COST CHARME SUMMER TRAINING SCHOOL
ISTANBUL – 2019

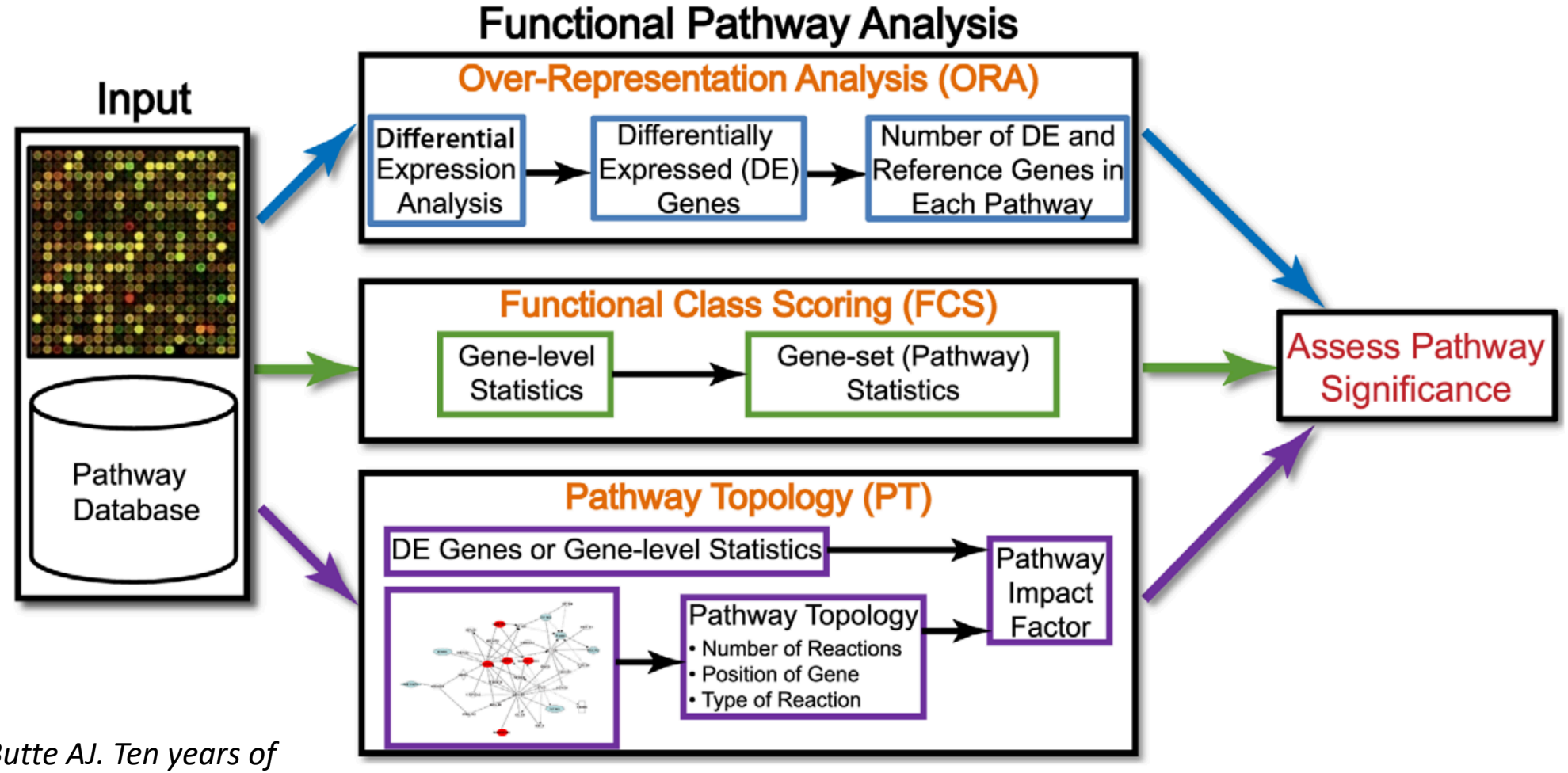
Overview

Background

- One of the most common use cases of NGS technologies is to perform experiments comparing two groups of samples (typically disease versus control) to identify **a list of significant (altered) genes**
- This list alone often falls short of providing mechanistic insights into the underlying biology of the disease being studied

- To **reduce the complexity of analysis** while **simultaneously providing great explanatory power**, one can investigate groups of genes that function in the same pathways/gene sets: **pathway analysis**

Background



Khatri P, Sirota M, Butte AJ. Ten years of pathway analysis: current approaches and outstanding challenges. PLoS Comput Biol. 2012;8(2):e1002375.

Motivation

- Utilizing protein-protein interaction information **enhances** pathway enrichment results
  - Successful applications include GNEA, EnrichNet, NetPEA

Liu M, Liberzon A, Kong SW, et al. Network-based analysis of affected biological processes in type 2 diabetes models. PLoS Genet. 2007;3(6):e96.
Glaab E, Baudot A, Krasnogor N, Schneider R, Valencia A. EnrichNet: network-based gene set enrichment analysis. Bioinformatics. 2012;28(18):i451-i457.
Liu L, Wei J, Ruan J. Pathway Enrichment Analysis with Networks. Genes (Basel). 2017;8(10)

- With pathfindR, our aim was likewise to **exploit interaction information** to extract the most relevant pathways

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pathfindR: An R Package for Pathway Enrichment Analysis Utilizing Active Subnetworks

Ege Ulgen, Ozan Ozisik, Osman Ugur Sezerman

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METHODS ARTICLE Provisionally accepted The full-text will be published soon. Notify me

Front. Genet. | doi: 10.3389/fgene.2019.00858

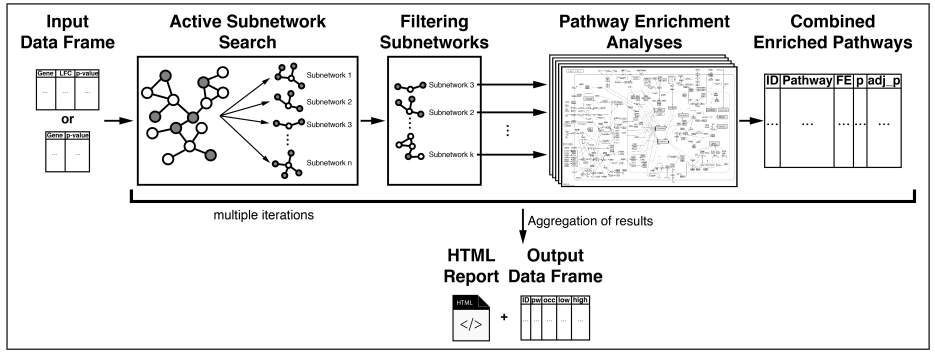
frontiers in Genetics

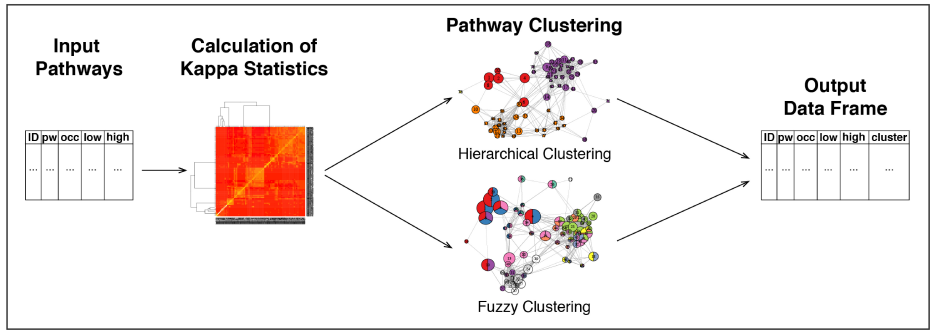
pathfindR: An R Package for Comprehensive Identification of Enriched Pathways in Omics Data Through Active Subnetworks

 **Ege Ulgen**[1*], **Ozan Ozisik**[2] **and Osman U. Sezerman**[1]

[1]Acıbadem University, Turkey

[2]Yıldız Technical University, Turkey



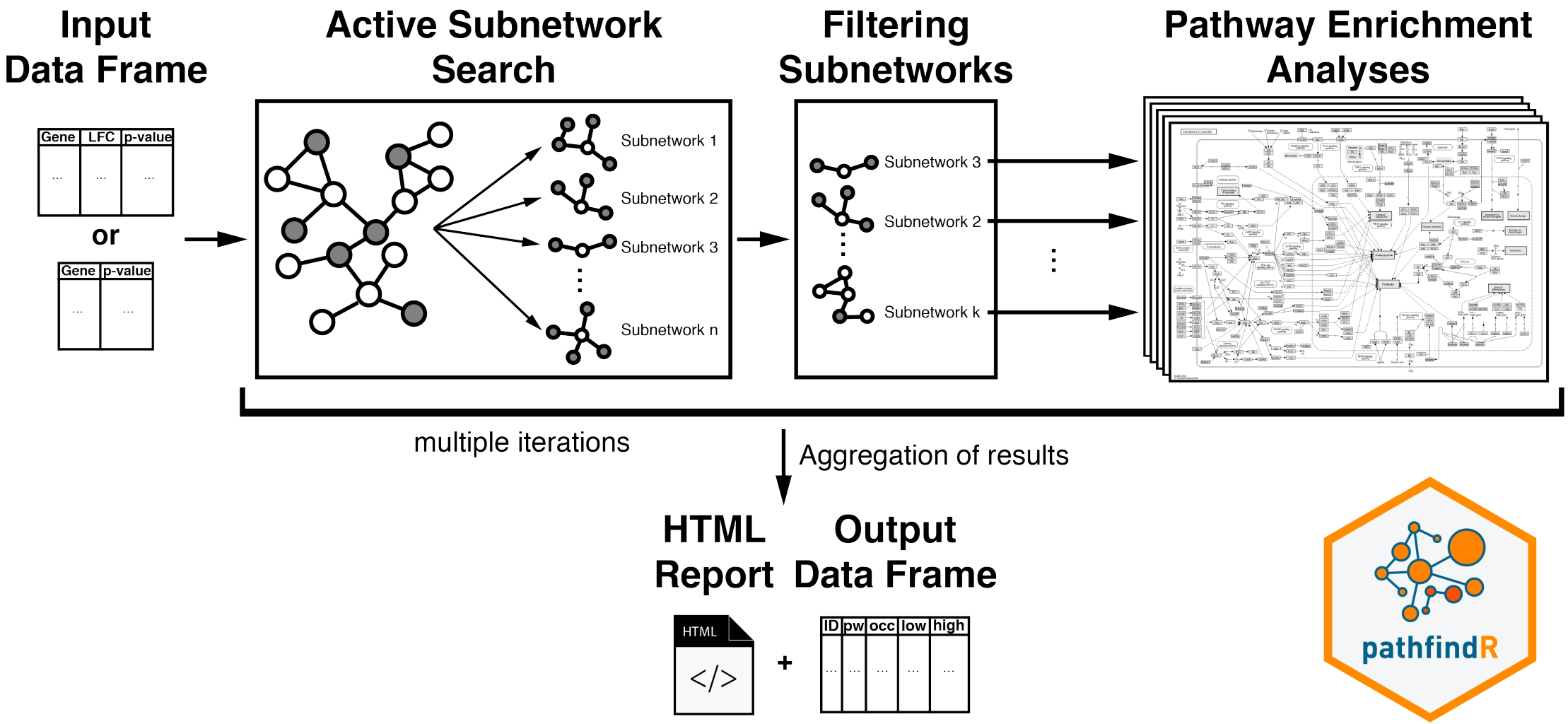


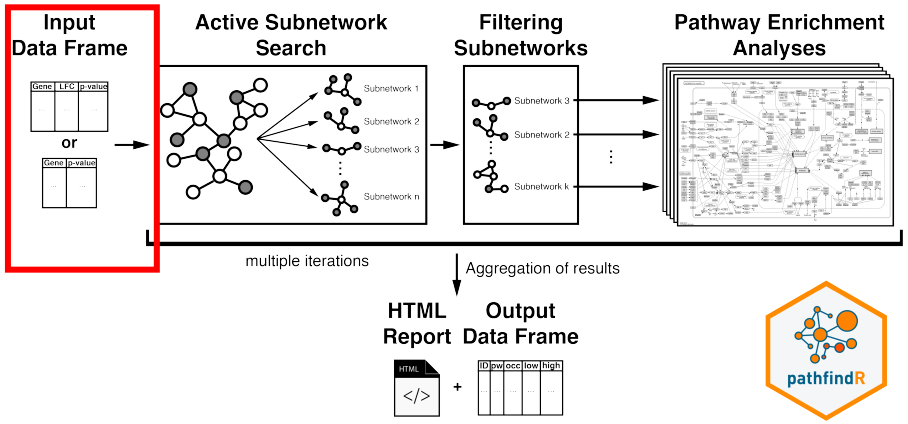
- Using input genes, pathfindR identifies sets of genes that form **active subnetworks** within a protein-protein interaction network
- It then performs **pathway enrichment analyses** on the identified active subnetworks

- Additionally, pathfindR provides functionality to:
  - **Cluster enriched pathways**
  - **Score overall pathway activity**
  - **Visualization of analyses**

pathfindR

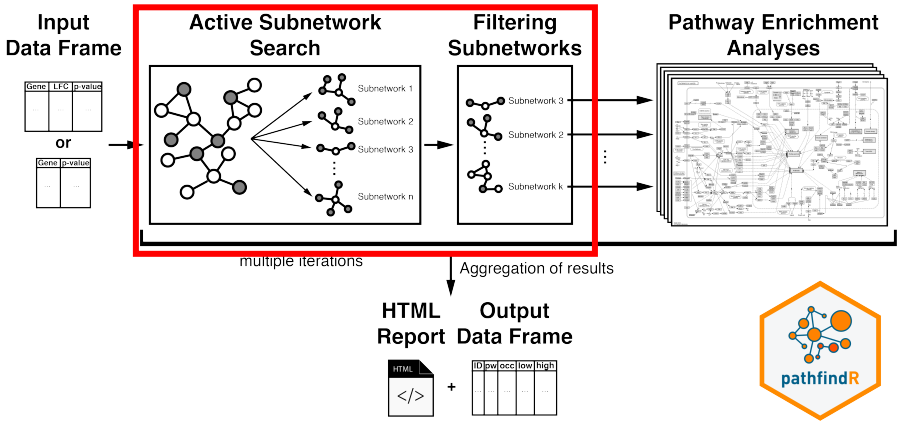
Active Snw.-oriented Enrichment Workflow





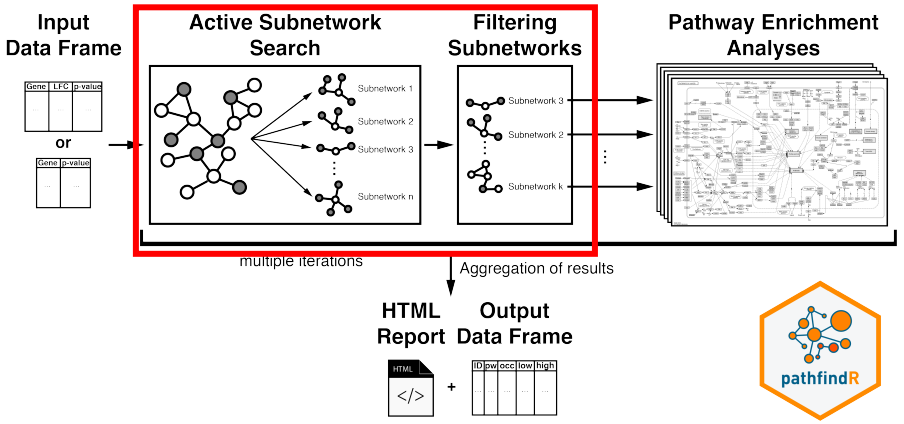
Gene Symbol	Change Value (OPTIONAL)	Adjusted p-value
FAM110A	-0.6939359	0.0000034
RNASE2	1.3535040	0.0000101
S100A8	1.5448338	0.0000347
S100A9	1.0280904	0.0002263
TEX261	-0.3235994	0.0002263
ARHGAP17	-0.6919330	0.0002708

⋮



- Active Subnetwork Search Algorithms:
  - Greedy Algorithm
  - Simulated Annealing
  - Genetic Algorithm

- Available Protein Interaction Networks (PINs):
  - Biogrid
  - GeneMania
  - IntAct
  - KEGG PIN*
  - **Custom PIN**



Active Subnetwork Search

Scoring of Subnetworks

In pathfindR, we followed the scoring scheme that was proposed by Ideker et al., 2002). The p value of each gene is converted to a z score using equation (1), and the score of a subnetwork is calculated using equation (2). In equation (1) Φ^{-1} is the inverse normal cumulative distribution function. In equation (2), A is the set of genes in the subnetwork and k is its cardinality.

$$z_i = \Phi^{-1}(1 - p_i) \quad (1)$$

$$z_A = \frac{1}{\sqrt{k}} \sum_{i \ A} z_i \quad (2)$$

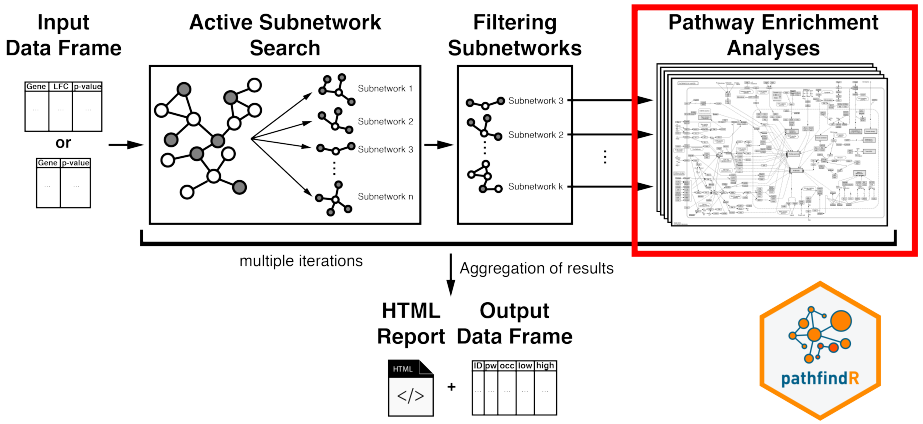
In the same scoring scheme, a Monte Carlo approach is used for the calibration of the scores of subnetworks against a background distribution. Using randomly selected genes, 2,000 subnetworks of each possible size are constructed, and for each possible size, the mean and standard deviation of the score is calculated. These values are used to calibrate the subnetwork score using equation (3).

$$s_A = \frac{(z_A - \mu_k)}{\sigma_k} \quad (3)$$

Subnetwork filtering

An active subnetwork passes the filter if it:

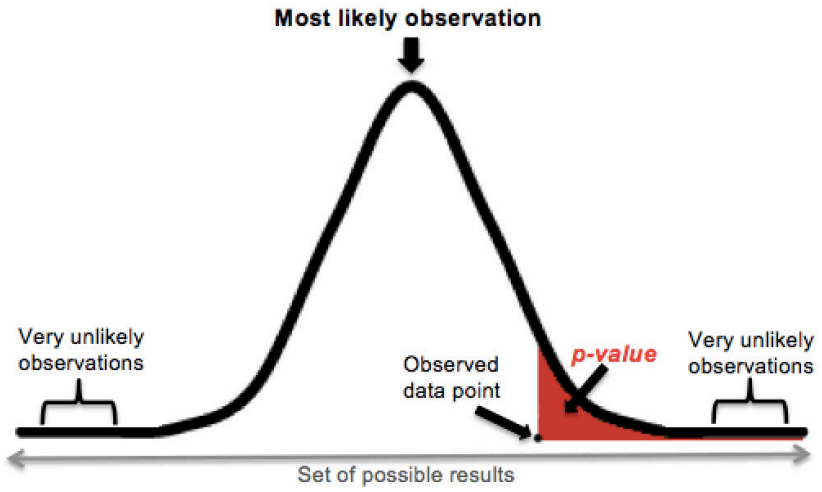
1. has a score larger than the given quantile threshold (default is 0.80) **and**
2. contains at least a specified number of 517 input genes (default is 10).



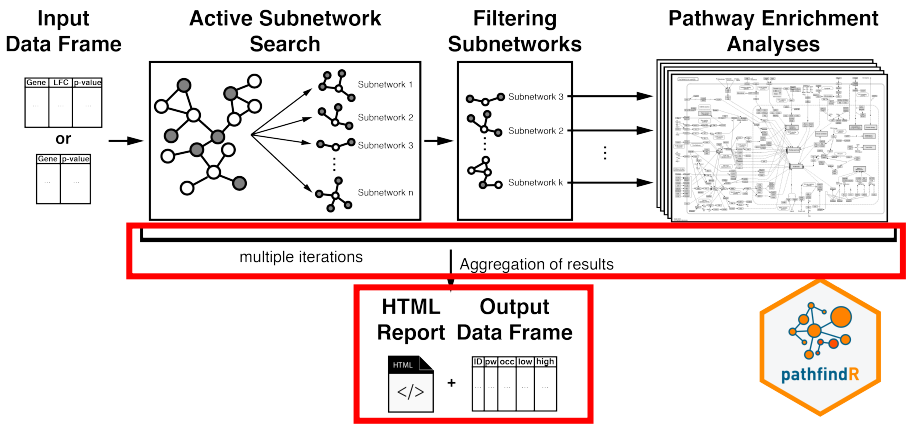
One-sided Hypergeometric Testing

- Available gene sets/pathways:
  - KEGG
  - Reactome
  - BioCarta
  - Gene Ontology gene sets
    - GO – All
    - GO – BP
    - GO – CC
    - GO – MF
  - **Custom gene sets/pathways**

$$P(X = k) = \frac{\binom{K}{k}\binom{N-K}{n-k}}{\binom{N}{n}}$$



A *p-value* (shaded red area) is the probability of an observed (or more extreme) result arising by chance



ID	Pathway	Fold_Enrichment	occurrence	lowest_p	highest_p	Up_regulated	Down_regulated
hsa00190	Oxidative phosphorylation	71.86252	10	3e-07	3e-07	NDUFB3, NDUFA1, COX7C, COX7A2, UQCRQ, COX6A1, ATP6V0E1, ATP6V1D	ATP6V0E2
hsa05012	Parkinson's disease	63.72714	10	4e-07	4e-07	NDUFA1, NDUFB3, UQCRQ, COX6A1, COX7A2, COX7C	SLC25A5, VDAC1, UBE2G1

⋮

pathfindR - Results

25 November, 2018

pathfindR-Enrichment results are presented below:

All pathways found to be enriched

A table that lists all pathways found to be enriched as well as lists of up- or down-regulated genes for each pathway. If it was requested, the pathway descriptions (names) are linked to the visualizations of these pathways with differentially-expressed genes colored according to change values.

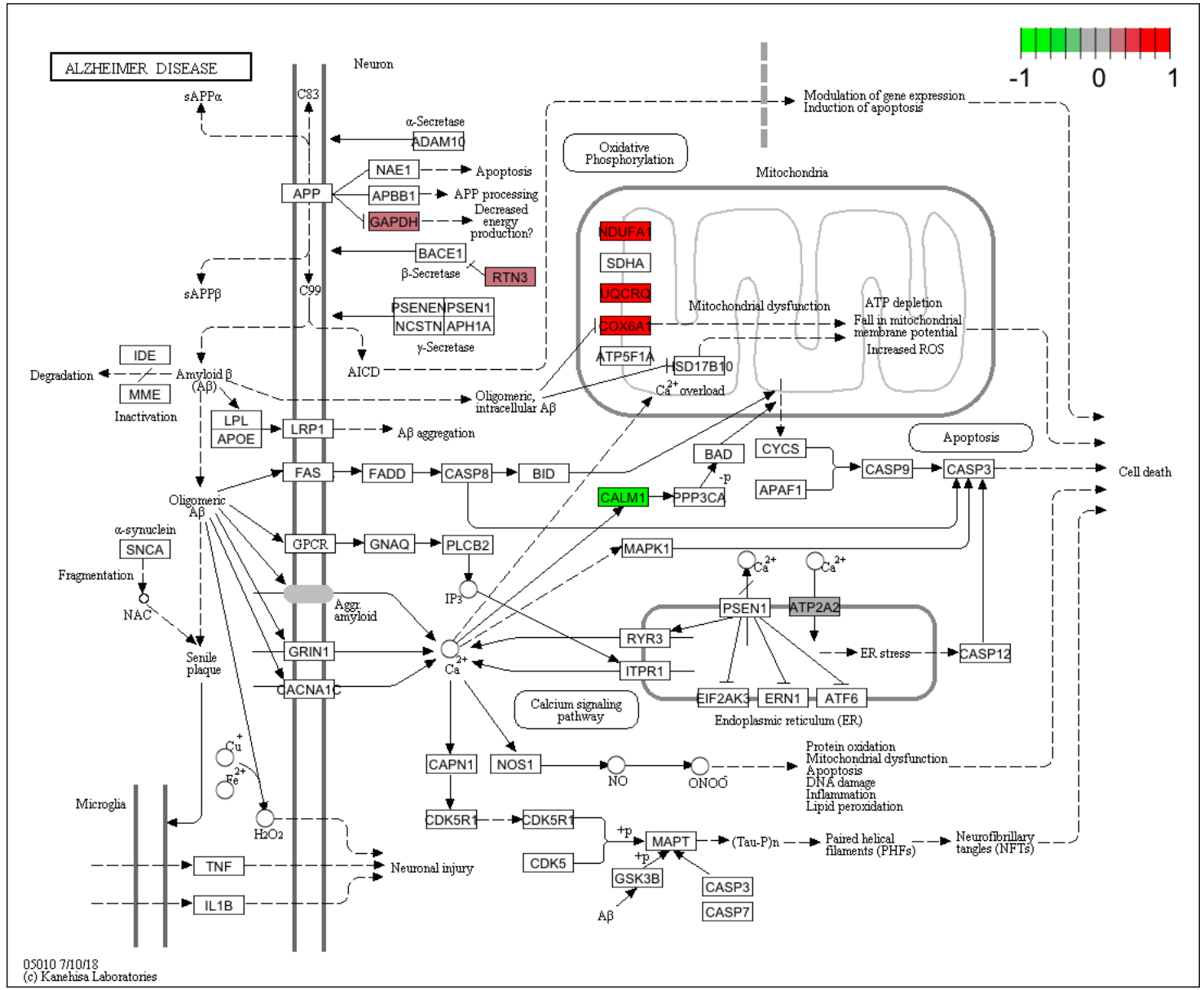
Tables of genes with converted gene symbols and genes without interactions

- A table listing the genes whose symbols (Old Symbol) were converted to aliases (Converted Symbol) that were in the protein-protein interaction network.
- A table listing the input genes for which no interactions in the PIN were found (after the aliases were also checked).

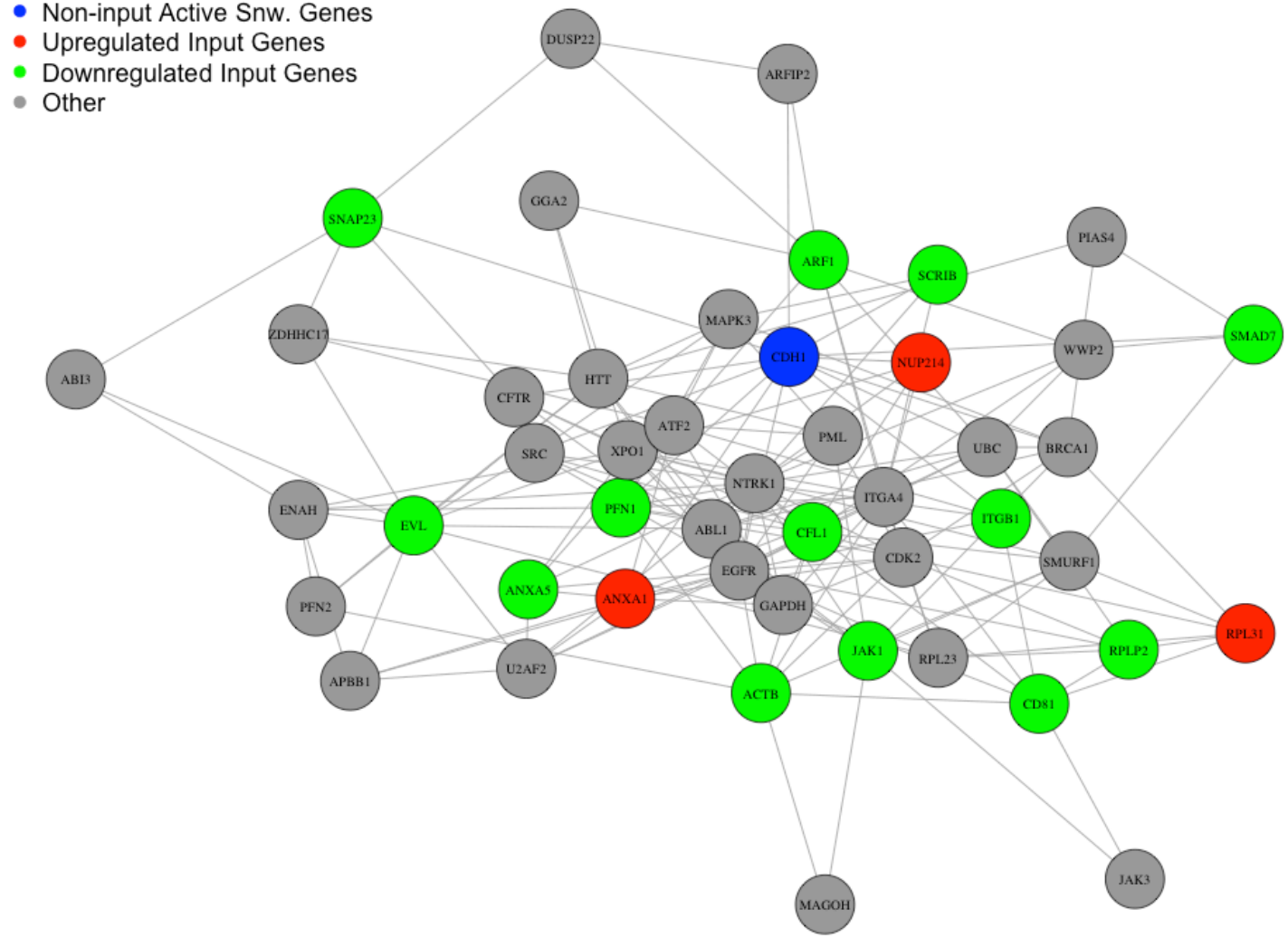
pathfindR - All Enriched Pathways - KEGG

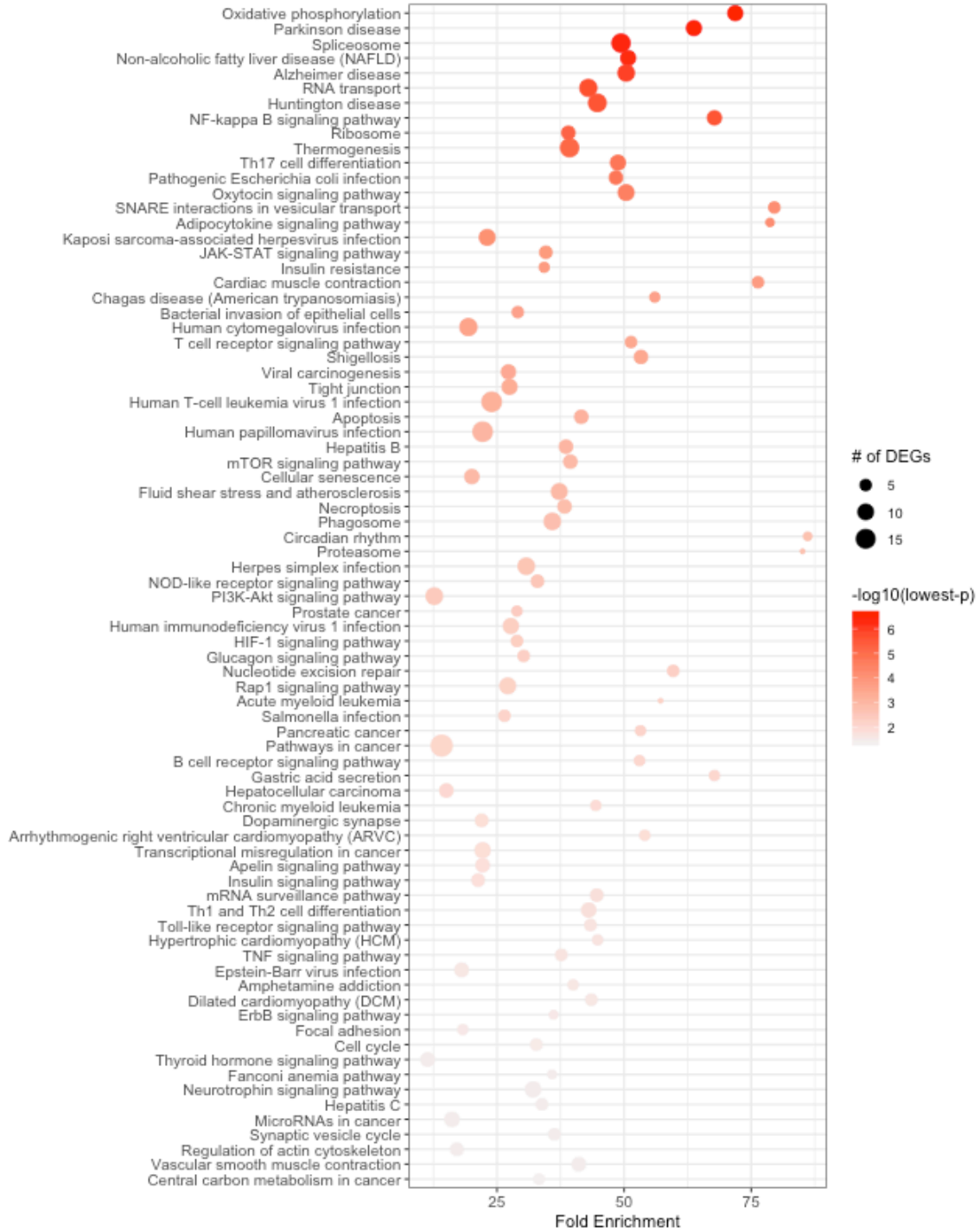
KEGG pathway visualizations were performed via the R Biocondutor package __pathview__ [1].

ID	Pathway	Fold_Enrichment	occurrence	lowest_p	highest_p	Up_regulated	Down_regulated
hsa00190	Oxidative phosphorylation	2.52036	10	2.6e-07	2.6e-07	NDUFA1, NDUFB3, UQCRQ, COX6A1, COX7A2, COX7C, ATP6V1D, ATP6V0E1	ATP6V0E2
hsa05012	Parkinson disease	2.23503	10	3.9e-07	3.9e-07	NDUFA1, NDUFB3, UQCRQ, COX6A1, COX7A2, COX7C	UBE2G1, VDAC1, SLC25A5
hsa03040	Spliceosome	2.90183	10	4.8e-07	4.8e-07	SF3B6, LSM3, BUD31	SNRPB, SF3B2, U2AF2, PUF60, SNU13, DDX23, EIF4A3, HNRNPA1, PCBP1, SRSF8, SRSF5
hsa04932	Non-alcoholic fatty liver disease (NAFLD)	1.78131	10	5.2e-07	5.2e-07	DDIT3, NDUFA1, NDUFB3, UQCRQ, COX6A1, COX7A2, COX7C	IKBKB, FASLG
hsa03010	Ribosome	1.47413	10	1.1e-06	6.4e-06	MRPS18C, RPS24, MRPL33, RPL26, RPL31, RPL39	RPLP2
hsa05010	Alzheimer disease	2.16090	10	1.2e-06	1.2e-06	GAPDH, RTN3, NDUFA1, NDUFB3, UQCRQ, COX6A1, COX7A2, COX7C	CALM3, CALM1, ATP2A2
hsa03013	RNA transport	2.16360	10	2.1e-06	7.7e-05	NUP214	NUP62, NUP93, RANGAP1, UBE2I, SUMO3, GEMIN4, EIF2S3, EIF2B1, EIF4A3, RNPS1, SRRM1


ALZHEIMER DISEASE
Neuron
-1
0
1
sAPPα
C83
α-Secretase
ADAM10
Modulation of gene expression
Induction of apoptosis
Oxidative Phosphorylation
Mitochondria
NAE1
Apoptosis
APP
APBB1
APP processing
GAPDH
Decreased energy production?
NDUFA1
SDHA
UQCRQ
COX6A1
ATP5F1A
SD17B10
Mitochondrial dysfunction
ATP depletion
Fall in mitochondrial membrane potential
Increased ROS
Ca2+ overload
BACE1
β-Secretase
RTN3
sAPPβ
C99
PSENEN
PSEN1
NCSTN
APH1A
γ-Secretase
AICD
IDE
Degradation
MME
Inactivation
Amyloid β (Aβ)
Oligomeric, intracellular Aβ
LPL
APOE
LRP1
Aβ aggregation
BAD
CYCS
Apoptosis
FAS
FADD
CASP8
BID
CASP9
CASP3
Cell death
APAF1
CALM1
PPP3CA
Oligomeric Aβ
α-synuclein
SNCA
Fragmentation
NAC
GPCR
GNAQ
PLCB2
MAPK1
Aggr. amyloid
IP3
Ca2+
PSEN1
ATP2A2
RYR3
GRIN1
ITPR1
ER stress
CASP12
CACNA1C
Senile plaque
Calcium signaling pathway
EIF2AK3
ERN1
ATF6
Endoplasmic reticulum (ER)
Cu+
Fe2+
CAPN1
NOS1
NO
ONOO-
Protein oxidation
Mitochondrial dysfunction
Apoptosis
DNA damage
Inflammation
Lipid peroxidation
Microglia
H2O2
CDK5R1
CDK5R1
+p
MAPT
(Tau-P)n
Paired helical filaments (PHFs)
Neurofibrillary tangles (NFTs)
CDK5
+p
GSK3B
CASP3
CASP7
Aβ
TNF
IL1B
Neuronal injury
05010 7/10/18
(c) Kanehisa Laboratories

anchoring_junction(3)
Involved Gene Interactions in Biogrid



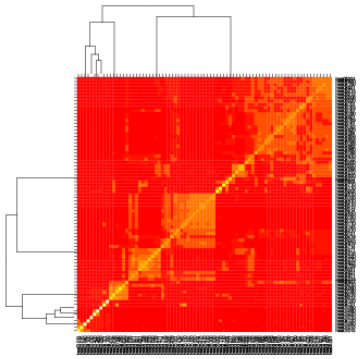

Oxidative phosphorylation
Parkinson disease
Spliceosome
Non-alcoholic fatty liver disease (NAFLD)
Alzheimer disease
RNA transport
Huntington disease
NF-kappa B signaling pathway
Ribosome
Thermogenesis
Th17 cell differentiation
Pathogenic Escherichia coli infection
Oxytocin signaling pathway
SNARE interactions in vesicular transport
Adipocytokine signaling pathway
Kaposi sarcoma-associated herpesvirus infection
JAK-STAT signaling pathway
Insulin resistance
Cardiac muscle contraction
Chagas disease (American trypanosomiasis)
Bacterial invasion of epithelial cells
Human cytomegalovirus infection
T cell receptor signaling pathway
Shigellosis
Viral carcinogenesis
Tight junction
Human T-cell leukemia virus 1 infection
Apoptosis
Human papillomavirus infection
Hepatitis B
mTOR signaling pathway
Cellular senescence
Fluid shear stress and atherosclerosis
Necroptosis
Phagosome
Circadian rhythm
Proteasome
Herpes simplex infection
NOD-like receptor signaling pathway
PI3K-Akt signaling pathway
Prostate cancer
Human immunodeficiency virus 1 infection
HIF-1 signaling pathway
Glucagon signaling pathway
Nucleotide excision repair
Rap1 signaling pathway
Acute myeloid leukemia
Salmonella infection
Pancreatic cancer
Pathways in cancer
B cell receptor signaling pathway
Gastric acid secretion
Hepatocellular carcinoma
Chronic myeloid leukemia
Dopaminergic synapse
Arrhythmogenic right ventricular cardiomyopathy (ARVC)
Transcriptional misregulation in cancer
Apelin signaling pathway
Insulin signaling pathway
mRNA surveillance pathway
Th1 and Th2 cell differentiation
Toll-like receptor signaling pathway
Hypertrophic cardiomyopathy (HCM)
TNF signaling pathway
Epstein-Barr virus infection
Amphetamine addiction
Dilated cardiomyopathy (DCM)
ErbB signaling pathway
Focal adhesion
Cell cycle
Thyroid hormone signaling pathway
Fanconi anemia pathway
Neurotrophin signaling pathway
Hepatitis C
MicroRNAs in cancer
Synaptic vesicle cycle
Regulation of actin cytoskeleton
Vascular smooth muscle contraction
Central carbon metabolism in cancer
25
50
75
Fold Enrichment
# of DEGs
5
10
15
-log10(lowest-p)
6
5
4
3
2

Pathway Clustering Workflow

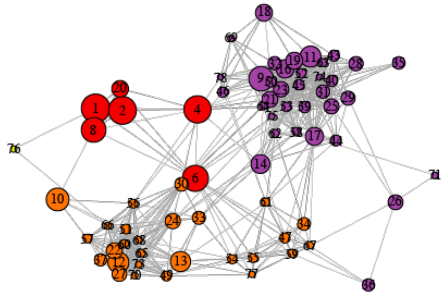
Input Pathways

ID	pw	occ	low	high
...	...	...	...	...

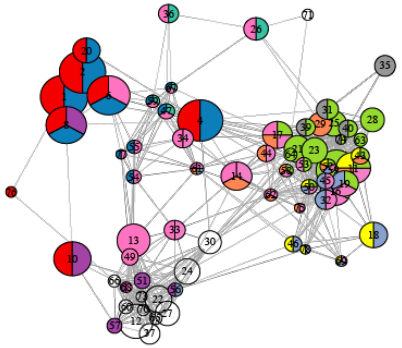
Calculation of Kappa Statistics



Pathway Clustering



Hierarchical Clustering

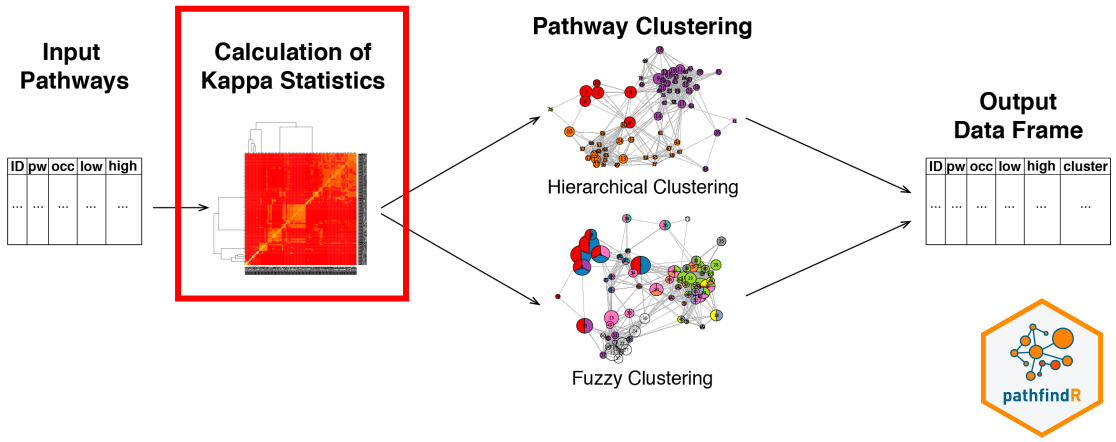


Fuzzy Clustering

Output Data Frame

ID	pw	occ	low	high	cluster
...	...	...	...	...	...

pathfindR



Using

1 – kappa similarity

as distance metric for clustering

(a)

	Cell death	Apoptosis	Ph domain	Sh2 domain	Apoptosis pathway	Membrane
Gene a	1	1	0	0	1	0
Gene b	1	1	0	1	1	0
Gene c	1	0	0	1	1	1
Gene d	1	1	0	0	1	1
Gene e	0	1	1	1	1	1
Gene f	0	0	1	1	0	1
Gene g	0	0	1	1	0	1

(b)

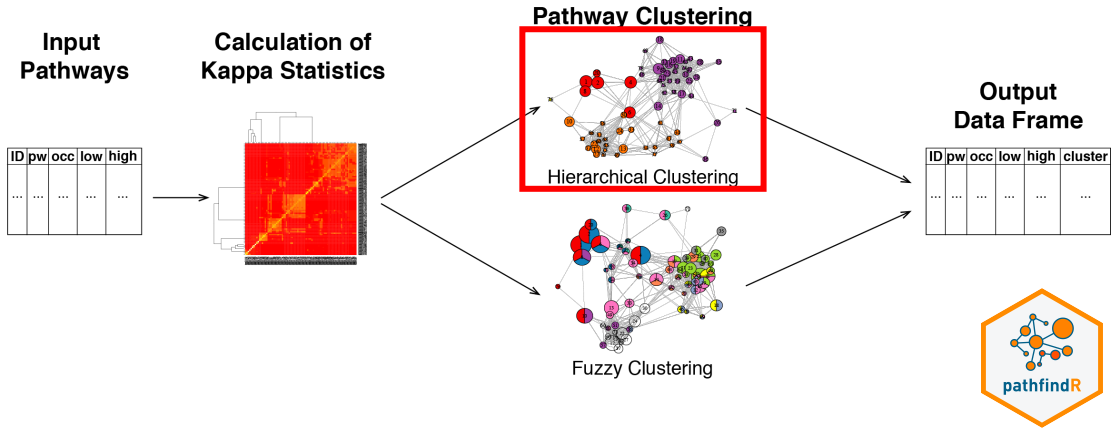
		Gene a: 1	Gene a: 0	Row total
Gene b	1	3 ($C_{1,1}$)	1 ($C_{0,1}$)	4 ($C_{1,\cdot}$)
Gene b	0	0 ($C_{0,1}$)	2 ($C_{0,0}$)	2 ($C_{0,\cdot}$)
Column total		3 ($C_{\cdot,1}$)	3 ($C_{\cdot,0}$)	6 (T_{ab})

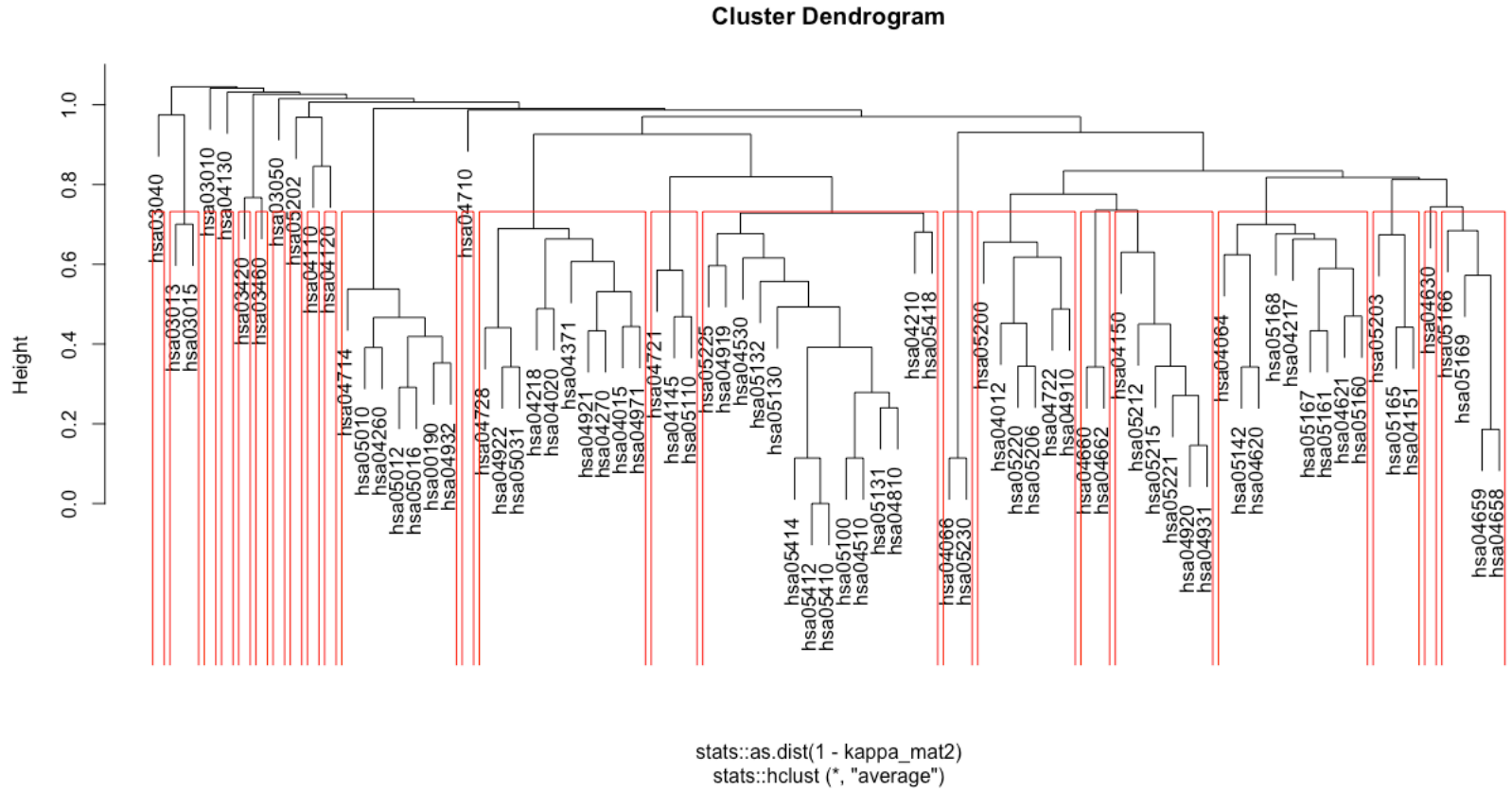
$$O_{ab} = \frac{C_{1,1} + C_{0,0}}{T_{ab}} = \frac{3+2}{6} = 0.83$$

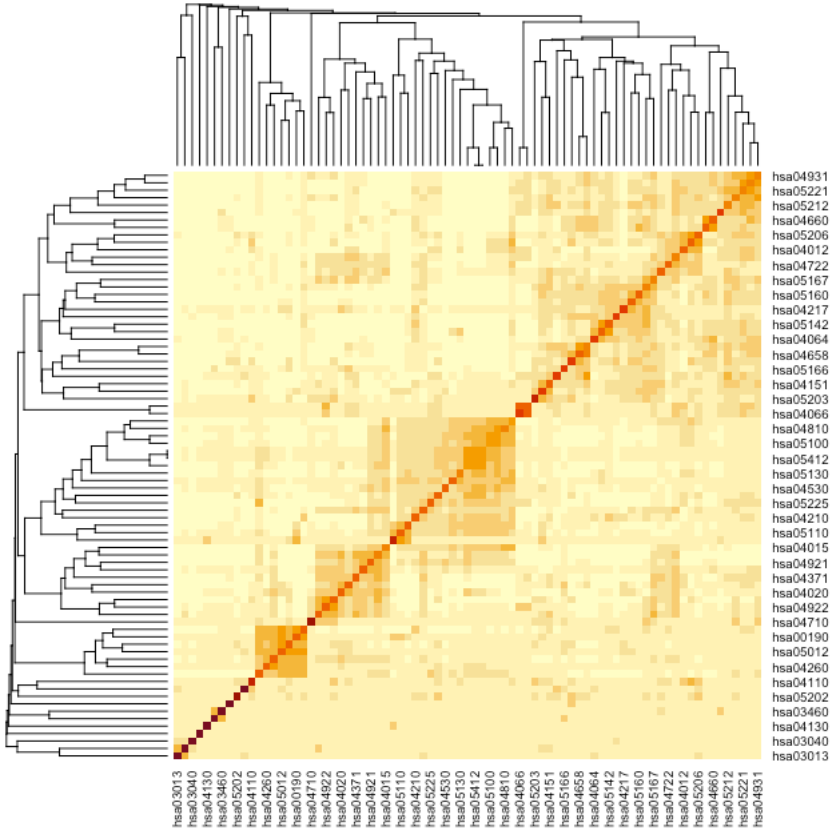
$$A_{ab} = \frac{C_{\cdot,1} \bullet C_{1,\cdot} + C_{\cdot,0} \bullet C_{0,\cdot}}{T_{ab} \bullet T_{ab}} = \frac{3 \bullet 4 + 3 \bullet 2}{6 \bullet 6} = 0.5$$

$$K_{ab} = \frac{O_{ab} - A_{ab}}{1 - A_{ab}} = \frac{0.83 - 0.5}{1 - 0.5} = 0.66$$

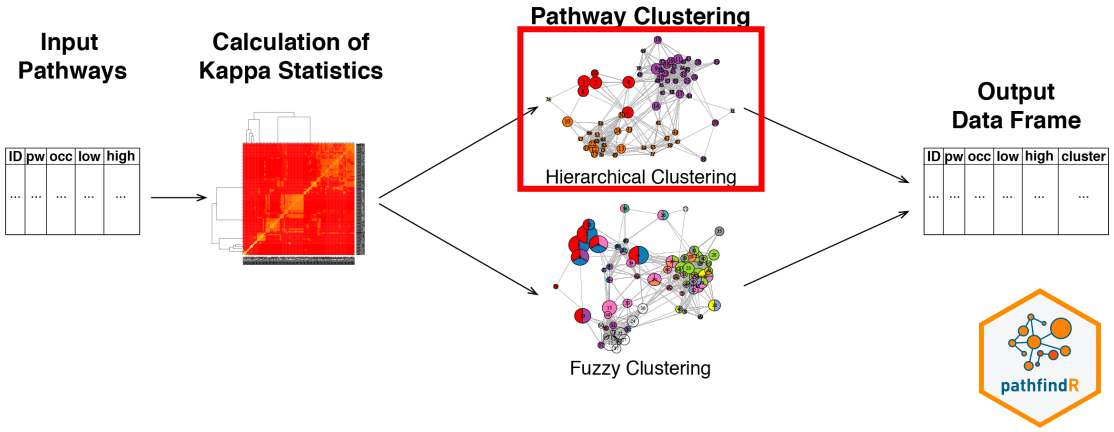
Huang DW, Sherman BT, Tan Q, et al. The DAVID Gene Functional Classification Tool: a novel biological module-centric algorithm to functionally analyze large gene lists. Genome Biol. 2007;8(9):R183.







The optimal number of clusters is automatically determined by maximizing the average silhouette width



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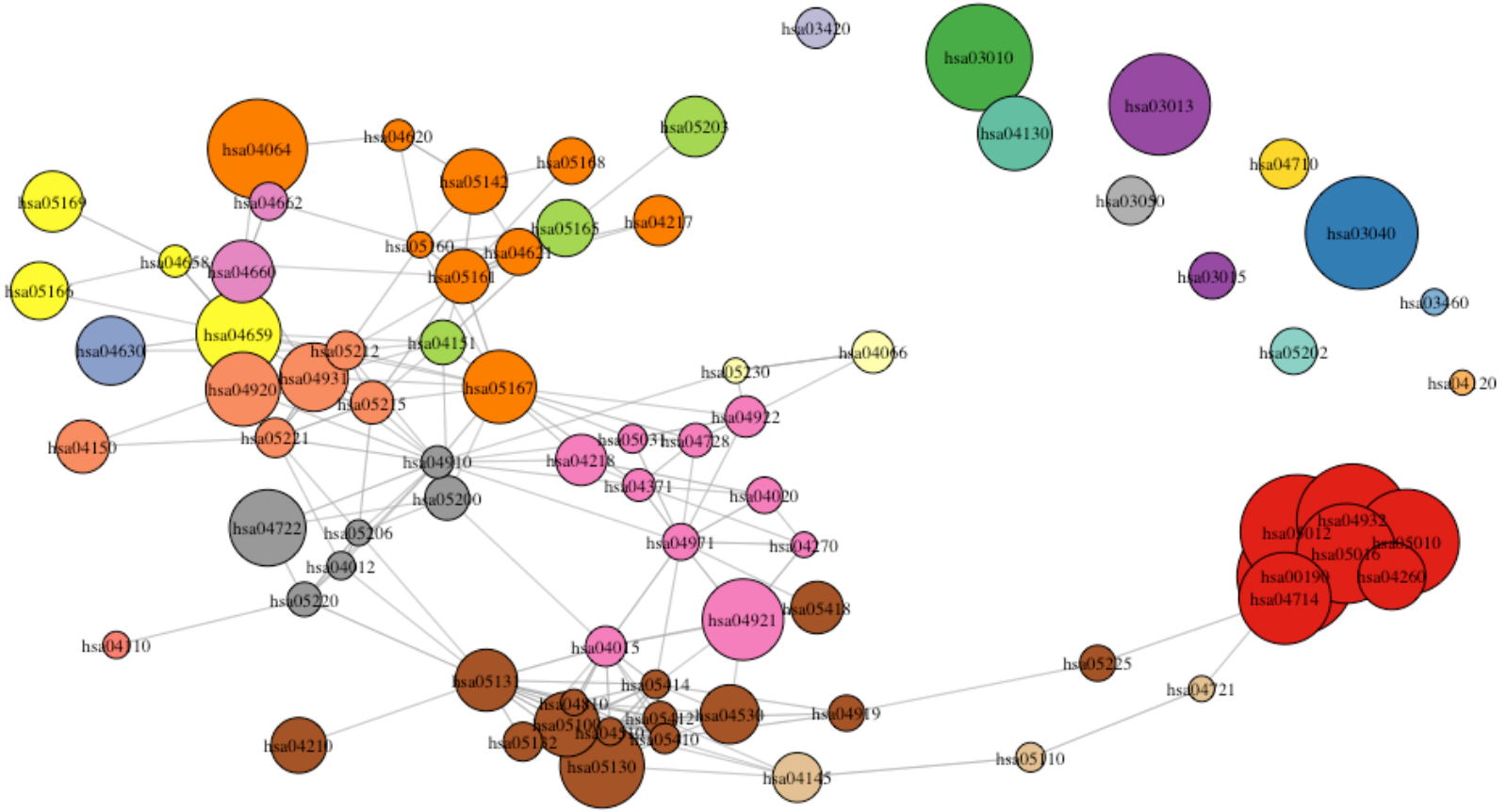

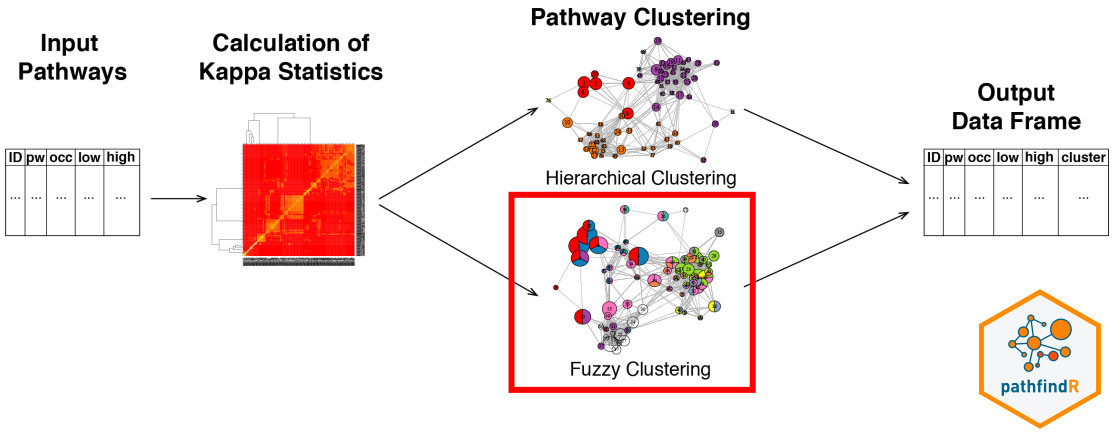

Using

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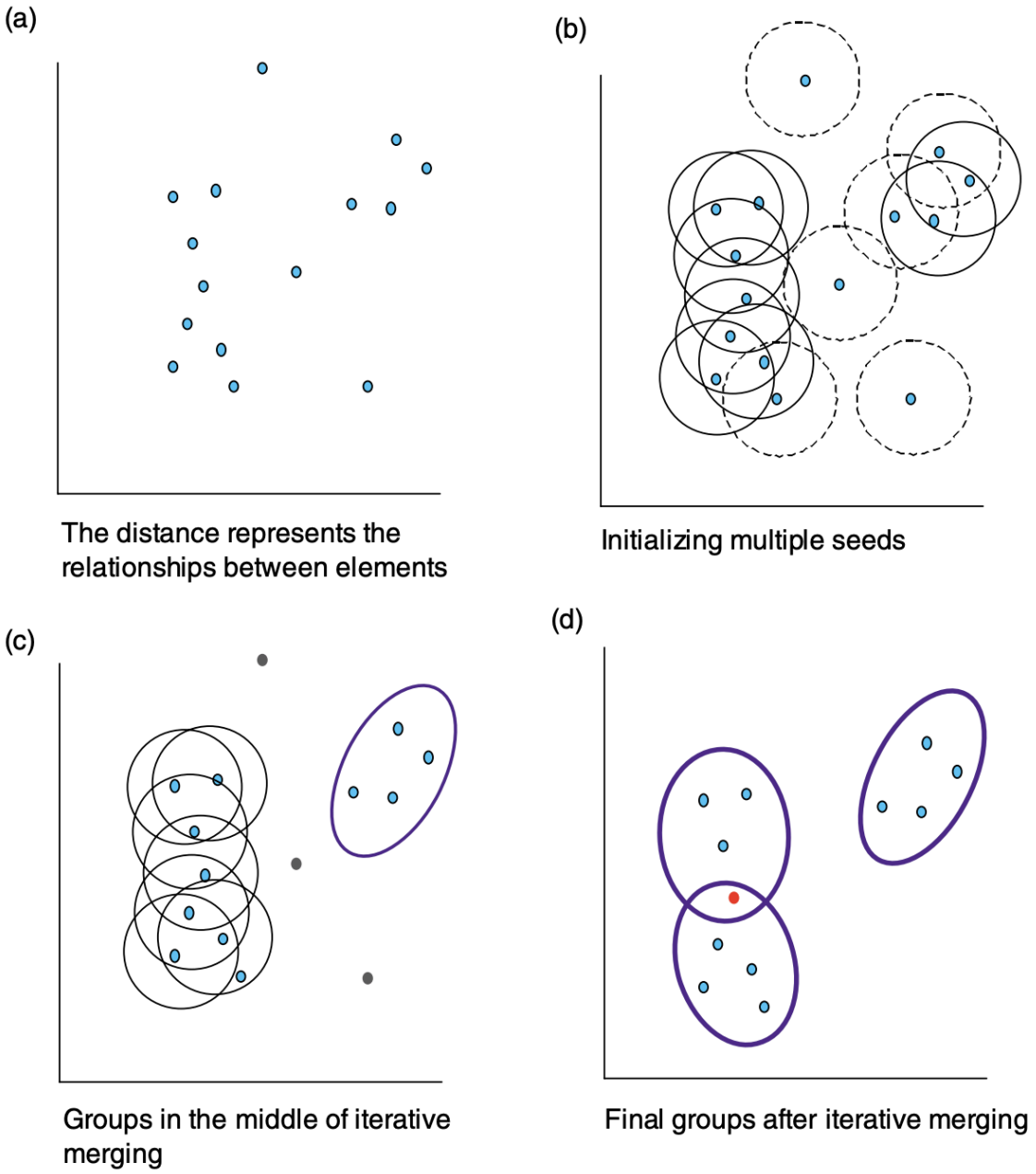
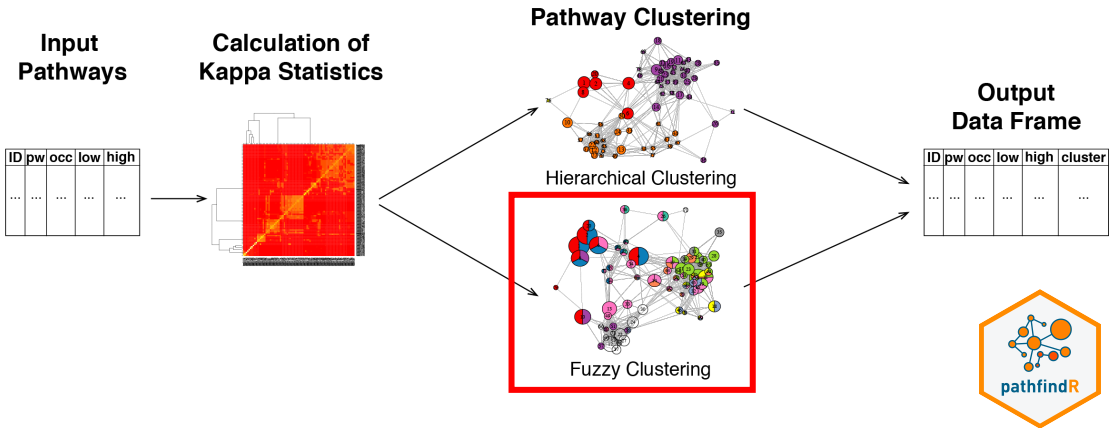
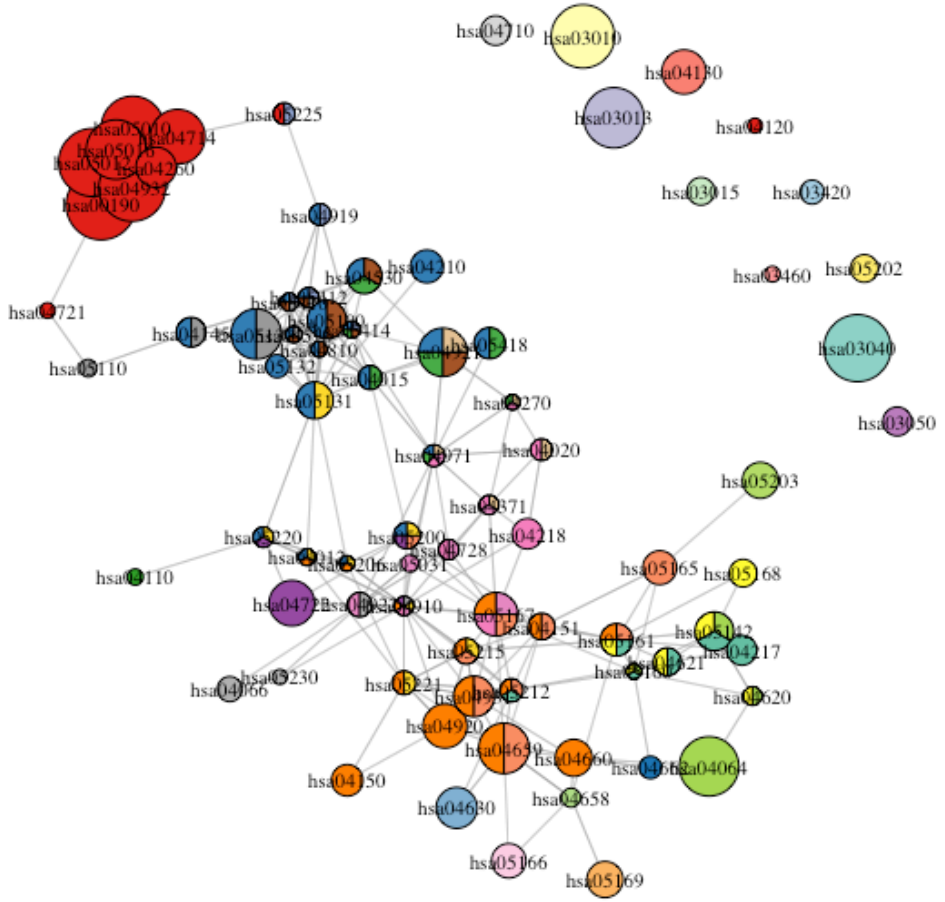
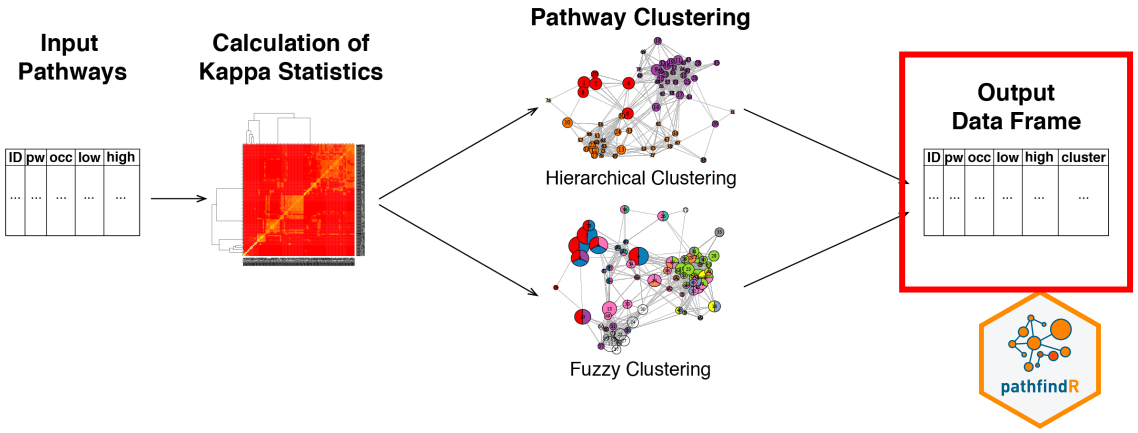
The heuristic fuzzy partition algorithm





No links shown for kappa < 0.35 (default)





Representative pathway selection

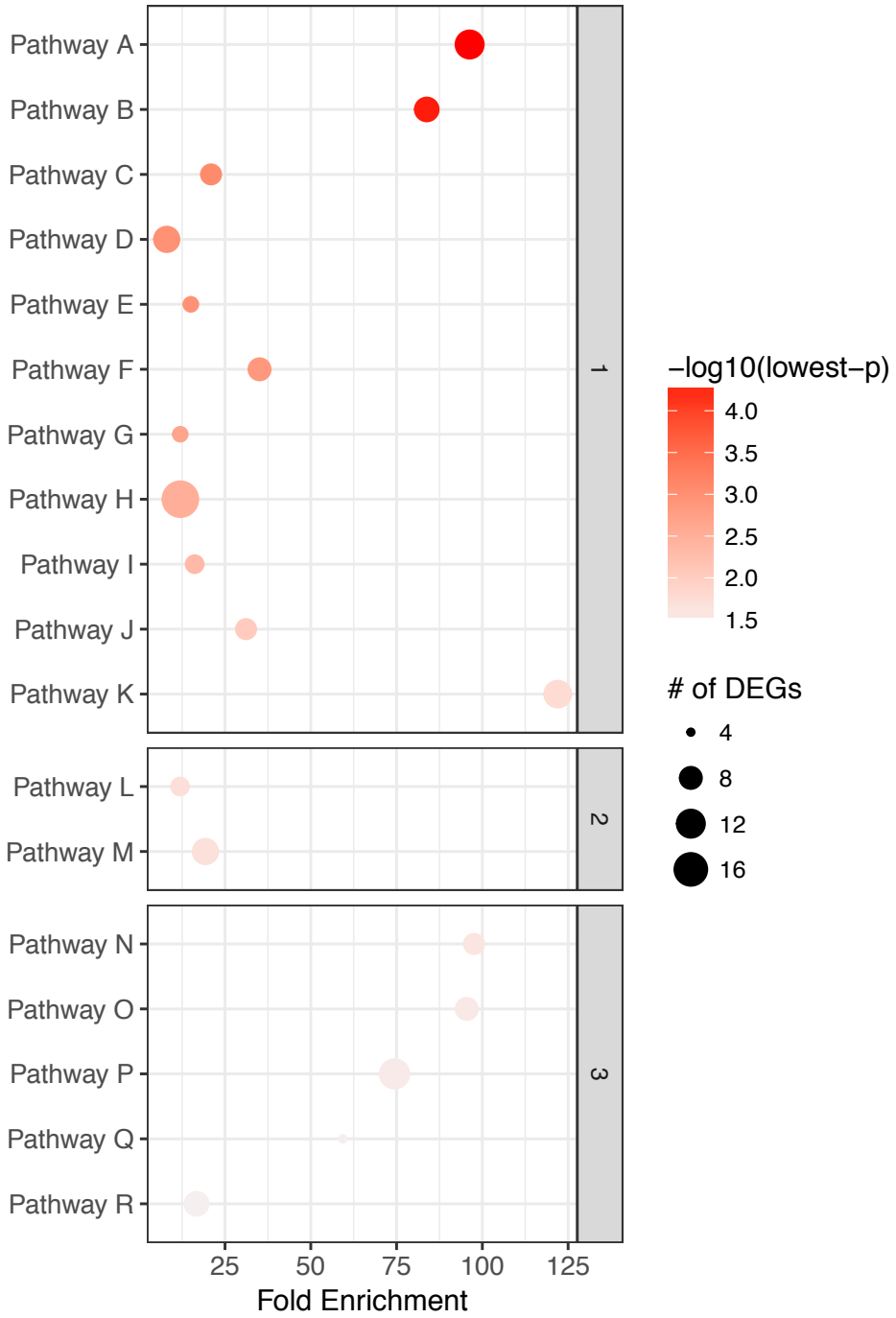
For each cluster, the representative gene set is chosen as the one with the lowest p value (default)

Note that this is an **ad hoc** decision and different approaches may be used:

- Highest fold enrichment
- The most biologically meaningful, etc.

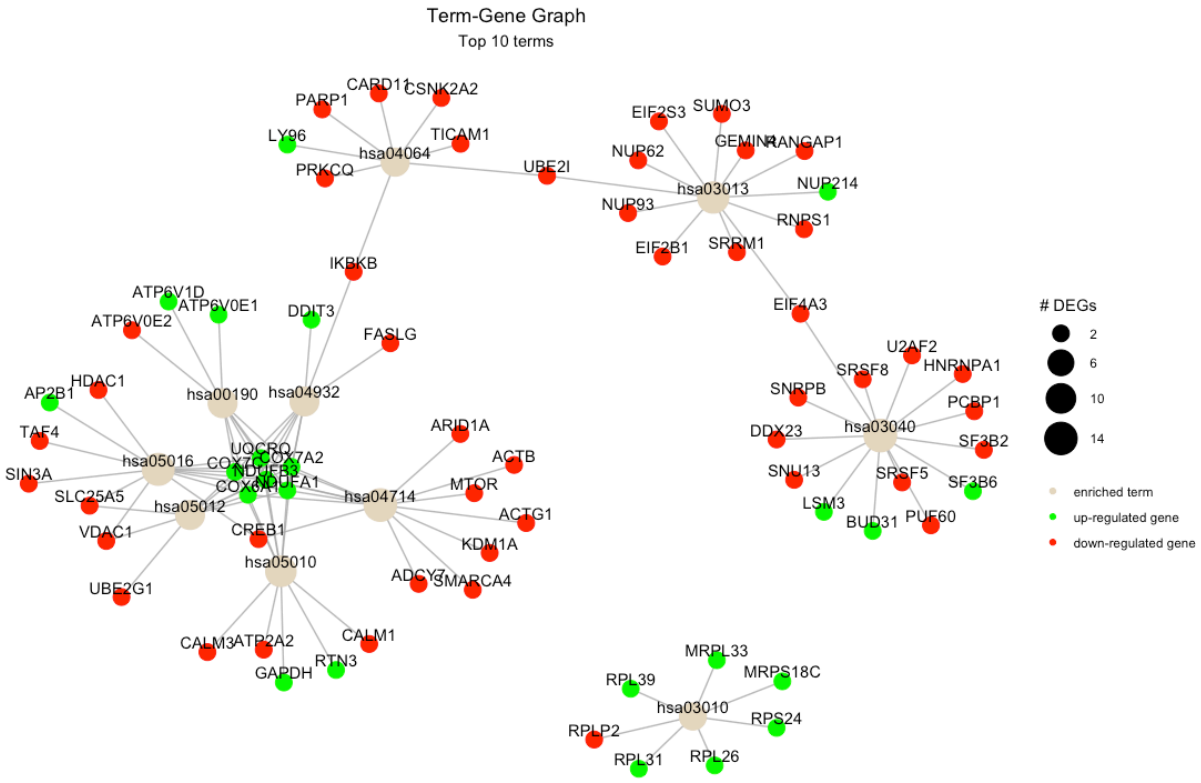
ID	Pathway	Fold_Enrichment	occurrence	lowest_p	highest_p	Up_regulated	Down_regulated	Cluster	Status
hsa00190	Oxidative phosphorylation	71.863	10	2.61E-07	2.61E-07	NDUFB3, NDUFA1, COX7C	ATP6V0E2	1	Representative
hsa05012	Parkinson's disease	63.727	10	3.88E-07	3.88E-07	UQCRQ, COX6A1, COX7A2	VDAC1, UBE2G1	1	Member
hsa04932	Non-alcoholic fatty liver disease (NAFLD)	50.79	10	5.19E-07	5.19E-07	DDIT3,COX6A1, COX7A2	FASLG, IKBKB	2	Representative

⋮

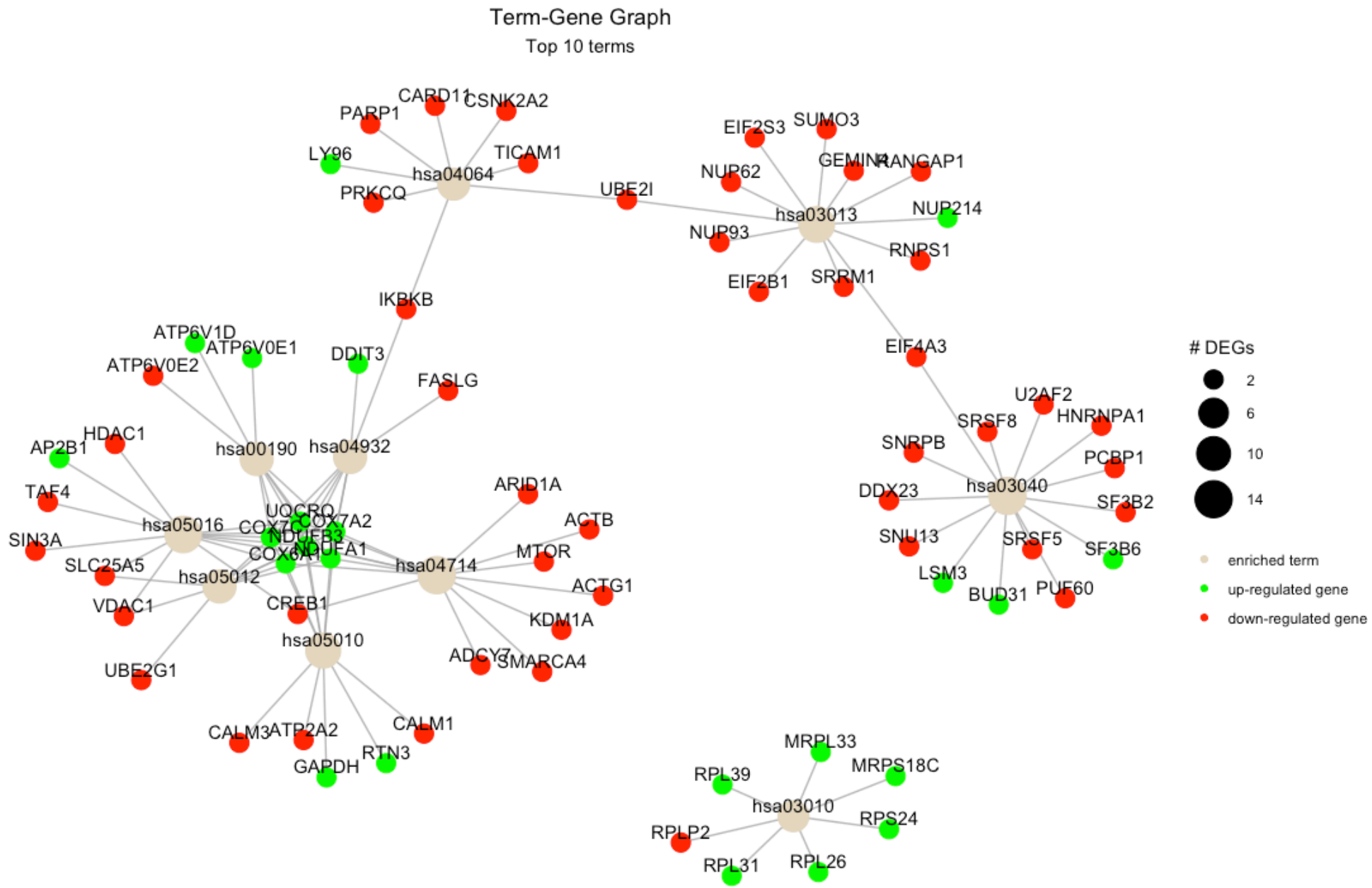

Pathway A
Pathway B
Pathway C
Pathway D
Pathway E
Pathway F
Pathway G
Pathway H
Pathway I
Pathway J
Pathway K
Pathway L
Pathway M
Pathway N
Pathway O
Pathway P
Pathway Q
Pathway R
1
2
3
25
50
75
100
125
Fold Enrichment
−log10(lowest−p)
4.0
3.5
3.0
2.5
2.0
1.5
# of DEGs
4
8
12
16

Term-Gene Graph

- Graph representation of enriched terms and related genes
  - Do different terms share common genes?
  - Is there a distinct set of genes that are related to a given term?



- Nodes:
  - Enriched terms (beige)
  - Up-regulated genes (green) or
  - Down-regulated genes (red)
- Edges:
  - Term-gene: the given term (pathway or gene set) involves the gene
- Sizes of term nodes are proportional to either:
  - the number of genes (default)
  - the $-\log_{10}$(p value)


Term-Gene Graph
Top 10 terms
CARD11
CSNK2A2
PARP1
LY96
TICAM1
hsa04064
PRKCQ
UBE2I
EIF2S3
SUMO3
GEMIN4
RANGAP1
NUP62
NUP214
hsa03013
NUP93
RNPS1
EIF2B1
SRRM1
IKBKB
EIF4A3
ATP6V1D
ATP6V0E1
ATP6V0E2
DDIT3
FASLG
U2AF2
HNRNPA1
SRSF8
SNRPB
PCBP1
HDAC1
AP2B1
hsa00190
hsa04932
DDX23
hsa03040
SF3B2
TAF4
ARID1A
UQCRQ
ACTB
SNU13
SRSF5
SF3B6
SIN3A
hsa05016
COX7C
COX7A2
NDUFB3
NDUFA1
COX6A1
MTOR
LSM3
BUD31
PUF60
SLC25A5
hsa04714
hsa05012
ACTG1
VDAC1
CREB1
KDM1A
hsa05010
ADCY7
SMARCA4
UBE2G1
CALM3
ATP2A2
CALM1
RTN3
GAPDH
MRPL33
RPL39
MRPS18C
hsa03010
RPS24
RPLP2
RPL31
RPL26
DEGs
2
6
10
14
enriched term
up-regulated gene
down-regulated gene

Pathway Scoring

For a set of pathways $P = \{P_1, P_2, ..., P_n\}$, where each P_i contains a set of genes, i.e. $P_i = \{g_1, g_2, ..., g_k\}$, the pathway score matrix *PS* is defined as:

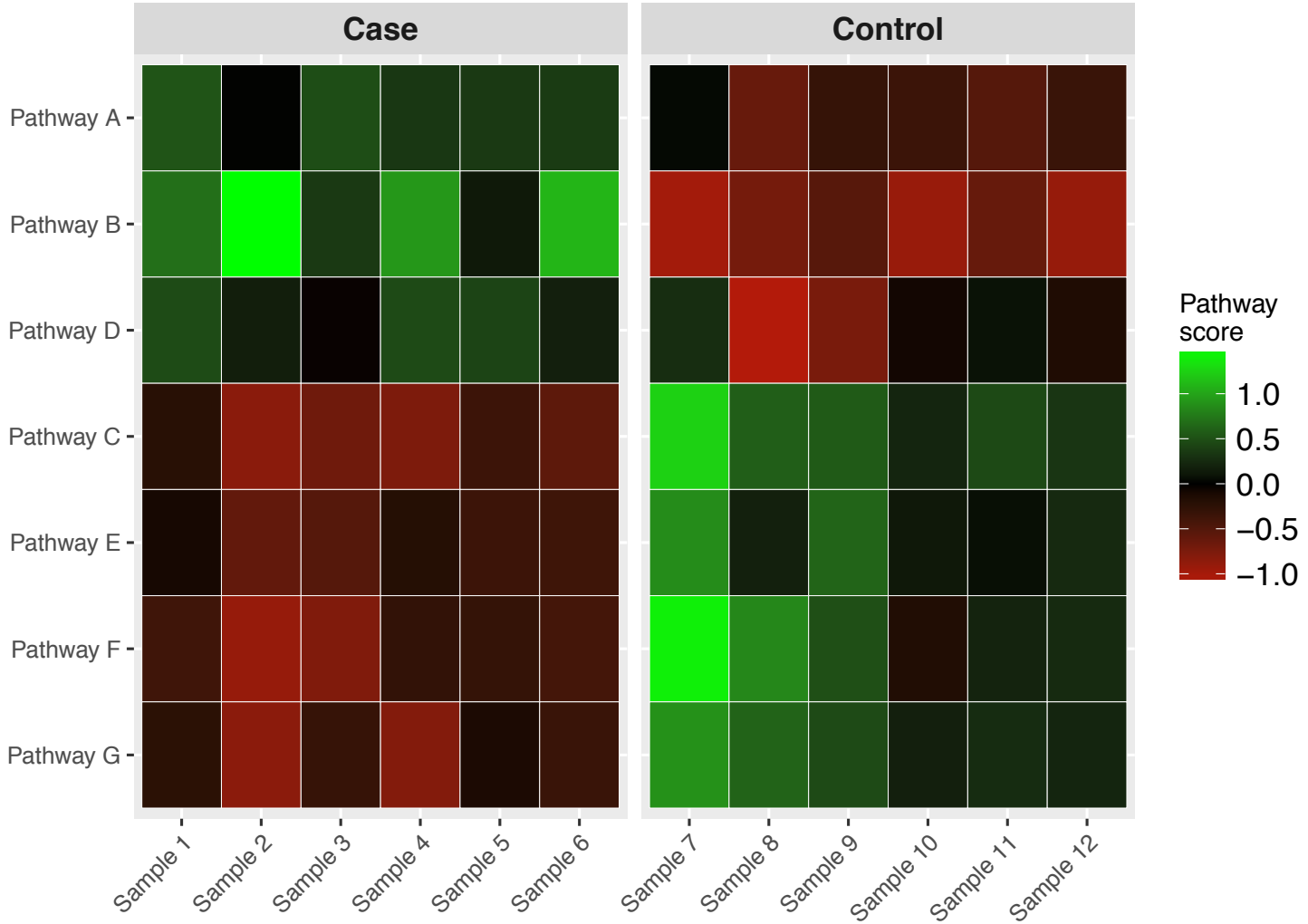
$PS_{p,s} = \frac{1}{k} \sum_{g \in P_p} GS_{g,s}$ for each pathway *p* and for each sample *s*.

GS is the gene score per sample matrix and is defined as:

$$GS_{g,s} = (EM_{g,s} - \bar{x}_g)/s_g$$

where *EM* is the expression matrix (columns are samples, rows are genes), $\bar{x}_g$ is the mean expression value of the gene and s_g is the standard deviaton of the expression values for the gene.

Pathway Scoring



Demonstration

Demo – I – Installation (CRAN release version)

Installation – Bioconductor Dependencies

```
if (!requireNamespace("BiocManager", quietly = TRUE))
        install.packages("BiocManager")
BiocManager::install("pathview")
BiocManager::install("AnnotationDbi")
BiocManager::install("org.Hs.eg.db")
```

Installation – pathfindR

```
install.packages("pathfindR")
```

OR from DockerHub

```
# pull image for latest release
docker pull egeulgen/pathfindr:latest
# pull image for specific version (e.g. 1.3.0)
docker pull egeulgen/pathfindr:1.3.0
```

Demo – II – Installation (Dev. version)

From GitHub

```
install.packages("devtools") # if you have not installed "devtools" package
devtools::install_github("egeulgen/pathfindR")
```

From DockerHub

```
# pull image for development version
docker pull egeulgen/pathfindr:dev
```

CRAN release 1.3.0

pathfindR: Pathway Enrichment Analysis Utilizing Active Subnetworks

Pathway enrichment analysis enables researchers to uncover mechanisms underlying the phenotype. pathfindR is a tool for pathway enrichment analysis utilizing active subnetworks. It identifies active subnetworks in a protein-protein interaction network using user-provided a list of genes. It performs pathway enrichment analyses on the identified subnetworks. pathfindR also offers functionalities to cluster enriched pathways and identify representative pathways and to score the pathways per sample. The method is described in detail in Ulgen E, Ozisik O, Sezerman OU. 2018. pathfindR: An R Package for Pathway Enrichment Analysis Utilizing Active Subnetworks. bioRxiv. <doi:10.1101/272450>.

Version:	1.3.0
Depends:	R (≥ 3.4), knitr, pathview
Imports:	AnnotationDbi, DBI, doParallel, foreach, rmarkdown, org.Hs.eg.db, ggplot2, fpc, grDevices, igraph
Published:	2018-11-20
Author:	Ege Ulgen, Ozan Ozisik
Maintainer:	Ege Ulgen <egeulgen at gmail.com>
BugReports:	https://github.com/egeulgen/pathfindR/issues
License:	MIT + file LICENSE
URL:	https://github.com/egeulgen/pathfindR
NeedsCompilation:	no
SystemRequirements:	Java JVM 1.8
Citation:	pathfindR citation info
Materials:	NEWS
CRAN checks:	pathfindR results

Downloads:

Reference manual:	pathfindR.pdf
Vignettes:	pathfindR - Step-by-Step (Manual) Execution of the pathfindR Workflow pathfindR - An R Package for Pathway Enrichment Analysis Utilizing Active Subnetworks
Package source:	pathfindR_1.3.0.tar.gz
Windows binaries:	r-devel: pathfindR_1.3.0.zip, r-release: pathfindR_1.3.0.zip, r-oldrel: pathfindR_1.3.0.zip
OS X binaries:	r-release: not available, r-oldrel: not available
Old sources:	pathfindR archive

Demo – III – Pathway Enrichment (w\ wrapper)

```
library(pathfindR)

RA_demo <- run_pathfindR(RA_input)

RA_demo <- run_pathfindR(RA_input,
                         gene_sets = "KEGG",
                         pin_name_path = "GeneMania",
                         output = "DEMO_OUTPUT")
```

Demo – IV – Pathway Clustering

```
# hierarchical clustering (default)
RA_demo_clu <- cluster_pathways(RA_demo)

# fuzzy clustering
RA_demo_clu <- cluster_pathways(RA_demo,
                                method = "fuzzy")
```

Demo – V – Term-Gene Graph

```
term_gene_graph(RA_demo) # top 10 terms (default)

# Graph using representative pathways
# selecting "Representative" pathways for clear visualization
RA_representative <- RA_clustered[RA_clustered $Status == "Representative", ]
term_gene_graph(RA_representative,
                num_terms = NULL, # to plot using all terms
                use_names = TRUE) # use pw names instead of IDs
```

Demo – VI – Pathway Scoring

```
# selecting "Representative" pathways for clear visualization
pws_table <- RA_clustered[RA_clustered$Status == "Representative", ]

## Expression matrix
exp_mat <- pathfindR::RA_exp_mat

## Vector of "Case" IDs
cases <- c("GSM389703", "GSM389704", "GSM389706",
"GSM389708", "GSM389711", "GSM389714", "GSM389716",
"GSM389717", "GSM389719", "GSM389721", "GSM389722",
"GSM389724", "GSM389726", "GSM389727", "GSM389730",
"GSM389731", "GSM389733", "GSM389735")

## Calculate pathway scores and plot heatmap
score_matrix <- calculate_pw_scores(pws_table, exp_mat, cases)
```

Resources

- Tutorial on Biostars:
  - https://www.biostars.org/p/322415/
- Vignettes
  - https://cran.r-project.org/web/packages/pathfindR/vignettes/
- pathfindR Wiki:
  - https://github.com/egeulgen/pathfindR/wiki

- To report any issues:
  - https://github.com/egeulgen/pathfindR/issues
- For all other questions:
  - egeulgen@gmail.com