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RESEARCH ARTICLE

The Furin Cleavage Site of Feline Coronavirus Type 1 (FCoV-1) and Its Structural Localization Within the S1 Domain D

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Abstract

Feline coronavirus type 1 (FCoV-1 is an alphacoronavirus (species *alphacoronavirus-1*) present in a distinct genetic clade. The FCoV-1 spike protein contains an identifiable furin cleavage site (FCS), which is highly unusual for an alphacoronavirus. FCoV-1 is a widespread and highly transmissible virus of both domestic and non-domestic felids. Notably, following infection with a low-pathogenicity virus, highly pathogenic variants of FCoV-1 are selected in individual cats by a process of 'internal mutation'; these variants appear to have robust tropism for macrophages and are strongly linked to the disease outcome known as feline infectious peritonitis (FIP). Also strongly linked to FIP disease outcome are a range of point mutations that are proposed to modify and disrupt the furin cleavage site. There is only a single structure of an FCoV-1 spike protein—for the UU4 variant sequenced from an FIP cat. Here, we structurally localized the FCS of FCoV-1 UU4 in comparison with that of a reference non-pathogenic variant (UU2). We show that the FCS is located within S1 domain D. Topology domain mapping revealed the FCoV-1 FCS to be within a solvent-exposed structural loop ("loop 2") located between two of the beta strands that comprise domain D of the spike protein—upstream of the interface of the S1 and S2 sub-domains. An equivalent "loop 2" of SARS-CoV-2 has also been identified as a proteolytic cleavage site for cathepsin L, suggesting conserved fusion-activation regulation of the coronavirus spike protein embedded within domain D.

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Introduction

Feline coronavirus (FCoV) is a common infection of domestic and wild cats, which presents as one of two biotypes classically referred to as feline enteric coronavirus (FECV) or feline infectious peritonitis virus (FIPV)[1]. "FECV" is what is presumed to cause initial localized infection in the enteric tract of an animal that causes only subclinical or mild

gastrointestinal signs. In contrast, “FIPV” causes a systemic infection and leads to the typically lethal (without antiviral intervention) disease known as feline infectious peritonitis (FIP). Development of FIP is complex and involves gaining monocyte/macrophage tropism, which allows for systemic spread. FIPV does not appear to be transmissible between cats, possibly due to changes in cell tropism.

According to the AAFP (American Association of Feline Practitioners) FIP guidelines there are three main outcomes after primary FCoV infection dictated by host immunology and viral shedding[2]. In the first scenario, 5% of cats are resistant to the virus and will quickly clear the infection with little to no shedding. In the second outcome, 70-80% of cats will intermittently shed low levels of virus for 2-3 months. Lastly, in 10-15% of cases cats become persistent shedders of high viral loads, sometimes for life. The fourth outcome is development of FIP. Here we refer to these virus biotypes as low pathogenicity (FECV) and high pathogenicity (FIPV).

Persistently infected, healthy carrier cats are also common. These animals shed FCoV in their feces and act as a source of infection. To determine sites of persistence and shedding, SPF cats were orally infected with non-virulent FCoV-1 (FECV biotype) using gut homogenates from healthy FCoV-1 positive cats. The major site of persistence and shedding are the colonic columnar epithelial cells in the colon[3], but other cell types and tissues may be involved and viral persistence remains largely unexplored.

FCoV-1 and FCoV-2 Terminology

FCoV has also been traditionally separated into two serotypes (serotype I and serotype II). These viruses were initially categorized as distinct “serotypes” due to their different responses to antibodies, but can be better separated by evolution, genome, prevalence, and molecular mechanisms[4]. We refer to these viruses here as FCoV-1 and FCoV-2. The differences in the spike gene between FCoV-1 and FCoV-2 are significant enough to warrant distinction of two separate genetic clades (previously referred to as A and B)[5]. Clade A/serotype-I viruses are FCoV-1 (and the related canine coronavirus, CCoV-1). The clade B/serotype II viruses are FCoV-2, CCoV-2 and transmissible gastroenteritis virus (TGEV), with CCoV seemingly the ancestor virus of this clade. FCoV-1 is much more prevalent than FCoV-2 in cat populations, but with both genotypes able to cause FIP.

The Feline Coronavirus Genome

Feline coronavirus is a pleomorphic, enveloped virus in the *Nidovirales* order, *Coronaviridae* family, and alphacoronavirus genus. FCoV genomes are large (29 kb) and contain 11 open reading frames (ORFs): ORF1a, ORF1b, S, 3a, 3b, 3c, E, M, N, 7a, and 7b. ORF1a and ORF1b encode for polypeptides 1a and 1b, which are subsequently enzymatically cleaved into 16 nonstructural proteins. The remaining ORFs encode structural and accessory proteins. The four structural proteins are spike (S), nucleocapsid (N), membrane (M), and envelope (E). The five accessory proteins are 3a, 3b, 3c, 7a, and 7b.

FCoV-1 and FCoV-2 Genomic and Structural Differences

Recombination

FCoV-2 typically arises through homologous recombination between FCoV-1 and CCoV. The two widely studied FCoV-2 strains (FECV II 1683 and FIPV II 1146) arose from distinct double recombination events, which produced chimeric viruses with the backbone (M, N, 7a, and 7b genes) of FCoV-1 and spike protein from CCoV[6]. Alphacoronaviruses, including FCoV-2, possess the conserved proteolytic processing activation site at S2', while FCoV-1 possesses two known activation sites at S1/S2 and S2' (see Figures 1 and 2).

Cell Culture Differences

FCoV-1 is much harder to study compared to FCoV-2, but it is more clinically relevant. FCoV-2 can be isolated and grown in conventional cell culture, while FCoV-1 cannot. There is a widely used and isolated strain of FCoV-1 (Black/TN406), but this is highly cell culture adapted[7].

Receptor

FCoV-2 binds to the aminopeptidase N (APN) receptor[8]; the receptor for FCoV-1 is unclear but it is now thought not to be APN[9]. However, both FCoV-1 and FCoV-2 are both able to infect non-permissive cell types when expressing exogenous lectins such as DC-SIGN[10]. Chimeric viruses made using the background of an FCoV-1 strain (cell culture adapted Black-TN406) with the spike protein from an FCoV-2 strain (FIPV 79-1146) were able to use fAPN for host cell entry. This study illustrates FCoV-1 is able to replicate in cell culture when an FCoV-2 spike protein is encoded. The spike protein is the main determinant of receptor usage[11].

Proteolytic activation FCoV-1 and FCoV-2

Currently FCoV (like other alphacoronaviruses) is known to contain a prominent S2' cleavage loop. Our early structural molecular modeling of FCoV spikes was based on the closest available structure at that time (HCoV-NL63) and illustrated that FCoV-1 also contained a protruding cleavage loop in the S1/S2 region, which makes it more accessible to proteases[4]. This loop contains a consensus sequence for cleavage by furin, with a prototype sequence of S-R-R-S-R-R-S predicted for low path viruses.

Mutations linked to low path and high path biotypes for FCoV-1

Mutations in the FCS that decrease furin cleavage of FCoV-1 spike are now though to be highly linked to the transition of low path viruses to the high path viruses causing FIP[12][13][14][15][16][17][18][19]. The S2’ cleavage site is less studied, and while this site can be mutated in FIP cases there is currently not a clear linkage with disease outcome. However, in addition to mutations at the S1/S2 furin cleavage site (FCS), there are numerous genomic differences associated with low

path and high path biotypes. Ultimately, it is likely a combination of multiple mutations that leads to the biotype switch. The early ground-breaking internal mutation theory focused on genetic changes in the 3c and 7b genes, however, we now know that the spike glycoprotein is the main driver of FCoV virulence. Initially, the M1058L mutation was believed to be an indicator of "FIPV." More research has shown that the M1058L mutation is associated with systemic spread (tropism changes) but not necessarily pathogenicity/virulence changes. In other words, this mutation alone is not sufficient to diagnose FIP[20].

As with all CoVs, the FCoV spike protein is broken into the two subunits S1 (receptor binding domain) and S2 (fusion domain). The S1 subunit contains two domains: N-terminal domain (NTD) and the C-terminal domain (CTD), both of which can function as receptor binding domains for proteins and sugar molecules in various coronaviruses[21][22]. For alphacoronaviruses, the NTD is comprised of two functionally equivalent domains with lectin-like folds but with low sequence identity (domains 0 and A). The CTD comprises domains B (containing the presumed RBD), C and D. The S2 subunit contains the fusion peptide (FP), heptad repeat 1 (HR1), heptad repeat 2 (HR2), transmembrane domain (TM), and an internal domain (ID) also known as an endodomain (E). The S1/S2 cleavage site has been presumed to sit at the interface between S1 and S2 domains, S2' is located upstream of the fusion peptide in the S2 domain. FCoV-1 also contains a linker (L) sequence between the S1/S2 and S2' activation sites.

Coronavirus Furin Cleavage Sites (FCSs)

FCoV-1 (along with CoV-1) is the only alphacoronavirus with an identifiable furin cleavage site. In contrast, an FCS is essentially universal in gammacoronaviruses and in the betacoronavirus sublineage embecovirus, and is widely found in other betacoronaviruses including SARS-CoV-2. Cleavage at the S1/S2 furin cleavage site is often predicted using the ProP 1.0 online server, which calculates a score of predicted cleavability based on sequences[23]. However, this server can oversimplify proteolytic cleavage as a biochemical reaction, without integrating more complex biochemical factors. A superior furin cleavage prediction site (PiTou) was designed using functional characterization of the 20-residue furin substrate motif. Compared to ProP, PiTou showed increased sensitivity and specificity[24][25]), however it is peptide-based and does not take into account secondary structural characteristics. We have recently documented the need for improved structural predictions and understanding of cleavage sites, using the model embecovirus/betacoronavirus MHV[26]. This leads to an improved need for prediction, which is in itself founded on structures rather than sequences, providing a role for what is known as structural phylogenomics.

Since our initial structural modeling of FCoV-1 and FCoV-2[4], there is an available cryo-EM structure of an FCoV-1 spike protein (UU4); pdb 6JX7[27], allowing more refined modeling of the FCoV cleavage sites. Here, we map the naturally mutated FCS of FCoV-UU4 (S-R-**S**-A-R-**G**-S) as a representative clinical high-path FCoV ("FIPV") (Figure 1) along with a structural model of FCoV-UU2, a representative low-path FCoV ("FECV"), containing a consensus FCS (Figure 2). These sequences were geographically and temporally; UU2/UCD from California, USA in 1993 and UU4 from the Netherlands in 2007, and as such accommodate genetic diversity of FCoV-1 (the two spike sequences are 90.8% identical/97.4% homologous). In both cases, we find the FCS is located within domain D, and not at the interface of the S1 and S2 sub-

domains. Domain topology mapping places the FCS in ‘loop2’ of domain D (Figure 3).

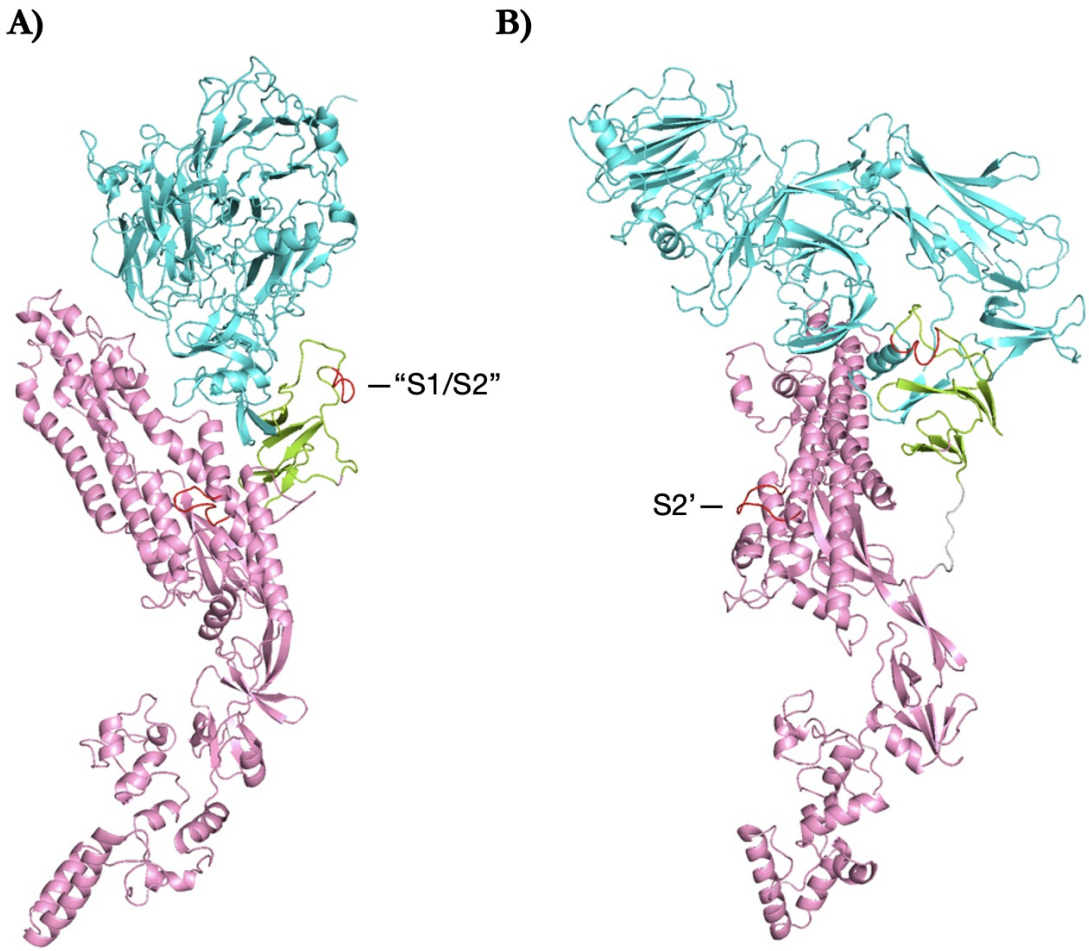

Figure 1. A) Structural model of the high path FCoV-1 UU4 spike protein in monomer form. Domain D is colored green, with the remainder of S1 in teal and S2 in pink. The two cleavage sites S2’ and “S1/S2” within Domain D are shown in red, and the interface of S1 and S2 is in white. **B)** Model as in A), rotated 90°. The GenBank accession number of UU4 spike is ACT10887.1

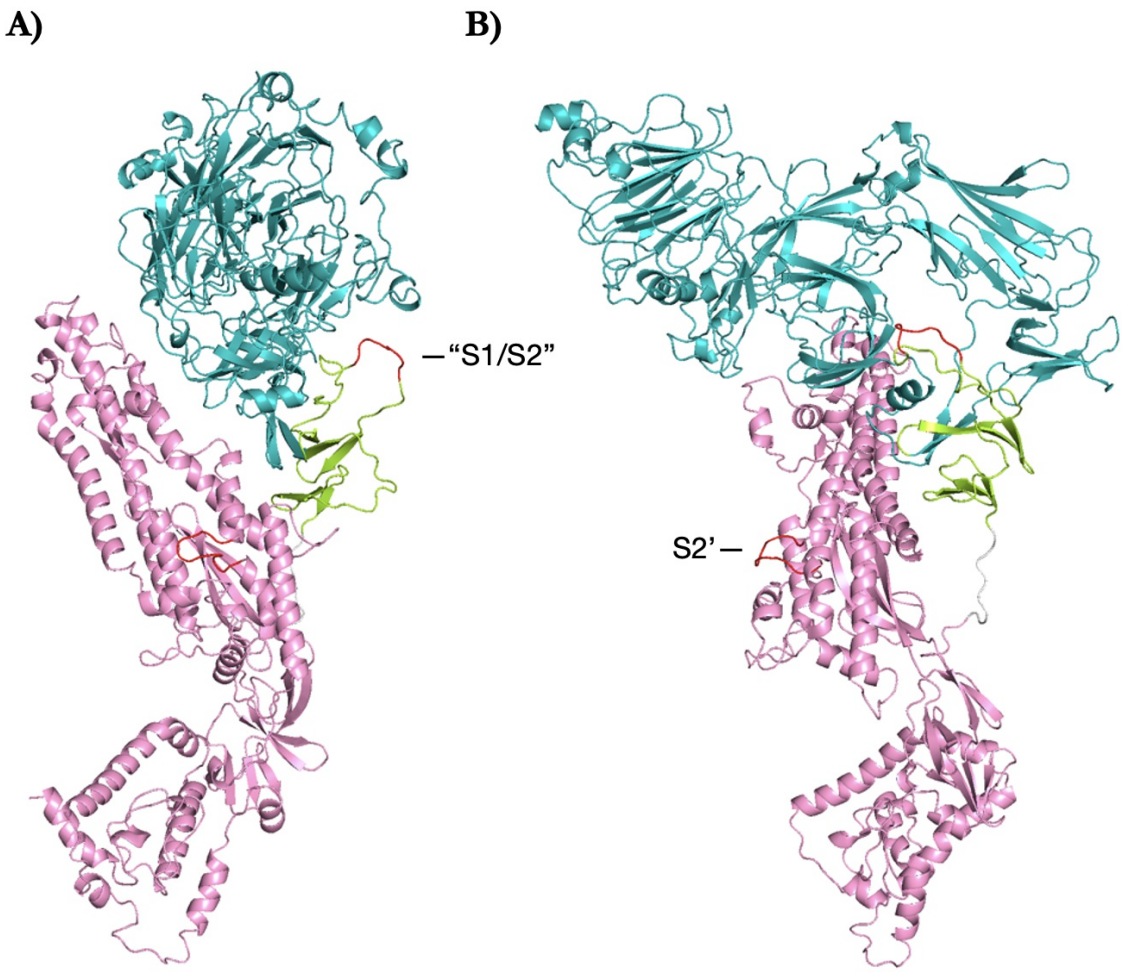

Figure 2. **A)** Structural model of the low path FCoV-1 UU2 spike protein in monomer form. Domain D is colored green, with the remainder of S1 in teal and S2 in pink. The two cleavage sites S2' and "S1/S2" within Domain D are shown in red, and the interface of S1 and S2 is in white. **B)** Model as in A), rotated 90°. The GenBank accession number of UU2 spike is ACT10948.1

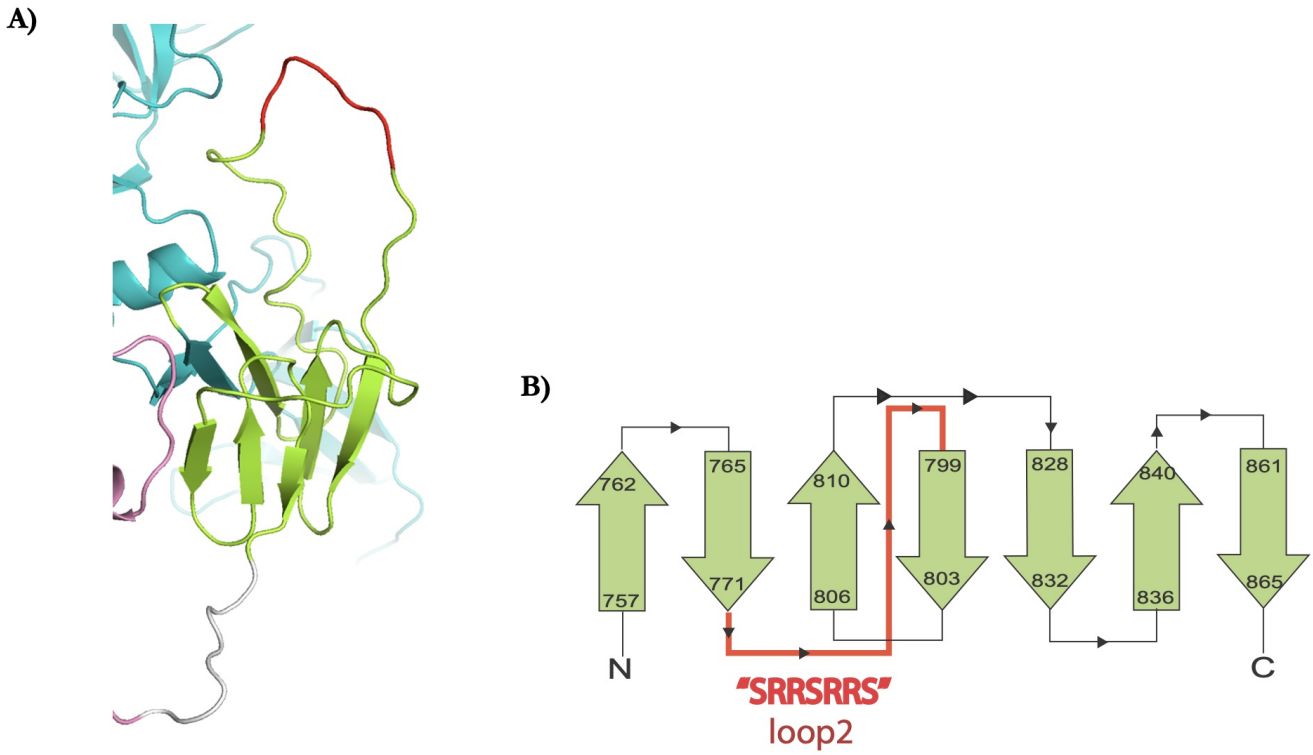

Figure 3. **A)** Enlarged view of domain D of the FCoV-1 UU2 spike protein. Domain D is colored green, with the remainder of S1 in teal and S2 in pink. UU4 shows an equivalent domain organization. The FCS (loop 2) is shown in red, and the interface of S1 and S2 is in white. **B)** Topology model of FCoV-1 spike domain D to show the location of the FCS (SRRSRRS consensus), with loop 2 in red. Amino acid numbering is based on the reference UU4 sequence.

Interestingly we find that domain D 'loop 2' is equivalent to CTSL-2 (Figure 4) , identified as an alternative cleavage site for SARS-CoV-2 upstream of the FCS, and cleaved by cathepsin L; see ref[28] and Fig. 2 of ref[29]. Domain D is also known as SD2 for SARS-CoV-2[30] and has been studied as a key regulator of protein stability for engineering stable spike derivatives[31], as well as containing the naturally occurring gateway mutations D614G and H655Y[32] that are now well recognized to work in concert with the FCS and the S2' cleavage site (and possibly also the NTD) to control fusion activation and cell entry.

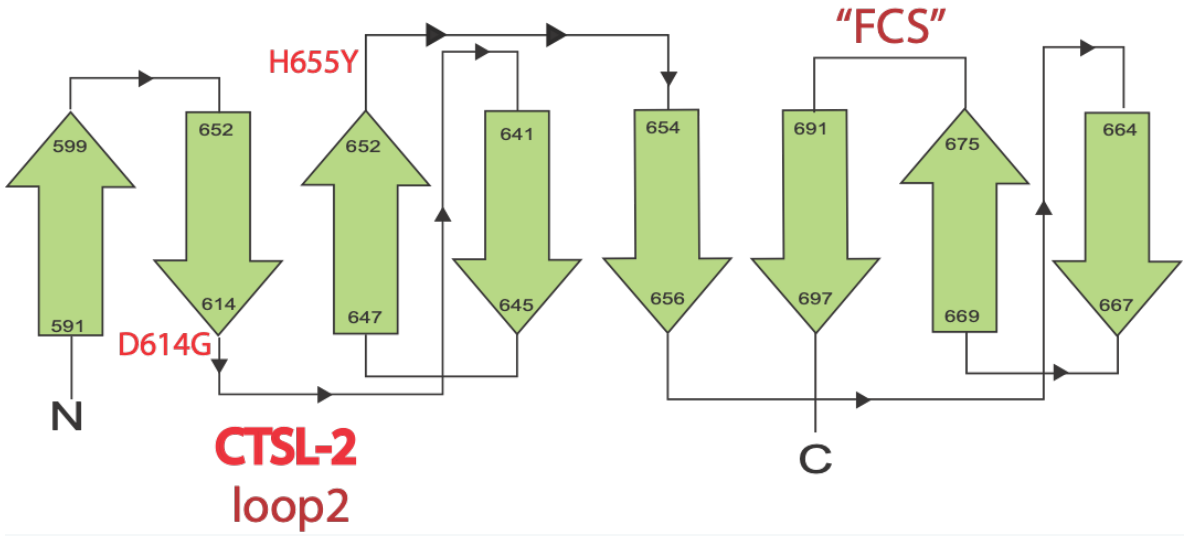

Figure 4. Topology model of SARS-CoV-2 spike domain D to show the location of the FCS and the proposed cathepsin cleavage site (CTSL-2) within loop 2, in red. Amino acid numbering is based on the reference Wuhan Hu-1 sequence. The locations of the 'gateway mutations' D614G and H655Y are indicated.

Methods

Protein Structural Modeling

Structural models of the FCoV-1 UU2 and UU4 spike proteins were generated using Robetta (https://robetta.bakerlab.org), using PDB: 6JX7 (UU4) as the template.

Topology Diagrams

Topology diagrams were obtained through PDBSum (https://www.ebi.ac.uk/thornton-srv/databases/pdbsum/), using the following templates: UU4, PDB: 6JX7; SARS-CoV-2, PDB: 6VXX. The topology diagrams generated were formatted in Adobe Illustrator.

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References

1. ^*Pedersen, N.C. History of Feline Infectious Peritonitis 1963-2022 – First description to Successful Treatment 2022.*

https://ccah.vetmed.ucdavis.edu/sites/g/files/dgvnsk4586/files/inline-files/Review%20FIP%201963-2022%20final%20version%20April%202022.pdf.

2. ^*Thayer V, et al. 2022 AAFP/EveryCat Feline Infectious Peritonitis Diagnosis Guidelines. J Feline Med Surg. 2022;24:905-933.*
3. ^*Kipar A, et al. Sites of feline coronavirus persistence in healthy cats. J Gen Virol. 2010;91:1698-707.*
4. a, b, c*Jaimes JA, et al. A Tale of Two Viruses: The Distinct Spike Glycoproteins of Feline Coronaviruses. Viruses. 2020;12:83.*
5. ^*Whittaker GR, Andre NM, Millet JK. Improving Virus Taxonomy by Recontextualizing Sequence-Based Classification with Biologically Relevant Data: the Case of the Alphacoronavirus 1 Species. mSphere. 2018;3:e00463-17.*
6. ^*Herrewegh AA, et al. Feline coronavirus type II strains 79-1683 and 79-1146 originate from a double recombination between feline coronavirus type I and canine coronavirus. J Virol. 1998;72:4508-14.*
7. ^*O'Brien A, et al. Characterizing replication kinetics and plaque production of type I feline infectious peritonitis virus in three feline cell lines. Virology. 2018;525:1-9.*
8. ^*Tresnan DB, Levis R, Holmes KV. Feline aminopeptidase N serves as a receptor for feline, canine, porcine, and human coronaviruses in serogroup I. J Virol. 1996;70:8669-74.*
9. ^*Dye C, Temperton N, Siddell SG. Type I feline coronavirus spike glycoprotein fails to recognize aminopeptidase N as a functional receptor on feline cell lines. J Gen Virol. 2007;88:1753-1760.*
10. ^*Regan AD, Ousterout DG, Whittaker GR. Feline lectin activity is critical for the cellular entry of feline infectious peritonitis virus. J Virol. 2010;84:7917-21.*
11. ^*Ehmann R, et al. Reverse Genetics for Type I Feline Coronavirus Field Isolate To Study the Molecular Pathogenesis of Feline Infectious Peritonitis. mBio. 2018 Sep 3;17:e1009910. doi:10.1371/journal.ppat.1009910.*
12. ^*Ouyang H, et al. Epidemiology and Comparative Analyses of the S Gene on Feline Coronavirus in Central China. Pathogens. 2022 Apr 12;11:460. doi:10.3390/pathogens11040460.*
13. ^*Murphy BG, et al. Serologic, Virologic and Pathologic Features of Cats with Naturally Occurring Feline Infectious Peritonitis Enrolled in Antiviral Clinical Trials. Viruses. 2024 Mar 17;16:462. doi:10.3390/v16030462.*
14. ^*Andre NM, et al. Distinct mutation in the feline coronavirus spike protein cleavage activation site in a cat with feline infectious peritonitis-associated meningoencephalomyelitis. J Feline Med Surg Open Rep. 2019;5:2055116919856103.*
15. ^*Andre NM, Miller AD, Whittaker GR. Feline infectious peritonitis virus-associated rhinitis in a cat. J Feline Med Surg Open Rep. 2020;6:2055116920930582.*
16. ^*Healey EA, et al. An outbreak of FIP in a cohort of shelter-housed cats: molecular analysis of the feline coronavirus S1/S2 cleavage site consistent with a "circulating virulent-avirulent" theory of FIP pathogenesis. J Feline Med Surg Open Rep. 2022. doi:10.1177/20551169221074226.*
17. ^*Licitra BN, et al. Mutation in spike protein cleavage site and pathogenesis of feline coronavirus. Emerg Infect Dis. 2013;19:1066-73.*
18. ^*Licitra BN, Olarte-Castillo XA, Whittaker GR. Persistent feline coronavirus infection in a cat with cardiac and*

gastrointestinal signs. Companion Animal. 2024. doi:10.12968/coan.2023.0054.

19. ^*Licitra BN, et al. Feline coronaviruses associated with feline infectious peritonitis have modifications to spike protein activation sites at two discrete positions. arXiv. 2014: https://arxiv.org/abs/1412.4034.*
20. ^*Barker EN, Tasker S. Advances in Molecular Diagnostics and Treatment of Feline Infectious Peritonitis. Advances in Small Animal Care. 2020;1:161-188.*
21. ^*Hulswit RJ, de Haan CA, Bosch BJ. Coronavirus Spike Protein and Tropism Changes. Adv Virus Res. 2016;96:29-57.*
22. ^*Millet JK, Jaimes JA, Whittaker GR. Molecular diversity of coronavirus host cell entry receptors. FEMS Microbiol Rev. 2021 Oct 28;45:fuaa057.*
23. ^*Duckert P, Brunak S, Blom N. Prediction of proprotein convertase cleavage sites. Protein Engineering, Design and Selection. 2004;17:107-112.*
24. ^*Tian S. A 20 Residues Motif Delineates the Furin Cleavage Site and its Physical Properties May Influence Viral Fusion. Biochemistry Insights. 2009;2:BCI.S2049.*
25. ^*Tian S, Huajun W, Wu J. Computational prediction of furin cleavage sites by a hybrid method and understanding mechanism underlying diseases. Sci Rep. 2012;2:261.*
26. ^*Choi A, et al. Analysis of the molecular determinants for furin cleavage of the spike protein S1/S2 site in defined strains of the prototype coronavirus murine hepatitis virus (MHV). Virus Res. 2023;340:199283.*
27. ^*Yang TJ, et al. Cryo-EM analysis of a feline coronavirus spike protein reveals a unique structure and camouflaging glycans. Proc Natl Acad Sci U S A. 2020;117:1438-1446.*
28. ^*Zhao MM, et al. Novel cleavage sites identified in SARS-CoV-2 spike protein reveal mechanism for cathepsin L-facilitated viral infection and treatment strategies. Cell Discov. 2022;8:53.*
29. ^*Meyer B, et al. Characterising proteolysis during SARS-CoV-2 infection identifies viral cleavage sites and cellular targets with therapeutic potential. Nat Commun. 2021;12:5553.*
30. ^*Wrapp D, et al. Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. Science. 2020;367:1260-1263.*
31. ^*Zhang X, et al. Disulfide stabilization reveals conserved dynamic features between SARS-CoV-1 and SARS-CoV-2 spikes. Life Sci Alliance. 2023 Jul 4;6: e202201796. doi:10.26508/lsa.202201796.*
32. ^*Yurkovetskiy L, et al. S:D614G and S:H655Y are gateway mutations that act epistatically to promote SARS-CoV-2 variant fitness. bioRxiv. 2023: p. 2023.03.30.535005.*