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SARS-CoV-2 3CLpro whole human proteome cleavage prediction and enrichment/depletion analysis

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**ABSTRACT**

A novel coronavirus (SARS-CoV-2) has devastated the globe as a pandemic that has killed millions of people. Widespread vaccination is still uncertain, so many scientific efforts have been directed toward discovering antiviral treatments. Many drugs are being investigated to inhibit the coronavirus main protease, 3CLpro, from cleaving its viral polyprotein, but few publications have addressed this protease’s interactions with the host proteome or their probable contribution to virulence. Too few host protein cleavages have been experimentally verified to fully understand 3CLpro’s global effects on relevant cellular pathways and tissues. Here, I set out to determine this protease’s targets and corresponding potential drug targets. Using a neural network trained on cleavages from 392 coronavirus proteomes with a Matthews correlation coefficient of 0.985, I predict that a large proportion of the human proteome is vulnerable to 3CLpro, with 4898 out of approximately 20,000 human proteins containing at least one putative cleavage site. These cleavages are nonrandomly distributed and are enriched in the epithelium along the respiratory tract, brain, testis, plasma, and immune tissues and depleted in olfactory and gustatory receptors despite the prevalence of anosmia and ageusia in COVID-19 patients. Affected cellular pathways include cytoskeleton/motor/cell adhesion proteins, nuclear condensation and other epigenetics, host transcription and RNAi, ribosomal stoichiometry and nascent-chain detection and degradation, ubiquitination, pattern recognition receptors, coagulation, lipoproteins, redox, and apoptosis. This whole proteome cleavage prediction demonstrates the importance of 3CLpro in expected and nontrivial pathways affecting virulence, lead me to propose more than a dozen potential therapeutic targets against coronaviruses, and should therefore be applied to all viral proteases and subsequently experimentally verified.

**1. Introduction**

Coronaviruses are enveloped, positive-sense, single-stranded RNA viruses with giant genomes (26–32 kb) that cause diseases in many mammals and birds. Since 2002, three human coronavirus outbreaks have occurred: severe acute respiratory syndrome (SARS) in 2002–2004, Middle East respiratory syndrome (MERS) from 2012 to present, and coronavirus disease 2019 (COVID-19) from 2019 to present. The virus that causes the latter disease, SARS-CoV-2, was first thought to directly infect the lower respiratory epithelium and cause pneumonia in susceptible individuals. The most common symptoms include fever, fatigue, nonproductive or productive cough, myalgia, anosmia, ageusia, and shortness of breath. More recently, however, correlations between atypical symptoms (chills, arthralgia, diarrhea, conjunctivitis, headache, dizziness, nausea, severe confusion, stroke, and seizure) and severity of subsequent respiratory symptoms and mortality have motivated researchers to investigate additional tissues that may be infected. One way to explain these symptoms and associated cellular pathways is to review enrichment and depletion in virus-host interaction networks, particularly those including the coronavirus proteases.

Angiotensin-converting enzyme 2 (ACE2), the main receptor for SARS-CoV-1 and – 2, has been shown to be less expressed in lung than in many other tissues. Respiratory coronaviruses likely first infect the nasal epithelium and tongue (Lechien et al., 2020) and then work their way down to the lung and/or up through the cribriform plate to the olfactory bulb, through the rhinencephalon, and finally to the brainstem (Baig et al., 2020; Lau et al., 2004; Netland et al., 2008; Li et al., 2020). Additionally, based on ACE2 expression and in vitro and in vivo models, multiple parts of the gastrointestinal tract (mainly small and large intestine, duodenum, rectum, and esophagus; less appendix and stomach) and accessory organs (mainly gallbladder, pancreas, liver, (Zhang et al., 2020; Chau et al., 2004) salivary gland (Liu et al., 2011); less tongue and spleen), (Zhan et al., 2006) kidney, (Naicker et al., 2020) male and female reproductive tissues, (Fan et al., 2021; Chen et al., 2020) heart,
Coronaviruses have two main open reading frames, orf1a and orf1b, separated by a ribosomal frameshift and resulting in two large polyproteins, pp1a and pp1ab, containing proteins including two cysteine proteases, (Ziebuhr et al., 2000) an RNA-dependent RNA polymerase, and other nonstructural proteins (nsp1–16). The main function of these proteases is to cleave the polyproteins into their individual proteins to form the transcription/replication complex, making them excellent targets for antiviral drug development. (Baez-Santos et al., 2015; Pil-layyar et al., 2016; Yang et al., 2005; Anand et al., 2003) The papain-like protease (PLpro) and 3 chymotrypsin-like protease (3CLpro) only have 3 and 11 cleavage sites, respectively, in the polyproteins, but it is reasonable to assume that both proteases may cleave host cell proteins to modulate the innate immune response and enhance virulence as in picornaviruses and retroviruses, such as human immunodeficiency virus (HIV).

PLpro is a highly conserved protein domain that has been shown to determine virulence of coronaviruses (Niemeier et al., 2018) and possess deubiquinating and deSGylating activity including cleaving ISG15 induced by interferon via the JAK-STAT pathway from ubiquitin-conjugating enzymes and potentially from downstream effects (Barretto et al., 2005; Yang et al., 2014; Bailey-Eskin et al., 2014; Li et al., 2011; Xing et al., 2013) PLpro deubiquination also prevents activating phosphorylation of IRF3 and subsequent type I interferon production, (Matthews et al., 2014; Devaraj et al., 2007) however the ubiquitinated leucine in human IRF3 is replaced by a serine in bats likely including Rhinolophus affinis (intermediate horseshoe bat), the probable species of origin of SARS-CoV-2 (Banerjee et al., 2020; Zhou et al., 2020).

3CLpro is also highly conserved among coronaviruses; SARS-CoV-2 3CLpro is 96.08% and 50.65% identical, respectively, to the SARS- and MERS-CoV homologs, the former with only 12 out of 306 amino acids substituted with all 12 outside the catalytic dyad or surrounding pockets. (Needle et al., 2015; Xue et al., 2008; Anand et al., 2002) Even the most distant porcine deltacoronavirus HKU15 3CLpro shares only 34.97% identity yet is similarly conserved in the these important residues. This conservation indicates that all these proteases are capable of cleaving similar sequences no matter the protease genus of origin. In addition to the 11 sites in the polyproteins, these proteases are known to cleave host proteins including STAT2, (Zhu et al., 2017) NEMO, (Wang et al., 2016) the innate immune modulators (Barretto et al., 2005; Yang et al., 2005; Anand et al., 2003) The papain-like protease (PLpro) and 3 chymotrypsin-like protease (3CLpro) only have 3 and 11 cleavage sites, respectively, in the polyproteins, but it is reasonable to assume that both proteases may cleave host cell proteins to modulate the innate immune response and enhance virulence as in picornaviruses and retroviruses, such as human immunodeficiency virus (HIV).

2. Methodology

2.1. Dataset preparation

A complete, manually reviewed human proteome containing 20,350 sequences (not including alternative isoforms) was retrieved from UniProt/Swiss-Prot (proteome:up000005640 AND reviewed:yes) (UniProt, 2019).

Coronavirus polyprotein sequences were collected from GenBank. (Benson et al., 2017) Searching for “orf1a,” “pp1a,” and “1ab” within the family Coronaviridae returned 391 different, complete polyproteins, and an additional polyprotein sequence from the monotypic Microhylla letovirius I was derived from accession number GECV01031551. (Bukhari et al., 2018) These polyproteins each contained 11 cleavages manually discovered using the Gustal Omega multiple sequence alignment server, (Sievers et al., 2011; Goughon et al., 2016; McWilliam et al., 2013) totaling 4312 balanced cleavages (Fig. 1). P1 glutamines and histidines were unambiguously conserved when aligned to known cleavages in SARS, SARS-CoV-2, MERS, IBV, etc., and all remaining glutamines and histidines were considered to be uncleaved. Although some of the ten amino acid sequences surrounding the cleavages were identical (805 different sites total), all 4312 balanced positive cleavages were used for subsequent classifier training in addition to all other different, uncleaved sequences with P1 glutamates (18,477) and histidines (12,128), totaling 34,917 samples.
3. Dataset characterization

Here I assumed that SARS-CoV-2 3CLpro is capable of cleaving all aligned cleavages between all genera of coronaviruses (Alpha-, Beta-, Gamma-, and Deltacoronavirus and the monotypic Alphaletovirus) because variation in cleavage sequences is greater within polyproteins than between them (Figs. 2 and 3) no matter the existence of protease/cleavage cophylogeny (Fig. 4). Wu et al. (2015) demonstrate that the same 11 clusters appear when a lower-dimensionality physiochemical encoding (with dimensionality 40 containing normalized volumes, interface and octanol hydrophobicity scales, and isoelectric points) is used, however this dataset is large enough that one-hot encoding (200 dimensional binary input) outperforms it.

4. Model and hyperparameter optimization

The NetCorona 1.0 server as in Kiemer et al.’s work, (Kiemer et al., 2004) my reproductions of their sequence logo-derived rules and NN, and my improved sequence logo-based logistic regression and naïve Bayes classification and NNs were optimized and compared to decide which model to use for prediction of human cleavage sites. (Pedregosa et al., 2011) Kiemer et al.’s seven genome sequence logo and multilayer perceptron used one-hot encoding for the 10 amino acid window surrounding each cleavage (linearizing 10 amino acids resulted in an input of 200 bits). (Kiemer et al., 2004) First, logistic regression was performed on the logit of the probability output of the sequence logo (as opposed to Chou et al.’s manual probability cutoff setting by maximizing an unbalanced measure of accuracy(Chou et al., 1993)) with a nonzero but optimally extremely small pseudocount and returned an MCC of 0.825 with 74.0% recall. Updating the sequence logo with all known cleavages (Fig. 1) improved its MCC to 0.931 with 94.1% recall. A naïve Bayes classifier was additionally constructed from both the positive and negative sequence logos and slightly improved the MCC to 0.935 with 94.0% recall. Fig. 5 demonstrates correlations (represented as the mutual information variant known as total entropy correlation coefficients or symmetric uncertainties) between positions that are not captured by simple sequence logos and classifiers assuming independence. (Bindewald et al., 2006; Maes et al., 2003) NNs, however, allow inclusion of 2D and higher-order correlations not easily visualizable and therefore often improve accuracy. Finally, in addition to information content, Fig. 6 shows a charge-polarity-hydrophobicity scale with no obvious trend, reaffirming why one-hot encoding performs can achieve a higher MCC than any physiochemical, lower-dimensional encoding for NNs when the training set is large enough.

As for my improvements to the NN, note that Kiemer et al.’s MCC of 0.840 is an average from triple cross-validation (CV). (Kiemer et al., 2004) Because the known cleavage dataset is small, no data went unused; the three NN output scores were averaged and similarly considered cleavages when greater than 0.5. Retraining the same NN structures (each with one hidden layer with 2 neurons) on the larger dataset...
resulted in three-average CV MCC of 0.968, a significant improvement even though the datasets are less balanced. This MCC was maintained after adding all other histidines (which precede 20/805 different cleavages) as negatives. Interestingly, two infectious bronchitis viruses (Igacovirus, Gammacoronavirus) and one wigeon coronavirus HKU20 (Andecovirus, Deltacoronavirus) contained cleavages following leucine, methionine, and arginine (VSKLL^AGFKK in APY26744.1, LVDY^-M^AGFKK and DAALR^-NNELM in ADV71773.1, and AIRCR^-NNELM in YP_005352870.1). To my knowledge, synthetic tetra/octapeptides have been cleaved following histidine, phenylalanine, tryptophan, methionine, and possibly proline residues, (Chuck et al., 2011; Goetz et al., 2007) but only one natural histidine substitution has been documented in HCoV-HKU1 (Woo et al., 2005) and likely does not affect function (Ma et al., 2015; Neuman et al., 2014; Fang et al., 2008).

To optimize hyperparameters, the whole dataset was repeatedly split into 80% training/20% testing sets with further splitting of the 80% training set for cross-validation. The optimal settings, no oversampling (within training folds (Santos et al., 2018)), limited-memory Broyden–Fletcher–Goldfarb–Shanno (lbfgs) solver, rectifier (ReLU) activation, 0.00001 regularization, and 1 hidden layer with 10 neurons, had an average 20% test set MCC of 0.976 when split and trained many times. Train/test sets repeatedly split with different ratios in Fig. 7 demonstrate that the entire dataset is not required for adequate performance for all three classification methods, although my final method used all the data to maximize accuracy. Note that any errors in these predictions are amplified when applied to the whole human proteome as below but that enrichment/depletion statistics proved robust against this variability. Similarly careful optimization and bias and variance characterization should again be performed if this type of analysis is to be repeated on other protease datasets. Also note that Fig. 7 displays a curve for a physiochemical encoding (also used in Figs. 1c and 1d) underperforming even at relatively small training sizes. Of the four physiochemical scales used, octanol hydrophobicity alone reached an MCC of 0.959, and, in the order of importance, addition of volume, interface hydrophobicity, and isoelectric point features increased the maximum MCC to 0.977.

Given that protease cleavage datasets are relatively small and training individual models is computationally inexpensive, combining...
multiple models into ensembles is recommended to reduce variability and at least slightly improve accuracy. The cross-validation described above is an ensemble that improves accuracy by introducing diversity in resampling like bootstrap aggregating. In addition to resampling methods, averaging ensembles of networks trained on the same dataset but initialized differently were able to improve accuracies as recently discovered in benchmark datasets. (Fort et al., 2020) Without an obvious upper bound on ensemble complexity, the final model used for subsequent analyses was an average from 10 sets of 100-fold cross-validated networks. The extremely few sequences incorrectly labeled with retraining and were not overrepresented in any lineage; in essence there was no distinction between easy-to-learn and hard-to-learn samples. The average 20% test MCC of this size ensemble was 0.985, although the final ensemble used the entire dataset.

5. Model robustness

Even with the extremely high accuracies of models trained on this large dataset, randomly train/test splitting does not account for any taxonomic biases. One can easily imagine that extending this training dataset to the entire order Nidovirales or even the class Pisoniviricetes may not improve SARS-CoV-2 protease prediction without some (co)phylogenetic weighting or complex resampling algorithms and experimental verification. A novel leave-one-(sub)genus-out resampling analysis (using the final NN architecture and one-hot encoding) summarized in Table 1 affirms that more divergent lineages are more difficult to accurately predict, but that leaving out whole Sarbecovirus and Betacoronavirus resulted in the MCCs 0.865 and 0.835, still rivaling accuracies in previous publications. Alternatively, initially training on only Sarbecovirus sequences and progressively expanding the training set phylogenetically to Milecovirus only reduced Sarbecovirus-specific MCCs from 0.996 to 0.989 while increasing all other subgenus-specific MCCs to similar values. This again affirms that the entire dataset should be used and that diversity between the 11 cleavages is more important than between lineage.

6. Cleavage prediction

Some predicted cleavage sites were close enough to the N- and C-termini that the ten amino acid window input into the neural network was not filled. These sites with P1 glutamine residue less than four amino acids from the N-terminus or less than five amino acids from the C-terminus were omitted because although they may be within important localization sequences, their cleavage kinetics are likely significantly retarded by truncation.

Of the 20,350 manually reviewed human proteins, 4898 were predicted to be cleaved at least once with a final average NN score greater than or equal to 0.5. To prove that the cleavages were nonrandomly distributed among human proteins, random sequences with weighted amino acid frequencies were checked for cleavages. Cleavages occurred at 1.28% of glutamines (4.77% of amino acids) (Kozlowski, 2017) or every 1640 amino acids in these random sequences. Most proteins are shorter than this and would, if randomly distributed, follow a Poisson distribution; this data’s deviation from this distribution indicates that
many cleavages are intentional.

7. Enrichment analysis

Protein annotation, classification, and enrichment analysis was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) 6.8 (Huang et al., 2009a, 2009b) Tissue (UP_TISSUE and UNIGENE_EST_QUARTILE), InterPro, direct Gene Ontology (GO includes cellular compartment (CC), biological process (BP), and molecular function (MF)), Reactome pathways, sequence features, and keywords annotations were all explored, and only annotations with Benjamini-Hochberg-corrected p-values less than 0.05 were considered statistically significant. Both enriched and depleted (no cleavages) annotations are listed in Tables S2-S10, and my training data, prediction methods, and results can be found on GitHub (https://github.com/Luke8472NN/NetProtease).

8. Discussion

Enrichment and depletion analyses are often used to probe the importance of annotations in many disease states, yet quantification is not possible without experimentation. Table 2 summarizes cleavages within and hypotheses about noteworthy pathways, however many cleavages exist. First, if a protein is central to a pathway, a single cleavage may be all that is required to generate equivalent downstream outcomes. Cleaved proproteins such as coagulation factors or complement proteins may even be activated by 3CLpro cleavage. Additional exhaustive analysis or inclusion of some measure of centrality is required to determine if any insignificantly enriched or depleted pathways are still affected at central nodes (as in false negatives). Second, protease-, substrate sequence-, substrate truncation-, pH-, temperature-, and time after infection-dependent cleavage kinetics convert this classification problem into a regression problem. Cleavage rates among the 11 cleavages per pp1ab vary by at least 50-fold and are uncorrelated with the scores from the classifier described here, so these predictions assume that 3CLpro exists in high enough concentrations and for a long enough time that rate constants do not matter because cleavage reactions are complete. Third, longer proteins are more likely to be randomly cleaved and may confound conclusions about annotations containing them. Cleavages in longer proteins (e.g. cytoskeletal or cell-cell adhesion components) are no less important than those in shorter sequences, and annotations containing proteins with multiple cleavages deviating from Poisson distributions are more likely due to highly conserved sequences than simply protein length. Lastly, convergent evolution within the host may also result in false positives and may be partially avoided by investigating correlations between domains, motifs, repeats, compositionally biased regions, or other sequence or structural similarities and other functional and

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**Table 1**

| Genus        | Subgenus | Positives | Negatives | % Positive | MCC  |
|--------------|----------|-----------|-----------|------------|------|
| Alphaletovirus| All      | 11        | 236       | 4.5%       | 0.628|
| AlphaCoV     | All      | 1241      | 9207      | 11.9%      | 0.881|
|              | CobaCoV  | 139       | 337       | 29.2%      | 0.986|
|              | DecaCoV  | 287       | 1108      | 20.6%      | 0.990|
|              | DuvinCoV | 246       | 905       | 21.4%      | 0.966|
|              | LuchCoV  | 68        | 374       | 15.4%      | 0.985|
|              | MinaCoV  | 239       | 661       | 26.6%      | 0.973|
|              | MinunaCoV| 243       | 772       | 23.9%      | 0.985|
|              | MyotaCoV | 179       | 453       | 28.3%      | 0.986|
|              | NycyaCoV | 267       | 871       | 23.5%      | 0.990|
|              | PedcCoV  | 360       | 1147      | 23.9%      | 0.977|
|              | RhinaCoV | 216       | 487       | 30.7%      | 0.986|
|              | SsetaCoV | 217       | 852       | 20.3%      | 0.964|
|              | SunaCoV  | 114       | 583       | 16.4%      | 0.837|
|              | TegaCoV  | 365       | 1716      | 17.5%      | 0.891|
| BetaCoV      | All      | 1200      | 10,433    | 10.3%      | 0.835|
|              | EmbcCoV  | 422       | 2741      | 13.3%      | 0.925|
|              | HibaCoV  | 42        | 378       | 10.0%      | 0.973|
|              | MerbcCoV | 330       | 3346      | 9.0%       | 0.829|
|              | NobeCoV  | 142       | 1488      | 8.7%       | 0.938|
|              | SarbeCoV | 381       | 2600      | 12.8%      | 0.865|
| GammaCoV     | All      | 1761      | 5724      | 23.5%      | 0.884|
|              | BrangaCoV| 163       | 321       | 33.7%      | 0.985|
|              | CegaCoV  | 45        | 337       | 11.8%      | 0.943|
|              | IgaCoV   | 1706      | 5157      | 24.9%      | 0.892|
| DeltaCoV     | All      | 111       | 2078      | 5.1%       | 0.799|
|              | AndeCoV  | 11        | 352       | 3.0%       | 0.664|
|              | BuldeCoV | 11        | 1403      | 6.0%       | 0.859|
|              | HerdeCoV | 11        | 369       | 2.9%       | 0.683|
neurological annotations. Ideally, a negative control proteome from an uninfected species could prevent false positives, but coronaviruses are extremely zoonotic. Here, depletions in the human proteome are taken as expected in this data, the most significant tissue enrichment of complex structural and cell junction proteins. It is noteworthy that major proteins associated with multiple neurodegenerative diseases (Alzheimer’s disease, Parkinson’s disease, amyotrophic lateral sclerosis, and spinocerebellar ataxia type 1) are also predicted to be cleaved. Testis has somewhat similar expression to epithelium and central and peripheral nervous tissues are also affected due to their similar expression and cleavage sites. Proteins with greater tissue specificity (3rd quartile) show enrichment is likely due to genes with immune function and mutagen detection and degradation (ZNF598, NEMF, and LIT1) in tissues including the brain, highly expresses ACE2, and is enriched in movement/motility-related (subset of structural proteins) and meiosis-related (chromosome segregation) proteins, further increasing the likelihood that this tissue is infectible. Spleen, however, does not express much ACE2, and its additional enrichments along the respiratory tract (tongue, pharynx, larynx, and trachea), in immune tissues (lymph node and thymus), and nasal cavity and respiratory epithelium are consistent with increased expression of ACE2 and increased risk of SARS-CoV-2 infection. Pulmonary and nasal epithelium is likely responsible for the majority of new cases of COVID-19.

9. Tissues

As expected in this data, the most significant tissue enrichment of 3CLpro cleavages are in the epithelium, but central and peripheral nervous tissues are also affected due to their similar expression and enrichment of complex structural and cell junction proteins. It is noteworthy that major proteins associated with multiple neurodegenerative diseases (Alzheimer’s disease, Parkinson’s disease, amyotrophic lateral sclerosis, and spinocerebellar ataxia type 1) are also predicted to be cleaved. Testis has somewhat similar expression to epithelium and central and peripheral nervous tissues are also affected due to their similar expression and cleavage sites. Proteins with greater tissue specificity (3rd quartile) show enrichment is likely due to genes with immune function and mutagen detection and degradation (ZNF598, NEMF, and LIT1) in tissues including the brain, highly expresses ACE2, and is enriched in movement/motility-related (subset of structural proteins) and meiosis-related (chromosome segregation) proteins, further increasing the likelihood that this tissue is infectible. Spleen, however, does not express much ACE2, and its additional enrichments along the respiratory tract (tongue, pharynx, larynx, and trachea), in immune tissues (lymph node and thymus), and
in other sensory tissues (eye and ear). Combining tissues, tobacco use disorder is the only significantly enriched disease, but acquired immunodeficiency syndrome (AIDS) and atherosclerosis were surprisingly depleted.

Cleavages are also surprisingly depleted in olfactory and gustatory pathways given the virus’ ability to infect related cells and present as anosmia and ageusia. Olfactory receptors are transmembrane rhodopsin-like G protein-coupled receptors that, when bound to an odorant, stimulate production of cAMP via the G protein and adenylate cyclase. The G proteins GNAL and GNAS are not cleaved, and some but not all adenylate cyclases are cleaved, likely resulting in an increase in cAMP. Camp is mainly used in these cells to open their respective ligand-gated ion channels and cause depolarization, but it is also known to inhibit inflammatory responses through PKA and EPAC. Multiple PDEs that degrade cAMP but not PDE4, the major PDE in inflammatory and immune cells, are cleaved. PDE4 inhibitors have been shown to reduce destructive respiratory syncytial virus—induces inflammation in lung, (Ikeura et al., 2000) but olfactory receptor neurons are quickly regenerated and sacrificed themselves when infected by influenza A virus. (Mori et al., 2002) The depletion in cleavages and resulting increase in cAMP in these neurons is likely to inhibit their programmed cell death long enough for the virus to be transmitted through the glomeruli to mitral cells and the rest of the olfactory bulb. Tongue infection may have similar mechanisms, and herpes simplex virus has been shown to be transmitted to the brainstem through the facial and trigeminal nerves (Thomander et al., 1988).

10. Gene ontology

Cleaved proteins are depleted in the extracellular space (except for structural collagen, laminin, and fibronectin mainly associated) and enriched in the cytoplasm and many of its components, indicating that the selective pressure for cleavage is weaker once cells are lysed and the protease is released. In the cytoplasm, the most obviously enriched sets are in the cytoskeleton, motor proteins, cell adhesion molecules, and relevant Ras GTPases, particularly in microtubule organizing centers (MTOCs) including centrosomes, an organelle central to pathways in the cell cycle including sister chromatid segregation. More specifically, cleavage of cilia-associated proteins may contribute to dyskinesia and reduced mucociliary escalator effectiveness associated with many respiratory viruses including HCoV-229E and SARS and their resulting bacterial pneumonias (Chilvers et al., 2001; Kuek and Lee, 2020). Additionally, ciliary dysfunction in olfactory cells in COVID-19 leads to anosmia, although the main reported mechanism is nsp13 (helicase/-triphosphatase)-centrosome interaction (Li and Li, 2020). Coiled coils account for many of these cleavages and are primarily expressed in corresponding cellular compartments in the epithelium, testis, and brain. Only the coronavirus nsp1, nsp13, and spike proteins have so far been shown to interact with the cytoskeleton, (Gordon et al., 2020; Lv et al., 2019; Rödiger et al., 2016) although many other viruses including influenza A virus, (Ohman et al., 2009) herpes simplex virus, rabies virus, vesicular stomatitis virus, and adeno-associated virus (Dohner and Sodeik, 2005) also modulate the cytoskeleton (Naghavi and Walsh, 2017). In neurons, this allows for axonal and trans-synaptic transport of viruses which can often be inhibited but sometimes exaggerated by cytoskeletal drugs often used in oncology (Kristensson et al., 1986; Solov’eva et al., 1988; Yi et al., 2017; Campbell et al., 2004).

Modulation of these structural and motor proteins is required for formation of the double-membrane vesicles surrounding replication complexes (Wolff et al., 2020; Neuman et al., 2014) and for egress. Similarly required for vesicular transport, the cargo COP1, clathrin, and caveolae pathways are untouched by 3CLpro, but COP1 components are likely cleaved due to their function in selecting cargo (Miller et al., 2002; Mancias and Goldberg, 2008) and contribution to membrane curvature preventing inward nucleocapsid engulfment (Stagg et al., 2006). Cleavage of many adaptor subunits often targeting degradation leaves only the poorly characterized AP4 or other unknown pathways to handle egress. Modulators of any of these vesicle trafficking pathways may be effective treatments for COVID-19.

The nucleus is enriched because its nuclear localization signals and scaffolding proteins are cleaved. Additionally, many nuclear pore complex proteins and importins/exportins associated with RNA transport are also cleaved. Lamins, which are cleaved by caspases during apoptosis to allow chromosome detachment and condensation, are also cleaved by 3CLpro. Chromatin-remodeling proteins including HATs often containing bromodomains, HDACs, SMC proteins also containing coiled coils, separase, and topoisomerase III alpha, but not CTCF nor any other topoisomerases are cleaved, complicating the effects on chromosome condensation and global gene expression. HDAC inhibitors have been shown to decrease or increase virulence depending on the virus, (Nagesh and Husain, 2016; Chen et al., 2019; Shulak et al., 2014; Feng et al., 2016; Mosley et al., 2006) and some but not all DNA methyltransferases and demethylases are cleaved, further complicating these effects. Viruses benefit from preventing programmed cell death and its corresponding chromosomal compaction in response to viral infection (pyknosis), but they also attempt to reduce host transcription by condensing chromosomes and reroute translation machinery toward their own open reading frames (Kaminsky and Zhivotovsky, 2010; Spencer et al., 2000). Relatedly, 28S rRNA has been shown to be cleaved by murine coronavirus, and ribosomes with altered activity are likely directed from host to viral RNAs (Banerjee et al., 2000). Ribosomal cleavages are depleted here because they are required for viral translation, but the few ribosomal proteins that are cleaved tend to be more represented in monosomes, not polysomes, (Slavov et al., 2015) indicating that ribosomes that initiate faster than they elongate are preferred because they likely frameshift more frequently, allowing for control of the stoichiometric ratio of pp1a and pp1ab (Plant et al., 2019). If slower ribosomes are not directly more likely to frameshift, they are still less likely to participate if frameshift-induced traffic jams, collision-stimulated translation abortion and splitting, (Park and Subramaniam, 2019) and subsequent 60 S subunit obstruction sensing and nascent-chain ubiquitylation, which is especially noteworthy because multiple proteins involved this quality control are predicted to be cleaved (Joazeiro, 2019). Signal recognition particle (SRP) subunits 68/72 kDa associated with the ribosome are also predicted to be cleaved, and the uncleaved SRP9/14 kDa are known to encourage translation elongation arrest to allow translocation including transmembrane domain insertion (e.g. coronavirus envelope protein) and have been associated with frameshifts (Siegel and Walter, 1985; Rottier et al., 1985; Young and Andrews, 1996) In fact, frameshifting is a highly enriched keyword in cleaved proteins mainly due to endogenous retroviral (ERV) elements, some of which can activate an antiviral response via pattern recognition receptors (PRRs). (Grandi and Tramontano, 2019) Some also resemble reverse transcriptases and may, like the GRIPSR system in prokaryotes, be capable of copying coronavirus genomic RNA to produce an RNAi response via the similarly cleaved DICER and AGO (Roy et al., 2020). If the latter is true, individuals with distinct ERV alleles and loci may differentially respond to SARS-CoV-2 infection and/or treatment, especially exogenous RNAi. Lastly, ribosomal proteins are also included in the nonsense-mediated decay (NMD) pathway, which is likely depleted in cleavages because NMD has been shown to be a host defense against coronavirus genomic and subgenomic RNAs’ multiple ORFs and large 3′ UTRs (Wada et al., 2019). It was also shown that the nucleocapsid protein inhibits this degradation, often cannot protect newly synthesized RNAs early in infection. The selective pressure on 3CLpro may be reversed by this nucleocapsid inhibition and the preferential degradation of host mRNAs such that host resources can again be directed toward viral translation.

In addition to affecting large organelles, 3CLpro is predicted to cleave all known components of vault. Vault function has not been completely described, but it has known interactions with other viruses (Wang et al., 2020; Steiner et al., 2006; Li et al., 2015). TERT, which is
associated with vault TEP1 is also cleaved, but is more frequently reported to be activated by other viral infections and/or promote oncogenesis. (Bellon and Nicot, 2008).

Other common viral process proteins are enriched in the epithelium and adaptive immune cells, and those cleaved may affect the heat shock response and other small RNA processing. Lactoferrin, an antiviral protein that is upregulated in SARS infection, (Reganathan et al., 2005) is also cleaved, although one of its fragments, lactoferricin, has known antiviral activity (Berlitelli et al., 2011). Many PRRs, their downstream effectors, and related pathways (PI3K/AKT/mTOR, MAPK, and nitric oxide synthesis, where nitric oxide has conflicting effects on viral infection (Adusumilli et al., 2020; Perrone et al., 2013)) and transcription factors are cleaved, yet no interferons nor their receptors are cleaved likely due to their redundancy. Downstream of interferon, however, multiple STATs and ISGs are cleaved. Finally, complicating the effects of infection on apoptosis, cleavages in both pro-apoptotic caspases and in the anti-apoptotic Bcl-2 and inhibitors of apoptosis exist.

11. Other pathways and keywords

Lipoproteins are a depleted keyword, but multiple apolipoproteins, lipid transfer proteins, and their receptors are predicted to be cleaved and, other than the proapoptotic APOL1, (Wan et al., 2008) are associated with chylomicrons, VLDL, and LDL as opposed to HDL, indicating that lipoproteins may contribute to the correlations between COVID-19 symptom severity, dyslipidemia, and cardiovascular disease. It was recently discovered that SARS-CoV-2 spike protein binds cholesterol, allowing for association with and reduced serum concentration of HDL. These findings combined with the 3CLpro cleavages show an opportunity for HDL receptor inhibitor treatment, especially antagonists of the uncleaved scavenger receptor SR-B1 (Peng et al., 2020). Cleavage of the adipokines leptin, leptin receptor, and IL-6 provide a mechanism for COVID-19 comorbidity with obesity independent of lipoproteins and indicate another potential treatment: anti-leptin antibodies (Rebello et al., 2020; Zhang et al., 2013).

Ubiquitinating and deubiquitinating (DUBs) enzymes are most enriched in the epithelium and the nucleus, and cleavages exist in E3 ubiquitin ligases such as NEDD4, E3-supporting cullins, and DUBs such as proteasomal base and lid subunits, but not in ubiquitin itself. NEDD4 has been shown to enhance influenza infectivity by inhibiting IFITM3 (Chesarino et al., 2015; Shi et al., 2018) and Japanese encephalitis virus by inhibiting autophagy, (Xu et al., 2017) but its ubiquitination of many diverse human viruses promotes their egress. IFITMs generally have antiviral activity (others include HIV-1, (Yu et al., 2015) dengue virus, (Zhu et al., 2015) and filoviruses (Huang et al., 2011)), but its use as a treatment for COVID-19 should be carefully considered given its varying effects among other coronaviruses (Zhao et al., 2018; Hachim et al., 2020) SARS-CoV-2 has two probable NEDD4 binding sites: the proline-rich, N-terminal PPAY and LPSY (Yang and Kumar, 2009) in the spike protein and nsp8, respectively. Although the former sequence is APNY and is likely not ubiquitinated in SARS-CoV, small molecule drugs targeting this interaction or related kinases may be useful treatments for COVID-19 as they have been for other RNA viruses. (Han et al., 2014; An et al., 2014; Maaroufi, 2020) Further research is required to compare these cleavages to the PLpro deubiquitinating activity and the specificity and function of distinct ubiquitin and other ubiquitin-like protein linkages. (Isaacsan and Ploegh, 2009 Jun; Zingrebe et al., 2013).

Helicases make up approximately 1% of eukaryotic genes and are enriched in cleavages with many containing RNA-specific DEAD/DEAH boxes. Most viruses except for retroviruses have their own helicase (nsp13 in SARS-CoV–2) and multiple human RNA helicases have been shown to sense viral RNA or enhance viral replication (Steimer and Klostermeier, 2012; Sharma and Boris-Lawrie, 2012; Umate et al., 2011). SARS nsp13 and nsp14 have been shown to be enhanced by the uncleaved human DDX5 and DDX1, respectively, (Xu et al., 2010; Chen et al., 2009) however subunits of the antiviral, RNA-degrading SKI and NEXT complexes and the catalytic subunit of the interacting exosome complex are cleaved. Additionally, DHX36 cleavage may be motivated by its importance in dsRNA sensing when complexed with DDX1 and DDX21, signaling through the similarly cleaved TRIF to type 1 interferons (Zhang et al., 2011). The remaining cleaved DEAD/DEAH-box helicases tend to interact with RIG-I-like receptor dsRNA sensing or are involved in ribosome biogenesis or translation initiation. Their varying proviral and antiviral activities make recommending possible therapeutic targets impossible without further characterization (Meier–Stephenson et al., 2018).

The coagulation cascade contains many predicted cleavages (coagulation factors II, III, VIII (also an acute-phase protein secreted in response to infection), XII, XIII, plasminogen), von Willebrand factor, plasma kallikrein, kininogen-1, and fibrinectin), but it is not trivial to predict if these cleavages are similar enough to those in the normal pathway to be activating or inhibiting even though 3CLpro is structurally similar to factors Ixa and Xa. (Biembengut and de Souza, 2020) Additionally, multiple cleaved serpin suicide protease inhibitors (PAI-2, megpin, A1AT, and the less relevant angiotensinogen, PZI, CBG, LEI, and HSP47) are related to coagulation, hinting that 3CLpro may increase both thrombosis and fibrinolysis rates or result in dose-dependent effects (Spiezia et al., 2020; Ji et al., 2020). Angiotensinogen is, however, unrelated to coagulation and is cleaved far from its N-terminus, so its effects on the renin-angiotensin system remain unknown. The structurally similar A2M has a predicted cleavage outside its protease bait region, however, the addition of a missense mutation Q694S would allow cleavage at the same site as factor XIII without reducing protease trapping ability as much as large deletions. (Sottrup-Jensen et al., 1989; Gettins et al., 1995) Additional support for this potential exogenous replacement includes presence of serine in the same position in PZP, which shares 71% identity with A2M and contains a neighboring GAG site resembling known PLpro cleavages in its primary bait region. Most other antiproteases, however, are too small to have many potential cleavage sites even though they are a very important response to respiratory virus infection. Serpin or alpha globulin replacement therapy or treatment with modified small, 3CLpro competitive inhibitors may be a useful treatment for COVID-19 (Meyer and Jaspers, 2015).

In addition to coagulation factors, the complement system can induce expulsion of neutrophil extracellular traps (NETs) intended to bind and kill pathogens (De Bont et al., 2019). NETs, however, simultaneously trap platelets expressing tissue factor and contribute to hypercoagulability. The complement pathway is not obviously enriched, but many central proteins (C1/3/4/5) are or have subunits that are cleaved, indicating viral adaptation to the classical, alternative, and likely lectin pathways (Noris et al., 2020; Agrawal et al., 2017; Ip et al., 2005). Neutrophilia and NET-associated host damage are known to occur in severe SARS-CoV-2 infection, so inhibitors of the pathway are currently in clinical trials: histone citrullination, neutrophil elastase, and gastermin D inhibitors to prevent release and DNases to degrade chromatin after release (Narasaraju et al., 2020; Zuo et al., 2020). Complement inhibition would likely similarly reduce the risks of hypercoagulability and other immune-mediated inflammation associated with COVID-19, but effects may vary widely between sexes and ages (Stahel and Barnum, 2020; Gaya da Costa et al., 2018).

Redox-active centers including proteins involved in selenocysteine synthesis are additionally depleted in cleavages likely because of their involvement in avoiding cell death and innate immune response. Respiratory viruses differentially modulate redox pathways, balancing lysis-enhanced virion proliferation and DUOX2-derived reactive oxygen species (ROS)-induced interferon response (Khomich et al., 2018). In addition to depleted antioxidant proteins, cleavage of DUOX1, NOX5, and XO, the former of which are upregulated in chronic obstructive pulmonary disease (COPD), (Schneider et al., 2010) indicates that coronaviruses prefer to reduce oxidative stress in infected cells, contrary to most COVID-19 symptoms. Given the diversity of responses to respiratory virus infections, each proposed antioxidant should be
thoroughly evaluated before being recommended as a treatment of COVID-19.

The impact of post-translational modifications on viral protease cleavage frequency remains uncharacterized. Glutamine and leucine, the two most important residues in the cleavage sequence logo, are rarely modified, but serine, the next most important residue, is the most frequently phosphorylated amino acid. Analysis of keywords showed enrichment of phosphoproteins and depletion of disulfide crosslinked, lipid-anchored, and other transmembrane proteins.

Lastly, the keywords polymorphism and alternate splicing were enriched, indicating that additional variability between cell lines and between individuals is likely. Once health systems are not so burdened by the quantity of cases and multiple treatments are developed, personalized interventions will likely differ significantly between individuals.

12. Conclusion

Many expected and novel protein annotations were discovered to be enriched in cleavages, indicating that 3CLpro is a much more important virulence factor than previously believed. 3CLpro cleavages are enriched in the epithelium (especially along the respiratory tract), brain, testis, plasma, and immune tissues and depleted in olfactory and gustatory receptors. Affected pathways with discussed connections to viral infections include cytoskeleton/motor/cell adhesion proteins, nuclear condensation and other epigenetics, host transcription and RNAi, coagulation, pattern recognition receptors, growth factor, lipoprotein, redox, ubiquitination, and apoptosis. These pathways point toward many potential therapeutic mechanisms to combat COVID-19: cytoskeletal drugs frequently used against cancer, modulators of ribosomal stoichiometry to enrich monosomes, upregulation of Dicer1 and Ago1/2, exogenous lactoferrin and modified antiproteases including alpha globulins, upregulation of serpins potentially via dietary antioxidants, complement inhibition, reduction of LDL and inhibition of HDL receptor (e.g. by antagonizing SR-B1), anti-leptin antibodies, and downregulating NEDD4 or related kinases and upregulating IFITMs. Pathway components with more complex disruption that may also deliver therapeutic targets but require elucidating experimental results include PDEs, histone acetylation, nitric oxide, and vesicle coasters. It is also worth further investigating how 3CLpro contributes if at all to the correlations between obesity and severity of infection or to viral infection of autoimmune and potentially oncological conditions.

Expansion of the training dataset to the whole order Nidovirales or class Pneumoviridae may provide more diversity to improve classifying methods if additional protease/cleavage coevolution does not invalidate the assumption of cross-reactivity. Issues requiring in vitro and in vivo experimentation include characterization of cleavage kinetics, any functional differences between proteases, the molecular effects of post-translational modifications, and the individual and population effects of polymorphisms in cleavage sequences on susceptibility to or severity of infection. Even though many caveats exist without experimentation, similar prediction, enrichment/depletion analysis, and therapeutic target identification should be performed for every viral protease.

Conflicts of interest statement

The author certifies that there is NO affiliation with or involvement in any organization or entity with any financial or non-financial interest in the subject matter or materials discussed in this manuscript titled “SARS-CoV-2 3CLpro whole human proteome cleavage analysis prediction and enrichment/depletion analysis.” This project was conducted in spare time outside my full-time employment and without any funding.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jmbiolchem.2022.107671.

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CRediT authorship contribution statement

Lucas Prescott: Conceptualization, Methodology, Data curation, Software, Validation, Formal Analysis, Writing, Visualization.

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