I antigen presentation; structure analyses by NMR spectroscopy.

Mutation of the highly conserved Ser-40 in p6 to Phe (S40F), but not the conservative mutation to Asp (S40D) or Asn (S40N), augments membrane association and K48-linked polyubiquitination of Gag. Phe-40, together with Tyr-36, causes the formation of a hydrophobic patch in the middle of the C-terminal α-helix of p6, providing a molecular rationale for the enhanced membrane association of S40F Gag observed in vitro and in HIV-1 expressing cells. Furthermore, the mutation of the highly conserved glutamic acids within p6 to alanine (E0A), like the S40F mutant, leads to an enhanced polyubiquitination and subsequent entry of Gag into the UPS, followed by increased MHC-I antigen presentation of Gag derived epitopes. The E0A mutant also exhibits elevated membrane association, but unlike the S40F mutant, it displays defective virus budding.

The cumulative data support a model in which p6, either by hydrophobic (S40F) or electrostatic (E0A) interactions with the plasma membrane, acts, in addition to matrix, as a membrane targeting domain of Gag. The extended exposure to so far unidentified, membrane-resident ubiquitin E3-ligases might augment the polyubiquitination, the entry into the UPS, and thus the immunogenicity of Gag.

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Virion ion channels, to Short-circuit the lipid membrane for survival.

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Virion ion channels are integral membrane proteins encoded by the viral genome and known to render the lipid membrane permeable for small ions and substrates. Most of them are just about 100 amino acids in length. For some viruses the channel proteins are essential, such as p7 of hepatitis C virus. For other viruses this type of protein is called ‘auxiliary’ protein, e.g. Vpu of HIV-1, because of its ‘supporting’ role during infectivity cycle of the virus. Structural and mechanistic features of how these viral proteins accomplish their roles are still to be elucidated. PPIs with host proteins serve as valuable template for drug development.

Biological relevant features of the proteins are investigated using two computational platform technologies which cover different time and size scales: coarse grained (CG) and classical molecular dynamics (MD) simulations. With these techniques (i) assembly of the proteins and (ii) mechanics of gating are investigated for bitopic Vpu and polytopic p7 of HCV. Distinct binding modes upon dimerization and oligomerization of the protein are observed for Vpu and compared with experimental results. Simulations of p7 reveal mechanical features of the TMDs as well as rectified ion dynamics.

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Introduction: Material surfaces that interact in a controlled manner with viruses can enable advanced tools in medicine, pharmacy, and environmental science; e.g., antiviral surfaces, tailored carrier particles in vaccines, or for water purification. However, can we engineer materials to interact specifically with viruses? Can we even correlate material-virus interactions to how viruses behave towards biological surfaces or membranes? A key aim of this contribution is to meet potential partners with excellent virology background to collaborate in a project (DFG Emmy Noether program) with focus on material-virus interactions.

Objectives: Preliminary work will be presented (Biomaterials, 34, 4203-13, 2013) showing how the surface composition of aluminium oxide particles (Al₂O₃) affects Hepatitis A, phage MS2, and PhiX174 adhesion onto the particles and how surface functionalisation and multifunctionality of materials influences the virus-material interactions.

Materials & Methods: Al₂O₃ particles with different functional groups (-NH₂, -COOH, -SO₃H, etc.) were physico-chemically characterized in detail (Acta Biomaterialia, 9, 5780-7, 2013). Adhesion of Hepatitis A, MS2, and PhiX174 was measured by plaque and immunofluorescence assays. Virus-surface interactions were correlated to virus structure models.

Results: Hepatitis A, PhiX174, and MS2 recognize molecular changes of surface chemistry of colloids. This provides insights in virus-surface interactions and allows controlling virus adsorption over 5 log values by tailoring the molecular particle surface composition.

Conclusion: The findings may support a specific design of selective virus-binding and virus-repellent materials for many applications.

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The inner tegument protein of herpes simplex virus type 1 (HSV-1) UL37 is an essential 120 kDa protein expressed late in the replication cycle. pUL37 interacts with cellular proteins, including the TNF receptor-associated factor 6 (TRAF 6), the serine/threonine kinase thousand and one kinase 3 (TAOK3) and dystonin. pUL37 has been shown to contain self-association domains within the N and C-terminus as well as a pUL36 binding domain in the C-terminal region. Lack of pUL37 abrogates the egress of viral particles and leads to the accumulation of viral capsids in the nucleus and the cytoplasm. To identify domains of pUL37 relevant for intracellular transport, assembly and egress, we generated a set of amino- (N-) and carboxy- (C-) terminal truncations of pUL37. Transient expression of full-length and EGFP-tagged pUL37 revealed that pUL37 predominantly localized in distinctive tubular and punctate structures adjacent to the nucleus. Truncation of the N- and C-terminal part of pUL37 showed that N-terminal residues 68-100 and C-terminal residues 899-901, respectively, are relevant for autoaggregation in transiently transfected cells. pUL37 containing deletions of the N-, or/and C-terminal autoaggregation motif was unable to autoaggregate in transient transcomplementation assays. These results demonstrate that residues 68-100 within the N-terminal part, and residues 899-901 within the C-terminal part of pUL37 mediate the formation of autoaggregates and are essential for pUL37 function, most likely by mediating assembly of HSV-1.

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Introduction: The class II fusion protein E of flaviviruses is the only known viral fusion protein with a double membrane anchor, consisting of two antiparallel transmembrane domains (TMDs) which execute important functions in the biosynthesis and processing of the viral polyprotein. Intra- and inter-TMD interactions were also shown to be essential for efficient membrane fusion.

Objectives: In the course of this project, we want to study the interactions within and/or between the double membrane anchors of E in the assembly processes of one of the major human pathogenic flaviviruses, tick-borne encephalitis virus (TBEV). This virus is closely related to the mosquito-borne yellow fever, dengue, Japanese encephalitis, and West Nile viruses.

Materials and Methods: Using the infectious clone of TBEV we have replaced the E TM regions by the homologous elements of the related Japanese encephalitis virus (JEV) in different combinations. This approach allows the investigation of TMD interactions required for flavivirus assembly without affecting polyprotein processing.

Results and Conclusions: The virus mutant containing the complete heterologous JEV E membrane anchor was impaired in its ability to release infectious virus particles suggesting that interactions with other viral proteins are required for efficient virus assembly. An even stronger reduction of virus production was observed for the mutants containing mixed TBEV-JEV E membrane anchors indicating a requirement for homologous TMD interactions. To identify such interactions more precisely, serial passaging experiments with the mutants will be performed to allow the viruses to acquire compensatory mutations.

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Introduction: There is a variety of different genotypes of HBV that differ with respect to their molecular virology and virus-associated pathogenesis. HBV genotype G (HBV/G) is characterized by very low HBsAg secretion and a lack of HBeAg secretion.

Objectives: To analyze HBV/G in comparison to HBV genotype A2 regarding morphogenesis and release of HBV-derived particles and to characterize factors contributing to HBV/G-associated pathogenesis.

Methods: HBV/G and HBV/A expressing hepatoma cells and infected primary human hepatocytes were analyzed by confocal laser scanning microscopy, western blot, rtPCR, density gradient centrifugation and electron microscopy.

Results: While release of viral particles is not affected in HBV/G replicating cells, secretion of subviral particles (svps) is impaired although svps are produced in high amounts. These svps that display an increased density and a predominantly filamentous morphology accumulate at the ER. The PreS1PreS2 domain of genotype G that forms aggregates causes the block in HBsAg-secretion at the ER and leads to decreased transcriptional activator function of LHBs. Intracellular accumulation of HBsAg and impaired induction of the cytoprotective transcription factor Nrf2 lead to an elevated level of ROI that results in activation of JNK and thereby to Ser-phosphorylation of IRS-1 that is known to impair insulin signaling, a key factor for liver regeneration.

Conclusions: Although competent for release of viral particles, secretion of svps is impaired in HBV/G expressing cells leading to ER-stress. Since in parallel HBV-induced Nrf2 activation is diminished, the capacity to inactivate ROIs is decreased. This might confer to genotype-specific pathogenesis.

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Introduction: Classical swine fever virus (CSFV), a member of the genus Pestivirus within the family Flaviviridae, induces an economically important disease of swine. The CSFV RNA genome encodes a single polyprotein that is proteolytically processed into 4 structural and 8 non-structural proteins. The essential structural glycoprotein ERNS is involved in inhibition of the host’s innate immune response. This function is presumed to rely on the partial secretion of ERNS, a process connected with the unusual membrane association of ERNS via an amphipathic helix. Thus, a deeper insight into structural properties of the membrane anchor could lead to a better understanding of ERNS secretion.

Question: The membrane anchors of several proteins including ERNS contain conserved sets of charged residues, the so-called “charged-zipper” motifs (Walther et al., Cell 2013). The influence of these charged residues on ERNS membrane insertion, partial secretion and processing of the ERNS-E1 precursor is analyzed.

Methods: A library of constructs with substitutions of the charged residues within the membrane-anchor of ERNS was established. The effects of these mutations were determined by immunoprecipitation analyses.

Results: The experiments have shown that mutations within the membrane anchor significantly influence ERNS membrane association, secretion and its proteolytic release. However, reciprocal exchanges of charged residues did not preserve ERNS membrane anchor functions.

Conclusions: The results show that the “charged-zipper” mechanism does not apply for ERNS membrane interaction or ERNS-E1 processing. Thus, the importance of the conserved distribution of charged residues in the ERNS anchor has to be further studied.

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More needles in the haystack - Hepatitis C virus can utilize various apolipoproteins with amphipathic alpha-helical repeats to produce infectious progeny

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Introduction: Hepatitis C virus (HCV) readily establishes chronic infections in fully immunocompetent human hosts. To this end HCV has developed various strategies of immune evasion including a tight association of the virion with human lipoproteins which protects virus particles from neutralizing antibodies.

Objectives: Here we aimed to determine which human apolipoproteins expressed in hepatocytes support HCV assembly and which determinants of these proteins are critical for this function.

Material and methods: Using whole transcriptome RNAseq analysis, mRNA expression of apolipoproteins in primary human hepatocytes of different donors was quantified. cDNAs encoding liver cell expressed apolipoproteins were overexpressed in 293T/miR122 cells which express very low endogenous levels of apolipoproteins and which do not sustain HCV assembly. Expression of apolipoproteins was monitored by ELISA assays. HCV replication and virus production were monitored by Western blots, luciferase reporter virus assays and by limiting dilution titration.

Results: Expression of ApoE and related apolipoproteins ApoA1, A2, A4, A5, C1, C2, C3, and C4 rescued virus production in 293T cells whereas unrelated apolipoproteins did not support HCV assembly. ApoC1 mutants in which the two amphipathic helices were swapped or in which the formation of only one helix was impaired by insertion of proline still enabled production of infectious HCV particles. In contrast, ApoC1 mutants carrying proline insertions into both amphipathic helices did not support virus assembly.

Conclusion: Various apolipoproteins compensate for lack of ApoE during HCV assembly, and virus production critically depends on the presence of amphipathic alpha helices within apolipoproteins.

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The genus *Pestivirus* with in the family *Flaviviridae* contains pathogens like Bovine Viral Diarrhea Virus (BVDV), which cause economically important animal diseases worldwide. BVDV is an enveloped virus with a single stranded RNA genome of positive polarity. The polyprotein encoded by the genomic RNA is cleaved by viral and host cell proteases into structural- and non-structural proteins. The glycoproteins E1 and E2 are associated with the envelope of BVDV particles. Both E1 and E2 are integral membrane proteins with a C-terminal transmembrane anchor.

Pestiviruses bud at intracellular membranes, but the exact compartment of budding and glycoprotein retention remains to be elucidated. In our studies we analyzed the subcellular localization and retention as well as the membrane topology of E1 and E2.

To analyze the localization of E1 and E2 we performed colocalization experiments using immunofluorescence microscopy. The topology was investigated by immunofluorescence microscopy with selective permeabilization. To characterize the requirements for intracellular retention we used chimeric and mutated proteins. The presence of these proteins on the cell surface was analyzed by flow cytometry, Western blot and immunofluorescence microscopy.

The results obtained so far show localization predominantly in the endoplasmic reticulum for both glycoproteins, with the ectodomain in the lumen and a short cytosolic tail. In addition to an already published E2 variant with increased surface localization, we could identify several mutants of the envelope proteins that show a change in retention behavior and an impact on virus growth.

Retention signals present in both E1 and E2 keep those proteins in the ER, which is most likely the place for virus budding. This retention is governed by specific residues in their transmembrane anchors.
The small proton channel protein M2 is a minor component of influenza A virus particles and has been implicated in the scission of budding virus particles from the plasma membrane. This action requires the amphipathic helix in the cytoplasmic part of M2, which inserts like a wedge into the membrane. Also, M2 is relevant for the accumulation of catabolic organelles termed autophagosomes, a feature of cells infected with influenza virus. It is not known whether the amphipathic helix of M2 is involved in autophagosome accumulation. We wondered whether the physiological amphipathic helix of M2 can be functionally replaced by unrelated helices from curvature-inducing or curvature-sensing proteins. We generated a panel of recombinant viruses with exchanged helices. These mutants did not show defects in one-step growth curves, but a steep decrease in virus titres was observed in multi-step growth curves at late time points for some of the mutant viruses but not the wildtype, hinting towards decreased environment stability of the mutant viruses. Along the same lines, infectivity of some mutant viruses but not wildtype dropped upon prolonged exposure to room temperature. Recently, the interaction of M2 with the autophagosome marker protein LC3-II was linked to virion stability. We assessed the accumulation of LC3-II in infected cells by a flow cytometry-based method, but found no significant differences between the different virus variants. This indicates that autophagy in infected cells is not critically dependent on the amphipathic helix of M2. Ongoing experiments aim at deciphering the potential molecular cause for M2-mediated virion stabilisation.

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Essential functions of the N-terminal Gag residues for Foamy Virus assembly and budding

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The N-terminal region of Prototype Foamy Virus Gag is responsible for particle budding via interaction with critical residues in Env/Elp while the function of this Gag region is ill-defined in the non-primate foamy viruses (FV) including feline FV (FFV). Mutagenesis of the FFV Gag N-terminus reveals key residues essential for either capsid assembly and/or viral budding via an interaction with FFV Env leader protein (Elp). Sedimentation analyses of cytosolic extracts from transfected HEK293T cells showed that wild-type Gag is mainly assembled into virus capsids and that un-assembled Gag and capsomeres of distinct size are undetectable. Proteolytic processing of Gag takes place only upon capsid assembly and in the presence of functional genomic RNA. This indicates that Pol encapsidation via interaction with genomic RNA is a prerequisite for Gag processing. An appended heterogeneous myr signal rescues Gag particle budding but fails to generate particles that co-package Pol, as shown by a lack of Gag processing. By use of an in vitro Gag-Elp interaction screen, Gag mutations abolishing particle assembly also interfered with Elp binding, indicating that Gag assembly is a prerequisite for this highly specific interaction. Overall, the different phenotypic changes of these N-terminal Gag mutants, including proteolytic Gag processing, intracellular Gag assembly, and particle budding and infectivity, highlight their essential, distinct and only partially overlapping roles during viral assembly and budding. The findings presented will be discussed based on a recent model developed for the PFV Gag-Elp interactions.

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Introduction: Previous studies revealed that mutations in the C-terminus of yellow fever virus (YFV) NS2A block virion assembly.

Objectives: To determine further regions in YFV NS2A important for virion assembly.

Methods: Charged-to-alanine scanning analysis on NS2A and coimmunoprecipitation analyses have been used to study the impact of NS2A mutants on virion assembly and protein-protein interactions, respectively.

Results: Five of fifteen NS2A mutants containing single, double or triple charged-to-alanine changes did not produce infectious particles. For two of those five mutants efficient RNA replication was still observed (R22A-K23A-R24A and R99A-E100A-R101A). Prolonged cultivation of transfected cells resulted in the recovery of pseudorevertants. Besides suppressor mutants in NS2A, a compensating second site mutation in NS3 (D343G) arose for the NS2A R22A-K23A-R24A mutant. We found this NS3 mutation previously to be suppressive for the NS2Aα cleavage site mutant Q189S also deficient in virion assembly. Here, the subsequently suggested interaction between NS2A and NS3 was proven. In addition, we could demonstrate that the regions encompassing R22A-K23A-R24A and Q189S in NS2A are localized to the cytoplasm where NS3 is also known to reside. However, the defect in particle production observed for the NS2A R22A-K23A-R24A and Q189S mutants was not due to a defect in physical interaction between NS2A and NS3 as the NS2A mutations did not interrupt NS3 interaction. In fact, a region just upstream of R22A-K23A-R24 was mapped to be critical for NS2A-NS3 interaction.

Conclusion: Our data support a complex interplay between YFV NS2A and NS3 in virion assembly and identify a basic cluster in the NS2A N-terminus to be critical in this process.

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Cleavage of HCMV genomes and packaging into preformed capsids is a coordinated process mediated by essential viral proteins. pUL56 and pUL89 were described to form the viral terminase and recently we found that pUL51 is also essential for genome cleavage-packaging. pUL51 interacts and co-localizes with pUL56/89, and seems to be necessary for their correct localization to nuclear replication compartments. Thus, pUL51 may represent a third subunit of the terminase complex. However, it remained elusive which regions of pUL51 are needed to fulfill its function or are important for the binding to pUL56/89.

Here, we mapped functional domains of pUL51 using linker-scanning mutagenesis by inserting a 5 amino acid sequence (GASAG) at several positions of pUL51. These mutated UL51 sequences were then introduced into the BAC-cloned HCMV genome, and permissive cells were transfected with the BACs using our efficient adenofection protocol. Viral growth was evaluated by plaque formation, the protein levels of pUL51 in the different mutants were analyzed using immunoblotting, and interactions between the terminase subunits as well as their subcellular localization were investigated.

Our results show that insertions within the conserved C-terminal domain forming putative α-helices lead to non-viable mutants, and pUL51 levels were markedly reduced in these mutants. Moreover, the terminase subunits pUL56 and pUL89 were no longer detected in nuclear replication compartments.

In sum, our data provide evidence that the C-terminal part of pUL51 is essential for viral growth and comprises the domains required for the formation of a functional terminase complex, as well as for its correct subnuclear localization.

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Secondary structure analyses of highly virulent bovine viral diarrhea virus genotype 2c genomes

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Introduction: In February 2013, bovine viral diarrhea virus 2c (BVDV-2c) infections caused severe clinical symptoms in calves, heifers, and dairy cattle in northern Germany and the Netherlands, with a mortality of up to 80%. Deep sequencing unveiled a mixture of distinguishable genome variants. While the majority (95%) harbored a duplication of the p7/NS2 region (dup⁺), the minority (5%) fit the known BVDV genome structure (dup⁻). Using a reverse genetic system it was shown that the dup⁻ variants are generated de novo during viral replication.

Objectives: Both for ss(+)RNA and ss(−)RNA secondary structures potentially enabling “polymerase jumping” and hence deletion of either of the copies, i.e. generation of dup⁻ genomes, were predicted. Here we aimed to confirm the predictions and to analyze the underlying mechanisms in more detail.

Materials and methods: To this end, we combined SHAPE (selective 2'-hydroxyl acylation analyzed by primer extension) with Illumina sequencing. SHAPE specifically modifies bases not involved in base pairing. Modified bases lead to termination of cDNA synthesis which can be detected by sequencing.

Results: Results obtained for ss(+)RNA clearly support the predicted secondary structures which enable “jumping” of the polymerase leading to the deletion of the first copy of the duplication. To prove the necessity and location of RNA secondary structures for copy deletion, synonymous mutants were generated based on the SHAPE results which should prevent formation of the RNA structures assumed to enable the deletion.

Conclusion: We were able to experimentally confirm the predicted RNA structures and in addition analyzed the impact of specific bases on secondary structure formation and copy deletion.

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The influenza A virus genome comprises eight single-stranded negative-sense RNA segments (vRNAs). Unique genome packaging signals have been found in the noncoding and terminal coding regions of both the 3' and 5' ends of each vRNA. We could show that the nucleoprotein (NP) of the recently discovered bat influenza virus H17N10 supports the polymerase activity and formation of viral like particles (VLP) of classical influenza A viruses, including A/SC35M. However, using a reverse genetics system, no infectious virus could be rescued combining NP segment of H17N10 and all other seven segments of SC35M. Surprisingly, we also failed to rescue SC35M with a NP H1710 segment harboring the packaging signals of SC35M. To understand the reason for this incompatibility, we created SC35M NP segments coding for bat/SC35M fusion proteins. Although all of these chimeras were functional in the SC35M polymerase reconstitution and VLP assay, only one fusion protein allowed viral rescue. This virus showed delayed viral growth properties, compared to wt SC35M. Primer extension analysis further revealed preferential amplification of one specific genome segment and determination of the genome content in viral particles revealed unequal packaging of genome segments. Finally, mutational studies further revealed that the incompatibility is caused by few H17N10-specific amino acids in bat NP. Taken together, these findings suggest that the failure to rescue SC35M with bat NP is caused by inefficient genome packaging that is caused by an imbalanced accumulation of specific genome segments in the infected cell.

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Structure and Assembly

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Prediction of conserved long-range RNA-RNA interaction in full viral genomes

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Introduction: Long range RNA-RNA interactions (LRIs) have been reported in several RNA viruses and play important roles in the viral genome replication. LRIs are often associated with local RNA structures, such as cis-acting elements, and therewith frequently located in loop regions or internal bulges. Currently, there exists no tool which is able to predict these pseudo-knot structures in full viral genome alignments.

Objectives:
We present LRIscan a tool to prediction conserved, genome-wide LRIs based on a multiple sequence alignment in only a few hours on an average computer.

Methods: LRIscan consists of three basic steps: (1) Identification of all possible LRIs based on a genome-wide alignment using a double sliding window approach (based on ViennaRNA Package). (2) The LRI-filter consists of a minimal distance of nucleotides (default: L>=100), a minimal number of interacting base-pairs (default: d>=5) called ‘seed’ without bulges, a conservation in >=55% of the considered sequences and a mean sequence complexity larger than 50%. (3) The final LRI score is a combination of the minimum free energy (MFE) and the structural accessibilities of the corresponding regions.

Results: We applied our method to an alignment consisting of 106 HCV genomes including all 7 genotypes in only 9.5h. We confirmed all previously known LRIs. Strikingly, we identified a conserved interaction between the apical loops of SLII and DLS. This possible initial interaction can be extended to include 62 interacting basepairs to build a potential genome circularization between the 5'UTR and 3'UTR.

Conclusion: With LRIscan, we are able to predict LRIs on multiple alignments based on hundreds of full viral RNA genomes. Our results facilitate the investigation of the role of LRIs in the viral replication.

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The herpesviral tegument arises from a sequential attachment of numerous viral proteins to the capsid. Although the exact mechanism is unclear, proteins, which are closely associated with capsids, are likely candidates regulating tegumentation. Here, we report a similar requirement of the human cytomegalovirus (HCMV) large tegument protein pUL48 and its complex partner, pUL47, for efficient tegumentation during infection. Mutant viruses either unable to express pUL47 (TBstop47) or lacking the UL48 gene (TBdelUL48) exhibited a comparable maturation defect in ultrastructural analysis. This maturation defect was characterized by an accumulation of non-enveloped DNA-containing capsids in the cell periphery accompanied by a decreased number of capsids at the viral assembly complex (vAC), the cytoplasmic site of tegumentation. The ultrastructure of non-enveloped capsids at peripheral accumulations suggested the lack of most of the tegument. Immunofluorescence stainings strengthened this assumption because these peripheral accumulations were positive for inner tegument protein pp150 but not for outer tegument protein pp28. An involvement of pUL47 and pUL48 in tegumentation was also indicated by their colocalization at the vAC during virus infection.

Our observation that pUL48 accumulated at the vAC in cells infected with TBstop47 and was also found at peripheral accumulations of non-enveloped capsids indicated that neither the intracellular localization nor the capsid association of pUL48 requires pUL47. In contrast, localization of pUL47 to the vAC was severely altered in absence of pUL48. Together, these results lead to an updated model of HCMV tegumentation that is orchestrated by pUL48 and pUL47 at the vAC.

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