BRIEF COMMUNICATION

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Distant sequence similarity between hepcidin and the novel coronavirus spike glycoprotein: a potential hint at the possibility of local iron dysregulation in COVID-19

Sepehr Ehsani [1,2,*]

[1] Theoretical and Philosophical Biology, Department of Philosophy, University College London, Bloomsbury, London, WC1E 6BT, United Kingdom
[2] Ronin Institute for Independent Scholarship, Montclair, New Jersey, 07043, United States

* E-mail: ehsani@uclmail.net / ehsani@csail.mit.edu

ABSTRACT

The spike glycoprotein of the SARS-CoV-2 virus, which causes COVID-19, has attracted attention for its vaccine potential and binding capacity to host cell surface receptors. Much of this research focus has centered on the ectodomain of the spike protein. The ectodomain is anchored to a transmembrane region, followed by a cytoplasmic tail. Here we report a distant sequence similarity between the cysteine-rich cytoplasmic tail of the coronavirus spike protein and the hepcidin protein that is found in humans and other vertebrates. Hepcidin is thought to be the key regulator of iron metabolism in humans. An implication of this preliminary observation is to suggest a potential route of investigation in the COVID-19 research field making use of an already-established literature on the interplay of local and systemic iron regulation, respiratory infections and the hepcidin protein. The question of possible homology and an evolutionary connection between the viral spike protein and hepcidin is not assessed in this report.

MAIN TEXT

As of the date of this paper, 175 countries and regions are tackling the challenge of the pandemic caused by the novel coronavirus.[1] Coronaviruses, first described in the 1960s,[2,3] are mostly present in birds and mammals, and there has thus far been seven coronavirus infectious disease outbreaks in humans causing respiratory illness.[4,5] The severe acute respiratory syndrome coronavirus (SARS-CoV) of 2002, the Middle East respiratory syndrome coronavirus (MERS-CoV) of 2012, and now the SARS-CoV-2 of 2019 (causing the 'COVID-19' disease) are three of the seven known coronavirus infections causing severe human disease.[6] This positive-sense single-stranded RNA virus family possesses the structural proteins spike (S), membrane (M) and envelope (E) proteins, along with the nucleocapsid (N) protein. It also has the largest genome among RNA viruses.[7] Much research interest is devoted to the spike (glyco)protein (forming the characteristic 'corona') and its importance in the development of vaccines and antivirals.[8,9] The angiotensin-converting enzyme 2 (ACE2) is thought to be its receptor on host cells.[10,11] The spike protein is formed of a receptor-binding subunit (S1), a membrane-fusion subunit (S2), a single-pass transmembrane (TM) domain, and a cytoplasmic/intracellular tail (CT).[7,12] Of note, the S1 domain has a similar fold as human galectins (galactose-binding lectins).[13] Lastly, in terms of the putative *primary* function of the spike protein, Li comments that "because coronaviruses must enter cells for replication, membrane fusion is the central function of coronavirus spikes".[7]

A basic question that might arise is: what exactly makes the pathobiology and disease course of these particular viruses *unique*? And, could it be that, in addition to viral replication inside the particular types of human host cells, other intracellular processes *specific* to these viruses are involved? Having this in mind, we wondered if there might be any sequence similarity (and thereby potentially structural similarity) between the SARS-CoV-2 spike protein (which has 1,273 amino acids)[14] and any vertebrate protein(s).

A simple BLAST search does not reveal any similarities with human proteins. However, based on previous experience with the pufferfish *Takifugu rubripes* proteome[15-17] and its unique evolutionary history, we restricted the search to this species. Interestingly, a query using the full-length SARS-CoV-2 spike protein (accession no. YP_009724390.1) revealed a sole hit with the pufferfish hepcidin[18] protein (XP_003965681.1; score: 32.7, E-value: 0.54). Given that SARS-like coronaviruses can be found in bats,[19] we also used a full-length bat coronavirus sequence (ANA96027.1) as the query, which showed a closer match with pufferfish hepcidin (score: 38.5, E-value: 0.005). The scores and E-values here are not meant to indicate any claims of statistical significance but rather are provided for the purpose of comparison. The similarity between the spike protein and hepcidin appears to be at the cytoplasmic tail[20] of the spike protein, or perhaps at the junction between the TM and CT domains. A multiple sequence alignment of this sequence region (generated using the AlignX feature of Vector NTI Advance 11.0, Invitrogen, Carlsbad, CA, USA), using three coronavirus spike proteins and four hepcidin proteins (from pufferfish, bat and human) is illustrated in **Figure 1A**.

The alignment depicts a number of conserved motifs, particularly between the first pufferfish hepcidin sequence (various pufferfish species have at least two hepcidin-like genes)[18] and the coronavirus spike proteins. In a sense, the pufferfish sequence seems to act as a 'bridge' between the coronavirus motifs and those in the human hepcidin sequence. The similar cysteine-rich motif takes the following form: '**L**XXX**T**X**CC**X**CCKG**XXX**CG**X**CC**(**R**/**K**)**F**'. Of note, the eight cysteines of the mature human hepcidin in the similarity motif, and the aligned cysteines of the SARS-CoV-2 spike protein, are not all specifically coded by one of the two cysteine-coding codons (TGC and TGT). Both codons are present in the respective gene segments. Also, for comparison purposes, as the coronavirus envelope protein[21] contains a related 'LCAYCCN' motif,[22] this sequence was also added as the last line of the alignment.

This is a distant and limited sequence similarity, and no claims about homology and sequence conservation can be made at present. Nevertheless, this raises a potential and intriguing question of whether there could be mimicry of human hepcidin (structural or otherwise), perhaps inside the host cell, by the TM-CT junction of the spike protein. It should be noted that it is unlikely for the sequence similarity reported here to be due to the relatively cysteine-rich composition of hepcidins. When searching all available teleost proteomes using the aforementioned bat coronavirus sequence, hepcidins are the only hits, even though they are clearly not the only cysteine-rich proteins. Moreover, in addition to various hepcidin orthologs containing 8 cysteines, 4-cysteine variants have been described in notothenioid fish species.[23]

Hepcidin is a small peptide hormone that was discovered in 2000/2001,[24-28] and initially named LEAP-1 (liver-expressed antimicrobial peptide). It has an antiparallel beta-sheet fold and contains four disulfide bonds, and is involved in iron trafficking and the host's response to infection.[29] In fact, it has been remarked by a number of commentators that "hepcidin is to iron, what insulin is to glucose".[30] Clear hepcidin orthologs appear to be missing in birds and invertebrates.[31] The human hepcidin (coded by the *HAMP* gene) is an 84-amino-acid prepropeptide, leading to a mature 25-amino-acid peptide that is detectable in blood and urine.[32] The proprotein convertase furin has been demonstrated to cleave prohepcidin at a polybasic site.[33,34] Of note, a furin-like cleavage site ('RRAR') has recently been reported to exist in the ectodomain of the SARS-CoV-2

spike protein, which is absent in coronaviruses of the same clade.[35] The protein-coding part of the *HAMP* gene is split over three exons, with the 25-amino-acid mature peptide occurring on the last exon. In terms of its putative function(s), Prentice notes that "although the hepcidin molecule does itself possess some antimicrobial activity, this is rather weak compared to peptides such as defensins, and its primary contribution to innate immunity is via regulation of iron".[31] Hepcidin binds to and mediates the degradation of ferroportin (encoded by the *SLC40A1* gene), the only known cellular iron exporter. The structural details of this interaction are being mapped and studied in ever more detail.[36-38]

There are a number of solved structures of hepcidin.[32,39] An NMR structure of human hepcidin is depicted in **Figure 1B** (visualized using 3-D Molecule Viewer, Vector NTI Advance 11.0, Invitrogen) including the locations of the four putative disulfide bonds. Available solved structures of the coronavirus spike glycoprotein, as far as our search could reveal, mostly utilize expression constructs that stop just short of the TM domain. As noted, this is partly because the protein's ectodomain is the main focus of studies on viral binding to host surface receptors.[11,40,41] For example, Wrapp, Wang and colleagues have recently reported the cryo-electron microscopy structure of ectodomain residues 1-1,208 of the spike protein (trimer in the prefusion conformation),[11] but this excludes the TM and CT domains. It goes without saying that the inclusion of transmembrane domains would require complicated structural elucidation protocols, and even then, one may still not be able to solve the structure of the protein in its entirety.

Moreover, we would like to report that using the Pfam-A (ver. 32) structural/domain database[42] in the HHpred remote homology and structure prediction toolkit,[43] the coronavirus spike protein regions analyzed here show some predicted structural similarity to lipolysis-stimulated receptor (LSR) lipoprotein receptor family (PF05624),[44] and hepcidin sequences show some predicted structural similarity to the Sar8.2 protein family found in Solanaceae plants (PF03058).[45] What, if any, significance these findings may hold is unclear at present. Of more importance right now would be the theoretical and/or actual elucidation of the structure of the spike protein TM-CT junction region and a comparison with the available hepcidin structures (the Pfam hepcidin entry, PF06446, currently references six PDB structures).

Given the prominence of cysteines in the aligned motif (**Figure 1A**), how are they utilized in the respective similar domains? At first pass, the usages appear to be different: as noted earlier, in hepcidin, the cysteines may give rise to a compact disulfide-bridged peptide (**Figure 1B**),[32,46] whereas in coronavirus spike glycoproteins, the cysteines in the TM-CT junction serve as palmitoylation acceptor residues[47] (**Figure 1C**) that facilitate membrane fusion.[48] At least a portion of the palmitoylation of the SARS-CoV spike protein has been reported to occur in a pre-medial Golgi compartment.[49] However, there is also the possibility of cross-disulfide bond formation with a non-homologous small cluster of cysteines within the envelope protein.[22] Moreover, if *S*-palmitoylation is a reversible and dynamic process,[50] it is to be determined if the spike protein junction cysteines might in fact have a different posttranslational modification in the host cytoplasmic environment (although there is no evidence of this at the moment). In the cited paper by McBride and Machamer, the authors conclude that the palmitoylation of the SARS-CoV spike protein "was not necessary for S protein stability, trafficking or subcellular localization" nor "for efficient interaction with M protein".[49] To what extent the posttranslational modifications of the spike protein and hepcidin, be it furin cleavage, disulfide bonds or palmitoylation, are in any way similar in an intracellular context, remains an open question.

In terms of the possibility of an evolutionary connection between the spike protein and hepcidin, one could imagine a scenario whereby an ancestral spike protein acquired a hepcidin-like sequence from a host organism, and the new sequence was palmitoylated to aid with membrane association. Li points out that "the

primordial form of coronavirus spikes might contain S2 only",[7] and the cytoplasmic motif features highlighted in the current report do not appear to be present in other class I viral membrane fusion proteins (which include the influenza virus),[51,52] although we have not performed an exhaustive search. However, a number of questions might then arise under such a scenario, such as the difference between the primary localizations of hepcidin (considering its putative interaction with extracellular and transmembrane regions of ferroportin)[36,37] versus the CT domain of the spike protein. Alternatively, it might be argued that perhaps a case of convergent sequence evolution is at play. For example, the influenza virus hemagglutinin glycoprotein appears to have a conserved 'CXICI' motif in its cytoplasmic tail domain,[53,54] and perhaps an ancestral spike protein with similar features convergently acquired hepcidin-like sequence motifs. These are of course speculations and remain open questions. Investigations pursuing these topics could also make use of studies that attempt to trace the evolutionary history of hepcidin itself.[23,55]

Given the central role of hepcidin in iron metabolism, it is important to point to a number of circumstantial but perhaps important findings (as pertaining to the topic at hand) in the literature. These include (i) a link between SARS and liver function abnormalities,[56] (ii) the association of pulmonary iron overload and restrictive lung disease,[57,58] (iii) the role of iron in pulmonary fibrosis,[59] and (iv) hepcidin's modulation of the proliferation of pulmonary artery smooth muscle cells.[60] Furthermore, using experiments on iron overload in European bass (*Dicentrarchus labrax*), Neves and colleagues discuss the functional partnership between hepcidin and ferroportin from an evolutionary perspective, and suggest that this may "open new possibilities for the pharmaceutical use of selected fish […] hepcidins during infections, with no impact on iron homeostasis".[61] Lastly, hepcidin upregulation along with serum iron reduction has been reported in influenza infections,[62,63] which may possibly suggest that a potential spike protein-hepcidin mimicry could allow the SARS-CoV-2 virus to exacerbate this response. Importantly, however, iron dysregulation changes may only be at a local cellular/tissue level and not reach a systemic response.[64]

Specific to COVID-19, a number of broader questions that could follow up from this work are: first, does the spike protein, similar to hepcidin, potentially promote iron sequestration in (alveolar) macrophages[65] and hence impede the host's immunological response? Second, could a recent report of the common presence of digestive symptoms in COVID-19 patients[66] be explainable in part by a link to hepcidin? And third, could systemic changes in serum iron levels[67,68] (with a possible view on the degree of above-normal serum ferritin in patients)[69] or levels of hepcidin itself be detected in patients with varying COVID-19 severities?

FIGURE LEGEND

Figure 1. Comparison of select hepcidin and coronavirus spike protein sequences. (**A**) A multiple sequence alignment of the C-terminal region of a number of coronavirus spike proteins (encompassing portions of the putative transmembrane and cytoplasmic tail segments), four hepcidins and the SARS-CoV-2 envelope protein is presented. The envelope sequence is provided only to demonstrate the cysteine residues with which the spike protein is proposed to form disulfide bridges.[22] The residue numbers are shown on the sides of each protein segment, and for proteins whose C-terminal sequences continue beyond the alignment, the full residue length is provided to the right. As per a color scheme used previously,[15] dark green, grey and black highlights depict conserved, similar and identical residues, respectively. 'Tr' stands for *Takifugu rubripes* (Japanese pufferfish), 'Rf' for *Rhinolophus ferrumequinum* (greater horseshoe bat) and 'Hs' for *Homo sapiens* (human). The protein accession numbers of the sequences shown are, in order: (1) AWH65954.1, (2) YP_009724390.1, (3) ANA96027.1, (4) XP_003965681.1, (5) XP_029694670.1, (6)

ENSRFET00010014064.1, (7) NP_066998.1 and (8) QHD43418.1. The domain illustration of the spike protein is based on Wrapp, Wang, *et al*.[11] (**B**) A solved NMR structure of human hepcidin[32] (PDB: 2KEF), adopting an antiparallel beta-sheet fold, is visualized with its putative four disulfide bonds formed between eight cysteine residues. (**C**) The position of the disulfide bonds in the sequence of the mature human hepcidin is illustrated along with the potential palmitoylation residues (ten cysteines) of the cytoplasmic tail of the SARS-CoV-2 spike protein. The palmitate visual is as per Linder and Deschenes.[70]

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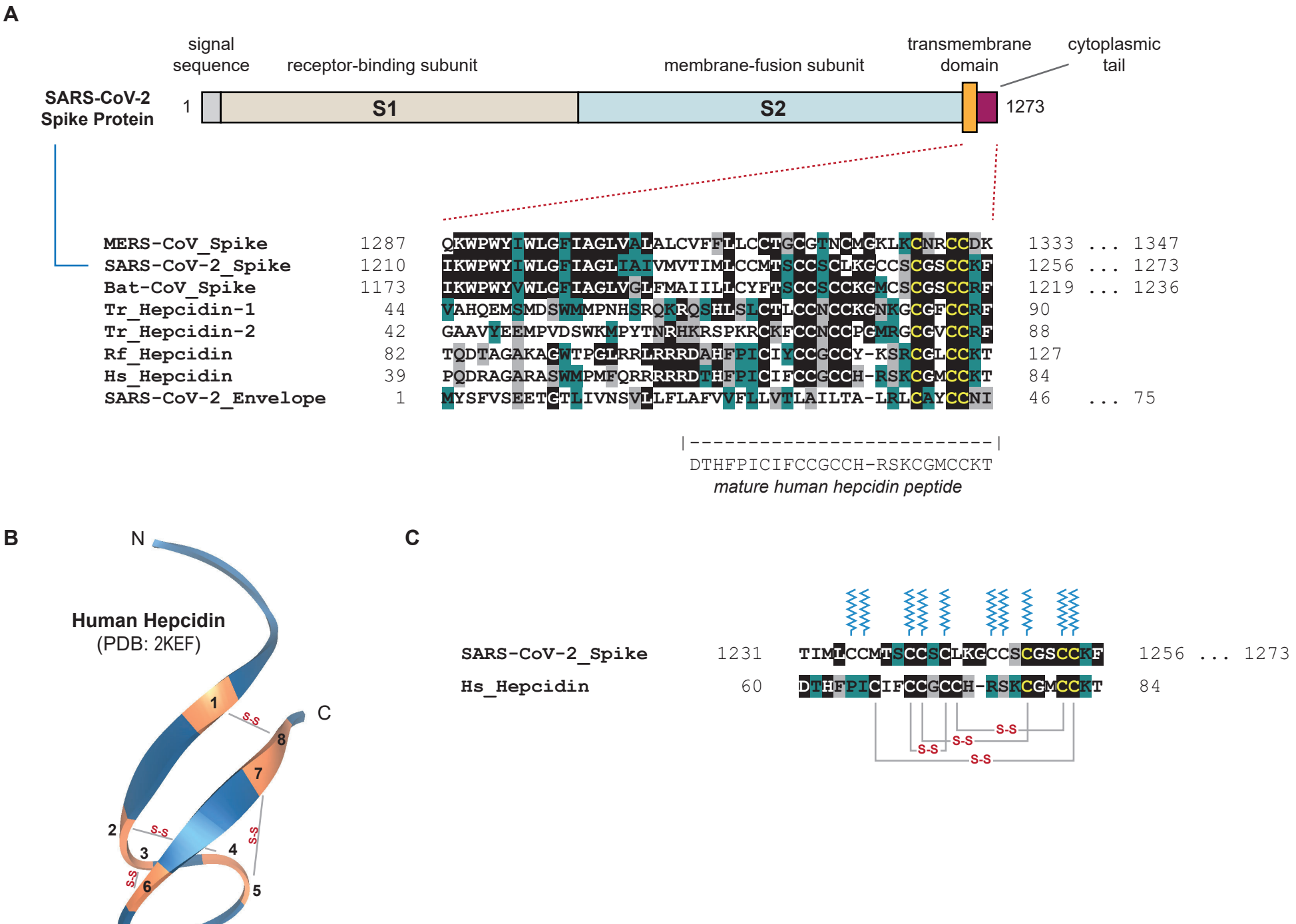
FIGURE 1


A
signal sequence
receptor-binding subunit
membrane-fusion subunit
transmembrane domain
cytoplasmic tail
SARS-CoV-2 Spike Protein
1
S1
S2
1273
MERS-CoV_Spike 1287 QKWPWYIWLGFIAGLVALALCVFFLLCCTGCGTNCMGKLKCNRCCDK 1333 ... 1347
SARS-CoV-2_Spike 1210 IKWPWYIWLGFIAGLIAIVMVTIMLCCMTSCCSCLKGCCSCGSCCKF 1256 ... 1273
Bat-CoV_Spike 1173 IKWPWYVWLGFIAGLVGLFMAIILLCYFTSCCSCCKGMCSCGSCCRF 1219 ... 1236
Tr_Hepcidin-1 44 VAHQEMSMDSWMMPNHSRQKRQSHLSLCTLCCNCCKGNKGCGFCCRF 90
Tr_Hepcidin-2 42 GAAVYEEMPVDSWKMPYTNRHKRSPKRCKFCCNCCPGMRGCGVCCRF 88
Rf_Hepcidin 82 TQDTAGAKAGWTPGLRRLRRRDAHFPICIYCCGCCY-KSRCGLCCKT 127
Hs_Hepcidin 39 PQDRAGARASWMPMFQRRRRRDTHFPICIFCCGCCH-RSKCGMCCKT 84
SARS-CoV-2_Envelope 1 MYSFVSEETGTLIVNSVLLFLAFVVFLLVTLAILTA-LRLCAYCCNI 46 ... 75
DTHFPICIFCCGCCH-RSKCGMCCKT
mature human hepcidin peptide
B
N
C
Human Hepcidin
(PDB: 2KEF)
1
2
3
4
5
6
7
8
S-S
C
SARS-CoV-2_Spike 1231 TIMLCCMTSCCSCLKGCCSCGSCCKF 1256 ... 1273
Hs_Hepcidin 60 DTHFPICIFCCGCCH-RSKCGMCCKT 84
S-S
S-S
S-S
S-S