ViralLink: An integrated workflow to investigate the effect of SARS-CoV-2 on intracellular signalling and regulatory pathways

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Abstract

The SARS-CoV-2 pandemic of 2020 has mobilised scientists around the globe to research all aspects of the coronavirus virus and its infection. For fruitful and rapid investigation of viral pathomechanisms, a collaborative and interdisciplinary approach is required. Therefore, we have developed ViralLink: a systems biology workflow which reconstructs and analyses networks representing the effect of viruses on intracellular signalling. These networks trace the flow of signal from intracellular viral proteins through their human binding proteins and downstream signalling pathways, ending with transcription factors regulating genes differentially expressed upon viral exposure. In this way, the workflow provides a mechanistic insight from previously identified knowledge of virally infected cells. By default, the workflow is set up to analyse the intracellular effects of SARS-CoV-2, requiring only transcriptomics counts data as input from the user: thus, encouraging and enabling rapid multidisciplinary research. However, the wide-ranging applicability and modularity of the workflow facilitates customisation of viral context, *a priori* interactions and analysis methods. Through a case study of SARS-CoV-2 infected bronchial/tracheal epithelial cells, we evidence the functionality of the workflow and its ability to identify key pathways and proteins in the cellular response to infection. The application of ViralLink to different viral infections in a cell-type specific manner using different available transcriptomics datasets will uncover key mechanisms in viral pathogenesis. The workflow is available on GitHub (https://github.com/korcsmarosgroup/ViralLink) in an easily accessible Python wrapper script, or as customisable modular R and Python scripts.

Author summary

Collaborative and multidisciplinary science provides increased value for experimental datasets and speeds the process of discovery. Such ways of working are especially important at present due to the urgency of the SARS-CoV-2 pandemic. Here, we present a systems biology workflow which models the effect of viral proteins on the infected host cell, to aid collaborative and multidisciplinary research. Through integration of gene expression datasets with context-specific and context-agnostic molecular interaction datasets, the workflow can be easily applied to different datasets as they are made available. Application to diverse SARS-CoV-2 datasets will increase our understanding of the mechanistic details of the infection at a cell type specific level, aid drug target discovery and help explain the variety of clinical manifestations of the infection.

Introduction

By mid-May 2020 at least 4000 scientific preprints and publications were released relating to Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2) and the disease it causes (COVID-19) (Kwon 2020). This fast uptake in research efforts is vital to decrease the health and economic impacts of this new pandemic. However, many questions remain unanswered regarding the molecular processes driving host responses to this coronavirus. One key challenge to utilisation of new findings is that published datasets are mostly unlinked to each other (due to parallel efforts by multiple research groups) and not always connected to community standard resources. An integrated and reusable method to interactively capture new data and connect it to existing data sources is needed. Such a comprehensive approach that can be run regularly when relevant new data is available, will increase and update our understanding of the mechanistic details of the SARS-CoV-2 infection. Further, it will aid drug target discovery by enabling identification of high confidence mediators through which the virus is affecting host cells (Barabási et al. 2011). Studying the effect of the virus at molecular level may explain the variety of clinical manifestations of the infection and the differences in susceptibility between different populations, and together with soon available human genomics data, could be used for identifying risk factors.

Upon entry of a virus into a human cell *via* surface receptors, viral RNA is released and translated into proteins (Oberfeld et al. 2020). In addition to their role in direct viral replication, these proteins are able to bind to human proteins creating a host-virus interface (Gordon et al. 2020). This interaction can lead to downstream signalling changes in the host cell, either as a result of viral hijacking or through a defined viral immune response by the host cell (Alto and Orth 2012). Ultimately, this signal flow results in intracellular gene transcription changes, cell-cell signalling and systemic host responses which drive the tug-of-war between the host and the virus (Fung et al. 2020). In order to understand and control this conflict, it is necessary to study each of these levels of host response in detail, including the intracellular response of the primarily infected cell.

Currently available data relating to intracellular SARS-CoV-2 infection includes human binding partners of viral proteins (Gordon et al. 2020) and transcriptomics datasets from infected cell lines/organoids (Blanco-Melo et al. 2020; Lamers et al. 2020), infected patients (Liao et al. 2020; Huang et al. 2020) and other infected animals (Pfaender et al. 2020; Blanco-Melo et al. 2020). Interdisciplinary and collaborative science can maximise the value of each of these datasets through data integration and comparison combined with application of different computational analysis approaches. One such computational analysis method is the utilisation of network approaches to model molecular interactions between the virus and human proteins

as well as within and between human cells (Guven-Maiorov et al. 2017). Network approaches have already been applied to study SARS-CoV-2 pathogenesis and to predict drug repurposing candidates and master regulators based on proteins in proximity to human binding proteins (which physically associate with SARS-CoV-2 proteins) (Gysi et al. 2020; Messina et al. 2020; Zhou et al. 2020; Guzzi et al. 2020).

Here we present a systems biology workflow, to study the effect of viral infections on host cells. ViralLink reconstructs and analyses a causal molecular interaction network whose signal starts with the binding of an intracellular viral protein to a human protein, travels via multiple signalling pathways, and ends at the transcriptional regulation of altered genes. Subsequently, the workflow investigates the causal network using betweenness centrality measures, cluster analysis, functional overrepresentation analysis and network visualisation. Using currently available datasets from SARS-CoV-2 infected bronchial epithelial cells we demonstrate that this workflow can identify biologically relevant signalling pathways and predict key proteins for potential drug interventions. As the workflow is built in a modular, standardised and updateable fashion, it can be used easily in the future to analyse new SARS-CoV-2 related datasets (from human biopsy data, multiple tissues, etc.).

Methods

ViralLink workflow overview

The ViralLink workflow investigates the effect of viral infection within cells by generating and analysing context-specific networks of intracellular signalling and regulatory molecular interactions. These networks link the intracellular binding of viral and human proteins to the transcriptional response of the infected cell (Figure 1). The context-specificity of the analysis is obtained through the choice of input transcriptomics datasets - it could refer to strain of virus, type of infected cell, severity of infection, age of host or any other context of interest. By default, the workflow is set up to analyse the intracellular effects of SARS-CoV-2, requiring only transcriptomics counts data as input and thus encouraging and enabling rapid multidisciplinary research. However, the wide-ranging applicability and modularity of the workflow facilitates customisation of viral context, *a priori* interactions and analysis methods. ViralLink contains three primary stages: 1) collection and input of data; 2) reconstruction of the network; and 3) investigation of results using functional analysis, clustering, centrality measures and visualisation.

Collection and input of data

Reconstruction of causal networks using ViralLink requires four separate input datasets (Figure 1): viral protein-human binding protein interactions, *a priori* human protein-protein interactions (PPIs), *a priori* human transcription factor (TF) - target gene (TG) interactions and an unnormalised counts matrix from a gene expression experiment. By default, all data except the transcriptomics counts are provided automatically. However alternative input files can be provided if desired.

The default workflow uses SARS-CoV-2 protein-human binding protein interactions obtained from an affinity-purification mass spectrometry study (Gordon et al. 2020) via Intact (Hermjakob et al. 2004; Orchard et al. 2014). This data was reformatted to contain one row per molecular interaction with 2 columns of UniProt IDs: SARS-CoV-2 proteins and human binding proteins. Alternative viral-human PPIs can be provided using the same data format. The workflow assumes all viral-human interactions have an inhibiting action on the human protein, unless a third column named “sign” is present in the input file containing “+” for activatory and “-” for inhibitory interactions. In addition, data is provided with the workflow containing the gene names corresponding to each of the SARS-CoV-2 proteins, to enable easy interpretation of the reconstructed networks.

For *a priori* human interactions, the workflow obtains and uses integrated collections of PPI and TF-TG interactions from OmniPath and DoRothEA, respectively (Türei et al. 2016; Garcia-Alonso et al. 2019). These interactions are obtained using the ‘OmniPathR’ R package (Türei et al. 2016; R Core Team 2013) to download and filter signed and directed interactions. For DoRothEA, only high and medium confidence level interactions are used (confidence scores A-C). In contrast to importing static input files, this script enables the use of up to date interaction data. Alternative interaction data can be used with the workflow provided it has the same format: specifically, it must contain source and target uniprot IDs in the columns ‘to’ and ‘from’ and if the transcriptomics data uses gene symbols, the interaction data must additionally contain gene symbols in the columns ‘source_genesymbol’ and ‘target_genesymbol’. Furthermore, the interactions must be directed and signed with the sign of the interaction given in the column ‘consensus_stimulation’ where the value ‘1’ represents a stimulation and anything else represents an inhibition.

The aforementioned *a priori* interactions are contextualised using transcriptomics data from any study of interest which compares viral infected to uninfected human cells or tissues. Correspondingly, the workflow requires unnormalised counts data from a transcriptomics experiment (containing Uniprot or gene symbols as IDs) and a corresponding mapping table

which lists the sample IDs (from the headers of the counts table) in the 'sample_name' column and the 'test' or 'control' status of the sample in the 'condition' column. This mapping table is used to carry out differential expression of a test condition (e.g. infected) compared to a control condition (e.g. uninfected). An example expression dataset and mapping table are provided with the workflow.

To process the transcriptomics data, the workflow uses 'DESeq2' in R to normalise the counts and to carry out differential expression analysis (Love et al. 2014). Any genes passing the log2 fold change and adjusted p value cutoffs, based on the provided parameters (default 1 and 0.05, respectively), are classed as differentially expressed genes (DEGs). Following removal of all genes with count = 0, normalised log2 counts across all samples are fitted to a gaussian kernel (Beal 2017). All genes with expression values above mean minus three standard deviations are considered as expressed genes. Subsequently, context-specific human PPI and TF-TG interactions are generated by filtering only interactions where both interacting molecules are expressed.

File paths to all input datasets and associated parameters (such as desired log2 fold change cut off) are specified in the parameters text file which is read in by the workflow.

Network reconstruction

The reconstructed causal network contains three layers of interactions, which are obtained, by default, from the three *a priori* interaction resources:

- Viral proteins interacting with human binding partners: from the SARS-CoV-2 collection in the IntAct database (Hermjakob et al. 2004; Orchard et al. 2014)
- Intermediary signalling protein interactions: from protein-protein interactions (PPIs) of the OmniPath collection (Türei et al. 2016)
- Transcription factors (TFs) regulating differentially expressed genes: from a transcriptomics dataset of interest and the DoRothEA collection (Garcia-Alonso et al. 2019)

A list of all TFs targeting the differentially expressed genes are obtained from the context-specific TF-TG interactions. The human binding proteins of viral proteins are connected to the listed TFs through the context-specific human PPIs using a network diffusion approach called Tied Diffusion Through Interacting Events (TieDIE) (Paull et al. 2013). As inputs for the TieDIE tool, the following information is used: (1) The signed, directed and expression based filtered PPIs is used as the input network. (2) Human proteins which are interacting partners of the viral proteins are used as the start nodes. The number of viral proteins bound to each of the

human proteins are assigned as the weights of the start nodes. (3) The TFs of the DEGs in the dataset are used as the stop nodes. The weights for each of the TFs in the set of stop nodes were calculated using the following formula (Equation 1) which considers both the log2 fold change of the DEGs as well as the sign (i.e stimulatory or inhibitory) of the relationship between the TF and the DEG.

$$Weight_{TF} = \frac{1}{N_{TFTG}} \sum_{n \in TG} LFC_n \cdot sign(TFTG_n)$$

Equation 1

$$sign(x) = \begin{cases} x = +1 & if\ x = activatory, \\ x = -1 & if\ x = inhibitory. \end{cases}$$

After running TieDIE, a custom R script is used to collate all the data into a final viral-initiated intracellular signalling network (causal network), outputting an edge table representation of the network, with a node table containing additional node annotations. Starting with the interactions output from TieDIE, viral protein-human binding protein interactions are added for each of the present human binding proteins. Similarly, TF-TG interactions (where the TG is a DEG) are added for each of the present TFs, creating a full network with three interaction types: SARS-CoV-2 protein-human binding protein, PPI and TF-DEG. All nodes of the network are added to a node table with annotations including heat values (output from TieDIE), Entrez IDs (obtained in R using the 'org.Hs.eg.db' package), gene symbols (obtained from UniProt (UniProt Consortium 2019)) and log2 fold change values from the differential expression analysis.

Network investigation

Following reconstruction of the causal network, ViralLink provides functionality to investigate the results using functional analysis, clustering, centrality measures and visualisation.

Centrality measures

To identify key molecules in the reconstructed network ViralLink uses a betweenness centrality measure - calculating the global importance of a node (in this case a protein) based on the number of shortest paths which pass through them when connecting all node pairs in the network (Koschützki and Schreiber 2008). Nodes with high betweenness centrality play a key role in transduction of signals through the network, and here represent proteins with biological importance in the cellular response to viral infection. Betweenness centrality is calculated for each node in the causal network using the R package 'igraph' and output as an annotation in

229 the node table (Csárdi and Nepusz 2006). Alternative centrality measures are available using
230 the 'igraph' package and can be integrated into the workflow by the user if required.

231 Cluster analysis

232 Clustering algorithms are commonly used in network biology to investigate the complex
233 structure of molecular interaction networks by extracting groups of densely connected
234 molecules (Bader and Hogue 2003; Brohée et al. 2008). Depending on the number of
235 molecules included, a cluster can represent a molecular complex or a group of molecules
236 which function closely with each other. Cluster analysis can identify subsets of a large network
237 with specific functions and indicate molecules that may have functional redundancy with each
238 other - potentially having implications for drug targeting. ViralLink employs the MCODE
239 clustering method to identify groups of densely connected nodes in PPI networks (Bader and
240 Hogue 2003). To carry out this analysis, ViralLink requires a local version of the Cytoscape
241 software to be open (Shannon et al. 2003; Su et al. 2014), which is controlled programmatically
242 using the R package 'RCy3' with the Cytoscape 'MCODE' app (v1.6.1) (Gustavsen et al.
243 2019). MCODE is run using default parameters: degree cut off =2, haircut=TRUE, node score
244 cut off=0.2, k-core=2, max depth=100. This analysis outputs the data as node annotations in
245 the node table, which are used for the functional analysis and visualisation steps of the
246 workflow. If Cytoscape is not running, this step of the workflow will be skipped.

247 Functional analysis

248 To further investigate important cellular functions and signalling pathways directly affected by
249 the virus of interest, ViralLink carries out functional overrepresentation analysis on different
250 parts of the causal network:

251 1. The DEGs of the network
252 2. The upstream human proteins (including human binding proteins, intermediary
253 signalling proteins and TFs)
254 3. Identified clusters (only those with ≥ 15 nodes are investigated)

255

256 Functional overrepresentation analysis is carried out in R using packages 'ClusterProfiler' (for
257 Gene Ontology annotations (Ashburner et al. 2000)) and 'ReactomePA' (for Reactome
258 annotations (Yu et al. 2012; Yu and He 2016; Fabregat et al. 2018). For analysis of the
259 upstream human signalling proteins and analysis of clusters, all proteins in the context-specific
260 human PPI interactions are used as the background. For analysis of the DEGs, all target
261 genes in the context-specific human TF-TG interactions are used as the background. For
262 Gene Ontology (Biological Process) analysis (except when running the compareCluster

263 command), the ‘simplify’ command is used (cutoff=0.1, select_fun=min) to remove redundant
264 functions. All functions with q val ≤ 0.05 are considered significantly overrepresented.
265
266 An additional R script is provided alongside the workflow which creates subnetworks of the
267 causal network based on functions of interest. These function-specific subnetworks highlight
268 how specific signalling pathways in the infected cell reach (and subsequently affect) specific
269 functions of the DEGs. For example, the subnetwork could be created to show how viral
270 proteins can affect different host toll-like receptor pathways, and how these pathways can
271 ultimately affect DEGs associated with interleukins. In this network the DEG nodes would be
272 replaced with nodes representing the interleukin functions (which must be overrepresented
273 based on the functional analysis). This script requires the output files from the functional
274 analysis, the node and edge tables of the causal network and a file of all Uniprot IDs
275 associated with all Reactome functions (which is provided with ViralLink, following download
276 from the Reactome website in April 2020). In addition, the script requires a list of
277 overrepresented DEG functions (Reactome) and a list of upstream signalling functions
278 (Reactome) to visualise. The script outputs an edge table, a node table and a Cytoscape file
279 (if Cytoscape is open locally at the time of running the script).

280 Visualisation

281 Data visualisation is often an important part of biological network interpretation, providing new
282 insights into the data and visually conveying analysis results (Pavlopoulos et al. 2008). As
283 such, ViralLink has the capability to import reconstructed networks into the open-source
284 Cytoscape network visualisation software (Shannon et al. 2003; Su et al. 2014). This
285 functionality requires that the user has Cytoscape installed and open locally. Specifically, the
286 workflow employs the ‘RCy3’ R package to interact with Cytoscape programmatically,
287 importing the node and edge tables to create network visualisations and saving the data as a
288 ‘.cys’ file. The causal network, the network clusters (where containing ≥ 15 nodes) and the
289 function-specific networks are visualised in this way. If calculated previously, the causal
290 network nodes are coloured based on their betweenness centrality, however further style and
291 layout customisation must be carried out by the user directly based on the data.

292 Implementation

293 The workflow consists of modular R and Python scripts which can be run separately or through
294 the provided Python wrapper script. If running for the study of SARS-CoV-2, the only required
295 input files are related to the transcriptomics data of interest: a raw counts table (using gene
296 symbols or UniProt protein IDs) and a two-column metadata table specifying test and control
297 sample IDs. One further script is provided to generate function-specific networks. This script

298 is not included in the wrapper because it requires the user to specify functions of interest from
299 the output of the functional analysis. To run everything, it is necessary that the user has R,
300 Python3 and Cytoscape installed. The only file the user needs to edit is the parameters text
301 file where input file paths and parameters are specified. All scripts, default input files and
302 details of how to run the scripts are freely accessible on GitHub
303 (https://github.com/korcsmarosgroup/ViralLink).

304 Use case

305 To demonstrate the application of this workflow for the study of SARS-CoV-2, we applied it to
306 a published transcriptomics dataset. We downloaded raw counts tables from a transcriptomics
307 study of SARS-CoV-2 infected (MOI 2, 24 hour incubation) NHBE cells (Normal Human
308 Bronchial/tracheal Epithelial cell line) with uninfected controls (Blanco-Melo et al. 2020) via
309 Gene Expression Omnibus (accession GSE147507) (Edgar et al. 2002; Barrett et al. 2013).
310 OmniPath and DoRothEA (v2, A-C) were downloaded on 15/04/2020. Any genes with log2
311 fold change ≥ |0.5| and adjusted p value ≤ 0.05 were classed as differentially expressed. All
312 networks were visualised in Cytoscape (v3.7.2).

313 Results

314 Use case: SARS-CoV-2 infection of lung cells

315 To demonstrate the application of this workflow for the study of SARS-CoV-2, we created
316 intracellular signalling networks of NHBE cells (from Normal Human Bronchial/tracheal
317 Epithelial cell lines) upon infection with SARS-CoV-2 based on data published by Blanco Melo
318 *et al.* (Blanco-Melo et al. 2020) and viral-human binding protein interactions published by
319 Gordon *et al.* (Gordon et al. 2020). The resulting causal network contains 804 nodes
320 (molecules) and 5423 interactions (Figure 2A, Supplementary Tables 1-2, Supplementary File
321 1). The 10 most central proteins of the reconstructed causal network (based on betweenness
322 centrality) are involved in a wide range of cellular functions (Figure 2B). Taken together these
323 proteins highlight the propensity for SARS-CoV-2 to affect cell proliferation, apoptosis, cell
324 adhesion, exocytosis and proinflammatory immune responses. These functions are influenced
325 through multiple cellular pathways, most notably MAPK/ERK and PI3K/AKT signalling
326 pathways.
327
328
329

330
331 Functional overrepresentation analysis of the causal network identified an enrichment of
332 interleukin and interferon related functions among the network DEGs, in line with previously
333 published findings (Supplementary Figure 1, Supplementary File 2) (Zhang et al. 2020; Chua
334 et al. 2020; Huang et al. 2020). Overrepresented functions and pathways of the upstream
335 signalling proteins (human binding proteins, intermediary signalling proteins and TFs) included
336 innate immunity-related functions, platelet signaling, PI3K/AKT signalling, MAPK activation,
337 estrogen receptor-mediated signalling, senescence and a number of growth factor receptor-
338 associated functions (such as VEGF signalling, receptor tyrosine kinases, stem cell growth
339 factor signalling (SCF-KIT) and neurotrophin receptor signaling). Therefore, we show that this
340 analysis highlights additional pathways through which SARS-CoV-2 could be affecting the lung
341 epithelial cells, which cannot be identified by looking at the transcriptomic results in isolation.
342
343 Based on functional overrepresentation analysis, we created a function-specific network by
344 sub setting the causal network. This visualisation was used to further explore the mechanisms
345 of how specific signalling pathways are affecting the DEGs (Supplementary Figure 2A,
346 Supplementary File 3). Specifically, we generated an innate-immunity associated subnetwork
347 containing all upstream human signalling proteins associated with Reactome functions
348 cytokine signalling in immune system, signaling by interleukins and MyD88-independent TLR4
349 cascade and all overrepresented functions of the DEGs (in place of the DEG nodes). These
350 pathways contain 9/10 of the top betweenness centrality nodes (all except RHOA), evidencing
351 the centrality and importance of the innate immune response to viral infection. Inspecting the
352 TF layer of this immune subnetwork, we find a number of key TFs including STAT proteins (3
353 and 4), IRF proteins (1 and 5) and NFKB-related proteins (NFKB1, NFKBIA).
354
355 Finally, we evidenced the application of MCODE clustering analysis to using the reconstructed
356 SARS-CoV-2-infected NHBE cell causal network. We identified four clusters containing 15 or
357 more nodes, making up 19% of the network (154/804) (Supplementary Figure 2B,
358 Supplementary Table 2, Supplementary File 1). Assuringly, 9/10 of the top betweenness
359 centrality nodes were included in these four clusters, further confirming the high connectivity
360 and importance of these nodes in the causal network. Functional overrepresentation analysis
361 of the cluster nodes highlighted a functional similarity between all four of the clusters
362 (Supplementary Figure C-D, Supplementary File 2). Likely this is due to the high number of
363 inter-cluster molecular interactions and because of the functional similarities between the top
364 central nodes.
365
366 Collectively, we show that our systems biology workflow, ViralLink, reconstructs a functionally
367 relevant intracellular signalling network affected by SARS-CoV-2 infection. Investigation of the

368 networks through functional analysis, centrality measures and cluster analysis, combined with
369 network visualisations, enables detailed study of the key proteins and pathways involved in
370 signal transduction.

Discussion

Infection by SARS-CoV-2 can cause a complex and systemic response by the human body. As such, a better mechanistic understanding of the effects of SARS-CoV-2 will aid identification of effective drug treatments and help to explain the differences in susceptibilities across different populations (Kirby 2020). This understanding can be gained using cross-disciplinary approaches which combine 'omics data generation, computational systems biology and validatory web lab experiments (Korcsmaros et al. 2017). Here we present a computational workflow that can be used to model the cellular response to infection by integrating knowledge of human binding proteins of viral proteins with the transcriptional response of a cell/cell type. Whilst set up primarily to run analyses based on SARS-CoV-2, ViralLink can be applied to any viral infection, provided data is available describing possible interactions between the viral proteins and human proteins.

ViralLink builds on our previously published resource MicrobioLink, which reconstructs networks representing the effect of extracellular and intracellular microbial proteins on cellular processes (Andrighetti et al. 2020). Differing from MicrobioLink, ViralLink inputs a predetermined list of viral-host PPIs and focuses only on pathways ending in transcriptional regulation: thereby reducing the complexity of the workflow (for accessibility and speed purposes) and increasing its predictive confidence. Furthermore, ViralLink extends the functionality of MicrobioLink with more advanced network analysis (functional enrichment, clustering and centrality measures) and visualisation options.

By exploiting previously collated and comprehensive collections of molecular interactions (Türei et al. 2016; Garcia-Alonso et al. 2019), ViralLink predicts how signal flows from the initial interaction with a viral protein or protein fragment to the ultimate transcriptional changes induced by the virus. Through mapping the direct intracellular effect of viral infection (using a network approach), this workflow enables further investigation into specific signalling pathways and transcription factors which play a key role in signal transduction. Signalling pathways are primarily regulated through post-translational modifications and thus would not be identified using transcriptomics datasets (Antebi et al. 2017). In addition, the resulting intracellular networks allow identification of differentially regulated genes that are affected as a direct result of viral recognition by protein-protein signalling pathways, rather than by secondary signals such as elevated cytokine levels. This permits a more focused analysis of possible drug targets and adds to the understanding of viral pathomechanisms. Functional analysis and visualisation methods included in the workflow are vital for interpretation of the generated intracellular networks, enabling detailed investigation of key proteins and signalling pathways.

408

409 Due to the modularity of the workflow, it can be easily adjusted or extended - different diffusion
410 and propagation algorithms, such as HotNet2 (Leiserson et al. 2015; Cowen et al. 2017), could
411 be implemented as required. The implemented diffusion tool, TieDIE, adds mechanistic value
412 by accounting for local causality (e.g. sign) but, on the other hand, has a reduced possible set
413 of input *a priori* interactions. If desired, a diffusion tool which does not need signed *a priori*
414 interactions can be implemented to increase the input dataset size. Alternatively, a different
415 method, such as an integer linear programming approach which identifies paths based on an
416 optimisation problem (as implemented in CARNIVAL), could be used for network
417 reconstruction (Liu et al. 2019). In addition, integration of CARNIVAL could extend the
418 workflow to permit network reconstruction without supplying upstream perturbations (in this
419 case the viral-host protein interactions). Whilst not currently integrated due to data availability
420 issues, the addition of phosphoproteomics data to the pathway propagation methods could
421 improve the prediction of active pathways (Dugourd et al. 2020) Alternatively, methods to
422 predict protein activity based on transcriptional signatures, such as VIPER and PROGENy
423 (Alvarez et al. 2016; Schubert et al. 2018) could be added to the workflow in addition to
424 network diffusion methods to increase the confidence of pathway predictions. Finally,
425 extension of the network to include additional regulatory molecule types (e.g. miRNAs) or to
426 study non-human hosts, could uncover further mechanisms by which SARS-CoV-2 can affect
427 host cells.
428

429 Accessible through GitHub, the workflow requires R and Python3 to be installed (and
430 Cytoscape for clustering and visualisation), however only a limited programming ability is
431 required to run the code. All code is wrapped into a Python script with a separate file where
432 all input file paths and parameters are specified. At a minimum, only two user specified input
433 files are required: a raw counts table from a transcriptomics study (using gene symbols or
434 UniProt protein IDs) and a two-column metadata table specifying test and control sample IDs.
435 All other files are provided or acquired directly within the workflow - but can be changed by
436 the user if required. However, one limitation of the current workflow is that creation of
437 Cytoscape visualisations and clustering analysis require the user to install and open the
438 Cytoscape app. If this is not possible, for example because the scripts are not being run on a
439 machine with a graphical interface, these steps are skipped. Furthermore, only basic
440 visualisation is possible programmatically, due to challenges applying one visualisation
441 strategy to all possible output networks, especially with regard to the function-based networks.
442

443 In addition to accessibility through a default emphasis on SARS-CoV-2, a key strength of this
444 workflow is the ability to use different input datasets: including different *a priori* molecular
445 interactions, viral-human binding protein interactions and expressed/differentially expressed

446 gene lists. This allows extensive customisation and permits rapid implementation to the most
447 cutting-edge data soon after publication. Running the workflow across different
448 transcriptomics datasets will allow comparison of intracellular viral responses between
449 different cell types, different species and across different conditions (such as severe vs
450 asymptomatic infection). For example, application of the workflow to transcriptomics data from
451 specific immune cell-types, such as macrophages, will likely uncover different host affected
452 signalling pathways and key TFs based on the infected cell-type. This, in turn, could increase
453 our understanding of the role of different immune populations in fighting the infection. In
454 addition, the workflow can be run on data from other SARS-CoV-2 strains when and if they
455 emerge, thereby aiding comparisons of mechanisms of action between the strains.
456
457 To evidence the use of this workflow, we applied it to study the effect of SARS-CoV-2 infection
458 in lung epithelial (NHBE) cells using transcriptomics data published by Blanco-Melo *et al.*
459 (Blanco-Melo et al. 2020). In the resulting causal network, DEGs directly affected by SARS-
460 CoV-2 initiated signalling are associated with functions that are known responses to SARS-
461 CoV-2 and other viral infections (Cao 2020; Shi et al. 2020; Sallard et al. 2020; Arvanitakis et
462 al. 1998). Upstream of these affected genes we identified a number of potentially important
463 signalling pathways relating to classical viral-immune responses, cell survival and cytoskeletal
464 rearrangements and cell adhesion. Previous investigation of the first SARS coronavirus
465 (SARS-CoV) identified an inhibition of cell proliferation and an increase in apoptosis regulated
466 to PI3K/AKT signalling (Mizutani et al. 2006; Tsoi et al. 2014). Our network of SARS-CoV-2-
467 initiated intracellular signalling suggests that the PI3K/AKT signalling and the AKT1 protein
468 itself are key mediators of SARS-CoV-2 initiated signal transduction and that apoptosis and
469 cell proliferation pathways are affected by SARS-CoV-2, thus highlighting similarities between
470 the two viruses. However, further experimentation and/or data curation is required to confirm
471 the direction of change of specific pathways (up- or downregulated) based on the results of
472 the presented workflow. Together our results indicate that SARS-CoV-2 can affect NHBE cells
473 through a variety of signalling pathways which have been previously associated with similar
474 viruses, including growth factor signalling, MAPK/ERK signalling and PI3K/AKT signalling.
475 Furthermore, centrality measures and cluster analysis identified proteins which likely play a
476 key role in transduction of these signals, and could be good targets for drug treatments.
477
478 Several other network reconstruction methods exist which could be and have been applied to
479 study SARS-CoV-2 infections. For example Messina *et al*. and Gysi *et al*. (Messina et al. 2020;
480 Gysi et al. 2020) use diffusion algorithms and other similar methods to investigate proteins in
481 close proximity to human binding proteins based on PPI interactions and gene co-expression
482 networks. Our workflow builds on these approaches by linking viral proteins to DEGs. Through
483 this method we can observe which signalling pathways mediate the effect of the virus on

484 cellular transcription levels, creating a systems level view of cellular changes as a result of the
485 virus. Using the functional analysis methods and network visualisation capabilities of the
486 workflow, it is possible to predict which viral proteins and host signalling pathways can affect
487 specific cellular functions, enabling more focused identification of drug targets. In addition to
488 protein mediators, this method describes TFs which are involved in the cellular response and
489 identifies which DEGs can be affected as a direct result of viral proteins hijacking host
490 signalling and which are affected through a different mechanism. In addition to the presented
491 workflow, at least one other method has been used to reconstruct SARS-CoV-2-initiated
492 intracellular signalling networks (Ding et al. 2020) corroborating the benefits of such analysis
493 methods. Differing from the here presented approach, this work uses an extended version of
494 the Signaling Dynamic Regulatory Events Miner method to reconstruct the networks, resulting
495 in a more mathematically complex but computationally heavy analysis (Gitter et al. 2013).
496 Furthermore, the workflow by Ding *et al.* is a less reusable and accessible workflow because
497 it was designed for a specific analysis.
498
499 In conclusion, ViralLink is an easily accessible, reproducible and scalable systems biology
500 workflow to reconstruct and analyse molecular interaction networks representing the effect of
501 the viruses on intracellular signalling. We believe it is the first available integrative workflow
502 for analysing the downstream effects of viral proteins using viral host interactions and host
503 response data. Application of this workflow to study COVID-19 based on a wide variety of
504 conditions and datasets will uncover mechanistic details about SARS-CoV-2 infection of
505 different cell types, providing valuable predictions for wet-lab and clinical validation.
506
507

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515

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Figure and Tables

Figure 1: ViralLink workflow overview.

Figure 2. Causal network of SARS-CoV-2-infected NHBE cells. A) Signalling flows from left to right: SARS-CoV-2 proteins/protein fragments (red triangles), human binding proteins (yellow parallelograms), intermediary signalling proteins (blue circles), transcription factors (green rectangles) and differentially expressed genes (grey rhombuses). Where a human protein/gene is acting in multiple layers of the network, it is only visualised once based on the following priority: DEGs, binding proteins, TFs, signalling proteins. **B)** Results of betweenness centrality analysis, which measures the global importance of nodes (molecules) in the network. Nodes coloured based on their betweenness centrality parameter, with the gene names of the 10 highest scoring (most central) nodes overlaid. DEGs have log2 fold change ≥ |0.5| and adjusted p value ≤ 0.05.

Supplementary Figure 1. Overrepresented Reactome functions (A, B) and Gene Ontology Biological Processes (C, D) of the causal network of SARS-CoV-2 infected NHBE cells. A) Top 10 overrepresented Reactome functions of upstream signalling proteins (including human binding proteins, intermediary signalling proteins and TFs) **B)** Top 10 overrepresented Reactome functions of network DEGs C) Top 10 overrepresented GO-BP functions of upstream signalling proteins (including human binding proteins, intermediary signalling proteins and TFs) D) All overrepresented GO-BP functions of network DEGs (q value ≤ 0.05). DEGs have log2 fold change ≥ |0.5| and adjusted p value ≤ 0.05.

Supplementary Figure 2: Function-specific network SARS-CoV-2- infected NHBE cells and cluster analysis on SARS-CoV-2-infected NHBE causal network. A) Function-specific subnetwork containing upstream signalling proteins related to the top overrepresented (q value ≤ 0.05) innate immunity-related Reactome functions (cytokine signalling in immune system, signaling by interleukins and MyD88-independent TLR4 cascade) and all overrepresented functions of the DEGs (in place of the DEG nodes). Layers of the network and node shapes same as in Figure 2. DEGs = differentially expressed genes. DEGs have log2 fold change ≥ |0.5| and adjusted p value ≤ 0.05. See Supplementary File 3. B) Cluster analysis results where clusters have ≥ 15 nodes. Position of clustered proteins shown within the causal network and to the right as isolated clusters. Nodes coloured by their cluster membership (black=unclustered, green=cluster 1, yellow=cluster 2, pink=cluster 3, blue=cluster 4). Presence of top 10 betweenness centrality nodes in the clusters is indicated to the right of the clusters. B) Gene Ontology (GO) overrepresentation analysis of the clusters.

564 Top five GO terms (by adjusted p value) displayed for each cluster. **C)** Reactome
565 overrepresentation analysis of the clusters. Top five Reactome terms (by adjusted p value)
566 displayed for each cluster. See Supplementary Table 2 and Supplementary File 2.
567
568 **Supplementary Table 1: Causal network of SARS-CoV-2-infected NHBE cell.**
569
570 **Supplementary Table 2: Node annotations for causal network of SARS-CoV-2-infected**
571 **NHBE cell.** Includes betweenness centrality measures and clusters identified by MCODE.
572 MCODE clusters 1,3,4 and 5 correspond to the clusters in the manuscript labelled 1,2,3 and
573 4 respectively. Clusters 2 and 6 were excluded due to size.
574
575 **Supplementary File 1: Causal network of SARS-CoV-2-infected NHBE cell, Cytoscape**
576 **file.**

577 **Supplementary File 2: Functional overrepresentation results.** Reactome and Gene
578 Ontology Biological Processes (q value <= 0.05) for differentially expressed genes (DEGs),
579 protein-protein (PPI) interaction nodes (human binding proteins, signalling proteins and
580 transcription factors) and the clusters of the causal network of SARS-CoV-2-infected NHBE
581 cell.

582 **Supplementary File 3: Function-specific network of SARS-CoV-2- infected NHBE cells,**
583 **Cytoscape file.**

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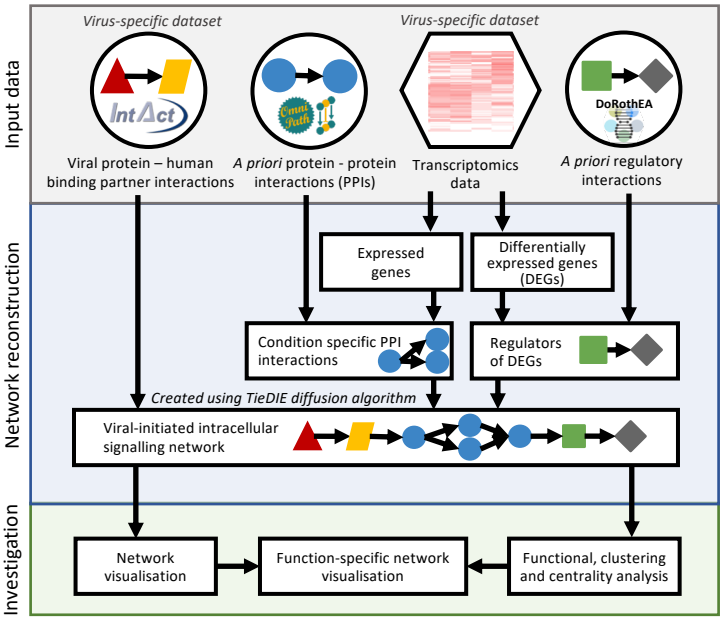
Figure 1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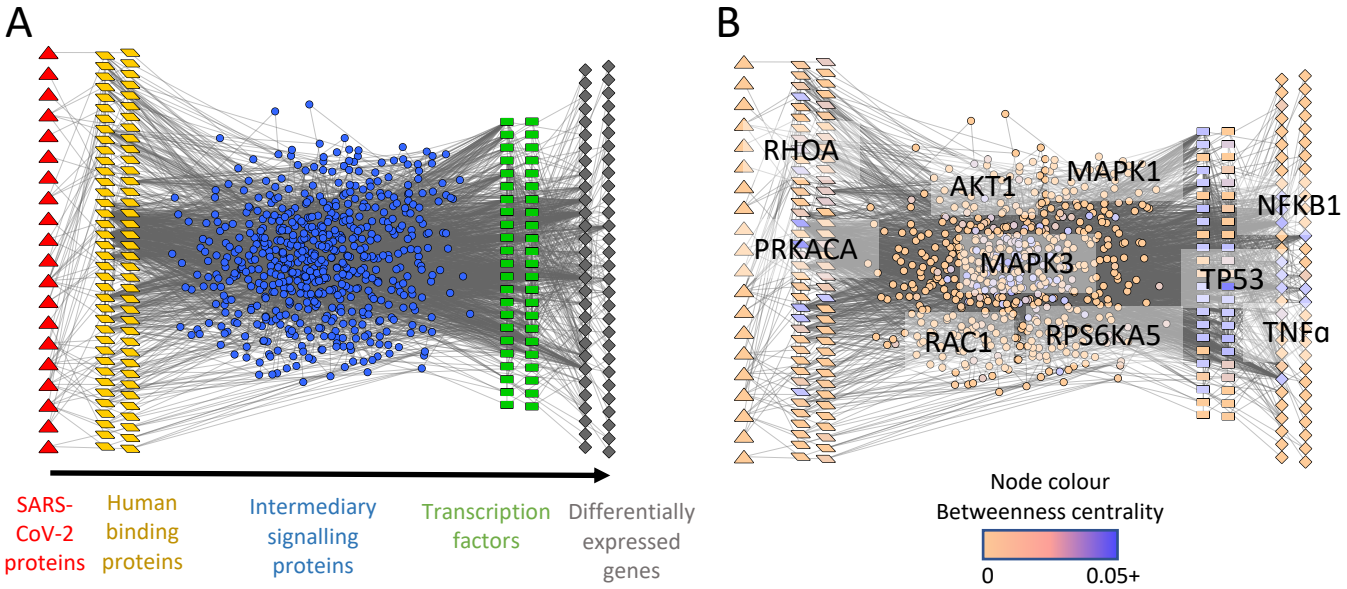
Virus-specific dataset
IntAct
OmniPath
DoRothEA
Viral protein – human binding partner interactions
A priori protein - protein interactions (PPIs)
Transcriptomics data
A priori regulatory interactions
Network reconstruction
Expressed genes
Differentially expressed genes (DEGs)
Condition specific PPI interactions
Regulators of DEGs
Created using TieDIE diffusion algorithm
Viral-initiated intracellular signalling network
Investigation
Network visualisation
Function-specific network visualisation
Functional, clustering and centrality analysis

Figure 2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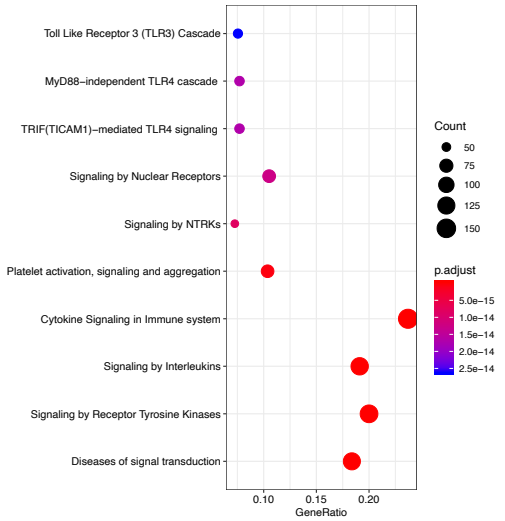
SARS-CoV-2 proteins
Human binding proteins
Intermediary signalling proteins
Transcription factors
Differentially expressed genes
B
RHOA
PRKACA
AKT1
MAPK1
MAPK3
RAC1
RPS6KA5
NFKB1
TP53
TNFα
Node colour
Betweenness centrality
0
0.05+

Supplementary figure 1

A



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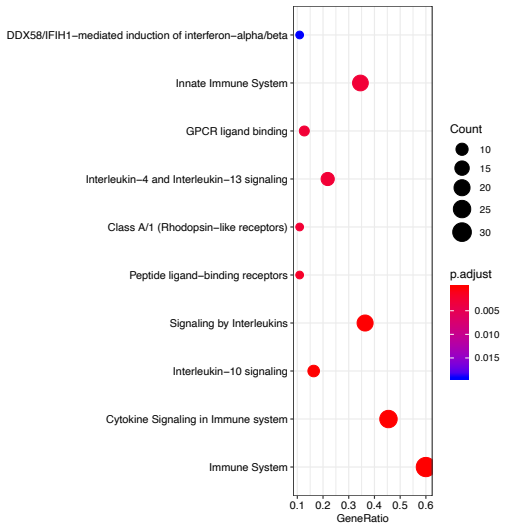

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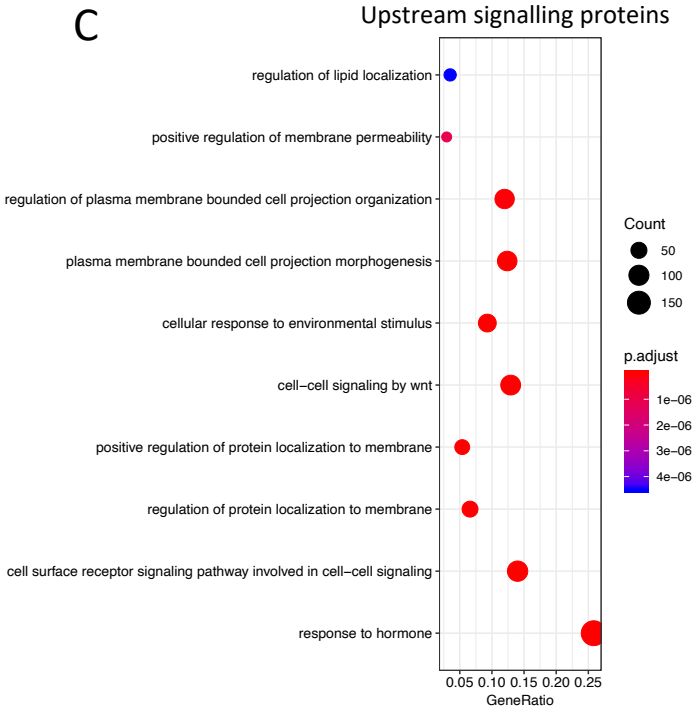

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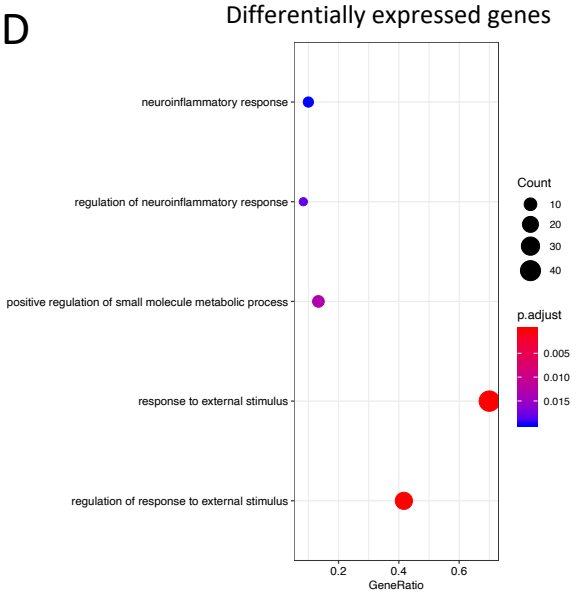
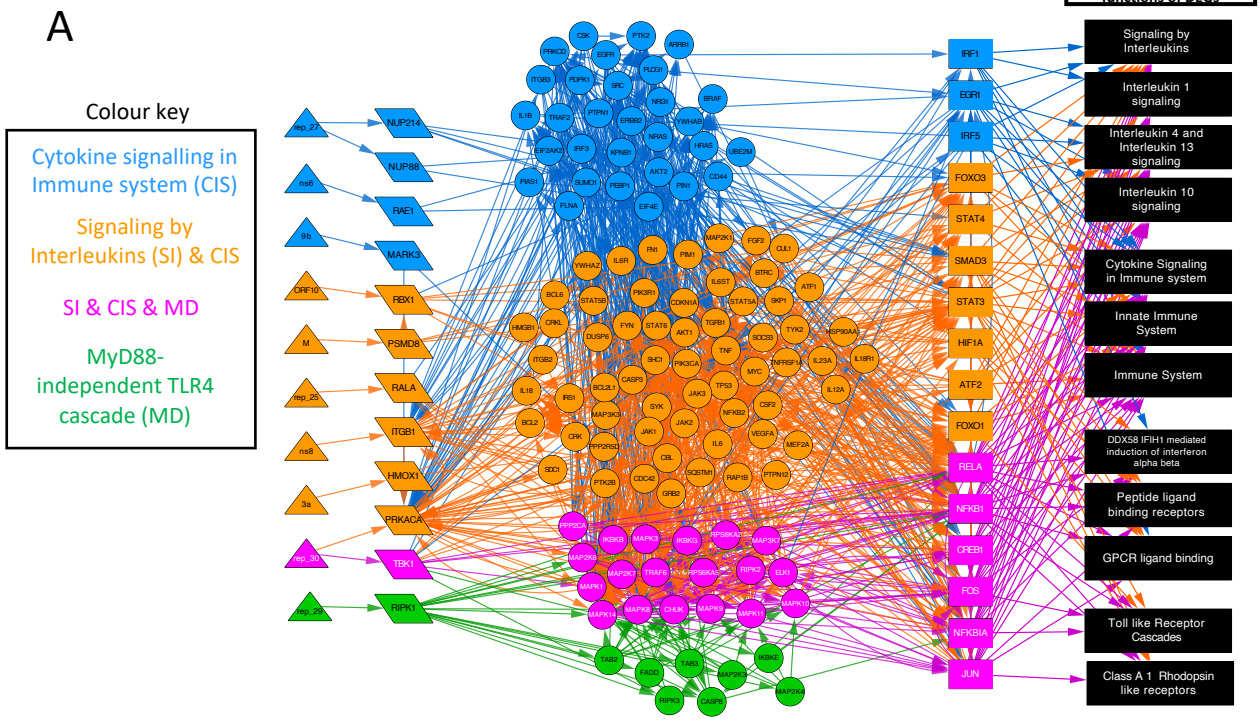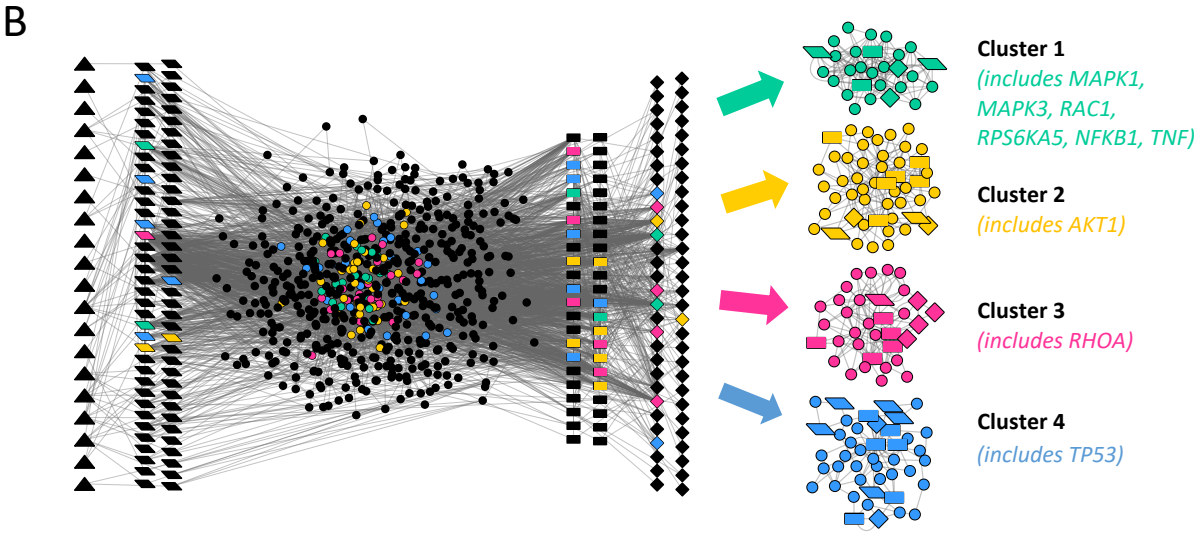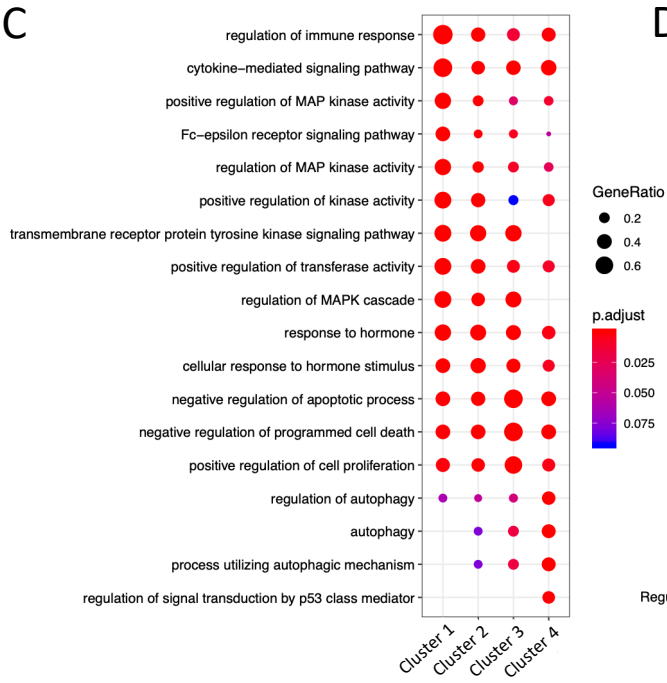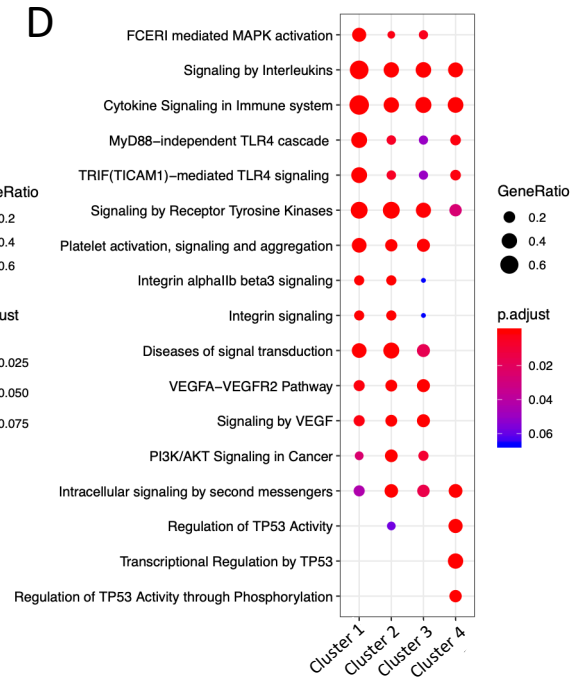
Supplementary figure 2