

Viruses go modular

Ariel Shepley-McTaggart¹, Hao Fan², Marius Sudol,^{3,4} and Ronald N. Harty^{1*}

¹Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, 3800 Spruce Street, Philadelphia, PA 19104; ²Bioinformatics Institute, Agency for Science, Technology and Research (A*STAR), 30 Biopolis Street, Matrix #07-01, Singapore 138671, and Department of Biological Sciences (DBS), National University of Singapore, and Center for Computational Biology, DUKE-NUS Medical School; ³Department of Physiology, National University of Singapore, Laboratory of Cancer Signaling & Domainopathies, Yong Loo Li School of Medicine, Block MD9, 2 Medical Drive #04-01, Singapore 117597 and Mechanobiology Institute, T-Lab, 5A Engineering Drive 1, Singapore 117411; ⁴Department of Medicine, Icahn School of Medicine at Mount Sinai, New York NY 10029.

* Corresponding author

Dr. Ronald N. Harty, Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, 3800 Spruce Street, Philadelphia, PA 19104. Phone: 215-573-4485, Fax: 215-898-7887, email: rharty@vet.upenn.edu

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Abstract

The WW domain is a modular protein structure that recognizes the proline-rich Pro-Pro-x-Tyr (PPxY) motif contained in specific target proteins. The compact modular nature of the WW domain makes it ideal for mediating interactions between proteins in complex networks and signaling pathways of the cell (e.g. the Hippo pathway). As a result, WW domains play key roles in a plethora of both normal and disease processes. Intriguingly, RNA and DNA viruses have evolved strategies to hijack cellular WW domain-containing proteins and thereby exploit the modular functions of these host proteins for various steps of the virus lifecycle, including entry, replication, and egress. In this review, we summarize key findings in this rapidly expanding field, in which new virus-host interactions continue to be identified. Further unraveling of the molecular aspects of these crucial virus-host interactions will continue to enhance our fundamental understanding of the biology and pathogenesis of these viruses. We anticipate that additional insights into these interactions will help support

strategies to develop a new class of small-molecule inhibitors of viral PPxY-host WW domain interactions that could be used as antiviral therapeutics.

Introduction

The modular, compact nature of the WW-domain contributes to its status as a prominent mediator of a wide array of protein-protein interactions in the cell. WW-domain ligands include short proline-rich motifs with the Proline-Proline-x-Tyrosine (PPxY) motif serving as the consensus ligand (1-3). In mammalian cells, interactions between WW-domain and PPxY-containing proteins regulate a plethora of pathways that are involved in processes such as protein ubiquitination, signaling, sorting, migration, and degradation (4-27). For example, key WW-domain/PPxY interactions are critical for mediating the formation of protein complexes in the Hippo signaling pathway, which regulates cell proliferation, migration, and apoptosis (18,24,26,28-30). In addition, all members of the HECT (Homologous to the E6AP Carboxyl Terminus) family of E3 ubiquitin ligases contain

multiple WW-domains involved in binding to specific host PPxY-containing substrates, leading to modulation of their stability, trafficking/localization, and/or signaling functions (14,31-37).

Intriguingly, a growing body of literature has demonstrated that modular WW-domain/PPxY interactions not only are critical for regulating cellular pathways, but also play key roles in regulating one or more steps in the lifecycles of many viral pathogens (38-46). Not surprisingly, select DNA and RNA viruses have evolved the ability to mimic these protein-protein interactions by using virally-encoded PPxY-containing proteins to compete with cellular counterparts for binding to specific host proteins bearing one or more of these small modular WW-domains, thus in essence, hijacking cellular pathways and networks which ultimately impact both the virus and the host (see Figs. 1, 2, Table 1). For example, the initial discovery that viruses encode PPxY motifs to hijack host proteins to facilitate virus egress had a tremendous impact on the field since it demonstrated that viruses usurp host proteins and machinery to complete the late stage of virus-cell separation most efficiently. This discovery opened up an entirely new field of research on virus-host interactions that regulate egress. For example, the PPxY motif contained within matrix proteins encoded by RNA viruses such as retroviruses and filoviruses was termed a Late (L) domain due to its role in facilitating the late step in virus-cell separation or pinching-off (budding). In addition to the PPxY motif, PT/SAP and YxxL were also identified as functional L-domain motifs in specific RNA viruses. Intriguingly, the RNA viral L-domains were found to be interchangeable and movable while retaining their function in recruiting host proteins to promote virus egress (47-50). In contrast, DNA containing viruses such as adenoviruses and herpesviruses express PPxY-containing proteins that interact with specific host WW-domain bearing proteins to facilitate virus entry/internalization and affect virus replication and cancer progression, respectively.

The viruses chosen for discussion here represent the best characterized examples of viruses that encode PPxY motifs that mediate

physical and functional interactions with host WW-domain bearing proteins to influence specific stages of the virus lifecycle. Part of the excitement about this field is that viruses that are very different from one another in terms of their genetic material, morphology, lifecycle, and disease all have evolved to encode one or more PPxY motifs that interact with specific host proteins to impact their lifecycles. We will discuss key findings that highlight the physical and functional nature of a wide array of virus-host PPxY/WW-domain interactions, describe how usurping of host proteins affects the biology and pathogenesis of these viruses, and finally describe recent progress to identify and develop antiviral therapeutics that target and block these modular virus-host interactions as part of a novel host-oriented strategy to combat and treat viral infections.

I. Modular Interactions and Virus Egress

A. Retroviruses:

1. Human Immunodeficiency Virus 1 (HIV-1)

Pioneering studies that first identified viral Late (L) domains and subsequently, their ability to interact with modular WW-domains of host proteins were undertaken with the Gag proteins encoded by HIV-1 and Rous Sarcoma Virus (RSV) (50-53). Indeed, the PPPY sequence located in the N-terminal p2b region of the Rous Sarcoma Virus Gag protein was the first PPxY type L-domain shown to play a key role in facilitating virion egress by interacting with cellular WW-domains (51). One of the first WW-domain bearing host proteins implicated in viral PPxY-mediated egress was Nedd4 (Neuronal precursor cell-Expressed Developmentally Down-regulated 4); the prototypic member of the HECT family of E3 ubiquitin ligases. Indeed, a plethora of reports soon followed that linked the budding pathways of several RNA viruses to either Nedd4, Nedd4 family members, and/or ubiquitin (39,43-45,54-68). Although HIV-1 Gag does not possess a PPxY motif, budding of HIV-1 was shown to be positively regulated by ubiquitin and E3 ubiquitin ligases such as Nedd4, likely via an indirect mechanism. For example, Strack et al, showed that 2–5% of the HIV-1 p6^{Gag} found in virions was monoubiquitinated, and that chimeric Gag constructs with L domains from RSV and HIV-1

both produced Gag-ubiquitin conjugates in virus-like particles (VLPs). Moreover, inhibition of the proteasome, which reduces the levels of free ubiquitin in the cell, limited Gag VLP formation (69,70). More recently, Mercenne et al. demonstrated that PPxY-containing host protein angiomin (Amot) could bind both Nedd4L and HIV Gag and serve as an adaptor protein to promote early stages of HIV-1 assembly and budding (71). While our understanding of the precise mechanistic role of ubiquitin and E3 ubiquitin ligases in facilitating virus budding still remains unclear, one possibility is that mono-ubiquitination of viral matrix proteins allows them to engage one or more members of the host Endosomal Sorting Complex Required for Transport (ESCRT) pathway, which subsequently mediates efficient virus-cell separation and egress of mature virions from the plasma membrane (72,73). In contrast, other reports suggest that different mechanisms may also be involved. For example, a functional link between Nedd4 family members and HIV-1 budding was revealed by studies demonstrating that overexpression of Nedd4L could rescue budding of an HIV-1 Gag protein lacking an L-domain motif (62,64,74). In addition, Nedd4-mediated ubiquitination of ESCRT proteins such as Tsg101, rather than PPxY-deficient viral proteins such as HIV-1 Gag, may serve to activate ESCRT and promote budding of HIV-1 (75). Alternatively, Weiss et al. reported that Nedd4-mediated ubiquitination of HIV-1 Gag was not sufficient to stimulate virus budding, but rather the synthesis of K63-linked ubiquitin chains in the vicinity of the viral bud was critical for E3 ligase-mediated virus budding (65). Indeed, the authors postulate that the K63-linked ubiquitin chains on viral or cellular substrates are sensed or recognized by components of the ESCRT machinery, which is subsequently recruited to facilitate virus egress (65).

2. Rous Sarcoma Virus (RSV)

Unlike HIV-1, retroviruses whose Gag proteins do contain PPxY motifs can physically and functionally interact directly with the modular WW-domains of either Nedd4, or Nedd4 family members to facilitate their egress and spread. For example, early work by

Kikonyogo and colleagues in which they screened a chicken embryo cDNA expression library with a peptide containing the PPxY L-domain sequence from RSV Gag-p2b, identified two WW domain interactors they named Late Domain-Interacting proteins 1 and 2 (LDI-1 and 2) (56). LDI-1 was found to share sequence homology with *Xenopus* Nedd4, while LDI-2 exhibited homology to other members of the Nedd4 family, specifically human WWP1 and the murine E3 ubiquitin ligase, ITCH (also known as AIP4) (56). The authors also demonstrated that budding of RSV Gag required the PPxY L-domain, and that overexpression of the isolated WW domains of LDI-1 acted in a dominant-negative manner to inhibit budding (56,76). In a follow-up study, Vana and colleagues demonstrated that a catalytically active LDI-1 protein was required to promote RSV Gag egress, as a catalytically inactive LDI-1 mutant blocked Gag release. While they were able to detect both mono- and polyubiquitinated forms of Gag within cells, the monoubiquitinated form of Gag was most prevalent in budded VLPs, and ubiquitination of RSV Gag was dependent on its PPxY L-domain (45).

3. Human T-Cell Leukemia Virus 1 (HTLV-1)

The PPPYVEPTAP sequence at the C-terminus of the HTLV-1 Gag protein contains a PPxY and PTAP L-domain motif, both of which were shown to play a role in facilitating release of HTLV-1 Gag VLPs; however, the PPxY motif appeared to be more important for efficient egress (39,43,59). HTLV-1 Gag was shown to interact with WW-domains of E3 ligases Nedd4, BUL1, and HECW2 (39,43,59), and results from Blot et al. suggested that the HTLV-1 PPxY motif first recruited host Nedd4, which then allowed the virus to engage the ESCRT complex, specifically Tsg101, to promote efficient assembly and egress of virus particles (59). Indeed, Tsg101 is a key component of the ESCRT-1 complex that can interact with viral PTAP L-domain motifs, and can also bind to ubiquitinated proteins, thus mediating their engagement of the ESCRT machinery. Later studies both confirmed and expanded the repertoire of host WW-domain bearing E3 ligases hijacked by the PPxY motif of HTLV-1

Gag to benefit egress and spread of virus particles. For example, HECT family E3 ubiquitin ligase WWP1, which possesses four WW-domains, was shown to ubiquitinate a lysine residue at position 74 of HTLV Gag to promote efficient virus egress (77,78). In contrast, a lysine-74 mutant of HTLV-1 Gag was not ubiquitinated and was budding defective. Dorjbal and colleagues (66) went on to test nine members of the Nedd4 family of E3 ubiquitin ligases (Nedd4, Nedd4L, BUL1, BUL2, WWP1, WWP2, ITCH, SMURF1, and SMURF2) for their ability to promote egress of HTLV-1, and found that ITCH was the main contributor to HTLV-1 budding. Also of interest was their finding that the physical binding efficiencies between the viral PPxY motif and the various host WW-domains did not correlate with their functional effect on HTLV-1 budding (66).

4. Murine Leukemia Virus (MLV)

Studies by Martin-Serrano and colleagues helped to solidify the functional link between HECT E3 ubiquitin ligases recruited by viral PPxY motifs and the ESCRT pathway during egress of wild type or chimeric Murine Leukemia Virus (MLV) Gag proteins. Using an array of WW-domain containing E3 ligases and chimeric MLV Gag proteins containing the PPxY motifs and flanking residues from either host amiloride-sensitive sodium channel ENaC, RSV Gag-p2b, or Ebola virus VP40 proteins, they demonstrated that the E3 ligase WWP1 was recruited to sites of MLV budding in a PPxY-dependent manner, and that enhancement of budding required a catalytically active HECT domain (79). They concluded that the HECT ubiquitin ligases likely served as co-factors for viral PPxY-mediated budding and provided a critical link to the ESCRT complex that mediated the final step of virus-cell separation. In a later study by the same group, they demonstrated that host PPxY containing arrestin-related trafficking (ART) proteins could function as adaptors, linking WW-domain containing HECT ubiquitin ligases to the ESCRT machinery (80). Indeed, they showed that the ARTs could be recruited to sites of PPxY-dependent viral budding and likely served as a platform for ubiquitination during the budding process (80). Thus, it is interesting to speculate that the

PPxY-containing viral matrix proteins have evolved to mimic and/or compete with cellular ART-like proteins for binding to modular WW-domains to regulate virus egress and spread.

B. Filoviruses:

1. Ebola (EBOV) and Marburg (MARV) Viruses

Filoviruses (Ebola [EBOV] and Marburg [MARV]) are emerging pathogens that can cause severe hemorrhagic disease with high mortality rates in primates and humans (81-83). There has been growing interest in elucidating the molecular mechanisms used by these viruses to bud and spread from cells, as well as in identifying novel virus-host interactions that contribute to the transmission and pathogenesis of these deadly viruses. The filovirus matrix protein, VP40, is the major structural component of filoviruses and plays a key role in directing virion assembly and promoting virion egress during late stages of filovirus replication (54,84-88). Intriguingly, a number of groups have shown that independent expression of filovirus VP40 leads to the production and egress of filamentous VLPs that mimic the morphology of authentic filoviruses (44,54,89-94). Thus, VP40 has been the focus of a plethora of studies to examine the molecular mechanisms and host interactors that regulate filovirus egress. As described above for the Gag proteins of many retroviruses, L-domains are highly conserved in the VP40 matrix proteins of all known EBOV and MARV species. Indeed, both EBOV and MARV VP40 proteins contain a PPxY-type L-domain motif that can compete with cellular counterparts for binding to specific WW-domain bearing host proteins, thus hijacking critical cellular pathways that impact both the virus and the host. It should be mentioned that EBOV VP40 and MARV NP (nucleoprotein) proteins contain a PT/SAP-type L-domain as well, and PT/SAP has been proposed to interact with and recruit host Tsg101 to facilitate virion egress (91,95-98). Recent findings describing key modular interactions between filoviral PPxY motifs and host WW-domain interactors will be discussed below.

Harty and colleagues were the first to demonstrate a role for the PPxY motif of EBOV VP40 in budding of VLPs and in interacting

physically and functionally with host WW-domains of E3 ligases Nedd4 and its yeast ortholog Rsp5 (54). A series of reports soon followed that demonstrated that the VP40 PPxY motifs of both EBOV and MARV functioned in a manner similar to that described earlier for both retroviral and rhabdoviral (55,89,99-101) PPxY motifs to recruit host interactors and ESCRT to facilitate egress (Fig. 2A) (44,54,58,60,91,96,97,102). Most of the early work on the filovirus VP40 PPxY motif was centered around its role in hijacking Nedd4 and ultimately the ESCRT complex to facilitate budding. More recent developments were based on utilization of a strategy involving screening of a specially prepared array composed of a complete set of bacterially-expressed and purified human WW-domains with either WT or PPxY-mutant peptides from EBOV or MARV VP40 to identify novel WW-domain interactors that may regulate filovirus budding (41,42,103). Interestingly, the authors found that there was an unprecedented degree of specificity, in that the filoviral WT PPxY motifs bound to only a select number of host WW-domains (41,42,103). In addition to the expected WW-domain interactors like Nedd4 and Rsp5, the newly identified VP40 hits from this screen included HECT family E3 ubiquitin ligases ITCH (AIP4) and WWP1 (41,42) previously identified as Gag interactors. In addition, BCL2 Associated Athanogene 3 (BAG3); a WW-domain bearing member of the BAG family of molecular co-chaperone proteins involved in regulating protein homeostasis and cell survival through chaperone-assisted selective autophagy (CASA), was identified as a novel VP40 interactor (103).

Han and colleagues confirmed that EBOV VP40 interacted with ITCH in a PPxY/WW-domain dependent manner as determined by co-immunoprecipitation of full-length proteins, and that the enzymatically active form of ITCH was required to mono-ubiquitinate EBOV VP40 leading to enhanced egress of VP40 VLPs (41). In addition, the authors used both siRNA knockdown/rescue assays and ITCH knockout cells to demonstrate further the biological importance of ITCH for VP40 budding in this VLP system and for egress of recombinant VSV engineered to express the PPxY L-domain of

EBOV VP40 (41,97). These data demonstrated that EBOV VP40 likely recruits host E3 ligase ITCH via the PPxY/WW-domain modular interaction to promote efficient egress of EBOV VLPs and VSV-EBOV recombinant viruses, and although yet to be proven, imply that a similar modular recruitment of ITCH may occur during authentic EBOV infection.

Similar findings were reported by Han and colleagues for E3 ligase WWP1 and EBOV VP40 (42), again demonstrating that the interaction between WWP1 and VP40 is PPxY/WW-domain dependent and that mono-ubiquitination of VP40 by enzymatically active WWP1 led to enhanced egress of VP40 VLPs (42). Not surprisingly, both ITCH and WWP1 interact with a plethora of cellular proteins to regulate multiple pathways and networks in mammalian cells (37). For example, ITCH plays a key role in regulating lymphocyte activation and differentiation; key processes in immune regulation and inflammatory signaling (104). Likewise, WWP1 functions in protein signaling and trafficking-related to TGF β and EGF signaling, as well as apoptosis and neurological diseases (37). While recruitment or hijacking of E3 ligases such as Nedd4, ITCH, and WWP1 may facilitate egress and modulate the pathogenesis of infectious EBOV, the potential effects of these modular interactions on normal host protein function remains unclear. In addition, whether the ability of filovirus VP40 and other PPxY containing viral proteins to interact with a wide array of WW-domain interactors is linked to the expression levels of these ligases in various cell types and reflects the cellular tropism of the virus, or is simply a mechanism of built-in redundancy used by the virus, remains to be determined.

Using the previously mentioned human WW-domain array, Liang and colleagues identified host BAG3 as a novel WW-domain interactor with the PPxY motifs of both EBOV and MARV VP40 (103). BAG3 is a stress-induced protein that regulates cellular protein homeostasis and cell survival through the CASA pathway (11,105). The authors confirmed the PPxY/WW-domain dependent interaction between BAG3 and both EBOV and MARV VP40, and intriguingly, demonstrated that expression of BAG3 *negatively* regulated budding of both

EBOV and MARV VP40 VLPs, as well as egress of infectious VSV-EBOV recombinants (103). This finding was novel since all previously identified host WW-domain interactors with VP40 *positively* regulated VLP and/or virus egress (103). The authors speculated that BAG3 and CASA may function as a host defense mechanism, whereby BAG3 recognizes and interacts with the viral PPxY motifs, thereby dampening virus egress by interfering with the budding function of viral PPxY-containing matrix proteins. Recently, BAG3 and the CASA machinery were reported to regulate both the synthesis and degradation of filamin; a key actin-crosslinking protein at the plasma membrane that modulates cell adhesion and migration (106-110). BAG3 is also involved in regulating the disassembly of the actin-based contractile ring during cytokinesis (cell-cell separation) (111,112). Cytokinesis is topologically equivalent to a virion budding from the cell surface (virus-cell separation), and both processes involve some of the same host pathways (113-121). One possibility is that BAG3 and the CASA machinery regulate actin-based dynamics, stress and membrane tension necessary for efficient pinching off of VLPs and/or viruses (122,123). Liang and colleagues used confocal microscopy to show that VP40 appeared to be sequestered away from the plasma membrane and accumulated in cytoplasmic aggregates in cells expressing WT BAG3, which correlated with a decreased efficiency of VLP egress (103). Although more studies are needed, it will also be of interest to investigate the potential interplay and binding competition between WW-domain bearing host proteins that positively regulate virus egress (*e.g.* ITCH) vs. those that negatively regulate virus egress (*e.g.* BAG3) as a means to better understand the biological impact of these dueling modular interactions.

II. Modular Interactions and Virus Entry/Replication

A. Adenoviruses:

Although most PPxY motifs encoded by RNA viruses hijack HECT-family, WW-domain interactors to promote virion egress, the adenovirus PPxY/host interaction was unique in that it was one of the first discoveries of a viral

PPxY motif functioning in virus entry rather than virus egress. Indeed, both the adenoviral membrane lytic internal capsid protein VI (PVI) and the capsid penton base protein possess PPxY motifs that play a role in Adenovirus (AdV) entry. AdV is a non-enveloped, double-stranded DNA virus, which enters cells by receptor-mediated endocytosis and utilizes the penton base protein for internalization, as well as escape from endosomes. In 2002, Galinier and colleagues screened a human lung expression library for host proteins that may influence adenoviral entry by interacting with the two PPxY motifs in the N-terminal region of the penton base polypeptide chain (40). They found that the first PPxY motif was critical for mediating interactions with WW-domain E3 ligases WWP1, WWP2 (also known as AIP2), and ITCH (40), providing the first demonstration that a non-enveloped DNA virus employed a PPxY motif to interact with host HECT-family ligases.

AdV requires exposure of the internal PVI capsid protein to breach the endosomal membrane and escape into the cytosol to complete the internalization step. Wodrich and colleagues identified a functional PPxY motif within PVI that recruited both Nedd4.1 and Nedd4.2 leading to ubiquitination of PVI (124). They went on to show that the PPxY motif within PVI was required for microtubule-dependent intracellular trafficking to the nucleus, and that PPxY mutants were able to escape the endosome, but were defective in trafficking to the nucleus (Fig. 2B) (124). Later findings by the same group suggested that recruitment of Nedd4 by the PPxY motif of PVI was a strategy used by the virus to divert the host E3 ubiquitin ligase from its physiological function in regulating autophagy, thus allowing the virus to evade the host autophagic response (125,126). Indeed, the authors observed that the WT PPxY motif of PVI prevented efficient formation of autolysosomes, and they speculated that the WT virus may interfere with elongation of the autophagosomal membrane, thereby preventing autophagosome formation (125,126). In sum, these novel findings were the first to demonstrate that the modular interaction between the adenoviral PPxY motif and the WW-domain(s) of Nedd4 not only promoted

efficient viral entry and trafficking, but also represented a unique strategy for the virus to reduce antigenic presentation and evade the host immune response (125,126).

B. Herpesviruses:

1. Epstein Barr virus (EBV; human gammaherpesvirus 4)

Epstein-Barr virus is a human herpesvirus that infects lymphoid and epithelial cells to cause infectious mononucleosis. EBV establishes a lifelong latent state in the absence of replicative gene expression in B-cells, and the viral Latent Membrane Proteins (LMP1, 2A, and 2B) are expressed in latency to antagonize the normal process of B-lymphocyte activation. Indeed, LMP2A plays a key role in enhancing the development of tumors and the transformation potential of cells. LMP2A, which helps maintain EBV latency, contains two highly conserved PPPPY motifs that were first identified by Longnecker and colleagues and were shown to interact with WW-domains (127-129). Two groups went on to show that the PPxY motifs of LMP2A recruited host proteins ITCH (AIP4), WWP2, Nedd4L and/or Nedd4 via one or more of their modular WW-domains (46,127). Indeed, Winberg and colleagues found that these E3 ubiquitin ligases formed PPxY/WW-domain dependent complexes with LMP2A in EBV infected B-cells, which they suggested may lead to ubiquitination of Lyn and Syk tyrosine kinases to ultimately block B-cell signaling (46). In support, Ikeda and colleagues demonstrated a reduction in the normal cellular levels of Lyn and LMP2A, suggesting that recruitment of Nedd4-like ubiquitin ligases via the viral PPxY motifs resulted in ubiquitin-mediated degradation of Lyn and LMP2A, thus modulating B-cell signal transduction (127).

More recently, Lan and colleagues described an intriguing interaction between the PPxY motifs of EBV LMP2A and a host WW-domain containing oxidoreductase (WOX1), that functions, in part, as a tumor suppressor (130). They report that WOX1 is important for cancer-promoting effects triggered by LMP2A, and specifically that the PPxY/WW-domain interactions between LMP2A and WOX1 were important for induction of matrix metalloproteinase 9 (MMP9). These findings

suggest that the modular interactions between the PPxY motifs of EBV LMP2A and the WW-domain on host WOX1 may be crucial for invasiveness and cancer progression associated with EBV infection (130).

2. Roseoloviruses (human betaherpesviruses; HHV-6A, HHV-6B and HHV-7)

Roseoloviruses, also known as human herpesvirus types 6A, 6B and 7 (HHV-6A, HHV-6B and HHV-7), have been linked to several neurological diseases, including encephalitis, epilepsy, and multiple sclerosis (MS). The roseoloviral protein U24 is a C-tail-anchored membrane protein shown to affect recycling and endocytosis of cell surface receptors (131,132). In addition, U24 function may be linked to MS, as it has been found to mimic the interaction of Myelin Basic Protein's (MBP) with its binding partner Fyn (133,134). The N-terminal region of U24 encoded by HHV-6A and HHV-7 contains a PPxY motif that was shown to interact with HECT-family E3 ligases SMURF2 and Nedd4; albeit more strongly to WW-domain #3 of Nedd4 (135). Importantly, since sodium channels have been found to be associated with MS lesions, investigators speculated that U24 may interfere with the endosomal recycling of sodium channels known to be regulated by Nedd4. Although still speculative, results from Sang and colleagues suggest that the PPxY motif of U24 and its strong interaction with WW-domain #3 of Nedd4 may play role in MS or other related demyelinating diseases (133).

C. Tombusviruses:

1. Tomato Bushy Stunt virus (TBSV)

Tomato Bushy Stunt virus is an RNA virus with a small positive-stranded RNA genome that infects plants, and the virus encodes a handful of viral proteins that are unable to support viral replication without contributions from host factors. Plants have developed innate and adaptive immune pathways, including cell-intrinsic restriction factors (CIRFs), that limit plant virus replication and disease (136). Indeed, the yeast E3 ubiquitin ligase Rsp5 was identified as a CIRF, as its WW-domains interact with the RNA binding sites on two TBSV replication proteins (p33 and p92pol), thus

blocking their ability to function in viral replication (38,137,138). Interestingly, the ability to block viral replication was independent of the catalytic HECT domain and was instead dependent on binding of the viral polymerase proteins by the WW-domain of Rsp5 itself (38). Although a canonical PPxY motif is not present in either p33 or p92pol, these two viral proteins do share structural similarity that likely renders them capable of interacting with the WW-domains of Rsp5. The Rsp5 WW-domains not only interfere with the formation and assembly of the viral RNA replicase, but also appear to inhibit subversion of additional host proteins required by the viral replicase to complete viral replication (138). In sum, these studies not only have identified host WW-domain interactors that can regulate replication of TBSV and perhaps other related plant viruses, but also have laid the groundwork for future studies to determine whether these modular interactions and WW-domains themselves could serve as the basis for new antiviral strategies.

III. Modular Interactions and Innate Immunity

A. Orthomyxoviruses:

1. Influenza A and B viruses.

Influenza viruses do not encode any functional PPxY motifs; however, a recent report described the indirect effect of a host PPxY/WW-domain modular interaction on the innate immune response and susceptibility to influenza virus infection (139). Interferon (IFN)-induced transmembrane protein 3 (IFITM3) is a PPxY-containing innate immune protein that restricts influenza virus infection, and levels of IFITM3 are regulated by ubiquitination following binding of IFITM3 to the WW-domains of E3 ligase Nedd4 (139). Chesarino and colleagues demonstrated that endogenous Nedd4 and IFITM3 colocalized with lysosomal markers in cells and that Nedd4-dependent regulation of IFITM3 levels affected the susceptibility of both cells and mice to infection with influenza A and B viruses (139). For example, knockdown of endogenous Nedd4 resulted in an increase in both IFITM3 levels and in cellular resistance to influenza virus infection and the PPxY motif of IFITM3 was required for Nedd4-mediated ubiquitination (139). Overall, these findings suggest that short term pharmacological

inhibition of Nedd4's modular interaction with IFITM3 could represent a novel therapeutic strategy to prevent infection by influenza viruses and perhaps other IFITM3-sensitive viruses as well.

B. Retroviruses and Filoviruses:

2. HIV-1 and Ebola virus.

Cellular innate immune responses have been identified that target the budding process of invading viral pathogens by affecting specific host proteins associated with virus egress, such as Nedd4 and ESCRT components. For example, interferon induced ubiquitin-like protein 15 (ISG15) was shown to interfere with release of HIV-1 by inhibiting host-mediated ubiquitination of Gag and Tsg101 (140). Indeed, follow-up studies on EBOV VP40-mediated budding demonstrated that ISG15 inhibited the ubiquitin ligase activity of Nedd4, which was required for efficient PPxY-mediated egress of EBOV VP40 VLPs (63,141,142). These studies were impactful, in that they identified an indirect mechanism of host inhibition of L-domain-mediated virus egress. Subsequent and elegant studies by the Leis group expanded on the ESCRT-associated antiviral role of innate immune protein ISG15 by showing that ISG15 interacted with and/or disrupted the activity of additional ESCRT-related proteins including CHMP5, LIP5, and Vps4 (143-145). Thus, ISG15 appears to be a key component of the host innate immune response that interferes with the assembly and budding processes of a broad array of viruses at multiple levels (146,147).

IV. Modular Interactions and Host-Oriented Antiviral Therapeutics

A. Filoviruses and Other PPxY-Containing Viruses.

The viral PPxY/host WW-domain interface represents an attractive target for the development of small molecule or peptide-based inhibitors that are often referred to as "host-oriented" therapeutics since they are designed to target a virus-host interface rather than a viral protein or viral function only. For some of the viruses discussed above, these therapeutics would be predicted to inhibit efficient virus egress and spread by preventing

the viral matrix protein from recruiting WW-domain partners and subsequent egress machinery (Fig. 3). Potential advantages of this host-oriented strategy include the widespread conservation of the viral PPxY, as well as other L-domain motifs in a wide array of viral matrix proteins, such that an effective therapeutic may have broad-spectrum activity against numerous viral pathogens. In addition, host-oriented inhibitors may diminish the emergence of drug-resistant viral mutations, particularly for RNA viruses, and may lead to a paradigm shift in the search for better antiviral drugs. Since these PPxY/WW-domain interactions represent a common mechanism for mediating egress for a wide range of RNA viruses, a combination of inhibitors targeting early stages of the virus lifecycle along with those targeting this late step in budding may represent a highly effective therapeutic cocktail. Considering the small size of functionally folded WW domains (less than 38 amino acids) and the rigid structure of PPxY L-domains, which form polyproline type II helices, engineering of their respective polypeptides and peptides into expression vectors may serve as competitors of L-domain/E3 ligase interactions. Indeed, a successful interference of Rous Sarcoma Virus budding from cells by *cis* overexpression of a WW domain support this avenue of potential interventions to harness retro- and filo-viremias *in vivo* (76).

Several recent studies support the feasibility of small molecule inhibition of PPxY/WW-domain interactions as an antiviral strategy for PPxY-containing RNA viruses. First, an *in silico* strategy was employed by Han et al. (148) to identify existing small molecule compounds from a ZINC database that could potentially block the interaction between the PPxY L-domain motif of EBOV and the modular WW-domain of host protein Nedd4 (149,150). Functional activity of the best candidate inhibitors was assessed by using filovirus VLP budding assays, as well as infectious virus budding assays employing VSV-WT, VSV recombinants expressing EBOV L-domain motifs, and rabies virus, all of which contain PPxY motifs. The authors showed that the most potent compounds (e.g. compound 5) had broad-spectrum anti-budding activity against PPxY-containing matrix proteins from

filoviruses, rhabdoviruses, and arenaviruses with little to no cytotoxicity at the inhibitory concentrations used (148). Importantly, the candidate inhibitors significantly reduced the budding of live infectious VSV and rabies virus in cell culture compared to that observed in the presence of structurally related negative controls.

To gain a better understanding of how the viral PPxY motif fits into the WW-domain pocket of Nedd4, and how inhibitors like compound 5 may antagonize this virus-host interaction, modeling and docking analyses were performed using the NMR structure of human Nedd4-WW3 domain and the Ebola VP40-derived PPxY peptide (PDB: 2KQ0), with and without compound 5 (Fig. 4). The peptide-binding site in the WW3 domain contains two sub-pockets. This study revealed that the second proline residue from the PPxY motif in the EBOV VP40 peptide occupies the first sub-pocket, surrounded by WW3 residues Asn24, Phe28, Thr37, and Trp39 (Fig. 4A), and that the tyrosine residue from the EBOV VP40 PPxY motif occupies the second sub-pocket, surrounded by Arg20, Ile30, His32, and Lys35 (Fig. 4A). Besides hydrophobic interactions, the peptide also forms one hydrogen bond and one salt bridge with WW3 residues Asn24 and Arg20, respectively (Fig. 4A). The docked conformation of compound 5 showed reasonable shape complementarity to this binding site, with its quinoxaline bicyclic moiety occupying the first sub-pocket, its phenyl ring occupying the second pocket, and its urea moiety forming hydrogen bonding interactions with Arg20 in the WW3 domain (Fig. 4B). Thus, compound 5 could directly compete with the EBOV VP40 peptide in binding to the human Nedd4-WW3 domain (Fig. 4C), which would correlate with its anti-budding activity observed by Han et al. (148). While the authors reported little to no cytotoxicity of compound 5 in these *in vitro* assays, additional experiments will be essential to assess any effects of the inhibitor on the normal cellular functions of Nedd4 and/or other related E3 ligases.

In a follow-up report by Loughran et al. (151), the authors described the preparation of a series of quinoxaline-2-mercapto-acetyl-urea

analogs of compound 5, which were evaluated for their ability to inhibit filoviral VLP and virus egress using assays described above. The new analogs inhibited both VP40 VLP and live recombinant VSV virus budding in the low nanomolar range, with little to no cytotoxicity, good human microsome stability, and no inhibition of P450 3A4 at 33 μ M concentration (151). The efficacy of lead candidate inhibitors to block egress of authentic hemorrhagic fever viruses *in vitro* and *in vivo* remains to be determined. Identification of inhibitors that block such interactions may be useful for a wide array of PPxY containing viruses that hijack or recruit WW-domain containing E3 ligases during the late step of budding. Such inhibitors may be particularly useful for the treatment of Ebola virus disease, as EBOV was shown recently to cross the blood-brain barrier and re-emerge months later in the CNS and eye, as well as in other immunologically privileged sites that are inaccessible to antibody therapy (152-165).

While progress to date on the identification and development of host-oriented, small molecule therapeutics that target virus-host interactions is still in the early stages, these studies indicate that this new therapeutic strategy is feasible *in vitro* and worth pursuing *in vivo*. However, a number of challenges and hurdles remain, one of which includes the potential for these therapeutics to disrupt key cellular protein WW-domain interactions leading to possible side effects from these compounds. Indeed, the precise target(s) of these compounds remains to be determined, as does their possible effects on normal host protein functions. Nevertheless, the demonstration that WW domains are potentially druggable (30), and the high specificity of viral L domain interactions with their cognate protein domains support further investigations into this approach to control egress of RNA viruses like EBOV. In support of strategic shifts in drug discovery efforts, recent successes have been achieved with the use of stapled peptides (166-168) and cell-penetrating cyclic peptides (169), which are designed to target specific intracellular protein-protein interactions. Indeed, Zhang et al. demonstrated that stapled peptides targeting dimer formation of the HIV-1 capsid protein showed potent antiviral activity against a broad

array of viral isolates, including strains that were resistant to reverse transcriptase and protease inhibitors (168). In sum, the continued pursuit of fundamental and new insights into the virus-host interface will ultimately lead to the development of novel antiviral targets and strategies.

V. Conclusions and Outlook

While studies described above highlight the key roles that host WW-domain interactors play in regulating various stages of RNA and DNA virus lifecycles, a number of questions remain. For example, are there additional *cis*- and/or *trans*-acting factors that influence or regulate the selective binding of the viral PPxY motif to a particular host WW-domain during a virus infection? Do host PPxY-bearing proteins that interact with cellular WW-domains compete with viral PPxY motif containing proteins in infected cells? With more than 1,200 PPxY motifs represented in over 1,000 human proteins, and with the number of predicted, functional human WW-domains being more than 90, the potential repertoire of WW domain-PPxY complexes in cells seems vast. Therefore, it will be important to understand the spatial, temporal, topological and structural levels of specificity that make signaling by PPxY L-domains and host proteins so discrete and biologically critical for the life cycles of many different viruses. As such, studies of highly conserved PPxY motifs and their shared interactions with modular WW-domains of host interactors will likely provide new and valuable insights into the mechanisms of viral budding and transmission, entry, immune evasion, and genome replication for a wide array of viral pathogens.

If indeed antivirals targeting PPxY/WW-domain interactions are to be pursued and developed, it will be critical to assess the potential cytotoxic effects of such antiviral compounds, and determine the specificity for these compounds to interfere with virus-host PPxY/WW-domain interactions, rather than with host-host PPxY/WW-domain interactions. Although the treatment regimen for such an inhibitor would likely be brief when used to target acute viral infections, the potential side effects on normal host protein function would still need to be assessed. In addition, the wide array of host WW-domains and their potential to

be affected by these host-oriented therapeutics must be taken into consideration when developing these compounds. To date, there does appear to be specificity in PPxY binding to select WW-domains, and a better understanding of the nature of this specificity will be crucial to ensure that any antiviral inhibitor will exhibit the same specificity for its target.

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List of Abbreviations:

AdV – Adenovirus
ART proteins - Arrestin-Related Trafficking proteins
BAG3 - BCL2 Associated Athanogene 3
BiMC - BiMolecular Complementation
BUL1 and 2 - Binds Ubiquitin Ligase proteins 1 and 2
CASA - Chaperone-Assisted Selective Autophagy
CIRFs - Cell-Intrinsic Restriction Factors
EBV - Epstein Barr Virus
EBOV – Ebola Virus
ENaC - Amiloride-sensitive sodium channel
ESCRT - Endosomal Sorting Complex Required for Transport
HECT - Homologous to the E6AP Carboxyl Terminus (E3 ligase family)
HECW2- HECT, C2 and WW domain containing E3 ubiquitin protein ligase 2
HHV - Human Herpes Virus
HIV-1- Human Immunodeficiency Virus1
HTLV-1 - Human T-Cell Leukemia Virus1
IFITM3 - Interferon-induced transmembrane protein 3
ISG15 - Ubiquitin-like protein ISG15
ITCH (AIP4) - Itchy E3 ubiquitin protein ligase
LDI-1 and 2- Late Domain-Interacting proteins 1 and 2
L domain – viral Late domain
LMP2A - Latent Membrane Protein 2A
MARV - Marburg Virus
MBP - Myelin Basic Protein
MLV - Murine Leukemia Virus
MMP9 - Matrix MetalloProteinase 9
Nedd 4 - Neuronal precursor cell- Expressed Developmentally Down-regulated 4
PPxY- Proline-Proline-x-Tyrosine
PVI – Pre-protein VI (membrane lytic internal capsid protein)
Rsp5- yeast Reverses SPT-phenotype protein 5 (E3 ubiquitin-protein ligase)
RSV - Rous Sarcoma Virus
SMURF 1 and 2 – Smad Ubiquitin Regulating Factors 1 and 2
TBSV - Tomato Bushy Stunt Virus
Tsg101 - Tumor susceptibility gene 101
VLPs - virus-like particles
VSV- Vesicular Stomatitis Virus
WOX1 - WW-domain containing Oxidoreductase
WWP1 and 2 - WW Domain Containing E3 Ubiquitin Protein Ligase 1 and 2

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Virus Family	Virus	Late Domain Containing Viral Protein**	WW-domain Bearing Host Interactor	Effect on viral lifecycle stage
Retroviruses	HIV-1	Gag	Nedd4, Nedd4.2/Nedd4L, Rsp5	Enhancement of budding
	RSV	Gag	LDI-1, LDI-2, Nedd4	Enhancement of budding
	HTLV-1	Gag	Nedd4, ITCH/AIP4, WWP1, HECW2	Enhancement of budding
	MLV	Gag	Nedd4, WWP1, WWP2, ITCH/AIP4, ART proteins	Enhancement of budding
Filoviruses	EBOV	VP40	Nedd4, Rsp5, ITCH, WWP1	Enhancement of budding
			BAG3	Inhibition of budding
	MARV	VP40	Nedd4	Enhancement of budding
			BAG3	Inhibition of budding
Herpesviruses	EBV	LMP2A	Nedd4, Nedd4.2/Nedd4L, WWP2, ITCH/AIP4	Viral latency
	EBV	LMP2A	WOX1	Cancer progression
	HHV (Roseolovirus)	U24	Nedd4.1, Nedd4.2/Nedd4L, SMURF-2	Neurologic disease progression
Adenoviruses	AdV	PVI	WWP1, WWP2, AIP4/ITCH, Nedd4.1, Nedd4.2/Nedd4L	Viral entry
Tombusviruses	TBSV	p92pol, p33 [#]	Rsp5p	Viral replication
Orthomyxoviruses	Influenza	IFITM3 (cellular)	Nedd4	Immune modulation

Table 1. Summary of virus-host interactions and effects on RNA and DNA viruses.

*all proteins listed are viral proteins, except for IFITM3

[#] p92pol and p33 do not contain canonical late domain motifs

Figure 1

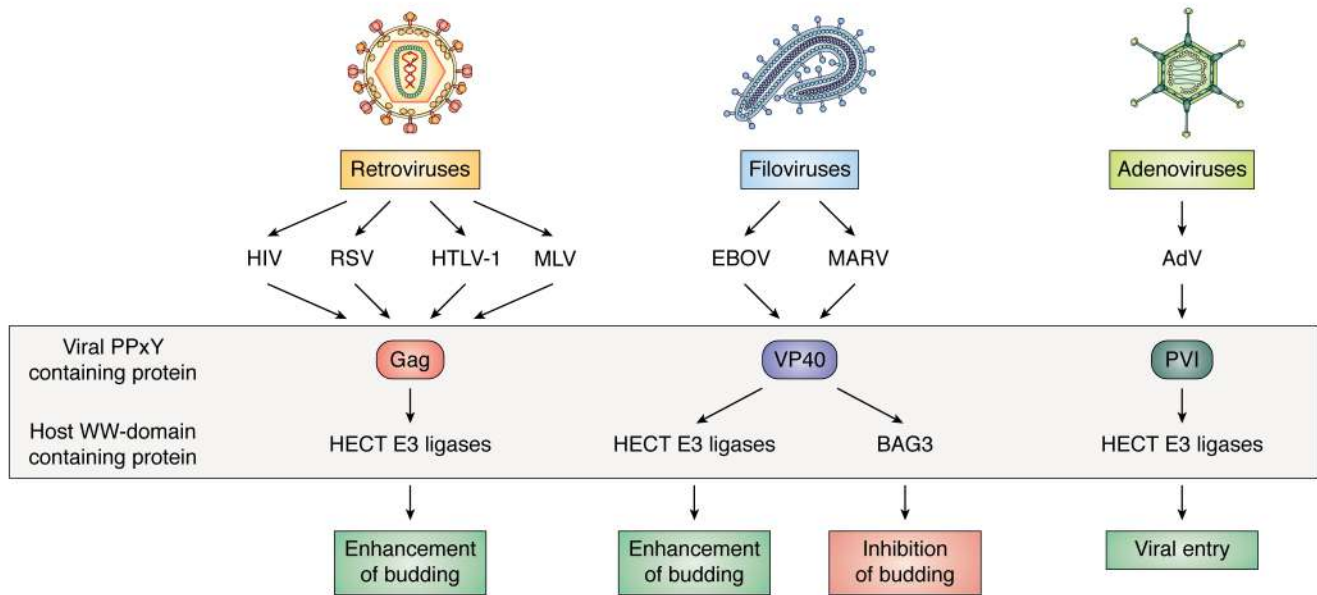


Fig. 1. Key modular PPxY/WW-domain interactions and outcomes for the Retroviruses, Filoviruses, and Adenoviruses.

Figure 2

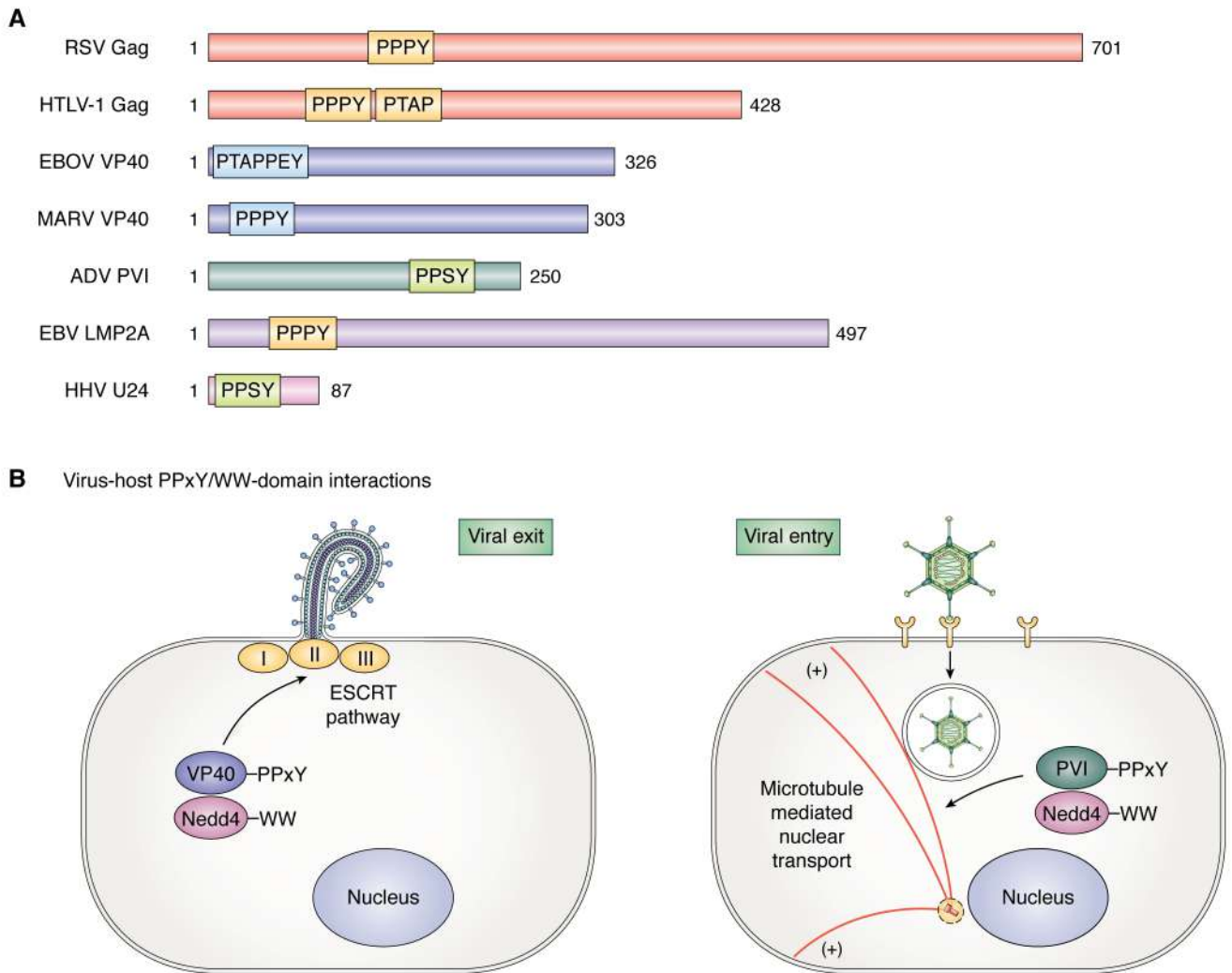


Fig. 2. A) Position and sequence of PPxY motifs in key viral proteins. **B)** Representative examples of virus-host PPxY/WW-domain interaction functioning during virus exit (left; EBOV VP40) and virus entry (right; AdV PVI).

Figure 3

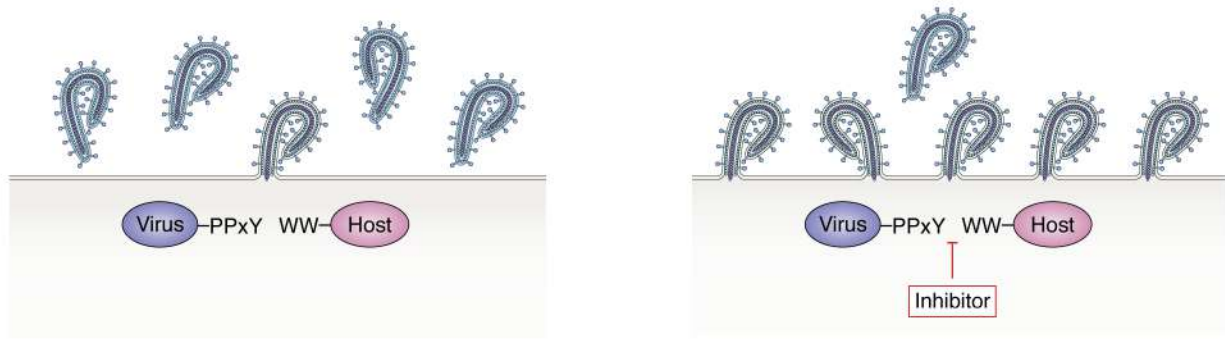


Fig. 3. Schematic diagram of filovirus budding in the absence (Left) or presence (Right) of host-oriented PPxY/WW-domain interaction inhibitors.

Figure 4

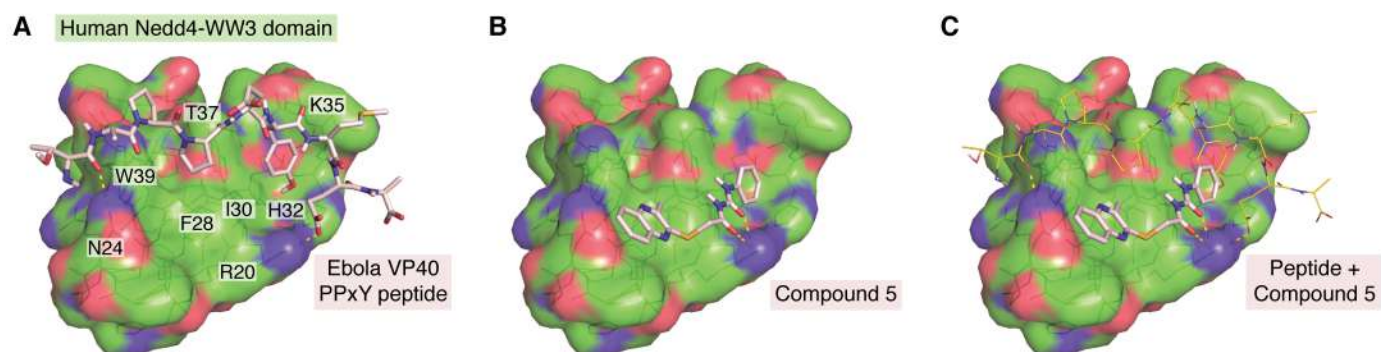


Fig. 4. *NMR structure of human Nedd4-WW3 domain in complex with: A) the Ebola VP40 PPxY peptide, B) the docked pose of compound 5, and C) both the Ebola VP40 PPxY peptide and compound 5.*

Viruses go modular

Ariel Shepley-McTaggart, Hao Fan, Marius Sudol and Ronald N Harty

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