

# Glycan-shielded homodimer structure and dynamical features of the canine distemper virus hemagglutinin relevant for viral entry and efficient vaccination

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## Abstract

Canine distemper virus (CDV) belongs to morbillivirus, including measles virus (MeV) and rinderpest virus, which causes serious immunological and neurological disorders in carnivores, including dogs and rhesus monkeys, as recently reported, but their vaccines are highly effective. The attachment glycoprotein hemagglutinin (CDV-H) at the CDV surface utilizes signaling lymphocyte activation molecule (SLAM) and Nectin-4 (also called poliovirus-receptor-like-4; PVRL4) as entry receptors. Although fusion models have been proposed, the molecular mechanism of morbillivirus fusion entry is poorly understood. Here, we determined the crystal structure of the globular head domain of CDV-H vaccine strain at 3.2 Å resolution, revealing that CDV-H exhibits a highly tilted homodimeric form with a six-bladed  $\beta$ -propeller fold. While the predicted Nectin-4 binding site is well-conserved with that of MeV-H, that of SLAM is similar but partially different, which is expected to contribute to host specificity. Five N-linked sugars covered a broad area of the CDV-H surface to expose receptor binding sites only, supporting the effective production of neutralizing antibodies. These features are common to MeV-H, although the glycosylation sites are completely different. Furthermore, real-time observation using high-speed atomic force microscopy revealed highly mobile features of the CDV-H dimeric head via the connector region. These results suggest that sugar-shielded tilted-homodimeric structure and dynamic conformational changes are common characteristics of morbilliviruses and ensure effective fusion entry and vaccination.

## eLife assessment

Both reviewers positively received the manuscript, in general. The agreement was that the manuscript presented **valuable** findings, using **solid** techniques and approaches, that shed additional light into how the canine distemper virus hemagglutinin might engage cellular receptors and how that engagement impacts host tropism. While both reviewers appreciated the X-ray crystallographic data, they also felt that the AFM experiments could have been performed at a higher standard and that the interpretation of the results ensuing from those AFM experiments could have been explained more thoroughly and in simpler terms. An additional missed opportunity of the current manuscript is the lack of comparison of the crystal structure to that of the already published cryo-EM structure, for context.

## Introduction

Canine distemper virus (CDV) infects dogs as well as other carnivores, and causes acute, highly contagious, febrile diseases that affect the respiratory, gastrointestinal, and central nervous systems, along with serious temporary immunodeficiency<sup>1,2,3</sup>. In 2008, the Chinese group reported a natural infection of CDV in rhesus monkeys, indicating that the CDV expanded its host range<sup>4</sup>. CDV is an enveloped virus with a non-segmented, negative-strand RNA genome that is classified in the genus *Morbillivirus* of the family *Paramyxoviridae*, to which the measles virus (MeV) also belongs. CDV has the attachment glycoprotein hemagglutinin (CDV-H) at the virus surface, which utilizes signaling lymphocyte activation molecule (SLAM) and nectin cell adhesion molecule 4 (Nectin-4) as specific entry receptors<sup>5,6,7,8</sup>. SLAM, which has two immunoglobulin (Ig)-like domains in the extracellular region, is expressed on thymocytes, activated lymphocytes, mature dendritic cells, macrophages, and platelets, reflecting the tropism and immunosuppressive nature of CDV<sup>8,9,10</sup>. On the other hand, Nectin-4 has three Ig-like domains as an extracellular region and is expressed on epithelial cells, permitting transmission from the basal to the apical side for the spread of viruses<sup>11,12,13,14,15,16</sup>. The mechanism of viral infection is similar to that of MeV, while CDV tends to cause more serious neurological disruption. Our recent report and other studies have demonstrated that the host species of CDV is partly determined by their SLAMs<sup>17,18</sup>, while the host innate immunity should also be considered<sup>19</sup>. Notably, some recent CDV strains can infect monkeys, such as rhesus and cynomolgus monkeys, raising the possibility of further expansion of host specificities<sup>20</sup>. Single amino acid substitutions (R519S, D540G, and P541S) can cause CDV-A75/17 or CYN07dV strains to infect Vero cells expressing human SLAM<sup>21,22,23</sup>. Therefore, these results highlight the need for caution regarding the evolution of CDV to acquire the infectious activity to human beings.

The crystal structures of the H protein of MeV (MeV-H) revealed a six-bladed  $\beta$ -propeller folded head domain similar to those of Hemagglutinin/Neuraminidase (HN) proteins of the other paramyxoviruses. On the other hand, the MeV-H forms a disulfide-linked homodimer in unique tilted orientation<sup>23</sup>. With regard to viral entry events, there exist proposed models in which a conformational change of the H protein induced by SLAM or Nectin-4 binding causes a structural change in the fusion (F) protein at the virus surface, leading to fusion of the virus envelope and the plasma membrane<sup>24,25</sup>. However, the detailed mechanism of fusion triggered by H proteins remains unclear.

Live attenuated vaccines of CDV developed for more than half a century are highly effective, while there are no officially available vaccines for other carnivores, such as lions, ferrets and earless seals<sup>26</sup>. Similarly, related MeV live vaccines are also effective, and the molecular mechanism for

efficient vaccination based on the crystal structures of MeV-H and its complexes has been proposed<sup>23,27–29</sup>. MeV-H has sugar shields that cover a large surface, except for the binding sites of human SLAM and Nectin-4. The anti-MeV-H antibodies induced by vaccines tend to target the receptor binding sites (RBSs), ensuring the efficient production of neutralizing antibodies<sup>28,29</sup>. However, the molecular mechanism underlying the efficient vaccination of CDV, which has different sugar modification sites, remains unclear.

Here, we report the receptor binding properties and crystal structure of CDV-H from a Kyoto Biken vaccine strain at 3.2 Å resolution. These results indicate that the homodimer structure conserved in MeV-H makes the sites for receptor recognition upward and easily accessible, which is consistent with the recently reported cryo-EM structure of the ectodomain of wild-type CDV-H<sup>30</sup>. The sugar modification sites of CDV-H are not conserved in sequence with those of MeV-H at all. Nevertheless, surprisingly, the glycan shields of CDV-H cover a large surface but expose the RBSs, essentially the same system structurally shared with MeV-H. Furthermore, real-time observation of CDV-H by high-speed atomic force microscopy was performed, showing unexpected dynamical features of CDV-H homodimers. These results allowed us to update the model for membrane fusion triggered by receptor binding to induce conformational changes in the fusion protein. Our findings provide insights into the molecular and dynamic events of viruses classified in the genus *Morbillivirus* for cell entry and effective vaccinations.

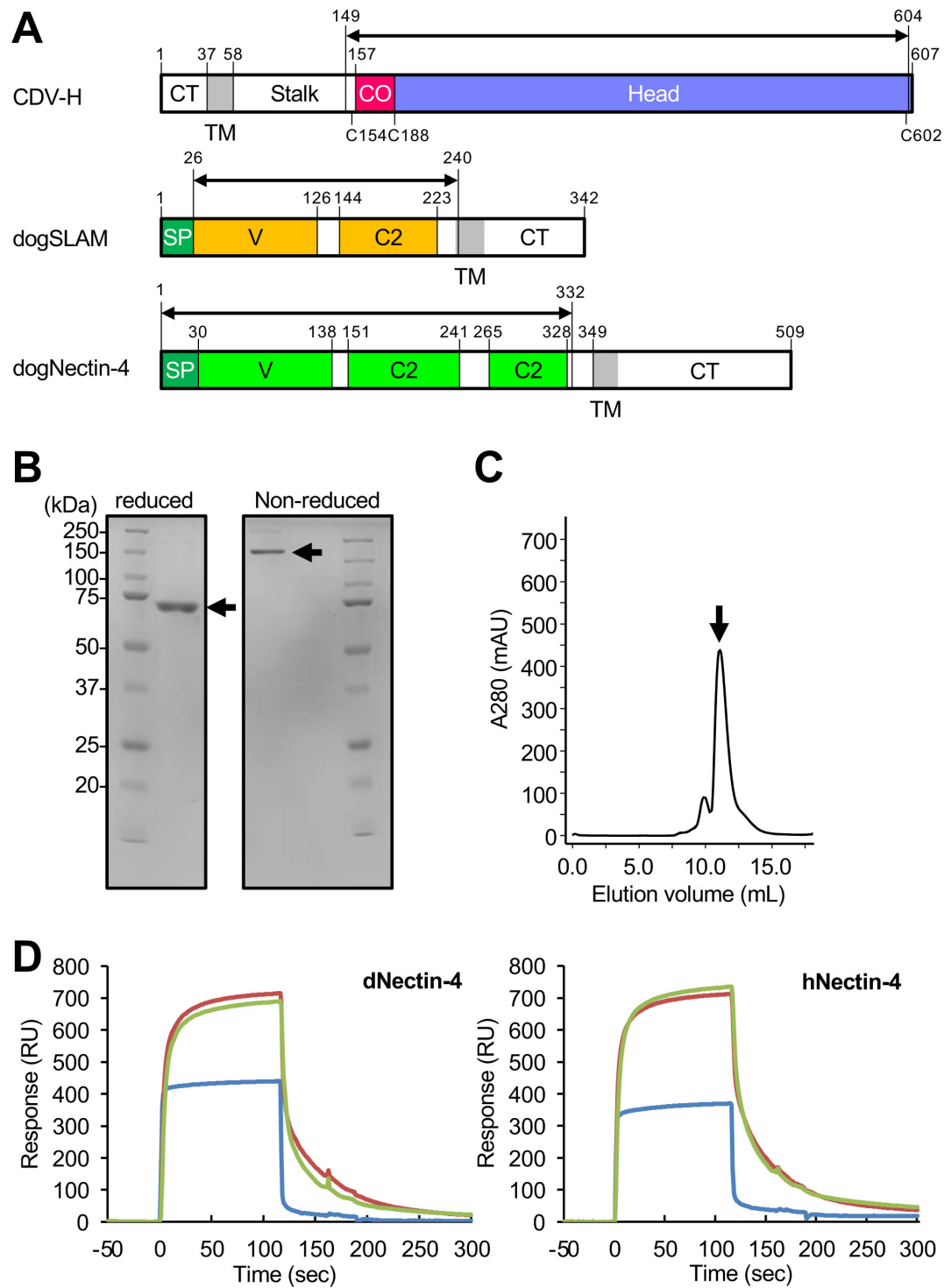
## Results

### Preparation and Nectin-4 binding characteristics of CDV-H

CDV-H consists of an N-terminal cytoplasmic tail, transmembrane region, stalk region, and C-terminal head domain (Fig. 1A). Surface plasmon resonance (SPR) analyses in two previous reports showed that the CDV-H protein can bind to dog SLAM with a  $\mu\text{M}$  range of  $K_d$ <sup>17,31</sup>, but there has been no observation that CDV-H binds to the Nectin-4 ligand at the protein level. Here, we examined the binding activity of CDV-H to human and dog Nectin-4. The soluble head domain of CDV-H (residues 149 to 604, Kyoto Biken strain) with a 6×Histidine tag at the C-terminal site was produced using HEK293T cells as described in our previous report (Fig. 1A-C)<sup>17</sup>. The ectodomains of human Nectin-4 (residues 1 to 333, designated as hNectin-4) and dog Nectin-4 (residues 1 to 332; dNectin-4) were expressed in HEK293T cells and purified by Ni-affinity chromatography, followed by size-exclusion chromatography (Fig. 1A, Supplementary Fig. 1). SPR analysis revealed that CDV-H binds to dNectin-4 with low affinity ( $K_d \sim 10^{-7}$  to  $10^{-6}$  M) and fast kinetics (Fig. 1D and Table 1). The binding characteristics of the CDV-H-dNectin-4 interaction were similar to those of CDV-H-SLAM, which is typical for receptor recognition of viral attachment proteins. Furthermore, we examined whether CDV-H could bind to hNectin-4. SPR experiments clearly demonstrated that CDV-H exhibits a sufficient response to hNectin-4 with similar binding kinetics to the CDV-H-dNectin-4 interaction, as shown in Fig. 1D and Table 1.

### Crystal structure of CDV-H

To determine the structure of the vaccine strain CDV-H by X-ray crystallography, the expression plasmid encoding soluble CDV-H head domain was transfected into HEK293S GnTI- cells, which lack N-acetylglucosaminyltransferase I (GnTI) activity to modify glycosylation with homologous oligomannose-type sugars ( $\text{Man}_5\text{GlcNAc}_2$ ) and was purified as described above. The molecular weight of the non-reducing soluble CDV-H determined by gel filtration chromatography and SDS-PAGE matched its dimer form (Fig. 1B), indicating that the head domain of CDV-H exists as a disulfide-linked homodimer. The crystals were obtained by sitting-drop vapor diffusion at 20°C in 100 mM tri-sodium citrate (pH 5.6), 10% polyethylene glycol-4000, and 10% isopropanol (Fig. 2A). We determined the crystal structure of CDV-H refined at 3.2 Å resolution by molecular replacement using the MeV-H structure<sup>23</sup>, which shares approximately 30% sequence identity



**Figure 1**

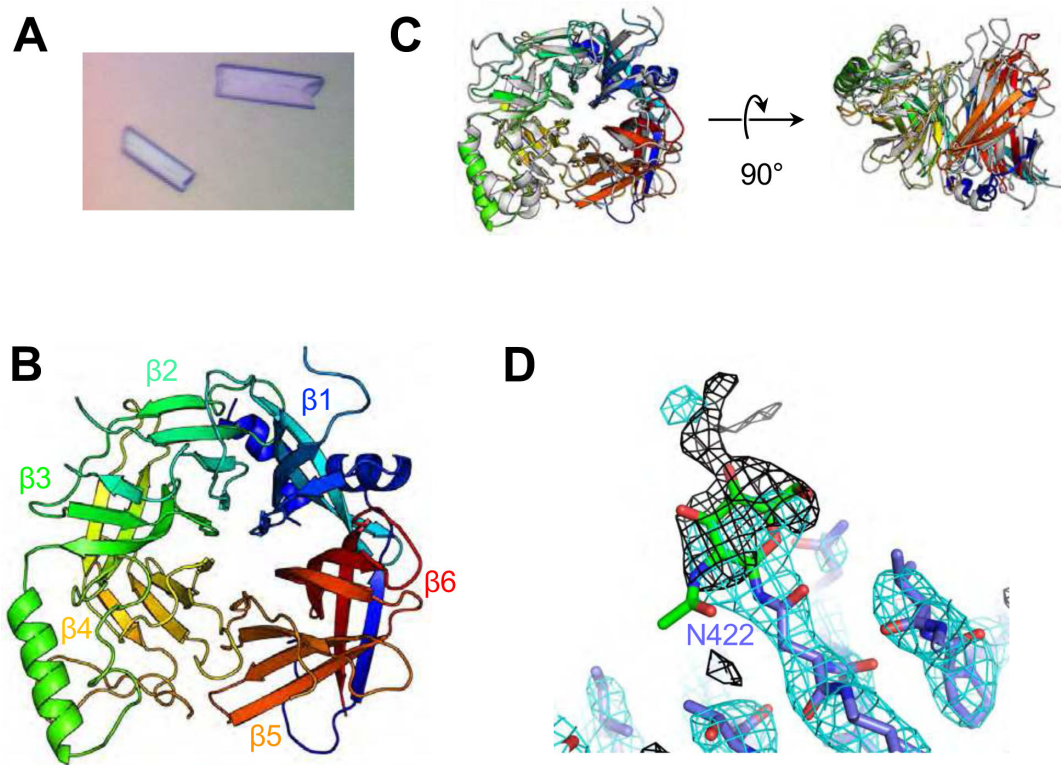
#### Protein Preparation and Nectin-4 binding to CDV-H.

A. Schematic images for expression region of CDV-H globular domain (top), dog SLAM ectodomain (middle) and dog Nectin-4 (bottom). CT, cytoplasmic tail; TM, transmembrane; CO, connector region; SP, signal peptide. B. SDS-PAGE for CDV-H protein in reducing (left) and non-reducing (right) conditions. The arrows indicate monomer (left) and disulfide-linked dimer (right) of CDV-H. C. Gel filtration chromatogram of CDV-H using Superdex 200 column. The arrow indicates the peak of CDV-H. D. Surface plasmon resonance analysis for the interactions between CDV-H and Nectin-4 receptors: dog (left) and human (right) Nectin-4. The Green, Red and Blue lines indicate CDV-Hwt, CDV-Hvac and  $\beta 2$  microglobulin as a negative control, respectively.

in the head domain with CDV-H as a search probe (**Fig. 2B** and **Table 2**). There are two CDV-H monomers (chain A and B) in the asymmetric unit of the crystals, which have only slight differences in the structures of some loops. Therefore, hereafter, we focus on chain A as a representative structure of CDV-H, in which a more extensive electron density is observed than that in chain B. The head domain of CDV-H of vaccine strain exhibits a six-bladed  $\beta$ -propeller structure, which is essentially the same as recent cryo-EM structure of wild-type CDV-H ectodomain<sup>30</sup>, similar to that of the MeV-H structures (**Fig. 2C**). Furthermore, CDV-H forms a homodimer structure facing the  $\beta 1$  propeller strand,  $\alpha 1$  and  $\alpha 2$  helices, resulting in a dimer orientation inclined to the horizontal plane (**Fig. 3A** and **Supplementary Fig. 4B**), similar to the MeV-H homodimer structures and distinct from other paramyxoviruses, such as parainfluenza virus 5 (**Fig. 3B**)<sup>23,25,32</sup>. The interactions at the homodimer interface are mainly composed of three parts: the top, middle, and bottom sections, as shown in **Fig. 3A**. The top section of CDV-H is a hydrophobic cluster consisting of Leu201, Val203, Trp259, Leu260, and Leu265, which interact with Leu205, Leu209, Leu234, and Ile248, respectively. Trp259 is substituted for Pro263 in MeV-H, compensated by the replacement of Leu209 with Tyr209, resulting in the maintained CH- $\pi$  interaction between Leu209 and Trp259 (**Fig. 3C top**). The middle section is a hydrogen-bond network formed by the main-chain O atoms of Thr268 and Thr269, with the N $\delta$  atom of Gln267 (**Fig. 3C middle**). Lys257 forms a hydrogen bond with the side-chain O atom of Asn270. Tyr271 interacts hydrophobically with Lys257, Leu265, and Gln267. Tyr271 further interacts with Trp259 at the interface between the top and middle sections. In MeV-H, His271 forms a hydrogen bond with the side-chain O atom of Met272 in another monomer. In the bottom section of CDV-H, Phe323 of each molecule is hooked by a  $\pi$ - $\pi$  interaction (**Fig. 3C bottom**). Phe323 further interacts with the hydrophobic residues, Phe255, Ile322 and Pro169. In MeV-H, Trp327 makes  $\pi$ -cation interactions with Lys328 of another monomer. The characteristics of these interactions at the homodimer interface are conserved in morbilliviruses, while some HN proteins in the paramyxoviruses have substitutions and lack middle or bottom sections, adopting different angles of homodimer formation (**Fig. 3B**). These findings suggest that the unique dimer orientation is a common feature of morbilliviruses and is appropriate for binding to the protein receptors SLAM and Nectin-4 (**Supplementary Fig. 2**).

## Receptor-binding sites

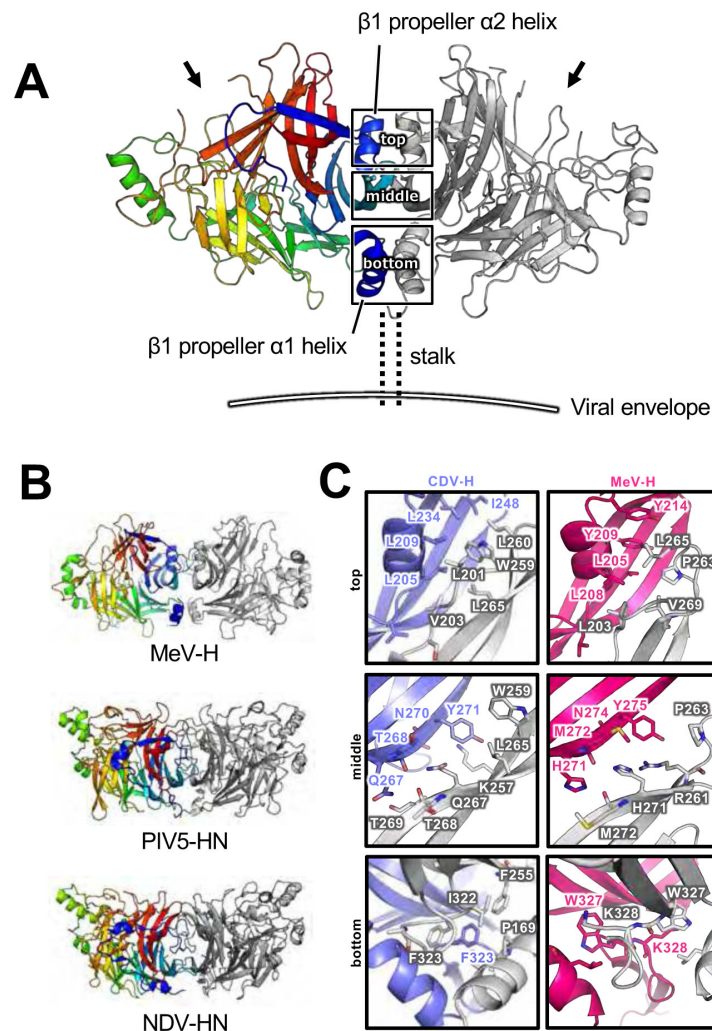
The previously reported complex structures of MeV-H with SLAM or Nectin-4 revealed that the  $\beta 4$ - $\beta 6$  blades are involved in their interactions<sup>28,29,33</sup>. The superposition of the current CDV-H structure onto each individual complex structure enabled the construction of a reasonable CDV-H-receptor complex model without any significant structural hindrance (**Supplementary Fig. 3**). Previous mutagenesis studies have reported that eight amino acids (Tyr525, Asp526, Ile527, Ser528, Arg529, Tyr547, Thr548, and Arg552) on the  $\beta 5$  blade of CDV-H are important for interaction with dSLAM<sup>34,35</sup>. The crystal structure of MeV-H complexed with tamarin SLAM showed that the H-SLAM interaction composes mainly 4 sites (**Fig. 4A**)<sup>28</sup>. At the corresponding site 1 on CDV-H, Asp501 is disordered in both asymmetric units of CDV-H. Asp501 and Asp503 on the acidic loop of the  $\beta 5$  blade are equivalent to Asp505 and Asp507 in MeV-H interacting with Lys77 and Arg90 of tamarin SLAM<sup>28</sup>. Both Lys77 and Arg90 are conserved in dSLAM. Next to the acidic loop, Arg529 of the  $\beta 5$  blade supported by Asp526 on CDV-H would interact with Glu123 of dSLAM in a manner similar to site 2 of the MeV-H-tamarin SLAM complex (**Fig. 4B** and **4C**). These interactions are likely strengthened by the surrounding hydrophobic interactions formed by Pro554 and Phe552 on MeV-H and His61 on tamarin SLAM, which correspond to residues Pro550 and Thr548 on CDV-H and His61 on dSLAM, respectively. Additionally, Leu63 of dSLAM, equivalent to Val63 of tamarin SLAM, is likely to be involved in hydrophobic interactions around site 2. Consistently, Ohno et al. also highlighted the importance of Leu63 in dSLAM for CDV infection<sup>36</sup>. While site 3 residues on the CDV-H edge of  $\beta$ -propeller 6 have a low conservation rate, this region interacts with  $\beta 8$  of tamarin SLAM in the complex structure of MeV-H-tamarin SLAM. Furthermore, the  $\beta 8$ -H contact-forming residues on dSLAM are conserved, except for His130. Therefore, this region can interact with dSLAM without side-chain



**Figure 2**

**Crystal structure of CDV-H.**

A. Crystals of CDV-H head domain. B. Overall structure of CDV-H with secondary structures in cartoon model, colored gradually from blue (N-terminus) to red (C-terminus). C. Structural comparison of CDV-H (rainbow color) and MeV-H (grey). D. Electron density map (2.5  $\sigma$  omit map shown in black mesh) around Asn422 of CDV-H attached with N-glycan.



**Figure 3**

### Homodimer structure and its interface of CDV-H.

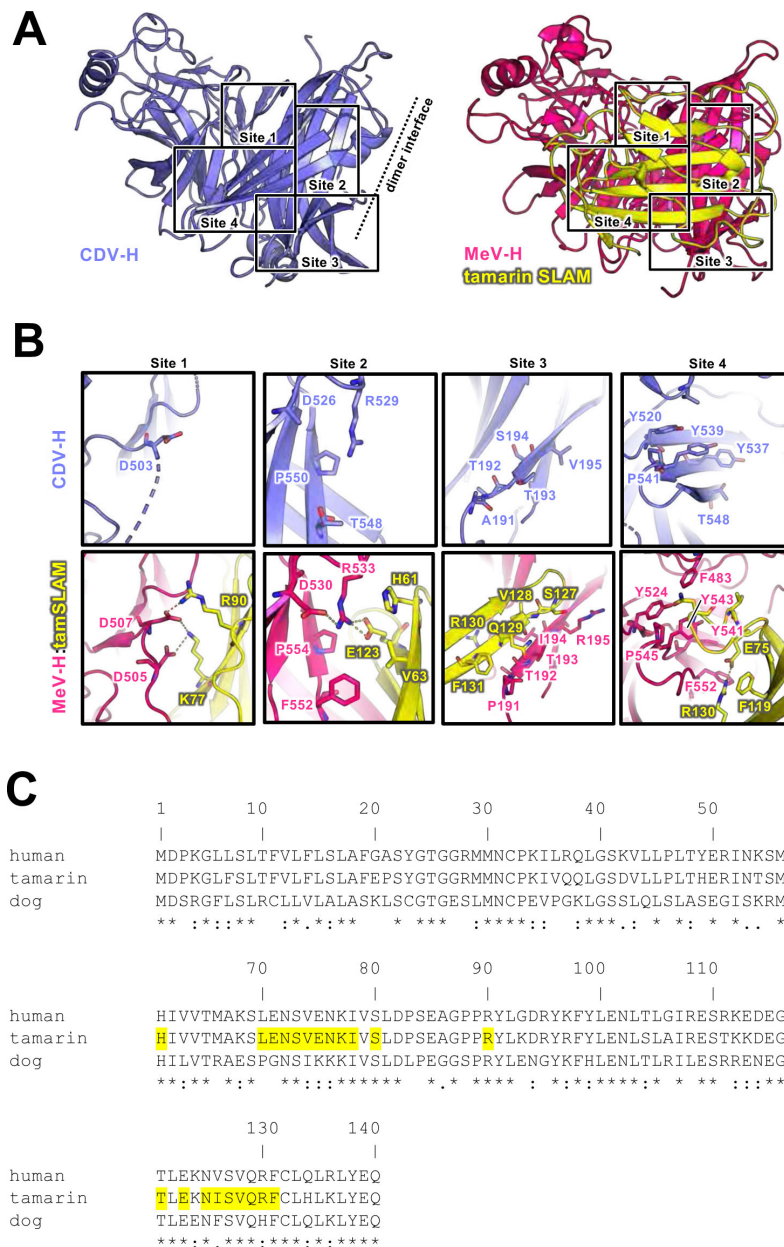
A. Homodimer structure of CDV-H with secondary structures in cartoon model as the same as **Fig. 2B** (rainbow: chain A and grey: chain B). The black arrows indicate the receptor binding sites. B. Structural comparison of CDV-H with other paramyxovirus hemagglutinin dimers. MeV-H (top, PDB code: 2ZB6), PIV5-HN (middle, PDB code: 4JF7) and NDV-HN (bottom, PDB code: 3T1E) are shown in similar direction with CDV-H of Fig. 3A. C. The closed-up images for three sites at the homodimer interface. One of the CDV-H dimer is shown in slate, asymmetric unit in grey. One of the MeV-H dimer is colored hot pink.

dependency. At site 4, the aromatic residues Tyr520, Tyr537, Tyr539, and Thr548 on CDV-H are conserved, except for the substitution of Phe552 with Thr548. Additionally, Ile74, of dSLAM equivalent to Val74, tamarin SLAM is stabilized by hydrophobic Leu464, Phe483, and Leu500 on CDV-H. Sequence comparison between dSLAM and tamarin SLAM showed that 7 of 21 residues at the H binding face (Pro70, Gly71, Ile74, Lys75, Lys76, Phe126, and His130 in dSLAM) are different (**Fig. 4C**). The amino acids corresponding to residues 74, 75, 76, 126, and 130 of dSLAM reportedly influence the host specificities of morbilliviruses<sup>37</sup>. Therefore, CDV-H binds to dSLAM in a manner similar to MeV-H-tamarin SLAM interaction.

Next, to examine CDV-H binding to dog Nectin-4, the superposition of CDV-H onto the MeV-H-hNectin-4 complex structure<sup>29</sup> revealed that 10 of the 24 interacting residues were identical in MeV-H with CDV-H, while all 20 interacting residues of hNectin-4 were identical to dNectin-4 (**Fig. 5B** and **5C**). As mentioned above, SPR analysis demonstrated that both dNectin-4 and hNectin-4 could bind to CDV-H (**Fig. 1D**). This result suggests that the binding mode of CDV-H to dNectin-4 is presumably the same as that of MeV-H to hNectin-4. Zhang et al. defined the interaction area as sites I to III in the complex structure of MeV-H with hNectin-4<sup>29</sup>. Along with the definition, the site I includes hydrophobic groove composed of 8 residues, which are maintained; the Leu464, Leu482, Phe483 and Leu526 of MeV-H are substituted to Phe460, Val478, Leu479 and Ile522 in CDV-H, respectively (**Fig. 5B**). The change from Leu500 of MeV-H to Thr496 suggests a lower contribution of site II, as supported by a previous report<sup>38</sup>. At site III, Gln391 and Tyr499 of MeV-H are substituted by Tyr387 and Gln495 of CDV-H, which interact with Tyr55 and Lys54 of Nectin-4 the receptors, respectively.

## Glycosylation sites

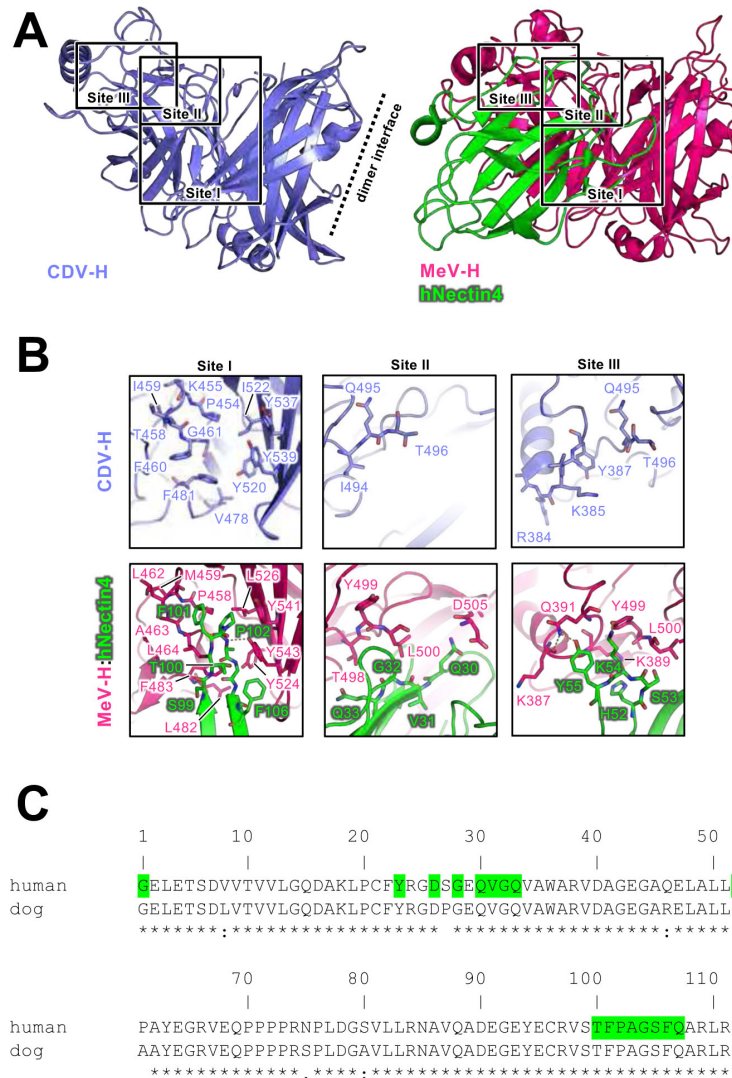
Previous studies have suggested that the H proteins of a wild-type strain isolated from cases of natural CDV infections in recent years and vaccine strains show approximately 90% identity in their open reading frame (ORF), but may have differences in antigenicity of viruses because the wild-type strain has more potential N-linked glycosylation sites in its H protein<sup>39–42</sup>. There were five potential glycosylation sites (Asn149, Asn391, Asn422, Asn456, and Asn587) in the H protein head domain of the Kyoto Biken vaccine strain (CDV-Hvac) and two additional sites (Asn309 and Asn603) in the 5VD wild-type strain (CDV-Hwt) (**Fig. 6A**). To identify the N-glycosylation sites, mutagenesis was performed on each site of CDV-Hs. Western blot analysis indicated that all asparagine CDV-H mutants except for the N309D and N603D mutants migrated further than the wild-type, revealing that both CDV-Hvac and CDV-H wt have five sugar chains in common glycosylation sites (Asn149, Asn391, Asn422, Asn456, and Asn587) (**Fig. 6B**), while Asn309 and Asn603 of CDV-Hwt are not glycosylated. This result is consistent with a previous report showing that five standard glycosylation sites exist in the ectodomain of the wild-type CDV-H protein<sup>43</sup>. The CDV-H structure, which contains five N-linked sugars as described above, exhibits electron density only for Asn422-linked GlcNAc (**Fig. 2D**). Asn149- and Asn422-linked sugars, which are located close to each other, appear to bury interspaces between the stalk and head domains, and Asn587-linked sugars might support the stabilization of the CDV-H dimer orientation (**Fig. 6C**, **Supplementary Fig. 4**). Sugars modified on Asn391, Asn456, and Asn587 seemed to cover the large pocket in CDV-H, and Asn456-linked sugars appeared to lean toward the center of the pocket (**Fig. 6C**). Finally, since the receptors, SLAM and Nectin-4, bind to the side surface of CDV-H conserved with MeV-H, as revealed by the above-mentioned mutagenesis study, the majority of CDV-H surfaces are covered with sugars, forcing only the RBSs to be exposed (**Fig. 6C**).



**Figure 4**

### Putative SLAM binding site on CDV-H.

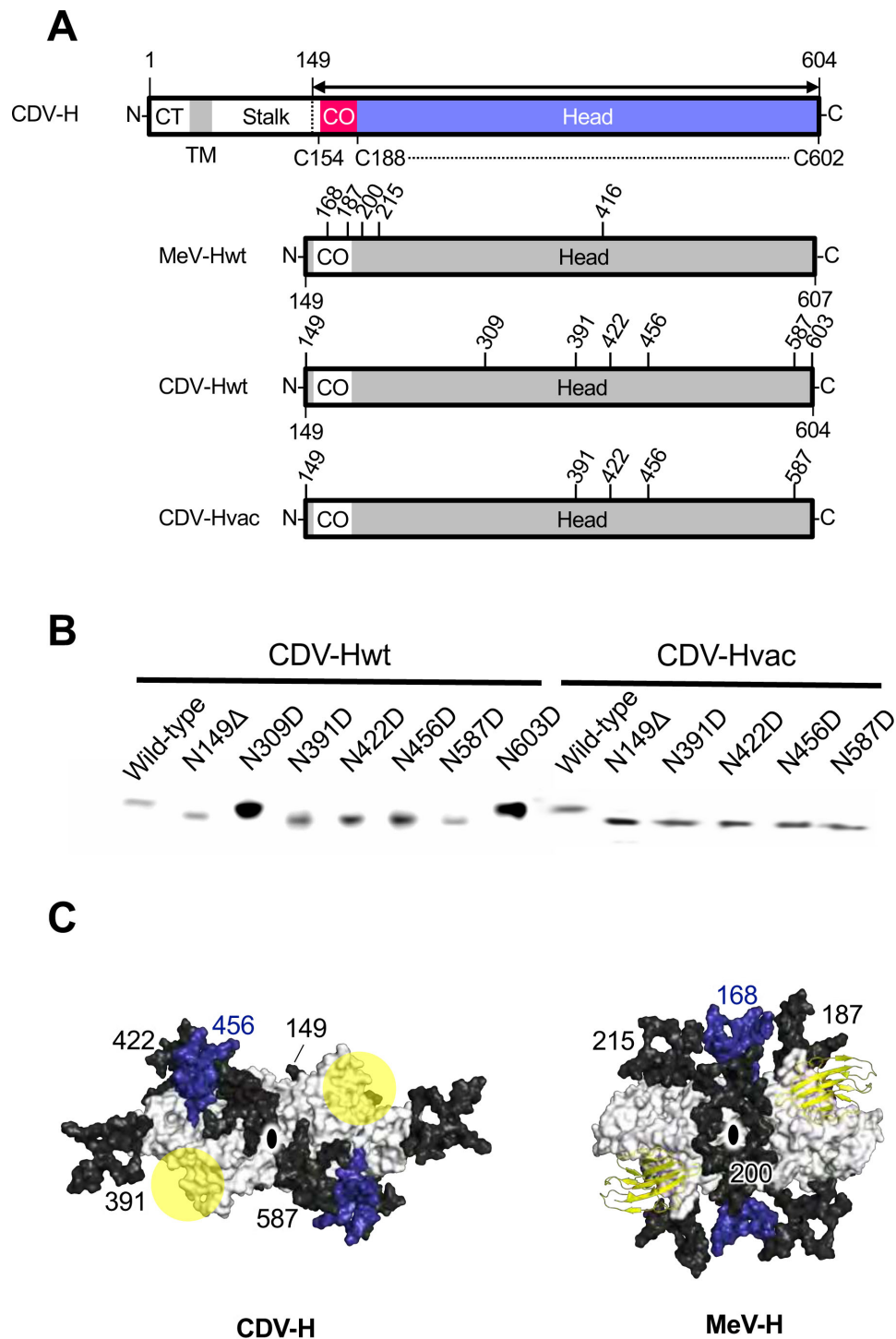
A. Structures of CDV-H with putative SLAM binding sites (left, slate) and SLAM complex of MeV-H (right, hot pink) are shown. B. The residues that are involved in SLAM receptor binding revealed by mutagenesis studies are shown in stick model on CDV-H (top) and MeV-H (bottom). C. Amino acid sequence alignment of v-type domains of SLAMs. Asterisks (\*) indicate fully conserved residue. A colon (:) indicates conservation between groups of strongly similar properties. A period (.) indicates conservation between groups of weakly similar properties. Interacting with hemagglutinin residues (<4.0 Å) are indicated in yellow-colored boxes.



**Figure 5**

### Putative Nectin-4 binding site on CDV-H.

A. Structures of CDV-H with putative Nectin-4 binding sites (left, slate) and Nectin-4 complex of MeV-H (right, hot pink) are shown. B. The residues that are involved in Nectin-4 receptor binding revealed by mutagenesis studies are shown in stick model on CDV-H (top) and MeV-H (bottom). C. Amino acid sequence alignment of v-type domains of Nectin-4s. Abbreviations and green-colored boxes are shown in the same manner with [Figure 4C](#).



**Figure 6**

### Glycan shield of CDV-H.

A. Schematic images of full-length and expression constructs of ectodomain of CDV-Hs (wild-type and vaccine strains), together with the of MeV-H. B. Mutagenesis studies for all potential glycan modification revealed to show that N390D and N630D showed clear western blot data. C. Differences in glycan shields. Glycosylation sites on the surface of CDV-H (left) and MeV-H (right) dimers shown in white, with common N-linked glycans modeled in black and deep blue. Receptor binding sites were shown in yellow circle on the CDV-H dimer. Tamarin SLAMs bound to the MeV-H dimer were shown in yellow. Two-fold symmetry axis was shown as black ellipse.

## Mobile interdomain features of CDV-H dimer revealed by the high-speed atomic force microscopy

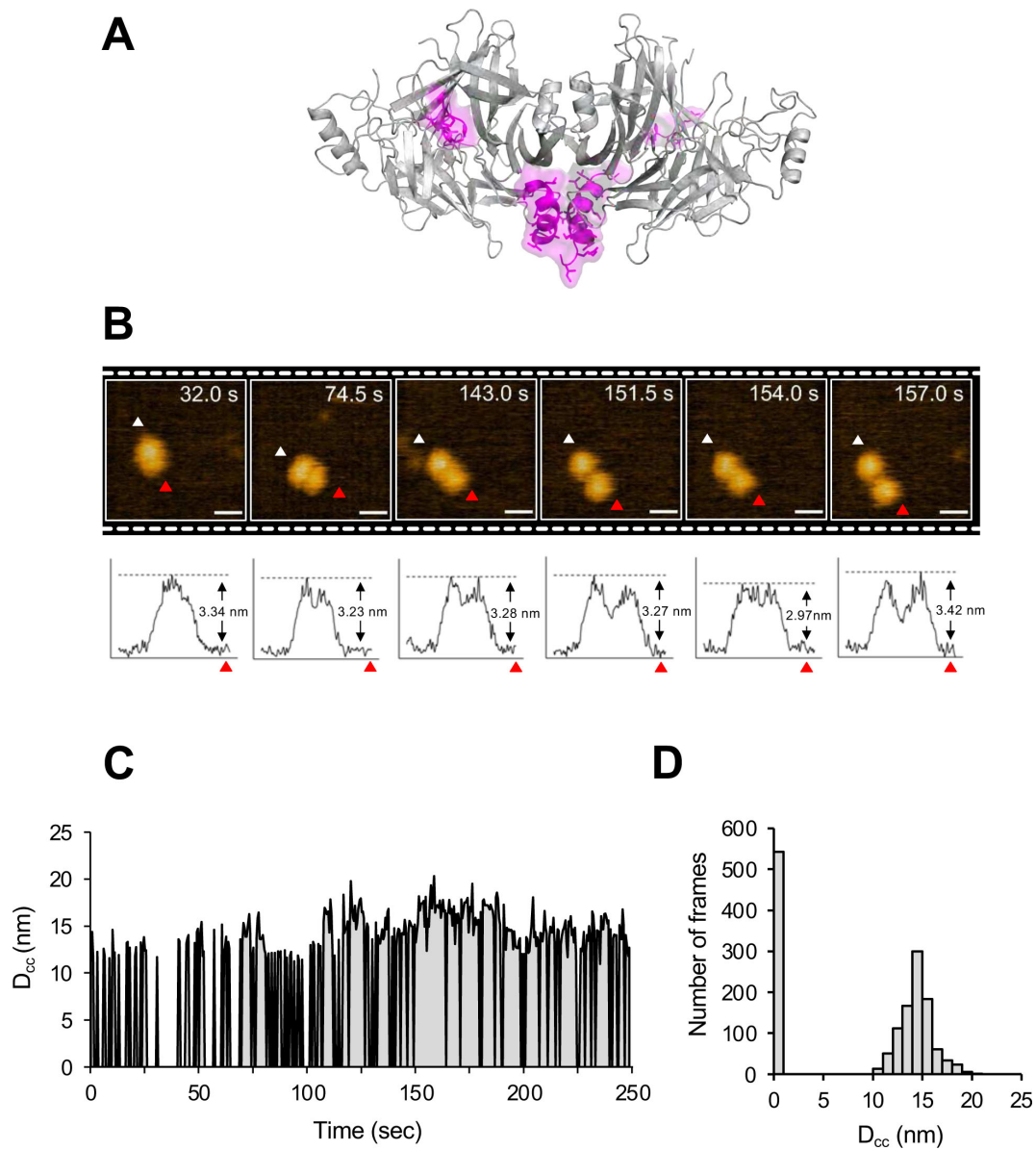
The crystal structure of CDV-H shows a connector domain (residues 158–187) that mediates a head domain to the stalk region, which facilitates homodimer formation with a set of disulfide bonds at Cys154 (**Fig. 1A** [↗](#) and **7A** [↗](#)). This region was mainly disordered in the previous MeV-H structures<sup>23</sup> [↗](#). Interestingly, there were no covalent bonds in the homodimer interface of the head domains, even though a conserved homodimer orientation was observed in the crystal structures of CDV-H and MeV-H, as described above. This observation raises the possibility that each head domain could dissociate and move flexibly. Therefore, here, high-speed atomic force microscopy (HS-AFM) was performed to directly visualize the real-time dynamic characteristics of the CDV-H under physiological conditions. CDV-H absorbed on the mica substrate was visualized as images of connected circles, and each domain moved freely to dissociate (**Fig. 7B** [↗](#) and **Supplementary Movie** [↗](#)). The distances between the domains were calculated by measuring those between the centers of mass of each domain. Time course analysis of the dynamics of the representative CDV-H dimer showed that CDV-H could adopt both associated and dissociated forms (**Fig. 7C** [↗](#)). Finally, the distribution of the distances between each head domain in the CDV-H dimers showed approximately 15 nm as a major peak (**Fig. 7D** [↗](#)). It is more than twice the distance between the centers of mass in the associated form.

## Discussion

In this study, we determined the crystal structure of the vaccine strain CDV-H head domain with a connector domain. The overall structure of the head domain shows a folding similar to that of MeV-H<sup>23</sup> [↗](#) and a homodimer with a tilted domain orientation is formed, essentially the same as the MeV-H homodimer, which is consistent with the cryo-EM structure of wild-type CDV-H ectodomain<sup>30</sup> [↗](#). The binding of CDV-H to dNectin-4 was demonstrated by surface plasmon resonance, revealing low-affinity and fast-kinetic interactions, similar to SLAM binding. The conventional idea that low-affinity and fast-kinetics interactions between viral attachment proteins and their receptors on target cells do not cause significant structural changes<sup>44</sup> [↗](#), suggesting that CDV-H-receptor recognition is essentially similar to that of MeV.

Our previous report and other studies have demonstrated that the host species of CDV are restricted by differences in SLAM between animals<sup>17</sup> [↗](#),<sup>18</sup> [↗](#). SLAM-interacting residues in CDV-H, predicted by superimposing the MeV-H-SLAM complex structure onto the CDV-H structure, are conserved. Notably, the mutations T496L and T548F in CDV-H were not sufficient to switch the SLAM receptor preference<sup>17</sup> [↗](#). Furthermore, a previous report demonstrated that the N-terminal region (position 29) of hSLAM contributes to binding to MeV but not to human-adapted CDV<sup>45</sup> [↗](#). These findings suggest that SLAM recognition sites are similar but somehow different between CDV and MeV. In contrast, interacting residues of hNectin-4 with MeV-H in the crystal structure of their complexes were completely conserved in dNectin-4 (**Fig. 5C** [↗](#)). In accordance with this result and crystal structures, the surface plasmon resonance study showed that CDV-H binds to hNectin-4 with low affinity (~ μM range) and fast kinetics, comparable to that of dNectin-4, as described above (**Fig. 1D** [↗](#)). Therefore, in contrast to SLAM binding, Nectin-4 recognition was highly conserved in MeV- and CDV-H. These results raise the possibility that future CDV-H variants acquiring human SLAM binding may have the potential to expand host specificity to infect human beings, and the cross-reactivity of antibodies targeting the receptor binding sites of CDV and MeV is expected.

While vaccines for CDV are quite effective and widely used, some groups insisted that the number of N-linked glycosylation sites in the wild-type CDV-H increased to weaken the immune responses induced by CDV vaccines<sup>39</sup> [↗](#),<sup>40</sup> [↗](#). However, a previous study together with this work clarified that the additional N-linked glycosylation sites, Asn309 and Asn603, of CDV-H are not modified



**Figure 7**

### Structural Dynamics of CDV-H analyzed by HS-AFM.

A. Homodimeric structure of CDV-H with a part of stalk region (residues 149 to 604). The globular head domain (grey) and the stalk region (magenta) are shown in cartoon model. B. Serial HS-AFM images of CDV-H homodimer at 2 fps are shown. Scale bar indicates 20 nm length. Graphs under each HS-AFM images indicate the z-axis parameter between red and white triangle. C. The distances between signal centers ( $D_{cc}$ ) of representative homodimer were monitored at time scales. D. The numbers of CDV-H homodimers are classified as several distances.

with sugars (**Fig. 6B**).<sup>43</sup> Thus, the number of sugars in the wild-type CDV-H was presumably maintained, ensuring that the CDV vaccine strains developed in the 1960s have been still effective. Additional passages could lose two N-glycosylation sites (Asn391 and Asn456) to produce CDV-H harboring three N-linked sugars.<sup>46</sup> The three sugars linked to Asn149, Asn422, and Asn587 were considered to be fundamental for the correct folding of CDV-H. However, the glycosylation sites mentioned above are not conserved in MeV-H (**Fig. 6A**). Surprisingly, however, we mapped glycans onto the CDV-H structure, showing that the glycan shield was established, except for putative receptor-binding sites, similar to MeV-H (**Fig. 6C**). Our findings provide structural and functional insights into the mechanism of the highly efficient induction of neutralizing antibodies, which are common to MeV-H, even though different sugar modifications<sup>23</sup>, contribute to the development of effective vaccines against other viruses.

Two glycoproteins, H and F, coordinate to achieve membrane fusion during viral entry as follows. The interaction between the H protein and the receptor induces activation of the F protein by H-F interaction. Subsequent irreversible refolding of the F protein causes its fusion peptide and transmembrane region to be closely located to form the fused pore. There are several kinds of the viral entry mechanisms for morbilliviruses proposed, such as the “safety-catch,” “bi-dentate” and “oligomerization” models<sup>47–50</sup>. The crystal structural analyses of MeV-H and MeV-H-SLAM complex<sup>28</sup> revealed two kinds of orientations (forms I and II) of the dimer of the MeV-H dimers, prompting us to propose a viral entry model in which form I (many interface areas between the dimers) can transform to form II (fewer interfaces of the dimers but stabilized upon SLAM complex formation). Considering further evaluation of the models, we recently proposed a possible model<sup>47</sup> as follows: (1) the stalk region of the H protein binds to the putative H protein binding site of F protein on the viral surface, (2) the H protein interacts with the host receptors at the virus-cell interface to facilitate the orientation shift of dimers of the H homodimers, (3) the H protein binds to an additional site of the F protein and/or opens the space to induce the refolding of F protein in pre- to post-fusion states. In this study, the crystal structure of CDV-H included a homodimer with unique intermolecular angles, common to MeV-H, exposing the presumed receptor binding site to the top side far from the viral membrane, which is likely beneficial for access to receptor binding. While the homodimer angles of CDV-H and MeV-H are different from those of other paramyxoviruses, such as parainfluenza virus (PIV5), the receptor binding sites are all exposed to the top site distal to the viral membrane (**Fig. 3A** and **3B**). The homodimer interface and flexible linkage between each head domain are conserved, suggesting that this structural feature of homodimers is relevant for the fusion step of viral entry. Next, the direct and real-time observation of viral glycoprotein structure using high-speed atomic force microscopy (HS-AFM) revealed that the homodimer of the CDV-H head domains showed relatively mobile intermolecular characteristics, even though the dimer configuration is dominant, which is in contrast with the static homodimer structures that are stabilized by several conserved interactions as observed in the crystals. In our previous report, Immunoglobulins were observed by HS-AFM in essentially the same tapping mode conditions, clearly indicating their dynamical and structural features without any dissociation of their domains<sup>51</sup>. On the other hand, the observation of mobility of the receptor binding domain (RBD) of SARS-CoV-2 S protein by HS-AFM was recently reported, showing that it forms open and closed conformation<sup>52</sup>. Therefore, these results supported that the mobility of CDV-H head domains reflected its dynamic features without any artificial forces. The previous model for morbillivirus entry only focused on the orientation shift of the dimers<sup>28</sup> or Cys154-centered pivot in MeV-H<sup>53</sup>; however, our results may suggest that further dissociation of the head domain homodimer via the connector region induced by receptor binding is relevant (**Fig. 8**). In fact, Navaratnarajah et al. conducted the mutagenesis study at the homodimer interface of MeV-H, revealing that the introductions of intermolecular disulfide-bonds reduced the membrane fusion activity<sup>53</sup>. On the other hand, the cryo electron tomography analysis of human parainfluenza virus 3 suggested that the head-up tetramer/oligomer formation of hemagglutinin-neuraminidase may change the interaction with the F protein to induce the fusion<sup>54</sup>, while the recent cryo-EM structure of wild-type CDV-H ectodomain itself revealed that the head dimers attach to one side of stalk region<sup>30</sup>. The other

possible models may include the monomer-dimer-tetramer transition facilitated by receptor binding for the fusion. Further biochemical/virological experiments are necessary to conclude the feasibility of these models. In addition, the close areas at the interface of the homodimer could be epitopes for anti-MeV-H antibodies, supporting the interface areas of the homodimer, which are good targets for neutralizing antibodies. These results provide significant insights into the viral fusion mechanism, as well as the rational design of antiviral drugs and vaccines for a wide range of infectious diseases.

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## Author contributions

H.F., M.S., T.H., Y.Y., and K.M. designed the study; M.S., M.K., T.H., and J.K. prepared and crystallized CDV-H; M.S., M.K., H.F., S.C., T.H., M.K., M.H.M., and M.T. performed binding, BN-PAGE, and MEV infection assays; M.S., T.O., M.K., T.H., N.M., and K.M. determined the crystal structures; M.K. and K.Y. analyzed the mobility by HS-AFM; H.F., K.Y., M.S., and K.M. wrote the manuscript.

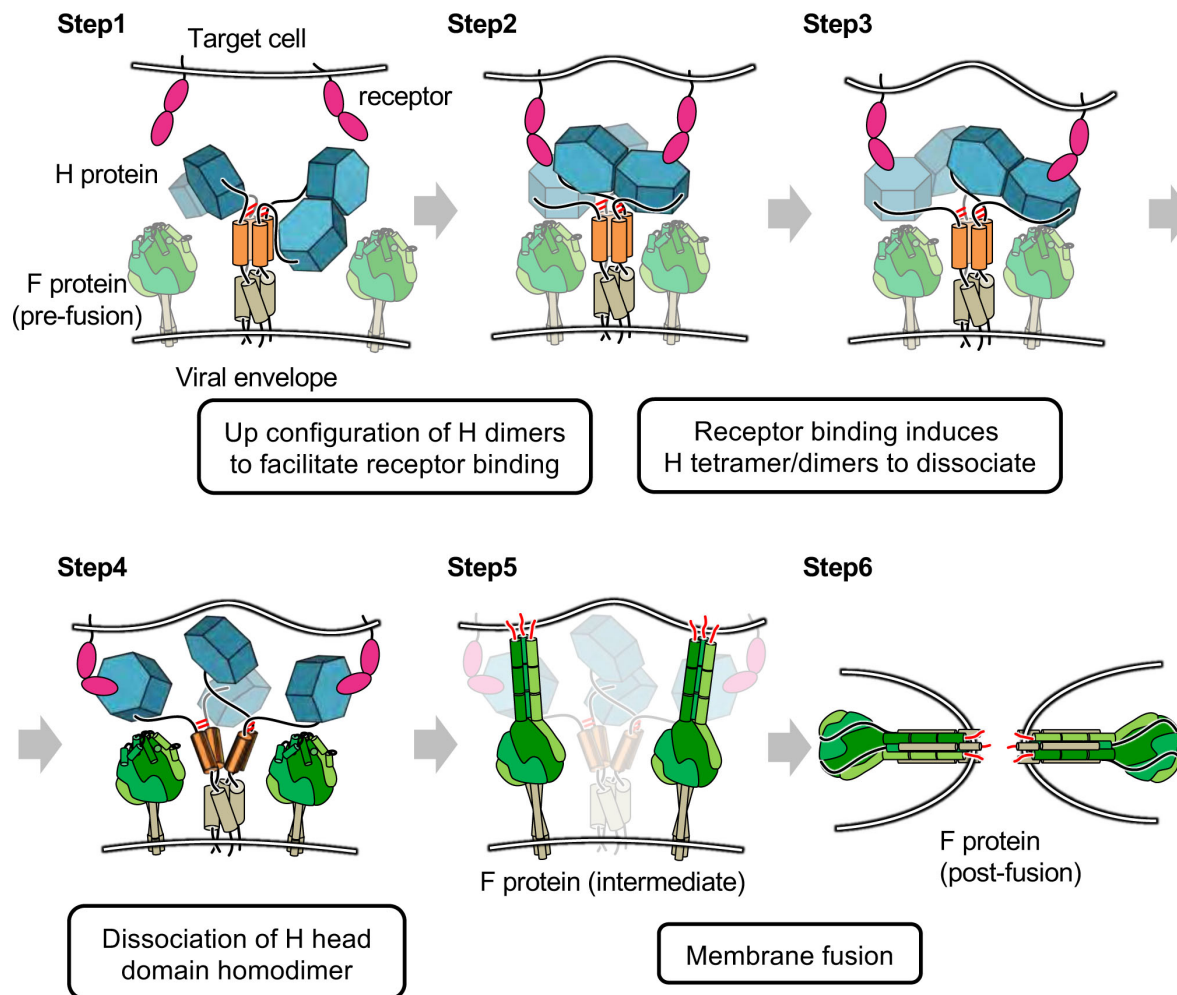
## Online methods

### Construction of Expression Plasmids

The DNA fragment encoding the ectodomain (amino acid residues Asn149 to Pro 607 (or to RArg604)) of CDV-H derived from a Kyoto Biken vaccine strain (or a 5VD wild-type strain) was cloned into pCA7 using the restriction enzyme sites *Eco*RI and *Xho*I<sup>56</sup><sup>55</sup>. The resultant plasmids contained CDV-H with a signal sequence (N-terminal) and His<sup>6</sup> tag (C-terminal), which were derived from the pHLsec vector<sup>56</sup>. The DNA fragment encoding the signal sequence and ectodomain of dog Nectin-4 (dNectin-4) was cloned into pCA7 with a His<sub>6</sub> tag sequence<sup>5</sup>.

### Protein Expression and Purification

HEK293S cells lacking N-acetylglucosaminyltransferase I (GnTI) activity<sup>56</sup><sup>57</sup> were grown in Dulbecco's modified Eagle medium (DMEM; Wako) supplemented with 10% (fetal bovine serum (FBS; Hyclone) at 37°C and 5% CO<sub>2</sub>. Eighty-% confluent cells were transfected with plasmids encoding CDV-H or dNectin-4 using polyethylenimine (Polyscience Inc.) at a ratio of 1:2. After transfection, the cells were cultured in DMEM containing 2% FBS for 4 days. The soluble proteins secreted into the culture medium were purified by Ni<sup>2+</sup>-NTA affinity chromatography (GE Healthcare) and Superdex 200 GL 10/300 gel filtration chromatography (GE Healthcare). The CDV-H protein for crystallization was further purified using Resource Q anion exchange chromatography (GE Healthcare).



**Figure 8**

**Proposed model of membrane fusion at CDV entry.**

Step1. The homodimer dimers have stalk regions (colored orange) attached to fusion proteins. Each H protein dimers is bridged by disulfide bond (shown in red bar) at "neck" region. Step2. Binding of receptor (SLAM or Nectin-4) to the head domains. Steps 3 and 4. Receptor pulling causes the head domains to dissociate a dimer of homodimers and completely collapse to individual domains. Step5 and 6. Fusion proteins are allowed to access the membrane of target cells and complete fusion.

## Western blot analysis

To determine the glycosylation sites on CDV-H, we disrupted the N-glycosylation potential sites by changing Asn to Asp (309, 391, 422, 456, 587, and 603) or deleting Asn149 of the CDV-H globular head domain. After they were subjected to SDS-PAGE and transferred to PVDF membranes, anti-His-tag antibodies and horseradish peroxidase-conjugated goat anti-mouse IgG were added. Horseradish peroxidase activity was detected using Amersham ECL Plus Western Blotting Detection Reagent (GE Healthcare).

## Crystallization and Structure Determination

The CDV-H protein (4 mg/ml in 20 mM Tris-HCl pH 8.0, 100 mM NaCl) was crystallized by sitting-drop vapor diffusion at 20°C in 100 mM tri-sodium citrate pH 5.6, 10% polyethylene glycol-4000, and 10% isopropanol in 0.4  $\mu$ l drops containing a 1:1 mixture of the protein to the reservoir solution. X-ray diffraction data sets were collected on NW-12 beamline at the Photon Factory (Tsukuba) and BL-41XU at Spring-8 (Harima) and integrated and scaled using HKL2000 (HKL Research, Inc.)<sup>58</sup>. The molecular replacement software, MOLREP, using the crystal structure of MeV-H (PDB ID code 2Z6B) as a search model, provided a clear solution<sup>23,59</sup>. Model refinement calculations were executed using REFMAC<sup>60</sup>, and model building was performed using COOT<sup>61</sup>. Figures were prepared using PyMOL<sup>62</sup>.

## Surface Plasmon Resonance (SPR)

Purified proteins were dissolved in HBS-EP buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.005% Surfactant P20) (BIAcore AB). SPR experiments were performed using a BIAcore3000 instrument (GE Healthcare). CDV-H was immobilized on a CM5 sensor chip (GE Healthcare) using the direct amine coupling method. dSLAM in HBS-EP buffer was injected over the immobilized CDV-H. Values obtained by subtracting the response in the negative controlled flow cell, which was immobilized bovine serum albumin from the response in sample flow cells, were regarded as the binding responses. The data were analyzed using the BIA evaluation version 4.1 (GE Healthcare) and ORIGIN version 7 software (Microcal Inc.). The dissociation constant ( $K_d$ ) was derived by the nonlinear curve fitting of the standard Langmuir binding isotherm. Kinetics data were fitted into rate equations derived from the simple 1:1 Langmuir binding model ( $A + B \rightleftharpoons AB$ ) using the curve-fitting model of BIA evaluation version 4.1.

## High-speed atomic force microscopy (HS-AFM)

HS-AFM images of CDV-H were acquired in the tapping mode at room temperature using an HS-AFM instrument, NanoExplorer (Research Institute of Biomolecule Metrology). The sample solution (2  $\mu$ l) was placed on the freshly cleaved mica surface and incubated for 5 min. The samples on the mica were scanned in phosphate buffer (5 mM phosphate pH 6.2) by HS-AFM using a cantilever BL-AC10DS-A2 (Olympus). Images of 200  $\times$  200 pixels from a 100  $\times$  100 nm area were obtained at a scan rate of two frames per second (fps). Images were analyzed using ImageJ v1.52p software (<http://rsbweb.nih.gov/ij/>)<sup>63</sup>.

## Data availability

Coordinates and structural factors were deposited in the Protein Data Bank under accession code 8IAY. All other data are available from the corresponding author upon request.

Methods describing structure determination, binding analysis, atomic force microscopy, and assays for MeV entry and infection are described in Supplementary Methods.

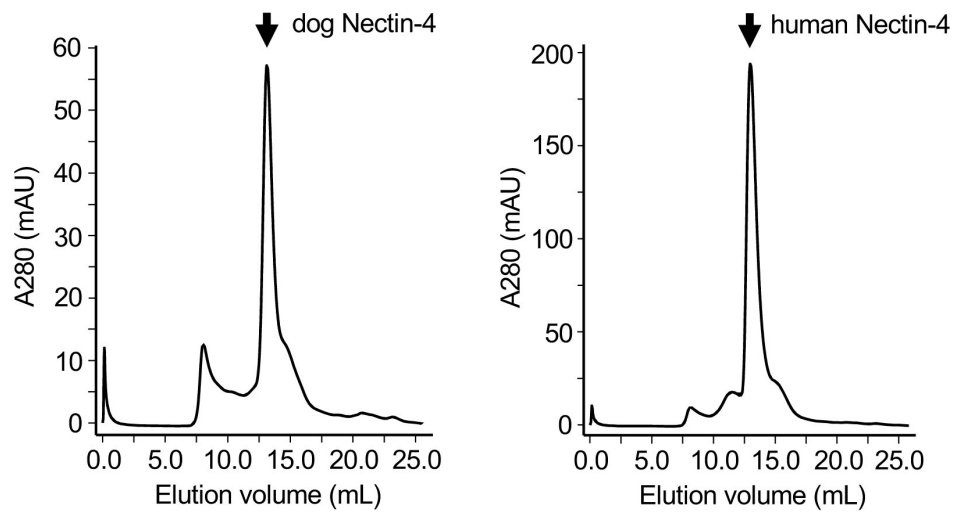
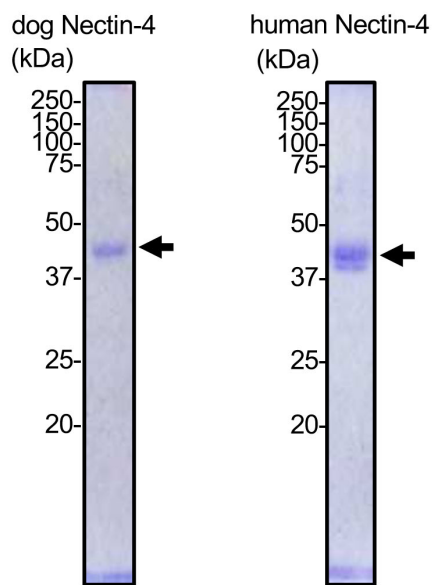
*Note: Supplementary Information is available on the Communications Biology website.*

## Supplementary Information

AFM image of CDV-H.CDV\_H, 100×100 nm. 5 fps5 frames per second100×100 nm scale, 200×200 pixelScale bar: 20 nm [↗](#)

AFM image of CDV-H.CDV\_H, 100×100 nm. 10 fps10 frames per second100×100 nm scale, 200×200 pixelScale bar: 20 nm [↗](#)

AFM image of CDV-H.CDV\_H, 500×500 nm. 5 fps5 frames per second500×500 nm scale, 200×200 pixelScale bar: 100 nm [↗](#)

**A****B**

### Supplementary Figure 1

#### Purification of dog and human soluble Nectin-4.

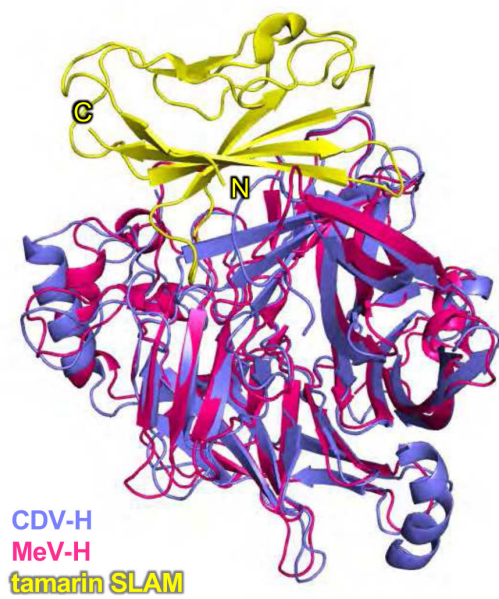
A. Gel filtration chromatogram of dog (left) and human (right) Nectin-4 using Superdex 200 column. The arrow indicates the peak of each Nectin-4. B. SDS-PAGE for dog (left) and human (right) Nectin-4 in reducing conditions. The arrow indicates the band of each Nectin-4.



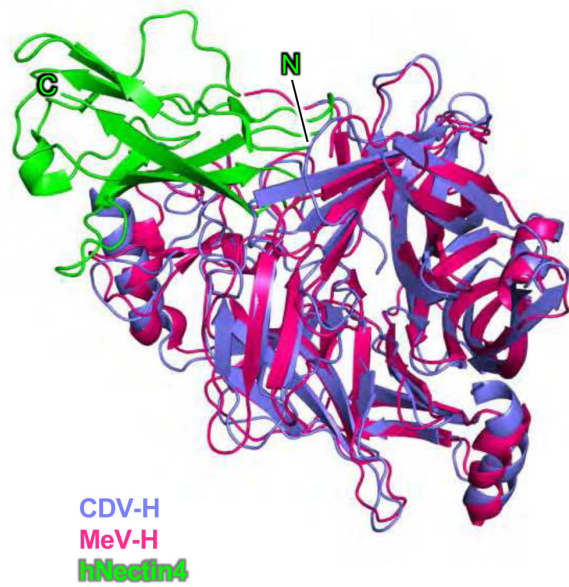
Supplementary Figure 2

## Structure-based alignment of receptor binding glycoproteins from various paramyxoviruses.

Attachment glycoproteins were aligned with the CDV-H structure. G proteins of Hendra virus; HeV (PDB code: 2VSK) and Nipah virus; NiV (PDB code: 3D12), HN proteins from Mumps virus; MuV (PDB code: 5B2C), Parainfluenza virus 5; PIV5 (PDB code: 4JF7), Newcastle disease virus; NDV (PDB code: 3T1E), Sosuga virus; SosV (PDB code: 6SG8), and human parainfluenza virus 3; hPIV3 (PDB code: 1V2I);, H protein of MeV (PDB code: 2ZB6). Green, yellow, and gray boxes indicate  $\alpha$ -helices,  $\beta$ -sheets, and disordered regions in the structure, respectively. Red letters indicate the residues involved in homodimer formation. The positions of the secondary structures of the six-bladed  $\beta$ -propeller CDV-H are shown underneath the alignment.



RMSD = 1.330 Å



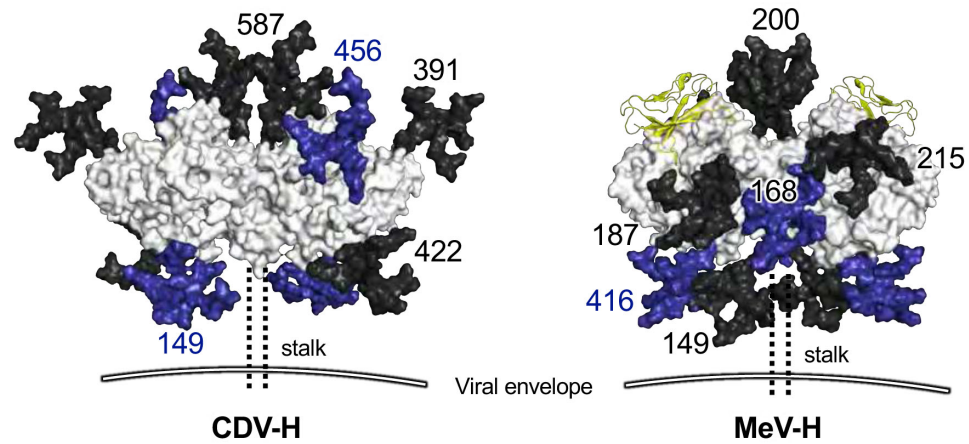
RMSD = 1.347 Å

### Supplementary Figure 3

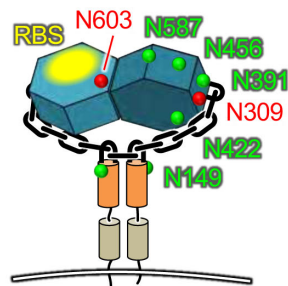
#### The superposition of CDV-H onto the MeV-H complex structure.

The superposition of CDV-H onto the MeV-H-tamarin SLAM complex (left), and onto MeV-H-hNectin-4 complex (right). Coloring is the same as that in [Figure 4](#) and [5](#).

**A**



**B**



#### Supplementary Figure 4

##### Glycan modifications onto CDV-H and MeV-H-SLAM complex.

A. Side view of glycan models (five residues) mapped on the surface presentation of CDV-H (left) and MeV-H-SLAM complex structure (right) was represented in the same manner with **Fig. 6C**. B. Schematic presentation of CDV-H dimer showing the locations of glycosylated (green) and unglycosylated (red) asparagine. RBSs was shown in yellow.

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## Editors

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### Reviewer #1 (Public Review):

Summary:

Fukuhara, Maenaka, and colleagues report a crystal structure of the canine distemper virus (CDV) attachment hemagglutinin protein globular domain. The structure shows a dimeric organization of the viral protein and describes the detailed amino-acid side chain interactions between the two protomers. The authors also use their best judgement to comment on predicted sites for the two cellular receptors - Nectin-4 and SLAM - and thus speculate on the CDV host tropism. A complementary AFM study suggests a breathing movement at the hemagglutinin dimer interface.

Strengths:

The study of CDV and related Paramyxoviruses is significant for human/animal health and is very timely. The crystallographic data seem to be of good quality.

Weaknesses:

While the recent CDV hemagglutinin cryo-EM structure is mentioned, it is not compared to the present crystal structure, and thus the context of the present study is poorly justified. Additionally, the results of the AFM experiment are not unexpected. Indeed, other paramyxoviral RBP/G proteins also show movement at the protomer interface.

### Reviewer #2 (Public Review):

Summary:

The authors solved the crystal structure of CDV H-protein head domain at 3.2 Å resolution to better understand the detailed mechanism of membrane fusion triggering. The structure clearly showed that the orientation of the H monomers in the homodimer was similar to that of measles virus H and different from other paramyxoviruses. The authors used the available co-crystal structures of the closely related measles virus H structures with the SLAM and Nectin4 receptors to map the receptor binding site on CDV H. The authors also confirmed which N-linked sites were glycosylated in the CDV H protein and showed that both wildtype and vaccine strains of CDV H have the same glycosylation pattern. The authors documented that the glycans cover a vast majority of the H surface while leaving the receptor binding site exposed, which may in part explain the long-term success of measles virus and CDV vaccines. Finally, the authors used HS-AFM to visualize the real-time dynamic characteristics of CDV-H under physiological conditions. This analysis indicated that homodimers may dissociate into monomers, which has implications for the model of fusion triggering.

The structural data and analysis were thorough and well-presented. However, the HS-AFM data, while very exciting, was not presented in a manner that could be easily grasped by readers of this manuscript. I have some suggestions for improvement.

1. The authors claim their structure is very similar to the recently published cryo-EM structure of CDV H. Can the authors provide us with a quantitative assessment of this statement?
2. The results for the HS-AFM are difficult to follow and it is not clear how the authors came to their conclusions. Can the authors better explain this data and justify their conclusions based on it?
3. The fusion triggering model in Figure 8 is ambiguous as to when H-F interactions are occurring and when they may be disrupted. The authors should clarify this point in their model.