Prenylation of viral proteins by enzymes of the host: Virus-driven rationale for therapy with statins and FT/GGT1 inhibitors

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Intracellular bacteria were recently shown to employ eukaryotic prenylation system for modifying activity and ensuring proper intracellular localization of their own proteins. Following the same logic, the proteins of viruses may also serve as prenylation substrates. Using extensively validated high-confidence prenylation predictions by PrePS with a cut-off for experimentally confirmed farnesylation of hepatitis delta virus antigen, we compiled in silico evidence for several new prenylation candidates, including IRL9 (CMV) and few other proteins encoded by Herpesviridae, Nef (HV-1), E1A (human adenovirus 1), NS5A (HCV), PB2 (influenza), HN (human parainfluenza virus 3), L83L (African swine fever), MC155R (molluscum contagiosum virus), other Poxviridae proteins, and some bacteriophages of human associated bacteria. If confirmed experimentally, these findings may aid in dissection of molecular functions of uncharacterized viral proteins and provide a novel rationale for statin and FT/GGT1-based inhibition of viral infections. Prenylation of bacteriophage proteins may aid in moderation of microbial infections.

Also see the video abstract here: https://youtu.be/0qVW3ym1R1g

Keywords:
- bacteriophages; CAAX; human viruses; PrePS; protein prenylation

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Introduction

S-prenylation is an important post-translational modification that covalently attaches isoprenoid farnesyl (15C) or geranylgeranyl (20C) groups to de novo synthesized polypeptides [1, 2]. When the protein is prenylated, it acquires hydrophobic properties allowing its non-covalent attachment to either cell or organelle membranes. Many proteins encoded in mammalian genomes are prenylated: among the most prominent examples are G-proteins of Ras and Rho families, nuclear lamins and intracellular membrane proteins Cenp-F, Cenp-E, and PXF [2, 3]. Substrate proteins for post-translational modifications are recognized by the respective prenyltransferases [4, 5] via a signal consisting of a physicochemically constrained hydrophilic linker followed by a cysteine-containing C-terminal motif CAAX (C = cysteine, A = aliphatic amino acid, X = terminal residue) [6, 7].

Abbreviations:
- aa, amino acid; ASFV, African swine fever virus; CMV, cytomegalovirus; DB, database; ER, endoplasmic reticulum; FFPS, farnesylyrophosphate synthase; PPP, farnesylpyrophosphate; FT, farnesylyltransferase; GB, genbank; GGPP, geranylgeranyl pyrophosphate; GGT, geranylgeranyl ytransferase; GI, gene id; GN, hemagglutinin-neuraminidase; GO, gene ontology; HA, hemagglutinin; HBV, hepatitis B virus; HCV, hepatitis C virus; HDV, hepatitis delta virus; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HSV, human herpesvirus; LHDAg, large delta antigen; NP, nucleocapsid protein; PrePS, prenylation prediction suite; RSA, relative surface accessibility.
Interestingly, recent experiments demonstrated that proteins of intracellular microbial pathogens of human cells can be post-translationally modified by eukaryotic prenyltransferases, thus, allowing these bacterial proteins to be anchored to host cell membranes [8]. In particular, prenylation events were detected in microbial proteins SifA of Salmonella typhimurium [9] and AnkB of intracellular invader Legionella pneumophila [3, 10, 11]. The latter event provides substantial selective advantage for Legionella by allowing rapid nutritional remodeling of the host cell [12]. In these and other intracellular bacteria, a number of additional prenylation targets were predicted computationally [3, 10]. Following the same logic, one can easily imagine that the proteins of another, more abundant, class of intracellular parasites, namely viruses, may also serve as prenylation substrates.

Previous studies reported prenylation of large antigen of hepatitis delta [13] and US2 tegument protein of pseudorabies virus [14], and computationally predicted a few more in the proteins of Mimivirus and in viral orthologs of Ras oncogenes acquired from eukaryotic cells by a number of retroviruses [7]. However, no systematic screen dedicated to prenylated viral proteins was attempted so far over the new wealth of sequence data since the genomic revolution. Here, we analyze potential prenylation events in proteins encoded by human viruses and present a number of hypotheses concerning the role of prenylation in viral biology.

By using the extensively validated PrePS algorithm [6–8], we detected potential prenylation candidates in the genomes of human immunodeficiency virus 1 (HIV), human adenovirus 1 (E1A), hepatitis C virus (NS5A), human para-influenza virus 3 (HN), and molluscum contagiosum virus (MC155R), and influenza virus (PB2). Finally, we report evidence suggesting the prenylation of the proteins encoded by bacteriophages of human-associated bacteria.

### Materials and methods

#### Human viral proteins dataset

The search for prenylated proteins was performed in two publicly available databases that contain partially overlapping sets of viral protein sequences.

**DB1 (Database 1)**

The complete UniProtKB/Swiss-Prot data set (Release 2015_02) of viral proteins (“uniprot_sprot_viruses”) was downloaded from the FTP server [15]. To limit our search, we included only viruses capable of replication in human cells. Dataset minimization was performed using C# script created in integrated development environment (IDE) Visual Studio 2012. Thus, an initial set of 16,427 viral proteins was reduced to 5875 proteins selected for further prediction of lipidation sites.

**DB2 (Database 2)**

Viral protein sequences were obtained from Genome Assembly/Annotation Projects FTP server [16]. This database consists of complete genomes/chromosomes, contigs and reference sequences of viral mRNAs and proteins. Its constituent datasets included:

A. Viral genomes [17], last updated: February 8, 2015: consist of 205,010 complete genomes sequences. In the process of its minimization by C# application, 1,766 human viral proteins were extracted.

B. Influenza database [18], last updated: August 20, 2015, with 512,155 proteins, including 469,398 proteins of influenza A strains, and a total of 42,461 proteins of B and C strains.

C. RefSeq [19] Release 69, last update: January 5, 2015. The minimization of this application identified 2,181 human viral proteins after sifting through a total of 212,488 viral proteins.

DB1 and DB2, with an exclusion of DB2B that was analyzed as is, were merged in one file then the duplicates were removed. The content of the generated database is shown in the supplemental data (Supplementary Table S1). Classification of viruses was performed according to ICTV 2015 taxonomy [20]. To validate the structure of database and lack of incompletely formatted/missing entries, all searches were performed in an original database, before its trimming, and resultant database, after the trimming. No human virus-related hits were missed in the trimmed set. For selected viral families, initial analysis was extended to non-minimized datasets A and C.

### Prenylation prediction

The viral proteins collected in databases were analyzed using PrePS (Prenylation Prediction Suite) [6]. There are three enzymes capable of protein prenylation [4, 5], including FT (CAAX farnesylation), GTT1 (CAAX geranylgeranylation), and GTT2 (Rab geranylgeranylation). PrePS computes prenylation sites from the amino acid sequences of query proteins from physicochemical properties and sequence conservation properties that aid in recognizing the substrate protein by prenyltransferases [21]. This process includes analyzing the potential presence of ~10 amino acid residues linker region located upstream of the cysteine-containing motif at the C-terminus and the presence of the motif itself. The PrePS computations are carried out independently for the three types of prenyltransferases, both CAAX-dependent FT and GTT1 as well as for the Rab-dependent GTT2. In all cases, algorithms produce the scores (positive scores indicate potential for prenylation and the more positive the score, the more likely it is) as well as an estimate of the probability of false positive prediction (p-values for the FT- and the GTT1-specific predictor, E-values for the GTT2-related predictor) [6].

### Protein sequence analysis

For multiple sequence alignments, we used Clustal Omega [22], then the sequences were analyzed by ClustalW2-Phylogeny using neighbor joining method. For displaying consensus sequences, we used “WebLogo” tool [23]. PROSPER (Protease Specificity Prediction server) [24] and PeptideCutter [25] were used to analyze protease recognition sites. Protein masses were calculated by a JavaScript program, published by Stothard P [26]. To predict signal peptide cleavage sites we used SignalP 4.1 Server [27]. To perform gene ontology analysis we used QuickGo.
Prenylation events are detected in many proteins of vertebrate Poxviridae, including molluscum contagiosum virus

Among the proteins of human dsDNA viruses, MC155R protein from molluscum contagiosum virus subtype 1 (Poxviridae) has been predicted to be prenylated with FT score $-1.812$ (p-value: 0.0543). MC155R is a nuclear-egress-membrane-like protein with molecular mass about 34 kDa, conserved domain PHA03325 and C terminus containing sequence CVLM. The function of MC155R protein is unknown [37]. Similarity to nuclear egress membrane proteins and relative position of MC155R gene within viral genome allows one to hypothesize that prenylation might have a role in molluscum contagiosum virus assembly and its release from the cell.

Interestingly, analysis of non-human poxviruses resulted in multiple hits predicting farnesylation of ankyrin (ANK) repeat containing proteins found in pigeonpox, poxvirus and canarypox viruses [38]. All ANK-containing proteins of avian poxviruses share the same prenylation motif CSIS, with exception of canarypox virus, which possess closely related motif CIIS. As ANK motif proteins are promiscuous drivers of protein-protein interaction, which connect many regulatory and structural functions, the finding of the CAAX-motif conservativeness in multiple avian poxvirus orthologs is intriguing. Interestingly, experimentally confirmed farnesylated protein AnkB of Legionella pneumophila [11] also possesses an ANK motif essential for its biological function.

In addition, C-terminal farnesylation motifs were also found in 127R protein of Yaba monkey tumor virus, myosin tail-containing protein of squirrelpox virus, CNPV023 protein of canarypox virus (Table 1) as well as the proteins of entomopoxviruses of Mythimna separate, Choristoneura biennis, Choristoneura rosacea and interferon resistance protein from Choristoneura rosaceana, and interferon resistance protein from Yoka poxvirus isolated from Central African mosquito pool (Supplementary Table S5).
Table 1. Prenylation of vertebrate proteins

| Virus name                      | Protein name                  | Reference number          | FT score and p-value | GGT1 score and p-value | CAAX |
|---------------------------------|-------------------------------|---------------------------|----------------------|------------------------|------|
| Adenoviridae                    |                               |                           |                      |                        |      |
| Duck adenovirus A               | ORF9, partial                 | AP_000098.1, NP_044718.1 | −1.759, <0.0519      | NS                     | CEIS |
| Turkey adenovirus 1             | Unnamed protein product, partial | YP_00933573.1            | −1.553, <0.0433      | NS                     | CSIS |
| Fowl adenovirus 5               | ORF1A, partial                 | YP_007986638.1           | −1.111, <0.0279      | NS                     | CVIL |
| Fowl adiavirdenovirus D         | ORF1A, partial                 | AP_000368.1              | −0.6, <0.0153        | 0.818, <0.0022         | CII  |
| Fowl adiavirdenovirus A         | ORF1A, partial                 | AP_000402.1              | −1.46, <0.0097       | −0.081, <0.0041        | CVIL |
| Pigeon adenovirus 1             | Protein ORF58                  | YP_009047119.1           | −1.615, <0.0460      | NS                     | CTVL |
| Porcine adenovirus 3            | 162R                          | AAFT78233.1              | −1.791, <0.0530      | NS                     | CCCC |
| Human adenovirus 1              | 6 kDa protein/E1A              | AAQ10564.1               | −1.795, <0.0530      | NS                     | CLLL |
| Asfarviridae                    |                               |                           |                      |                        |      |
| African swine fever virus Malawi LIL 20/1 | Uncharacterized protein L83L | VF83L_ASFM2              | 0.789, <0.0015       | 0.539, <0.0027         | CTII |
| African swine fever virus wart hog/ Namibia/Wart80/1980 | Uncharacterized protein L83L | VF83L_ASFWA; VF83L_ASF4 | 0.961, <0.0010       | 0.515, <0.0028         | CTII |
| African swine fever virus pig/ Kenya/KEN- 50/1950 | Uncharacterized protein L83L | VF83L_ASK5               | 0.52, <0.0026        | 0.202, <0.0034         | CTII |
| African swine fever virus BA71V | Uncharacterized protein L83L | VF83L_ASBF7; NP_042705.1 | 0.844, <0.0013       | 2.911, <0.0004         | CTIL |
| Deltaviridae                    |                               |                           |                      |                        |      |
| Hepatitis delta virus (ISOLATE 7/18/83) | Large delta antigen        | LHDAG_HDV83              | −1.876, <0.0573      | NS                     | CTPQ |
| Hepatitis delta virus (ISOLATE AMERICAN) | Large delta antigen        | LHDAG_HDVAM              | −1.602, <0.0453      | NS                     | CRPQ |
| Hepatitis delta virus (ISOLATE D380) | Large delta antigen        | LHDAG_HDV3               | −1.691, <0.0490      | NS                     | CRPQ |
| Hepatitis delta virus (ISOLATE ITALIAN) | Large delta antigen        | LHDAG_HDVIT              | −1.602, <0.0453      | NS                     | CRPQ |
| Hepatitis delta virus (ISOLATE LEBANON-1) | Large delta antigen        | LHDAG_HDVL1              | −1.504, <0.0414      | NS                     | CRPQ |
| Hepatitis delta virus (ISOLATE JAPANESE M-1) | Large delta antigen      | LHDAG_HDVM1              | −1.902, <0.0585      | NS                     | CRPQ |
| Hepatitis delta virus (ISOLATE JAPANESE M-2) | Large delta antigen      | LHDAG_HDVM2              | −1.902, <0.0585      | NS                     | CRPQ |
| Hepatitis delta virus (ISOLATE NAURU) | Large delta antigen          | LHDAG_HDVNA             | −1.504, <0.0414      | NS                     | CRPQ |
| Hepatitis delta virus (isolate Peru-1) | Large delta antigen      | LHDAG_HDVP1              | −0.398, <0.0117      | NS                     | CTQQ |
| Hepatitis delta virus (ISOLATE JAPANESE S-1) | Large delta antigen      | LHDAG_HDVS1              | −1.876, <0.0573      | NS                     | CTQQ |
| Hepatitis delta virus (ISOLATE JAPANESE S-2) | Large delta antigen      | LHDAG_HDVS2              | −1.876, <0.0573      | NS                     | CTQQ |
| Hepatitis delta virus (isolate TW2476) | Large delta antigen        | LHDAG_HDVTS              | −1.876, <0.0573      | NS                     | CTQQ |
| Hepatitis delta virus (isolate US-2) | Large delta antigen        | LHDAG_HDVU3              | −1.602, <0.0453      | NS                     | CRPQ |
| Hepatitis delta virus (isolate VnzD8624) | Large delta antigen        | LHDAG_HDV1               | −0.398, <0.0117      | NS                     | CTQQ |
| Hepatitis delta virus (isolate VnzD8349) | Large delta antigen        | LHDAG_HDV2               | −0.398, <0.0117      | NS                     | CTQQ |
| Hepatitis delta virus (isolate VnzD8379) | Large delta antigen        | LHDAG_HDV3               | −0.398, <0.0117      | NS                     | CTQQ |
| Hepatitis delta virus (isolate woodchuck) | Large delta antigen | LHDAG_HDW0               | −1.602, <0.0117      | NS                     | CRPQ |
| Flaviviridae                    |                               |                           |                      |                        |      |
| Hepatitis C virus               | Non-structural 5A protein     | AAB87527.2               | −1.727, <0.0510      | NS                     | CSMS |

(Continued)
| Virus name                                    | Protein name                                      | Reference number | FT score and p-value | GGT1 score and p-value | CAAX |
|----------------------------------------------|--------------------------------------------------|------------------|----------------------|------------------------|------|
| **Herpesviridae**                            |                                                  |                  |                      |                        |      |
| Human herpesvirus 5 strain AD169             | Uncharacterized protein IRL9                     | IR09_HCMVA       | 0.187, <0.0047       | NS                     |      |
| Suid herpesvirus 1 (strain NIA-3)            | Protein US2 homolog                              | US02_SUHVN       | 0.969, <0.0010       | NS                     |      |
| Bovine herpesvirus type 1.2 strain st        | Protein US2 homolog                              | US02_BHV1S       | -0.04, <0.0069       | 2.087, <0.0008         |      |
| Bovine herpesvirus 5                         | Virion protein US2                               | YP_003662530.1   | -0.013, <0.0066      | 1.91, <0.0010          |      |
| Bovine herpesvirus 1                         | Virion protein US2                               | NP_045367.1      | 0.033, <0.0061       | 2.608, <0.0005         |      |
| Panine herpesvirus 2                         | Membrane protein UL20                            | NP_612664.1      | -0.904, <0.0222      | NS                     | CTI1 |
| Suid herpesvirus 1                           | Virion protein US2                               | YP_068391.1      | 0.969, <0.0010       | NS                     | CTIS |
| Suid herpesvirus 2                           | Protein U4                                       | YP_008492590.1   | -0.783, <0.0192      | NS                     | CVPA |
| Felid herpesvirus 1                          | Protein UL24 homolog                             | UL24_FHV1        | 0.311, <0.0038       | NS                     |      |
| Gallid herpesvirus 3                         | Deoxuridine triphosphatase                       | NP_066883.1      | -0.118, <0.0078      | NS                     | CSLQ |
| Alcelaphine herpesvirus 2                    | orf40                                            | YP_00904422.1    | -0.029, <0.0068      | NS                     | CKIV |
| Anatid herpesvirus 1                         | UL19                                             | YP_003084403.1   | -1.196, <0.0305      | NS                     | CLPM |
| Cyprinid herpesvirus 1                       | Protein ORF144                                   | YP_007003797.1   | -1.759, <0.0520      | -0.373, <0.0049        |      |
| Murid betaherpesvirus 1                      | TS9 protein                                      | AAB6475.1        | -0.497, <0.0130      | NS                     | CSTQ |
| Marek’s disease virus serotype 2 (MDV2)      | dUTPase                                          | BAA32587.1       | -0.118, <0.0078      | NS                     |      |
| **Iridoviridae**                             |                                                  |                  |                      |                        |      |
| Infectious spleen and kidney necrosis virus  | ORF105R                                         | NP_612327.1      | 0.875, <0.0013       | NS                     | CVVQ |
| Rock bream iridivirus                        | ORF097R                                         | AAT71912.1       | 0.338, <0.0036       | NS                     | CVLQ |
| **Orthomyxoviridae**                         |                                                  |                  |                      |                        |      |
| Influenza A Virus (A/Fiji/15899/83) (H1N1))  | PB2 protein                                      | CAD92257.1       | -1.726, <0.0510      | NS                     | CRI1 |
| **Papillomaviridae**                         |                                                  |                  |                      |                        |      |
| Crocata crocuta papillomavirus 1             | E6 protein                                       | YP_006666514.1   | -1.09, <0.0273       | NS                     | CCLC |
| **Paramyxoviridae**                          |                                                  |                  |                      |                        |      |
| **Human parainfluenza virus 3**              | Hemagglutinin-neuraminidase                      | NP_067152.1      | -1.156, <0.0293      | NS                     | CSQS |
| **Paroviridae**                              |                                                  |                  |                      |                        |      |
| Chicken parovirus ABU-P1                    | NS1                                              | YP_009046818.1   | 1.641, <0.0002       | NS                     | CLVQ |
| Picoanoviridae                               |                                                  |                  |                      |                        |      |
| Caprine kobovirus                           | VP3                                              | YP_009001372.1   | -1.597, <0.0451      | NS                     | CTLQ |
| **Poxviridae**                               |                                                  |                  |                      |                        |      |
| Molluscum contagiosum virus subtype 1        | MC155R                                          | NP_044106.1      | -1.812, <0.0544      | -1.416, <0.0087        |      |
| Yaba monkey tumor virus                     | 127R                                             | NP_038383.1      | -1.229, <0.0316      | NS                     | CKLC |
| Squirrelpox virus                            | Hypothetical pox protein-similar to SQPV115      | YP_008658391.1   | 0.042, <0.0060       | NS                     | CVTS |
| Penguinpox virus                             | Ankyrin repeat protein                           | YP_009046026.1   | 0.378, <0.0034       | NS                     | CSIS |
| Pigeonpox virus                              | Ankyrin repeat protein                           | YP_009046267.1   | 0.378, <0.0034       | NS                     | CSIS |
| Fowlpox virus                                | Ankyrin repeat gene family protein               | NP_038986.1      | 0.378, <0.0034       | NS                     | CSIS |
| Canarypox virus                              | CNPV041 ankyrin repeat protein                   | NP_055064.1      | 0.165, <0.0049       | NS                     |      |
| Canarypox virus                              | CNPV023 vaccinia C4L/C10L-like protein           | NP_955046.1      | -1.082, <0.0073      | NS                     | CIIL |
| Yoka poxivirus                               | Interferon resistance protein                    | YP_004821369.1   | 0.094, <0.0055       | NS                     | CKLC |
| Reoviridae                                   |                                                  |                  |                      |                        |      |
| Great Island virus                           | VP6(dBP)                                         | YP_003890671.1   | 0.258, <0.0042       | NS                     | CVVQ |

(Continued)
Cytomegalovirus and other vertebrate herpesviridae encode multiple hits for prenylation

In 2003, prenylation of the protein US2 encoded by pseudorabies (Alphaherpesvirinae) has been described by Clase et al. [14], in line with observations that its replication is suppressed in presence of farnesyl transferase inhibitors. PrePS-guided analysis of human Herpesviridae genomes has shown just one possibly prenylated protein candidate, namely, IRL9 from human herpesvirus 5 (CMV) (FT score: 0.187, \( p < 0.0047 \)). This protein with unknown function consists of 143 aa, with no conserved domains identified.

In light of his observation, it is important to note that CMV replication is restrained in presence of statins, well-known inhibitors of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase [39]. Activity of HMG-CoA is a bottleneck in the production of mevalonate, a precursor to both farnesylpyrophosphate (FPP) and geranylgeranylpolyporphosphate (GGPP), the short-chain isoprenoids utilized by prenylation machinery. In presence of statins, the lack of FPP and GGPP leads to decreased expression of viral antigens, the suppression of viral DNA synthesis, and particle production [39]. It is tempting to speculate that high-confidence prediction of the farnesylation in CMV protein IRL9 points at potential drug-gable target, which disruption by specific small molecular inhibitor may provide anti-cytomegalovirus breakthrough.

Extending the analysis for possible prenylation of the proteins encoded by Herpesviruses of other vertebrates has shown multiple hits for prenylation (Table 1; Supplementary Table S3). Functions are not yet established for a majority of these proteins.

In several animal herpesviruses, including bovine herpesviruses 1 and 5 and Suid herpesvirus 1, we detected prenylation of a conserved tegument polypeptide US2, known for its interactions with ubiquitin-conjugated proteins, and probable participation in the trafficking of endosomal membranes in infected cells [40]. Interestingly, previous study of Clase et al. [14] reported prenylation of Pseudorabies, a Class D herpesvirus ortholog of US2 protein. However, when US2 was isolated from purified Pseudorabies virions, prenylation was not confirmed [14]. Later, in 2013, Kang et al. [40] studied another US2 ortholog found in herpes simplex virus 2, and pointed that it does not contain CAAX, and is not prenylated, while being capable of association with membranes by a completely different mechanism, through hydrophobic amino acids between residues 121 and 137 [40]. Confirmed lack of prenylation in membrane-associated US2 protein of some herpesviruses, alongside with PrePS-predicted prenylation of its orthologs in other herpesviruses indicate possible evolutionary change of function, or change of anchoring mechanism serving as a prerequisite for its effective functioning.

Some previous in silico studies reported that UL32 protein of Human herpesvirus (HSV) may be prenylated on its C-terminal CXXX box [41, 42]. Indeed, in both HSV1 and HSV2 UL32 proteins possess C terminal box CTY. However, according to high-confidence predictions by PrePS, physicochemical properties preclude UL32 from serving as a substrate for any prenyltransferase. Consecutively, extensive search for any experimental data on prenylation of UL32 protein had not been successful.

E1A product of human adenovirus 1 is likely target for prenylation

Analysis with PrePS detected a potential for being prenylated in the 6 kDa E1A product of human adenovirus 1 with terminates with conserved C-terminal sequence CLLS (FT Score: \(-1.791, p\)-value \(=0.0530\)). These 55-aa proteins are present in many human Mastadenoviruses of type C, capable of causing respiratory, gastrointestinal, and urinary illnesses. Notably, human adenovirus 5, which is ancestral for Mastadenovirus type C clade, ends with non-prenylated RLLS sequence [43], thus indicating that the emergence of C-terminal prenylation motif is relatively new event in evolution of type C clade. In other vertebrate adenoviruses, prenylation events were recognized in partial protein sequences only.
Prenylation of HIV-1 platform protein Nef may augment its ability to bind membranes

Human immunodeficiency virus 1 (HIV-1) protein Nef was identified as high-confidence target for human farnesyltransferase (FT Score: −0.641, p-value = 0.0005). C-end farnesylation of Nef relies on its CVVS motif, previously shown to be especially efficient guide for eukaryotic prenylation enzymes [44]. Interestingly, protein Nef is dispensable for the spread of HIV-1 virus in cell culture systems, while being critical for high titre viral multiplication in living hosts and for the development of AIDS [45, 46]. In a nutshell, Nef is a platform which brings viral and host proteins in proximity to each other, thus, allowing derangement of a variety of host cell pathways. In particular, Nef modulates numerous membrane trafficking regulators [47]. We hypothesize that prenylation augments Nef with membrane binding ability, which may facilitate its previously demonstrated role as membrane signaling disruptor.

Importantly, HMG-CoA reductase inhibitors statins induce the resistance of CD4 + T cells to HIV-1 infection [48] and suppress HIV-1 virion release through inhibition of protein geranylgeranylation [49]. It is likely that the farnesylation of Nef will be amenable to suppression by statins along with host cell-encoded prenylation targets.

Prenylated versions of HCV protein NS5A may enhance their EPR anchoring and escape binding to antiviral proteins of the host

Hepatitis C virus (HCV) NS5A is a phosphoprotein which anchors in cytoplasmatic face of the endoplasmatic reticulum by its amphipathic N-terminal helix [50]. We showed that many variants of HCV protein NS5A end with C-terminal sequence CSMS (FT score: −1.727, p-value = 0.0510). These findings indicate that clonal evolution of HCV may result in NS5A mutants capable of additional, or “rescue” anchoring with its prenylated C-end. Genes for prenylated versions of NS5A were found in sequenced HCV specimens of genotypes 2, 3, 4, 5, and 6.

NS5A interacts with viperin, interferon-inducible inhibitor of farnesyl pyrophosphate synthase (FPPS). This interaction depends on residues within both N-terminal amphipathic helix and C-terminus of NS5A and takes place both at the lipid-droplet interface and at the HCV replication complex [51]. Notably, FPPS is an essential enzyme in isoprenoid biosynthesis pathway, which is also suppressed by statins, capable of inhibiting replication of HCV and enhancing anti-HCV effects of interferon when used in combination [52]. It is plausible that prenylation of NS5A may protect viral protein from interactions with viperin or other antiviral proteins, explaining selective support for these CSMS mutants evident in sequenced clonal populations of HCV.

Farnesylation of parainfluenza HN may also contribute to the fusion of viral envelop with host cell membrane

The C-terminus of hemagglutinin-neuraminidase (HN) encoded by a genome of human parainfluenza virus 3 ends in
amino acid motif CSQS (FT score = –1.156, p-value: 0.0293). Previously, the glycosylation of HN protein of Influenza was shown as critical to the recognition of the attachment receptor, cleavage of the F protein, and the promotion of the fusion [53]. Therefore, it is plausible that the farnesylation of parainfluenza HN may also contribute to the fusion of viral envelop to target cell membrane.

An analysis of a total of 512,155 Influenza protein sequences resulted in the PrePS-guided prediction of 17 candidates for farnesylation and 9 potential substrates for GGT1. No candidates for GGT2 were detected. All predicted prenyltransferase substrates were identified in influenza A viral strains only, but not in clades B and C. However, it appears that all except one influenza protein candidates are annotated only as partial sequences with termini probably coming from sequencing/PCR boundaries and, therefore, unlikely to represent...
African swine fever (ASF) virus infection is critically dependent on prenylation and may be suppressed by statins

African Swine Fever (ASF) virus, the only member of the dsDNAASFviridaefamily, causes a high mortality hemorrhagic fever in domesticated pigs as well as persistent asymptomatic infections of wild swine, including warthogs and bushpigs [54]. In multiple strains of ASFV, C-ends of the protein L83L are predicted to be prenylated on either CTIL or CTII motifs (Table 1). Interestingly, ASFV genome also encodes membrane-bound B318L enzyme that converts farnesyl pyrophosphate into all-trans-geranylgeranyl diphasphate [55], which may be then incorporated into cellular or viral proteins. This enzyme is essential for ASFV infection.

We propose that functioning of the ASF L83L protein, a target for eukaryotic FT and GGT1, is directly dependent on viral B318L enzyme, which augments cellular pools of prenylation precursors. This prediction aligns well with cell-culture based observations that ASFV requires host protein prenylation at several stages of the infectious cycle [56]. Moreover, we speculate that domesticated and wild swine demonstrate differential susceptibility to ASF due to the variance in intrinsic activity of HMG-CoA reductase, which is known to be induced by obesity-promoting diet [57, 58], and therefore, in relative availability of prenylation precursors. This hypothesis may be directly tested in in vitro/in vivo experiments evaluating the statins as potential suppressors of ASFV infection.

Widespread prenylation in phages of human associated bacteria may aid in moderation of microbial infections

Some bacterial proteins, such as SifA (Salmonella typhimurium) and AnkB (Legionella pneumophila), are shown to be modified by the host cell prenylation system. These modifications were confirmed experimentally, pointing that bacterial proteins come into contact with prenylation machinery of the host. Therefore, the proteins of bacteriophages may also come to proximity of host prenylation enzymes and end up prenylated. We hypothesize that the prenylation of bacteriophage proteins may aid in moderation of microbial infection in human host, thus ensuring long-term survival of bacterial population available for bacteriophage.

Prenylation events detected in a variety of bacteriophage proteins produced in ecosystems of human bodies are presented in Table 2. For a majority of these proteins, their functions remain unknown (Supplementary Table S4). The majority of the phages with prenylated proteins infect Gram-negative bacteria, except for these replicating in Staphylococcus, Lactobacillus, Clostridium, and Mycobacterium. Prenylated proteins were encoded by the genomes of phages grouped into four viral families: Siphoviridae (15), Myoviridae (13), Leviviridae (3), Podoviridae (3), and some unclassified phages (2) (Table 2). Prenylation hits were detected predominantly within dsDNA viruses, with only three prenylation candidates found in ssRNA viruses.

As an example, we present PrePS-guided analysis of dsDNA Siphoviridae bacteriophages [61, 62], with 15 prenylation candidates identified. Many Siphoviridae infect human-compatible host bacteria of Lactobacillus, Mycobacterium, Pseudomonas, Salmonella, Staphylococcus, and Vibrio genera (Table 2). In particular, marine bacteria Vibrio paraaeremolyticus, capable of establishing itself in a cytoplasmatic niche within human epithelial cell and causing seafood-associated gastroenteritis [63, 64], may host a Siphoviridae phage vB_VpaS_MAR10. This phage encodes 196-aa protein YP_007111899 with C-terminal prenylated sequence SSA-AS. This protein is similar to putative endolysin of Vibrio phage SSP002 (GB AFE86379.1), and is predicted to have lytic transglycosylase activity [63]. Enzymes of this kind usually are involved in phage-mediated lysis of infected bacterial cells through hydrolysis of β-1,4-linked polysaccharides [65]. Interestingly, prenylation candidate YP_007111899.1 is likely to be directed to cellular membrane as it includes N-terminal signal peptide (SignalP scores SSA-AS D = 0.826 D-cutoff = 0.5 for Gram-negative bacteria), with a cleavage site located between positions 21 and 22. Same peptide could also be cleaved by signal peptidase of eukaryotic cells with even higher SignalP scores (SSA-AS D = 0.915 D-cutoff = 0.450). These observations indicate that YP_007111899.1 may be, indeed, anchored in one or another membrane. In concert with this hypothesis, PSORT [31] analysis of YP_007111899 indicates that this protein is located in the bacterial periplasmic space. Prenylated transglycosylase enzyme comes to proximity with bacterial cell wall and weakens it to provide an exit for bacteriophage, resulting in the spread of phage infection and the decrease of bacterial burden in eukaryotic host.

Interestingly, the genome of V. parahaemolyticus encodes its own FT (GB Q87IR2.1). For now, its function in protein farnesylation in vivo remains hypothetical. If proven active, this bacterial FT may prenylate bacteriophage protein YP_007111899.1, thus facilitating its anchoring in bacterial cell

Preynylation is common in proteins encoded by diverse groups of relatively large viruses

Protein isoprenylation system of invertebrates is operative in phylogenetically diverse organisms, and its components are often exchangeable [59, 60]. Hence, novel insights in prenylation-driven elements of invertebrate viral lifecycles may be relevant to human viruses. High-confidence farnesylation or geranylgeranylation events were detected in proteomes of multiple species of Nima-viridae, Baculoviridae, Extomopoxviridae, Iridoviridae, Ichnoviridae, and others, as well as in proteins of the viruses isolated from various Protozoa, including gigantic Pandoraviruses and Megaviruses as well as moumouviruses and mimiviruses (Supplementary Table S5).
| Reference number | Protein | Virus | FT score and p-value | GGT1 score and p-value | CAAX |
|------------------|---------|-------|----------------------|------------------------|------|
| **Leviviridae**  |         |       |                      |                        |      |
| P09674.1        | RNA-directed RNA polymerase beta chain**** | Enterobacteria phage BZ13 | –0.216 <0.009 | NS | CVLS |
| P07393.1        | RNA-directed RNA polymerase beta chain**** | Enterobacteria phage GA | –0.317 <0.011 | NS | CVLS |
| **Myoviridae**  |         |       |                      |                        |      |
| YP_004009483.1  | Hypothetical protein Ac42p121 | Acinetobacter phage Ac42 | –0.598 <0.015 | 0.19 <0.0034 | CSLL |
| YP_004009657.1  | Hypothetical protein Acj61p040 | Acinetobacter phage Acj61 | –1.403 <0.047 | NS | CSIW |
| YP_009005300.1  | Head vertex assembly chaperone | Enterobacter phage PG7 | –1.651 <0.047 | NS | CTTC |
| YP_007006240.1  | Hypothetical protein FV3_00069* | Enterobacteria phage vB_EcoM-FV3 | –1.659 <0.048 | NS | CALG |
| YP_008530315.1  | Hypothetical protein Ec2_0070* | Escherichia phage 2 JES-2013 | –1.659 <0.048 | NS | CALG |
| YP_002003573.1  | Hypothetical protein* | Escherichia phage rv5 | –1.659 <0.048 | NS | CALG |
| YP_009030978.1  | Hypothetical protein | vB_EcoM_FFH2 | –1.659 <0.048 | NS | CALG |
| YP_214419.1     | Hypothetical protein PSSM2_187 | Prochlorococcus phage P-SSM2 | NS | –0.366 <0.0048 | CAII |
| YP_001957133.1  | Hypothetical protein 201phi2-1p413 | Pseudomonas phage 201phi2-1 | –1.932 <0.0599 | NS | CLTP |
| NP_835688.1     | Hypothetical protein Rm378p101 | Rhodothermus phage RM378 | NS | –1.612 <0.009 | CKIL |
| YP_004251105.1  | Hypothetical protein | Vibrio phage ICP1 | 0.487 <0.0028 | 2.207 <0.0007 | CVIL |
| YP_004934235.1  | g001 | Yersinia phage phiR1-37 | –0.851 <0.021 | NS | CAIQ |
| YP_007236076.1  | Helicase loader Dnal | Yersinia phage phiR1-RT | –0.851 <0.021 | NS | CKIL |
| **Podoviridae** |         |       |                      |                        |      |
| YP_007010599.1  | Hypothetical protein BPABA456_00180 | Acinetobacter phage Bphi-B1251 | –1.526 <0.042 | NS | CVIN |
| YP_007001429.1  | Hypothetical protein* | Escherichia phage TL-2011c | –1.486 <0.041 | NS | CVQY |
| YP_009100301.1  | Antirepressor protein | Shigella phage POCJ13 | –1.518 <0.042 | NS | CVQY |
| **Siphoviridae** |         |       |                      |                        |      |
| YP_008058943.1  | Hypothetical protein phiCP51_0020 | Clostridium phage vB_CpeS-CP51 | NS | 0.546 <0.0027 | CFIL |
| NP_046921.1     | UmuD | Enterobacteria phage N15 | –1.977 <0.0114 | NS | CPVL |
| YP_004934058.1  | UmuD | Escherichia phage HK639 | –1.086 <0.0073 | NS | CPVM |
| YP_002455801.1  | Fiber tail protein | Lactobacillus phage Lv-1 | –1.489 <0.0408 | 1.415 <0.0087 | CGVL |
| YP_009013376.1  | gp18 | Mycobacterium phage | –0.953 <0.0237 | NS | CGVS |
| YP_008409844.1  | Hypothetical protein PHELEMICH_18*** | Mycobacterium phage Phelemich | –0.953 <0.023441 | NS | CGVS |
| YP_008409939.1  | Hypothetical protein REPROBATE_18*** | Mycobacterium phage Reprobate | –0.953 <0.023441 | NS | CGVS |
| YP_001294574.1  | Hypothetical protein ORF066 | Pseudomonas phage M6 | –1.001 <0.024746 | NS | CLLQ |
| YP_006561030.1  | Hypothetical protein MP1412_23** | Pseudomonas phage MP1412 | –1.001 <0.024746 | NS | CLLQ |
| YP_001595847.1  | Hypothetical protein** | Pseudomonas phage YuA | –1.772 <0.052555 | NS | CLLY |

(Continued)
membrane. Alternatively, after the lysis of bacterial cell, bacteriophage protein may get exposed to eukaryotic FT and acquire not yet undiscovered function.

**Bacteriophage encoded prenylated proteins share common physical properties or sequence similarity**

Conservation across species indicates that a sequence has been maintained by evolution despite speciation. Therefore, similarity of protein sequences is commonly used as evidence of structural and functional importance [66]. The phages of related bacteria *Escherichia* and *Shigella*, named TL-2011c and POCJ13, encode prenylated proteins YP_007001429.1 and YP_009100301.1, respectively. These proteins share AntA/AntB antirepressor domain (Pfam08346) [67], GO-predicted localization in the bacterial cytoplasm, overall similarity of 68% (*E*-value = 2e-50) and an identical C-terminal motif CVIQ. Its conservation between two proteins found in somewhat related phages suggests possible importance of posttranslational lipidation for bacteriophage life cycle.

Table 2. (Continued)

| Reference number | Protein | Virus | FT score and p-value | GGT1 score and p-value | CAAX |
|------------------|---------|-------|----------------------|------------------------|------|
| YP_008239633.1   | Hypothetical protein SP031_00180 | Salmonella phage FSL-031 | -0.647, <0.016220        | NS              | CKIQ |
| YP_001742066.1   | Major tail subunit | Salmonella phage Vi II-E1 | -0.517, <0.013701        | NS              | CTVS |
| YP_239931.1      | ORF155  | Staphylococcus phage 42E | -1.8, <0.053787          | NS              | CITS |
| YP_002332369.1   | Repressor | Staphylococcus phage Ipla35 | -1.865, <0.056781        | NS              | CCSF |
| YP_007111899.1   | Protein containing transglycosylase domain | Vibrio phage vB_VpaS_MAR10 | -1.599, <0.045141        | NS              | CVIQ |
| Unclassified     |         |       |                      |                        |      |
| YP_009015303.1   | Hypothetical protein MT_57068 | Pseudomonas phage phiPto-bp6g | -1.623, <0.046130        | NS              | CVIP |
| YP_007878015.1   | Hypothetical protein VPDG_00043 | Vibrio phage henriette 12B8 | NS, -0.106, <0.004157    | CNLL             |      |

FT: CAAX farnesylation. GGT1: CAAX geranylgeranylation. No GGT2 (Rab geranylgeranyltransferase) substrates were identified. NS: Non-significant. Proteins marked with asterisks are 100% identical to each other, **95% identical, ***80% identical, ****100 % identical.

GO analysis of the subcellular localization of the unique prenylated proteins of bacteriophages showed that 81.48% of them are distributed in the cytoplasm, 11.11% in the bacterial inner membrane, and 7.41% in the bacterial periplasmic space (Fig. 3A). To understand the structure of the C termini of the predicted proteins, we calculated relative surface accessibility (RSA) (Fig. 3B). Within 20 C-terminal amino acids, bacteriophagal prenylated proteins demonstrated substantial similarity of their RSAs. For example, Cysteine (position 17, Fig. 3B) had RSA values in range of 0.479 and 0.885, indicating common physical properties of bacteriophage-encoded proteins identified as targets for prenylation machinery.

**Predicted prenylation events may aid in deciphering functions of uncharacterized viral proteins**

For making use of sequence information for biological and biomedical application, the mapping of function to genome regions is necessary; yet, the large body of functionally uncharacterized genome regions is the main bottleneck in life science research at this moment [68, 69]. Many proteins encoded by viral genomes are not yet associated with particular biological functions. The prenyl group accepting property points toward membrane association, thus, providing an important clue for subsequent wet-lab analysis.

Indeed, GO analysis of unique prenylated proteins showed that just 27% of them have been associated with any functional annotation (Supplementary Table S4). About 50% of characterized proteins are predicted to be a cellular component, 44% have assigned molecular functions, and only 6% are involved into one or another biological process (based on GO groups). Remarkably, approximately 50% of annotated proteins are GO-associated with membranes (GO:0016021; GO:0016020) (Supplementary Table S4), in line with predicted prenylation events.

**Conclusions and outlook**

Many important viral pathogens, including Mastadenoviruses Type C, HCV, HIV1, ASLV, and a number of Poxviridae and Herpesviridae viruses,
including CMV, encode the substrates for mammalian prenylation enzymes.

We hypothesize that viral protein prenylation events are advantageous for the propagation of the virus. Our findings support virus-driven rationale for the development of antiviral FT/GGT and FPPS inhibitors, many of which are already being evaluated in preclinical and clinical trials under assumption of predominant targeting of the host cells [35, 70–73]. At least in some infections, antiviral activities of HMG-CoA inhibiting statins, which are presumed to act by suppression of small GTPases encoded by host cell, may be also dependent upon direct disruption of the functioning of viral protein target. In case of ASFV, which is highly dependent on cholesterol biosynthesis pathway and prenylation system of the host being intact [56], we surmise that the propagation of this virus may depend on diet-induced upregulation of HMG-CoA reductase commonly seen in domesticated, but not in wild swine.

The follow-up studies of shortlisted viral prenylation candidates may take advantage of a variety of recently developed techniques for rapid experimental assessment of prenylome, including thin layer chromatography (TLC) [74, 75], total metabolic prenylome labeling coupled with either flow cytometry or more established in-gel fluorescence [76] and a combination of Stable Isotope with Amino acids in Cell Culture (SILAC)-mass spectrometry (MS) with isoprenoid affinity tagging in vitro [77]. These and other experimental investigations may aid the development of specific inhibitors targeting the interface between viral protein and host enzyme.

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Figure 3. Functional annotation of the prenylated proteins encoded by bacteriophages. A: The subcellular localization of prenylated bacteriophage’s proteins. B: Relative surface accessibility (RSA) of the 20C terminal amino acids. X-axis: amino acid number, Y-axis: protein number. RSA values less than 0.2 predicts the amino acid to be buried from the surface.

Amino acids in Cell Culture (SILAC)-mass spectrometry (MS) with isoprenoid affinity tagging in vitro [77]. These and other experimental investigations may aid the development of specific inhibitors targeting the interface between viral protein and host enzyme.

High-confidence prenylation targets have been identified in many bacteriophages infecting microbes inhabiting human body. Given that bacteriophages are becoming increasingly attractive as clinically acceptable means for controlling of multi-drug-resistant bacteria, a possibility of molecular crosstalk between representatives of all three kingdoms of life requires further investigation.

Figure 3. Functional annotation of the prenylated proteins encoded by bacteriophages. A: The subcellular localization of prenylated bacteriophage’s proteins. B: Relative surface accessibility (RSA) of the 20C terminal amino acids. X-axis: amino acid number, Y-axis: protein number. RSA values less than 0.2 predicts the amino acid to be buried from the surface.
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