# Unheeded SARS-CoV-2 protein? Look deep into negative-sense RNA

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**Abstract:**

SARS-CoV-2 is a novel ssRNA+ virus from the *Coronaviridae* family, which has caused the COVID-19 pandemic. The genome of SARS-CoV-2 is one of the largest of RNA viruses, comprising of 26 known protein-coding loci. This study aimed to explore the coding potential of negative-strand RNA intermediate for its potential to contain additional protein coding-loci. Surprisingly, we have found several putative ORFs and one brandt new functional SARS-CoV-2 protein-coding loci and called it *Avo1* (Ambient viral ORF1). This sequence is located on negative-sense RNA intermediate and *bona fide* coding for 81 amino acid residues long protein and contains a strong Kozak sequence for translation on eukaryotic ribosomes. *In silico* translated protein Avo1 has a predominantly alpha-helical structure. The existence of the *Avo1* gene is supported also by its evolutionarily and structural conservation in RaTG13 bat coronavirus. The nucleotide sequence of *Avo1* also contains a unique SREBP2 binding site which is closely related to the so-called "cytokine storm" in severe COVID-19 patients. Altogether, our results suggest the existence of still undescribed SARS-CoV-2 protein, which may play an important role in the viral lifecycle and COVID-19 pathogenesis.

## Introduction:

Coronavirus SARS-CoV-2, as a causative agent of COVID-19 disease, is intensively studied worldwide. It is generally considered that the SARS-CoV-2 genome is coding for 16 non-structural proteins (NSP1-16), 4 structural proteins (surface glycoprotein, membrane glycoprotein, envelope protein, nucleocapsid phosphoprotein), and 6 – 9 "accessory factors" (still designated as "open reading frames", ORFs) (Gordon et al. 2020). The length of the SARS-CoV-2 proteins varied from 1945 aa to 13 aa (Huang et al. 2020; Li et al. 2020, 6; Helmy et al. 2020). The shortest SARS-CoV-2 protein has only 13 aa (nsp11) and its function is unknown (Chen, Liu, a Guo 2020), followed by ORF10 (38 aa), ORF7b (43 aa), ORF6 (61 aa), and envelope protein (75 aa) (Wu et al. 2020). The longest SARS-CoV-2 protein is a multi-domain protein nsp3 (1945 aa) (Helmy et al. 2020). All to date described SARS-CoV-2 proteins are coded by positive-sense viral RNA strand. Overall, positive-sense RNA viruses are generally accepted to encode proteins solely on the positive strand, but in the current work by Dinan et al., they demonstrated that also negative-sense viral RNA strand has the potential to be protein-coding (Dinan et al. 2020). We have decided to inspect the negative-sense intermediate viral RNA strand of SARS-CoV-2 and look for new potential ORFs. As an indicator for *in vivo* validity, we have chosen a combination of complementary bioinformatics approaches, e.g. the presence of the so-called Kozak sequence, which is considered strong supportive evidence for being translated on host ribosomes (Gong et al. 2014), transcription factor binding site analysis, homology search, and 3D structure prediction. Our analyses propose a novel SARS-CoV-2 ORF, which has a high probability to be translated in host cells.

## Methods:

The SARS-CoV-2 reference genome (NC_045512.2) was searched by NCBI ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/), to predict all potential ORFs longer than 200 nucleotides (we have chosen an arbitrary threshold of 200 nt to make this analysis feasible and to minimize false-positive results). We have focused especially on the negative strand intermediate (i.e. frames -1, -2, and -3). Using ATG$^{pr}$ web-based tool (https://atgpr.dbcls.jp/) (Salamov, Nishikawa, a Swindells 1998), we have inspected, if they contain the so-called Kozak sequence, which is considered an important signal in the 5´ end of mRNAs to be translated on eukaryotic ribosomes (Noderer et al. 2014). To compute Mw and isoelectric point (pI), we used the Expasy Compute pI/Mw tool (https://web.expasy.org/compute_pi/) (Gasteiger et al. 2005). For the transcription factor binding site analysis, the RegRNA 2.0. webserver was used (Chang et al. 2013). To find possible domain homologs we used NCBI´s Conserved Domains Database (CDD) webserver (Marchler-Bauer et al. 2015) with an E-value cut-off set to 10. For *ab initio* structural modeling, the QUARK tool was used (Xu a Zhang 2012) and the resulting structures were visualized within UCSC Chimera 1.15 workflow (Pettersen et al. 2004).

Supplementary materials contain all nucleotide and predicted protein sequences (SM1). Predicted PDB structures are enclosed in SM2. CDD domain hits are included in SM3. Prediction of Kozak sequences is enclosed in SM4. Predicted negative-sense RNA ORFs longer than 200 aa (searched in the whole group of coronaviruses https://www.ncbi.nlm.nih.gov/genome/?term=coronavirus) are enclosed in SM5 together with estimated pI values (Gasteiger et al. 2005) and N-glycosylation prediction (http://www.cbs.dtu.dk/services/NetNGlyc/) (Blom et al. 2004).

## Results and discussion:

We have predicted all putative SARS-CoV-2 ORFs longer than 200 nucleotides, resulting in a set of 8 protein candidates (all ORFs are enclosed in SM1, the longest was 303 nt long, and the shortest 201 nt in size). Then we have inspected if they contain the so-called Kozak sequence. We have found one highly promising candidate locus and called it *Avo1* (Ambient viral ORF1). *Avo1* nucleotide sequence (Figure 1 Top) contains a very well-conserved Kozak signal in the form of RYMRMVAUGGC (Noderer et al. 2014), which is a nucleic acid motif that functions as the protein translation initiation site in most mammalian

mRNA transcripts (Noderer et al. 2014). The strongest Kozak signals found in the SARS-CoV-2 genome are depicted in Figure 1 Bottom (five of six Kozak signals are located in RNA+ strand, one is located on the RNA- strand).

*Avo1* locus is situated on nucleotide positions 422 – 667 of the reference SARS-CoV-2 genome (NC_045512.2) and putative protein is translated in reading frame -2 (i.e. only from negative-sense SARS-CoV-2 RNA intermediate). Protein product would be 81 amino acid residues long and its molecular weight was computed to be 9586.06 Da with an isoelectric point (pI) 9.22. Interestingly, the proposed *Avo1* gene contains 12 nt long SREBP2 binding site (Figure 1 Top). Recently it was found that "COVID-19-activated SREBP2 disturbs cholesterol biosynthesis and leads to cytokine storm" (Lee et al. 2020).

*>Avo1*

Kozak sequence TSS SREBP2 binding sequence

GTAACTATGGCCACCAGCTCCTTTATTACCGTTCTTACGAAGAAGAACCTTGCGGTAAG

CCACTGGTATTTCGCCCACATGAGGGACAAGGACACCAAGTGTCTCACCACTACGACC

GTACTGAATGCCTTCGAGTTCTGCTACCAGCTCAACCATAACATGACCATGAGGTGCAG

TTCGAGCATCCGAACGTTTGATGAACACATAGGGCTGTTCAAGTTGAGGCAAAACGCCT

TTTTCAACTTCTACTAA

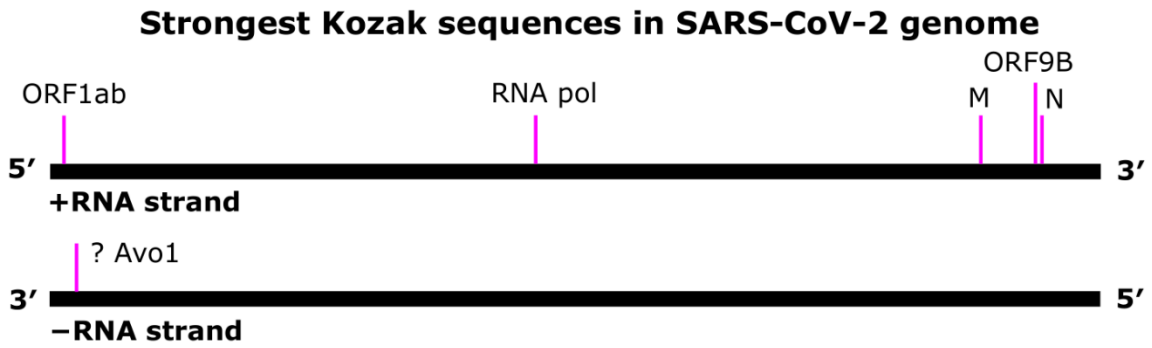


**Figure 1.** (Top) Genomic sequence of putative *Avo1* gene. Kozak sequence is depicted in blue color, start codon ATG in green, stop codon TAA in red. SREBP2 binding sequence is highlighted by pink. TSS stands rather for "translation start site", because we are dealing already with viral RNA. (Bottom) Six highly significant Kozak sequences in the SARS-CoV-2 genome, the *Avo1* signal is the second strongest one (see SM4).

Homology searches revealed that *Avo1* ORFs are evolutionarily conserved in several coronaviruses, e.g. in bat coronaviruses RaTG13, RpShaanxi2011, BtRs-BetaCoV/HuB2013, Rc-o319, or in pangolin coronavirus GX/P2V (Figure 2 Top). On the other side, human SARS-CoV and MERS-CoV *Avo1* loci are truncated by stop codons - dynamic loss or gain of specific accessory proteins may be an explanation for different viral properties (virulence, pathogenesis, etc.). Three weak domain homology signals were found in the CDD database: B-12 dependent methionine synthase, Herpes envelope superfamily, and adenosine deaminase.

Another evidence for possible Avo1 protein biological significance is that instead of some disordered clumps, an independent *ab initio* prediction of tertiary structures of SARS-CoV-2 Avo1 and RatG13 Avo1 has shown five alpha-helices forming conserved structures (Figure 2 Bottom).

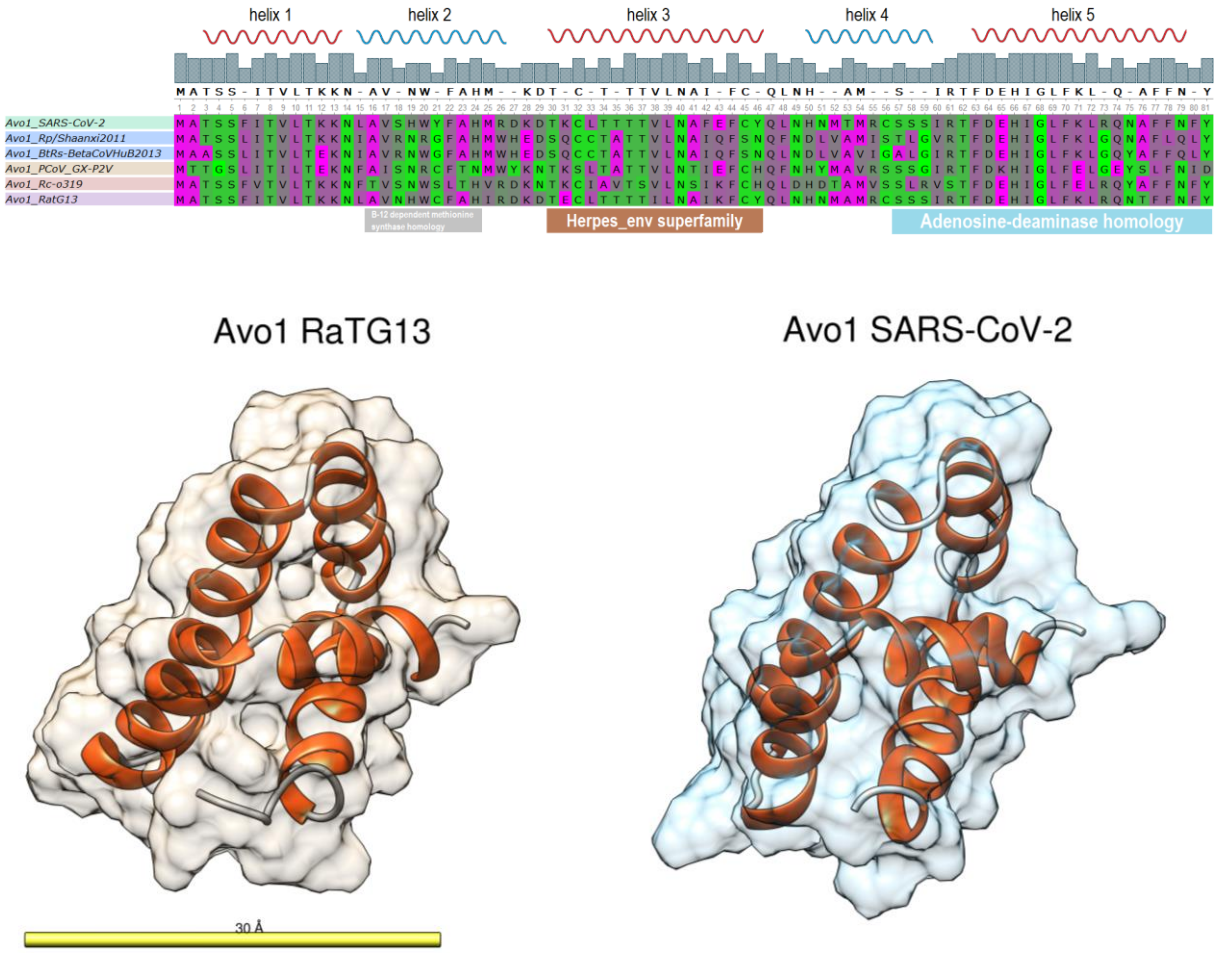


**Figure 2.** (Top) Alignment of Avo1 putative proteins from homologous coronaviruses. Coloring express helix propensity, magenta – high, green – low. Consensus sequence inferred from the alignment bar plotted. Domain hits for the SARS-CoV-2 sequence are inside the rectangles. (Bottom) *De novo* modeled Avo1 structure using QUARK workflow (Xu a Zhang 2012), yellow scale bar is 30 Å long.

Our subsequent searches reveal a couple of interesting ORFs located on negative sense RNA in other Coronaviruses, so it seems that the negative-sense coronaviral RNA can serve as an additional pool for their coding potential. For example, Miniopterus bat coronavirus HKU8 (NC_010438.1) has a 438 aa long ORF and Alphacoronavirus Bat-CoV/P.kuhlii/Italy/3398-19/2015 (NC_009988.1) has a 313 aa long ORF on its negative sense RNA. All the longest 13 hits (over 200 aa) are listed in SM5. Analyzes of their predicted pI revealed, that all of them have pI higher than 8.4 (with the average value of 10.07) - it is very surprising because there was no significant sequence homology between them. Nine of these long ORFs also contains potential nuclear localization signal(s) (see SM5). Following prediction of N-glycosylation suggests that the vast majority (12/13) of these ORFs are posttranslationally modified by N-glycosylation. N-glycosylation is a common phenomenon, how viral proteins can be recognized by an innate immune response, which is the first line of defense against infection (York, Stevens, a Alymova 2019). Our findings are summarized and depicted in the figure below (Figure 3).

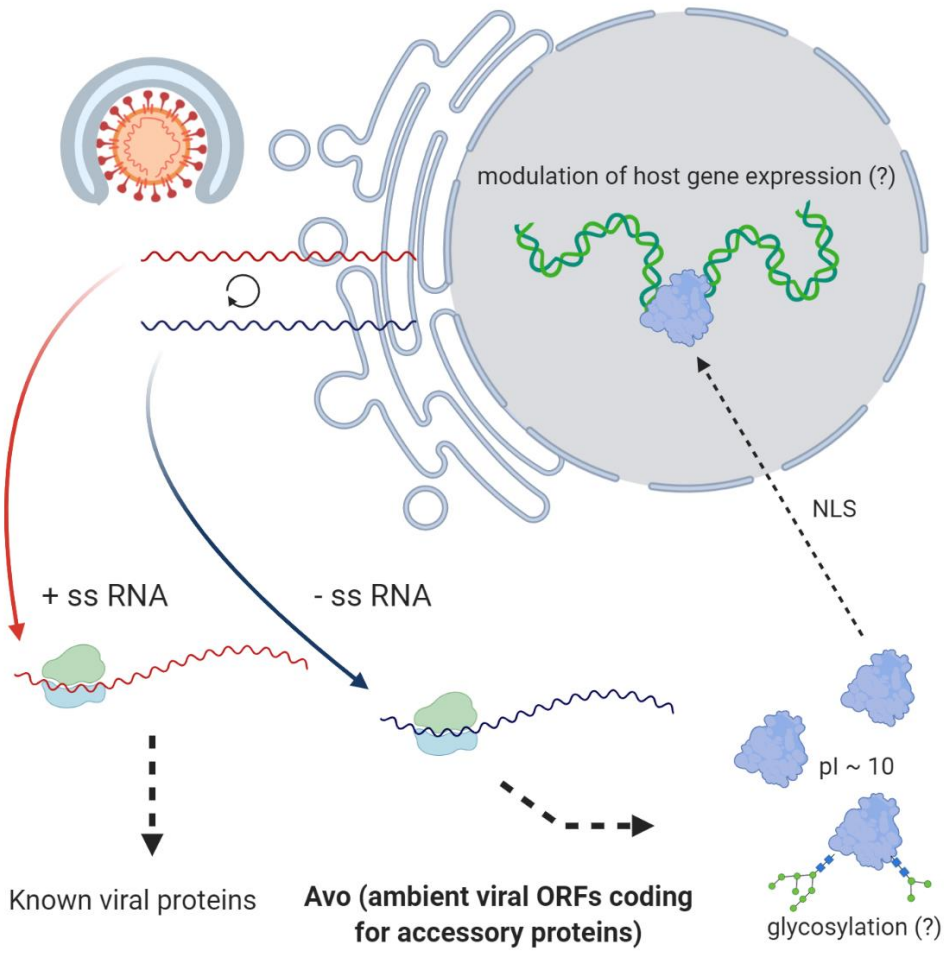


**Figure 3. Proposed hypothesis about accessory viral proteins coding on negative sense RNA strand.** The replication of coronaviral RNA takes place inside special compartments connected to the endoplasmic reticulum. We propose the hypothesis, that also negative sense RNA has a coding potential and most of the resulting proteins are likely to be glycosylated. In addition, some of them are possibly translocated into the nucleus, where can modulate host gene expression via nucleic acid binding.

It was found that the SARS-CoV-2 transcriptome is very diverse and rich (Kim et al. 2020) and therefore, it is likely that also SARS-CoV-2 proteome will follow this trend and still unheeded viral proteins or protein variants wait for its discovery. Every newly described SARS-CoV-2 protein (and its host interacting proteins) could be a potential target for a drug repurposing or development of novel therapies (Gordon et al. 2020), for instance it was found, that SARS-CoV-2 accessory proteins ORF6 and ORF8 inhibit type I interferon signaling pathway, and thus play a critical role in innate immune suppression during viral infection (Li et al. 2020, 6). Altogether, our results suggest the existence of a still undescribed SARS-CoV-2 protein, which may play an important role in the viral lifecycle and COVID-19 pathogenesis.

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**Author contribution:**

M.B. carried out most of the analyses and wrote the manuscript, A.V. has conceived the presented idea and help with data curation, V.B. and J.C. have contributed to writing and revisions, P.P. supervised the work.

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