

An introduction to Functional analysis of gene lists using pathways information

Bareket Dassa, Bioinformatics Unit

Some slides were adjusted from Shifra Ben-Dor and Ester Feldmesser

Introduction to Deep Sequencing Analysis course

2019



Dan ((Graur))

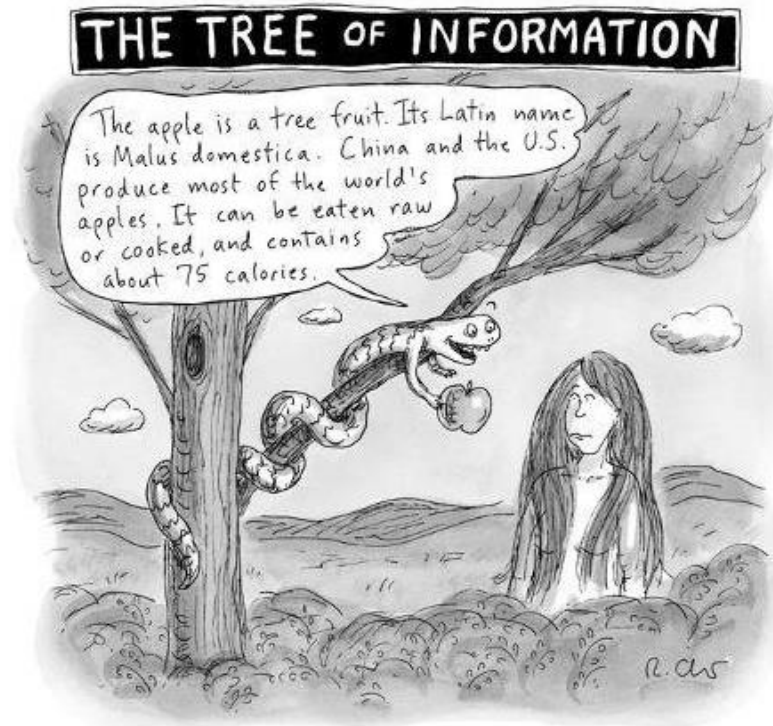
@DanGraur

Follow



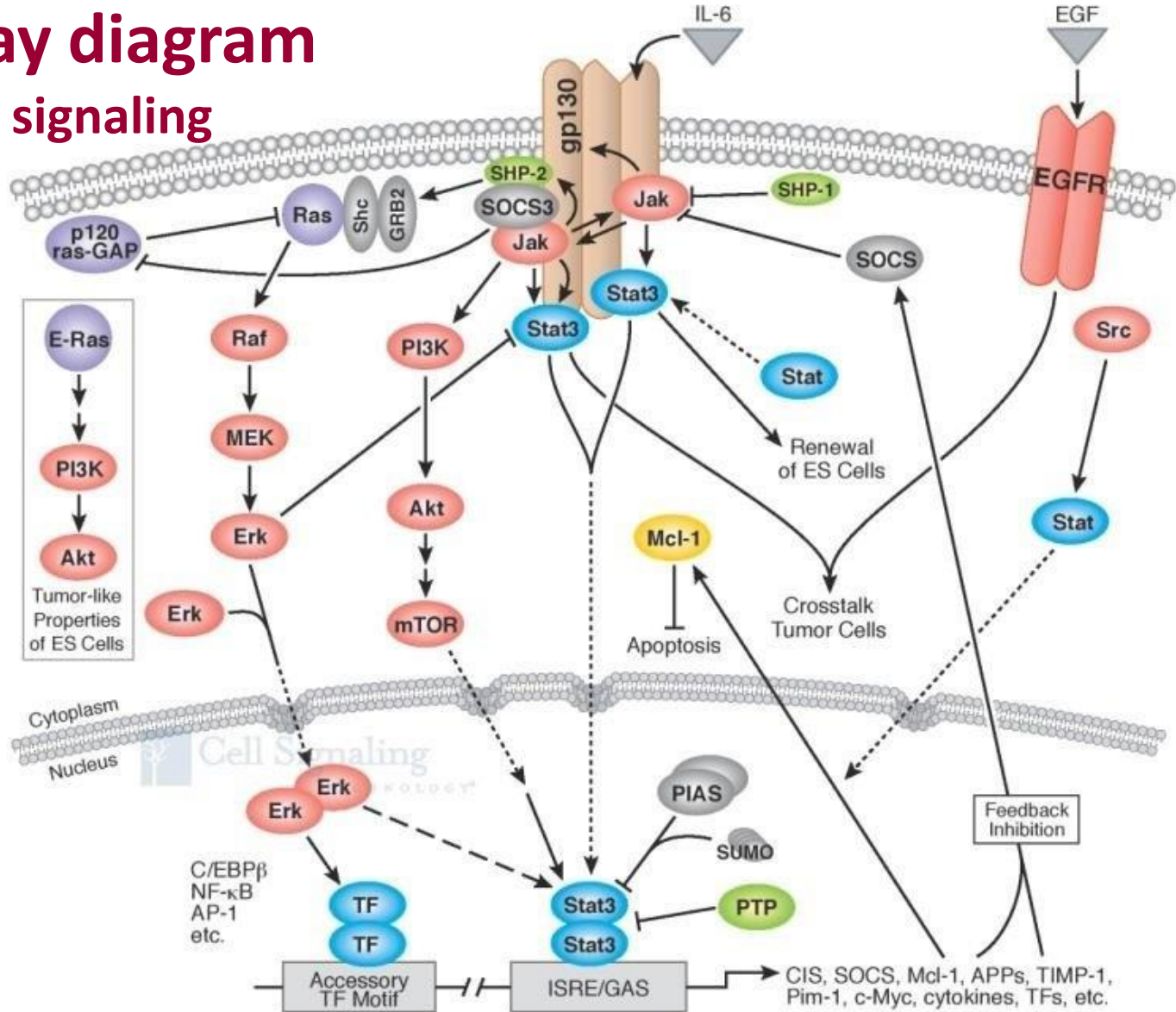
“Data is not information,
information is not knowledge,
knowledge is not wisdom,
wisdom is not truth.”

Robert Royar (1994) paraphrasing Frank Zappa (1979). The saying is beautifully illustrated by imagining Eve & the Tree of Information instead of the Tree of Knowledge. [#BigData](#)



Pathway diagram

JAK/STAT signaling



Pathway Diagram Keys

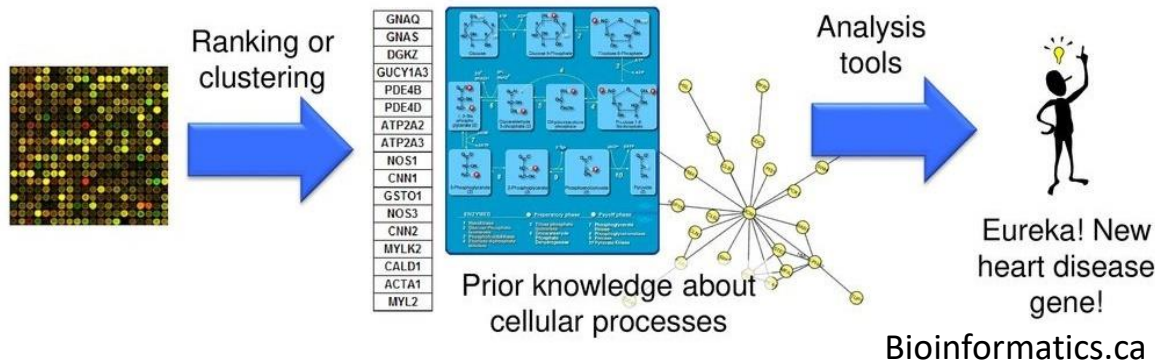
Kinase	Enzyme	G-protein	Direct Inhibitory Modification	Tentative Inhibitory Modification	Translocation
Phosphatase	pro-apoptotic	Acetylase	Multistep Stimulatory Modification	Separation of Subunits or Cleavage Products	Transcriptional Stimulatory Modification
Transcription Factor	pro-survival	Deacetylase	Multistep Inhibitory Modification	Joining of Subunits	Transcriptional Inhibitory Modification
Caspase	GAP/GEF	Ribosomal subunit	Tentative Stimulatory Modification		
Receptor	GTPase	Direct Stimulatory Modification			

Why pathway analysis?

- To understand how genes and molecules are networked to form a biological system
- All pathways are **networks** of interactions between gene products and metabolites, that describes a **known** biological phenotype
But not all networks are pathways

In NGS experiments we start with:

1. A gene list
2. A pathways database



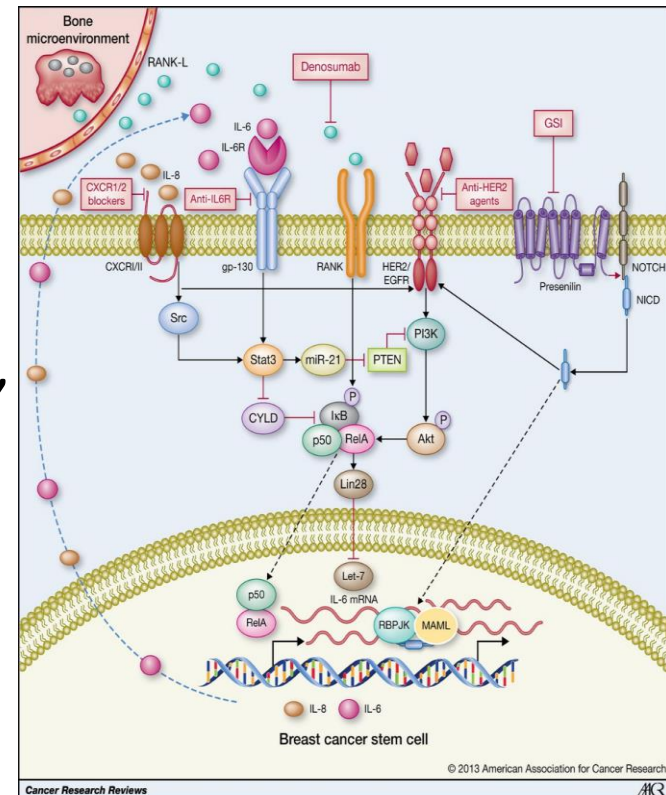
And aim to:

Identify the pathways which are significantly impacted in a given condition

What's behind the pathway diagram?

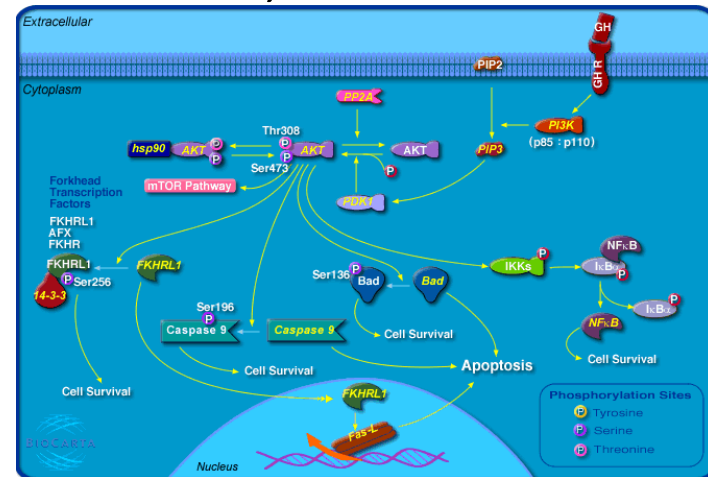
- Constructed from experimental biology data, usually **man curated**
- Capture the **dependencies and interactions** between:
 - 1) enzyme – ligand
metabolic pathways
 - 2) protein – protein
cell signaling pathways
complexes for cell processes
 - 3) gene regulatory elements – gene products
genetic/ transcription regulatory networks

Using **various sources**: Gene Ontology, Phenotypes, Diseases, Drugs, Chromosomal location, Networks



What's behind the pathway diagram?

- **Intuitive**, easy to interpret
- **Reduces complexity** of information (from thousands of genes to hundreds of pathways)
- Improves statistical power (fewer test)
- **Pathway enrichment** analysis generates a list of over-representated pathways in a gene list
- There are **multiple** methods and pathways databases, it is a rapidly evolving field



Challenges in pathways analysis

There is no *precise* biological definition of a pathway, no pathway analysis method is perfect:

1. Annotation challenges

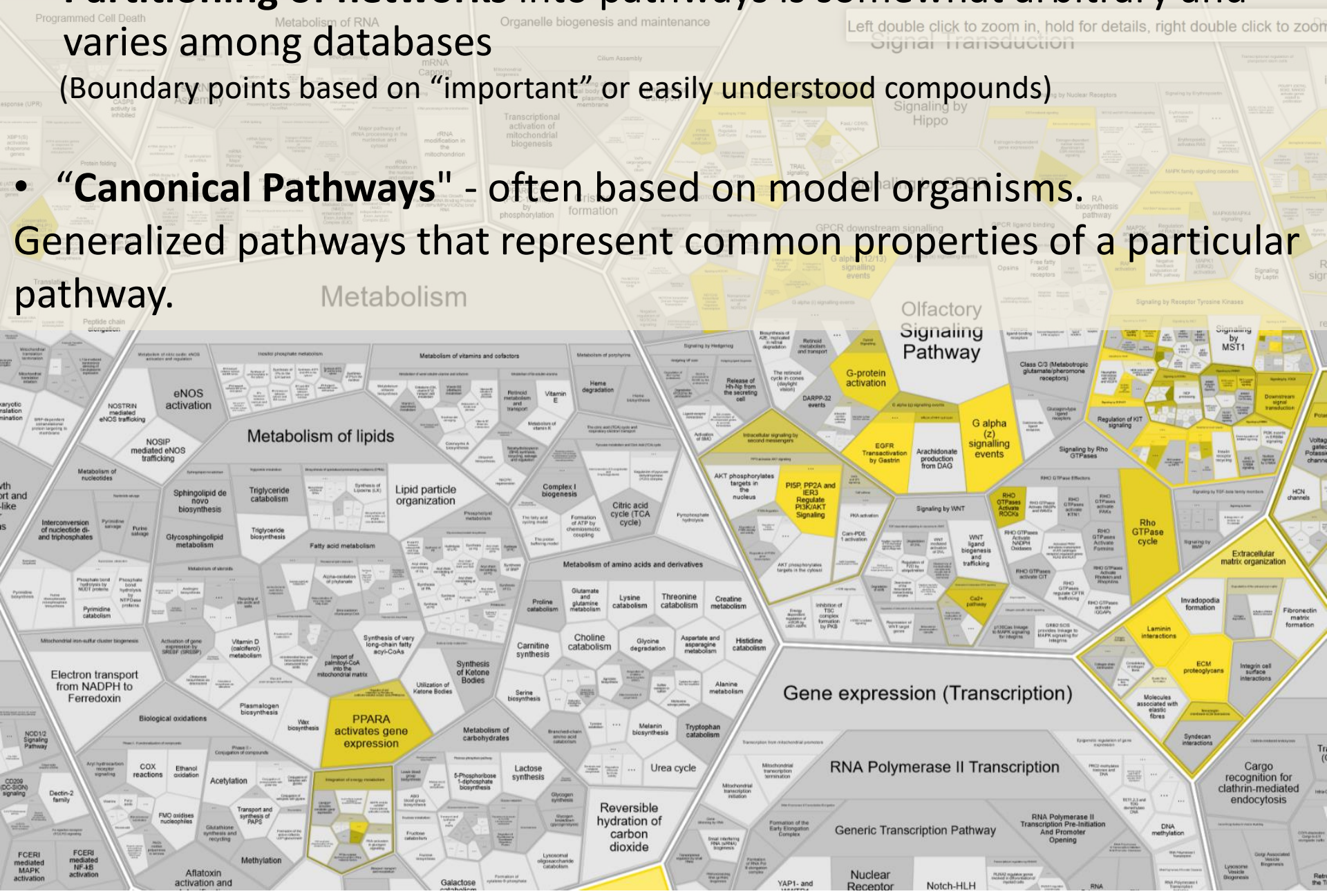
- **Incomplete** and inaccurate annotations
- **Missing condition-**, tissue- and cell-specific information
- **Manual curation** of genomes takes a long time

- Partitioning of networks
- Pathways are inter-linked

• **Partitioning of networks into pathways is somewhat arbitrary and varies among databases**

(Boundary points based on "important" or easily understood compounds)

• **"Canonical Pathways"** - often based on model organisms. Generalized pathways that represent common properties of a particular pathway.

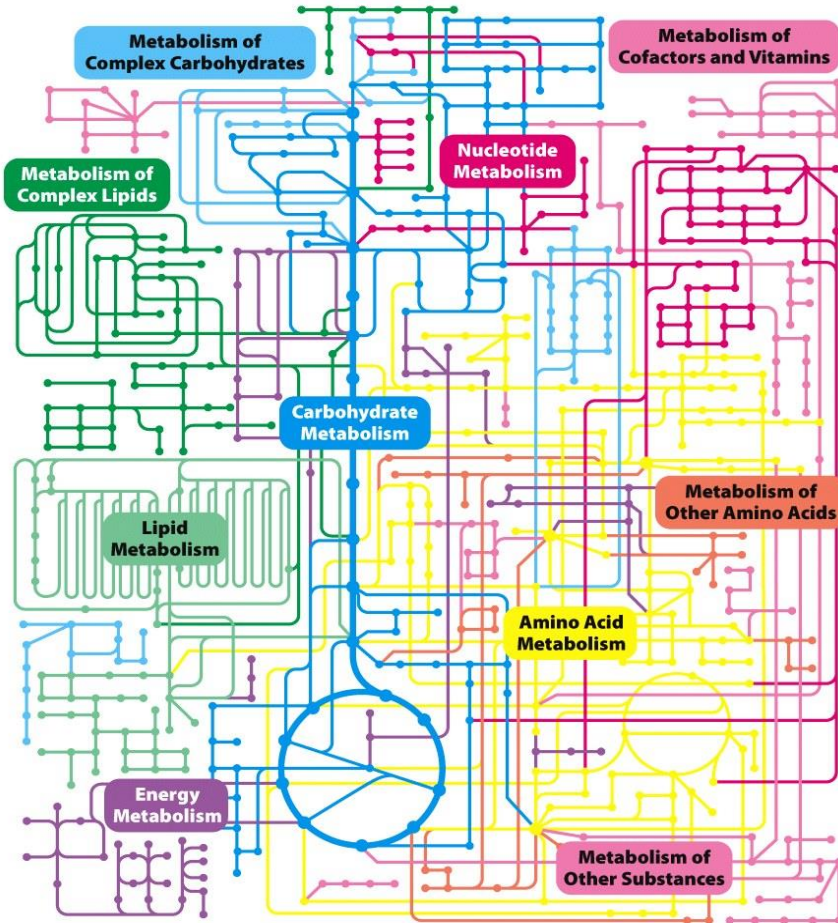


Pathways are inter-linked

How to display information about genes that appear in multiple pathways, and thereby interconnect those pathways?

In Signaling, Metabolic or Regulatory pathways

metabolic pathways:



Glutamate signaling pathways in melanoma:

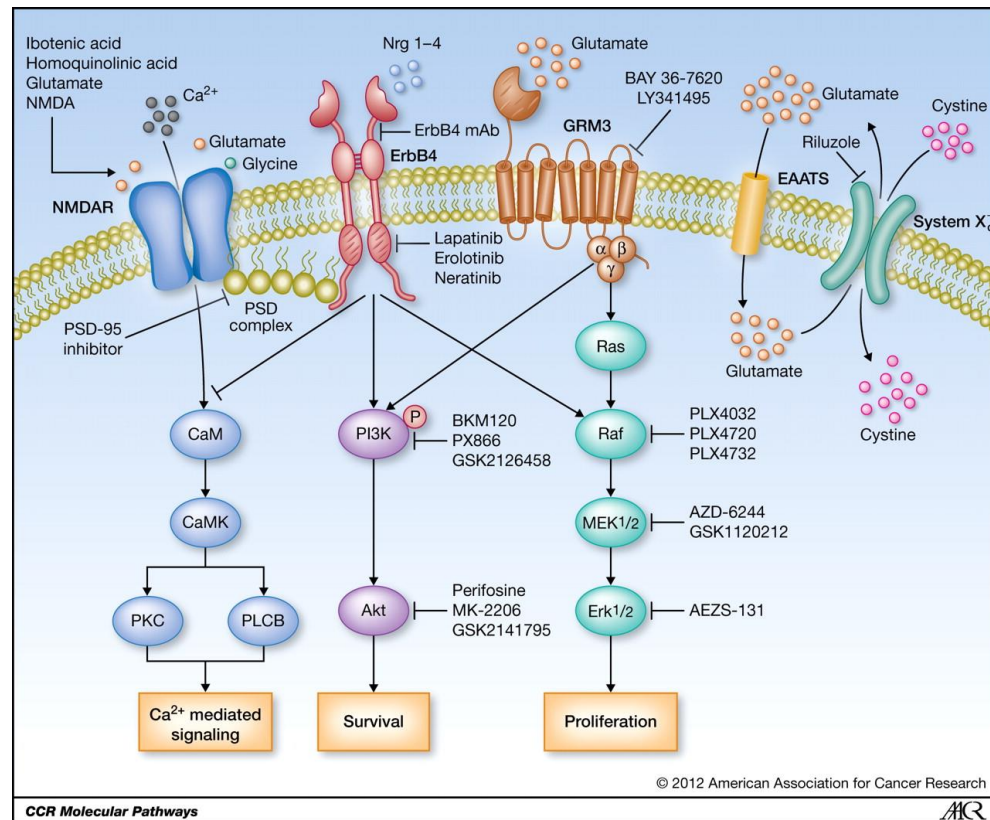


Figure 15.2
Biochemistry, Seventh Edition
© 2012 W. H. Freeman and Company

<https://clincancerres.aacrjournals.org/content/18/16/4240>

Challenges of pathways analysis (continued)

2. methodological challenges

- Inability to model **dynamic response**, or effects of an external stimuli (perturbations)
- Most approaches assume that all genes have a **similar** weight in the pathway, which is often not true

How to assess performance of pathway analysis methods?

Compare different methods against a set of benchmark data sets

Additional reading:

Ten Years of Pathway Analysis: Current Approaches and Outstanding Challenges:

<https://doi.org/10.1371/journal.pcbi.1002375>

Pathway Analysis: State of the Art <https://doi.org/10.3389/fphys.2015.00383>

Selected pathways databases

- KEGG (Kyoto Encyclopedia of Genes and Genomes)
- BioCyc and MetaCyc
- Reactome
- BioCarta
- WikiPathways
- PANTHER
- MSigDB
- Pathway Commons
- ...



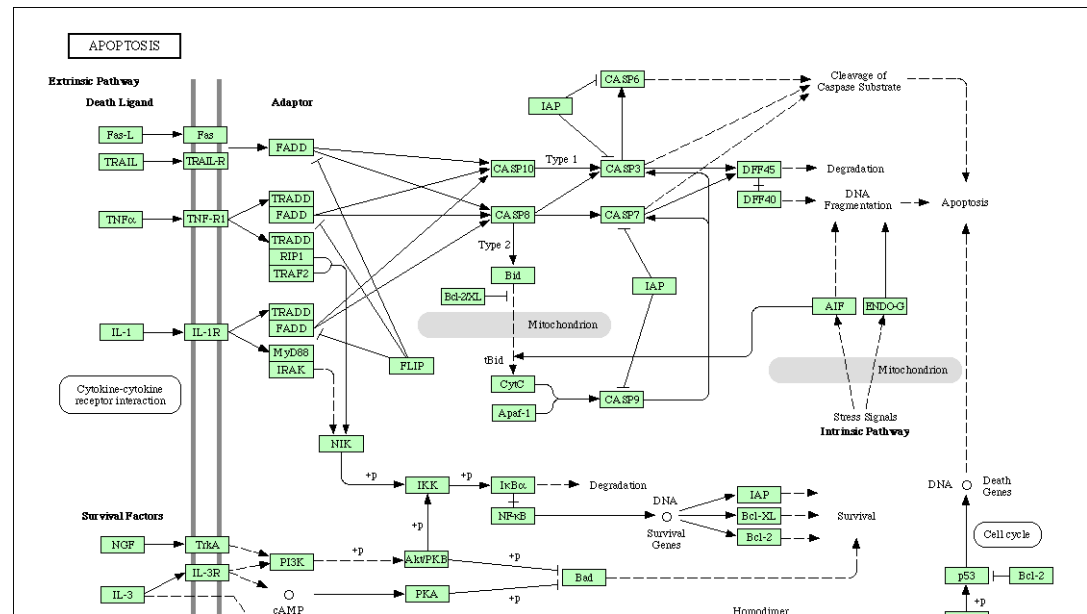
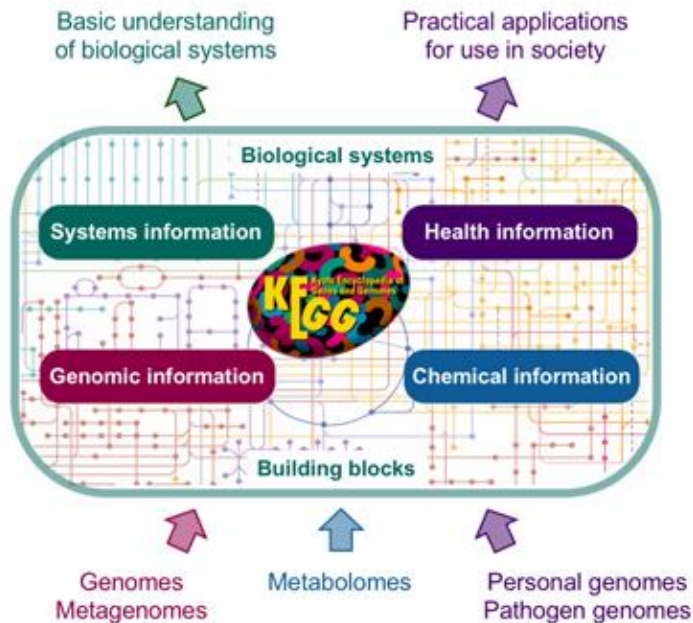
KEGG: Kyoto Encyclopedia of Genes and Genomes

www.genome.jp/kegg/

KEGG PATHWAY

is a comprehensive source of **manually drawn** pathway maps

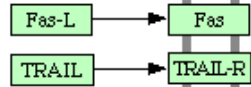
Representing our knowledge on the molecular interaction, reaction and relation networks, using fully sequenced genomes, genes, proteins, pathways, and chemical compounds



APOPTOSIS

Extrinsic Pathway

Death Ligand



Adaptor



KEGG PATHWAY:

537 reference pathways (manually drawn)
 generating 673,233 pathways (organism-specific pathway maps that are computationally generated by matching functional ortholog)

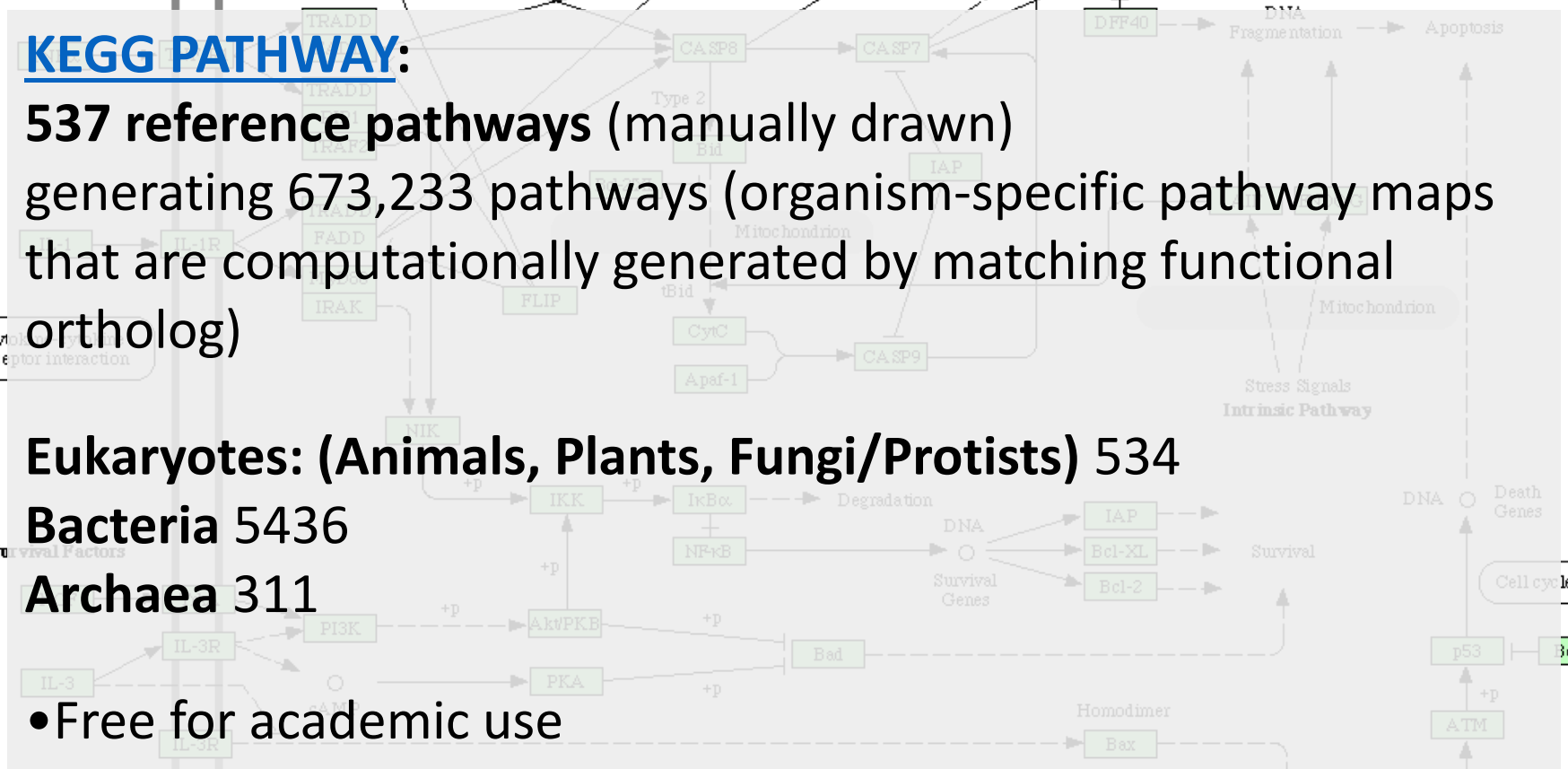
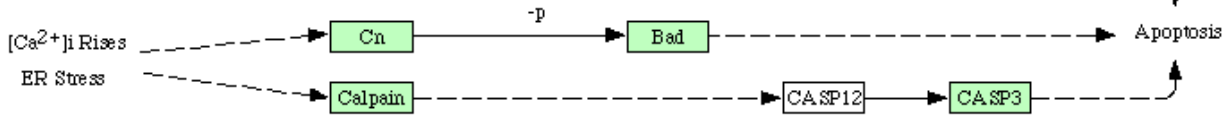
Eukaryotes: (Animals, Plants, Fungi/Protists) 534

Bacteria 5436

Archaea 311

• Free for academic use

Ca²⁺-induced Cell Death Pathways





KEGG PATHWAY includes relation networks for:

1. Metabolism

2. Genetic Information Processing

3. Environmental Information Processing (Membrane transport, Signal transduction)

4. Cellular Processes (Transport, Cellular community, Cell motility...)

5. Organismal Systems (Immune system, Endocrine system...)

6. Human Diseases

7. Drug Development



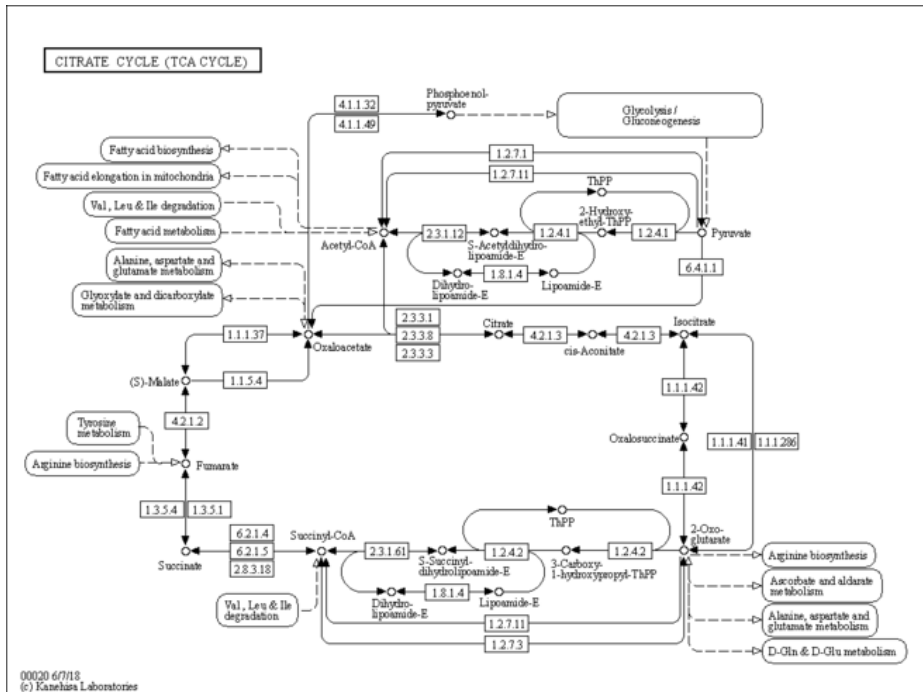
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2. Genetic Information Processing

3. Environmental Information Proc

4. Cellular Processes (Transport, Ce



1.2 Energy metabolism

- 00190 Oxidative phosphorylation
- 00195 Photosynthesis
- 00196 Photosynthesis - antenna proteins
- 00710 Carbon fixation in photosynthetic organisms
- 00720 Carbon fixation pathways in prokaryotes
- 00680 Methane metabolism
- 00910 Nitrogen metabolism
- 00920 Sulfur metabolism

1.3 Lipid metabolism

- 00061 Fatty acid biosynthesis
- 00062 Fatty acid elongation
- 00071 Fatty acid degradation
- 00072 Synthesis and degradation of ketone bodies
- 00073 Cutin, suberine and wax biosynthesis
- 00100 Steroid biosynthesis
- 00120 Primary bile acid biosynthesis
- 00121 Secondary bile acid biosynthesis
- 00140 Steroid hormone biosynthesis
- 00561 Glycerolipid metabolism
- 00564 Glycerophospholipid metabolism
- 00565 Ether lipid metabolism
- 00600 Sphingolipid metabolism
- 00590 Arachidonic acid metabolism
- 00591 Linoleic acid metabolism
- 00592 alpha-Linolenic acid metabolism
- 01040 Biosynthesis of unsaturated fatty acids

1.4 Nucleotide metabolism

- 00230 Purine metabolism
- 00240 Pyrimidine metabolism

1.5 Amino acid metabolism

- 00250 Alanine, aspartate and glutamate metabolism
- 00260 Glycine, serine and threonine metabolism
- 00270 Cysteine and methionine metabolism
- 00280 Valine, leucine and isoleucine degradation
- 00290 Valine, leucine and isoleucine biosynthesis
- 00300 Lysine biosynthesis



KEGG PATHWAY includes relation

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2. Genetic Information Pro

3. Environmental Information P

4. Cellular Processes (Transport,

5. Organismal Systems (Immune

6. Human Diseases

7. Drug Development

2. Genetic Information Processing

2.1 Transcription

- 03020 RNA polymerase
- 03022 Basal transcription factors
- 03040 Spliceosome

2.2 Translation

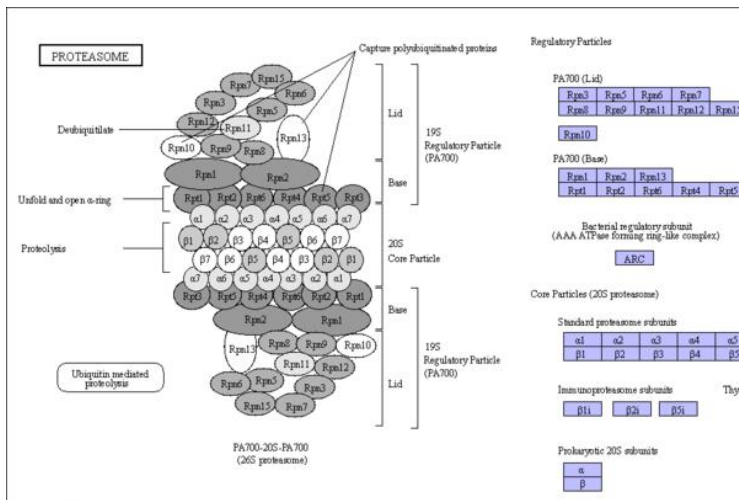
- 03010 Ribosome
- 00970 Aminoacyl-tRNA biosynthesis
- 03013 RNA transport
- 03015 mRNA surveillance pathway
- 03008 Ribosome biogenesis in eukaryotes

2.3 Folding, sorting and degradation

- 03060 Protein export
- 04141 Protein processing in endoplasmic reticulum
- 04130 SNARE interactions in vesicular transport
- 04120 Ubiquitin mediated proteolysis
- 04122 Sulfur relay system
- 03050 Proteasome
- 03018 RNA degradation

2.4 Replication and repair

- 03030 DNA replication
- 03410 Base excision repair
- 03420 Nucleotide excision repair
- 03430 Mismatch repair
- 03440 Homologous recombination
- 03450 Non-homologous end-joining
- 03460 Fanconi anemia pathway



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1. Metabolism

2. Genetic Information Processing

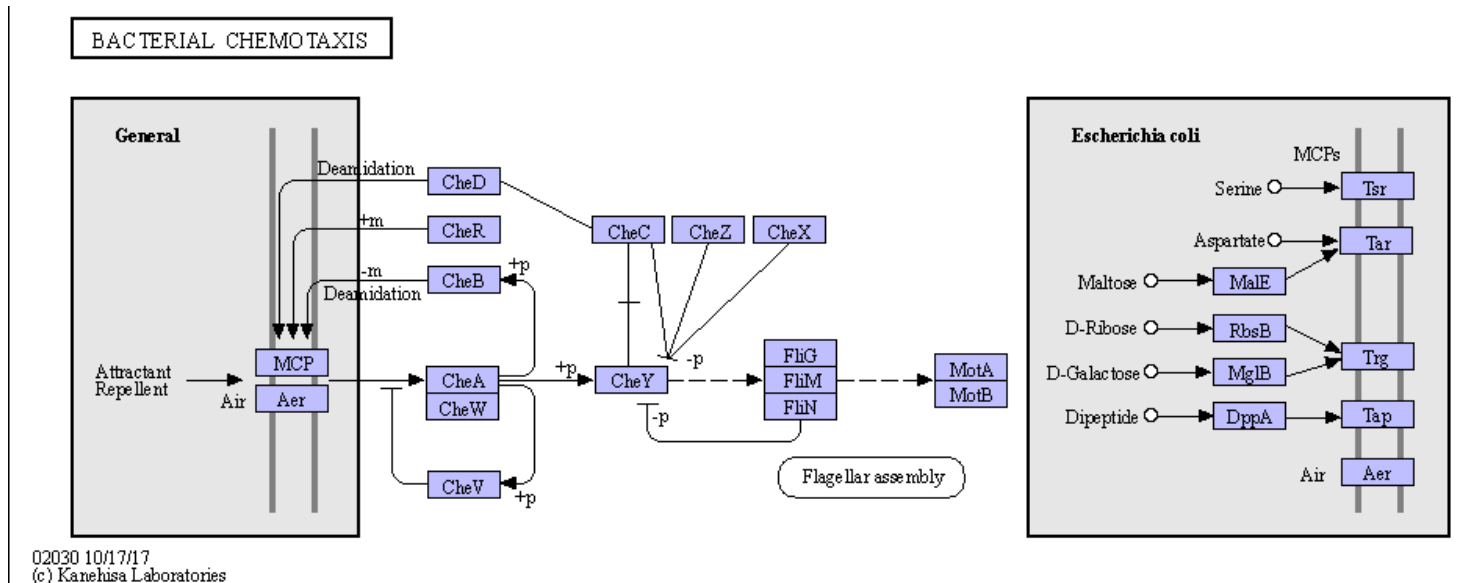
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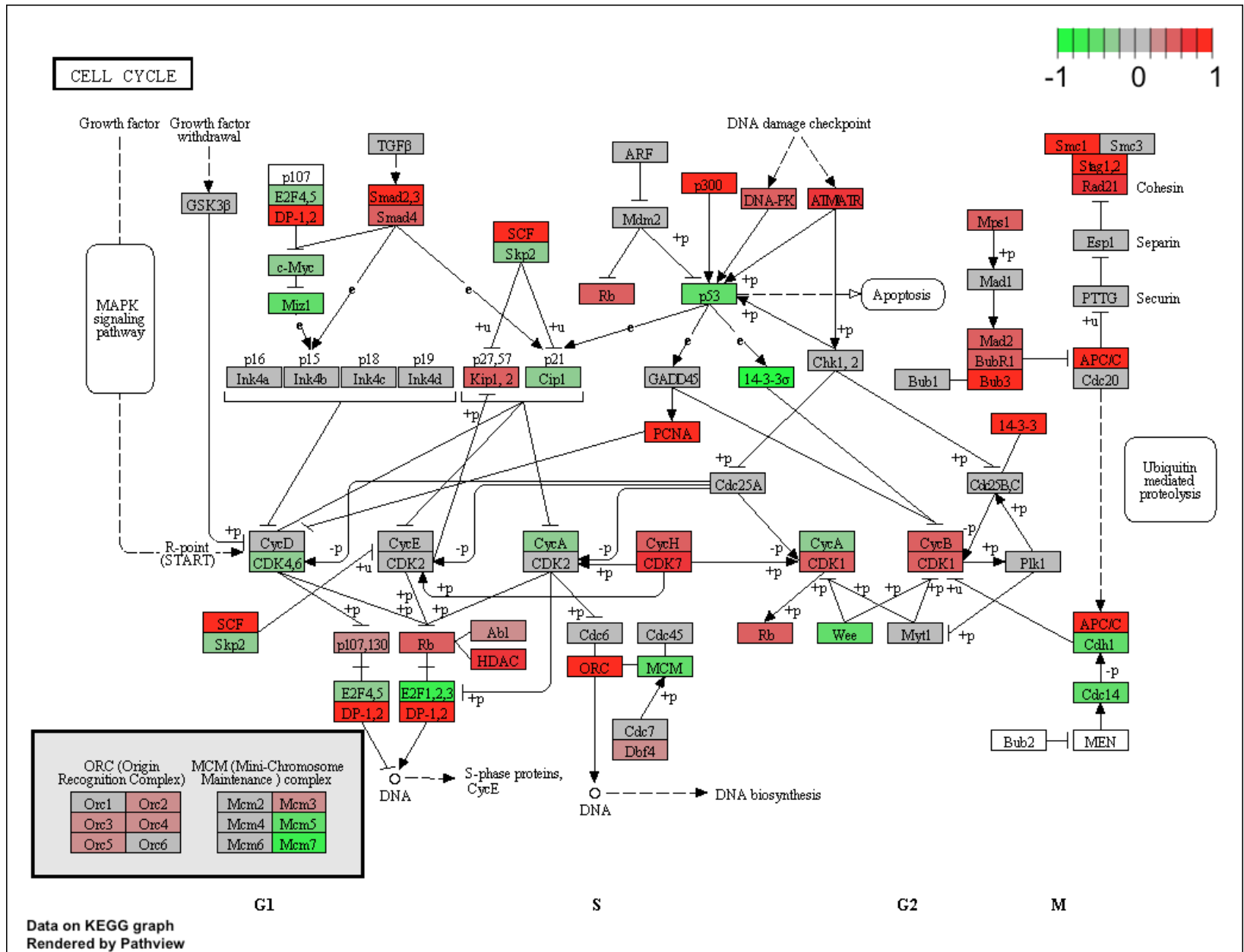
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Color pathways with KEGG Mapper

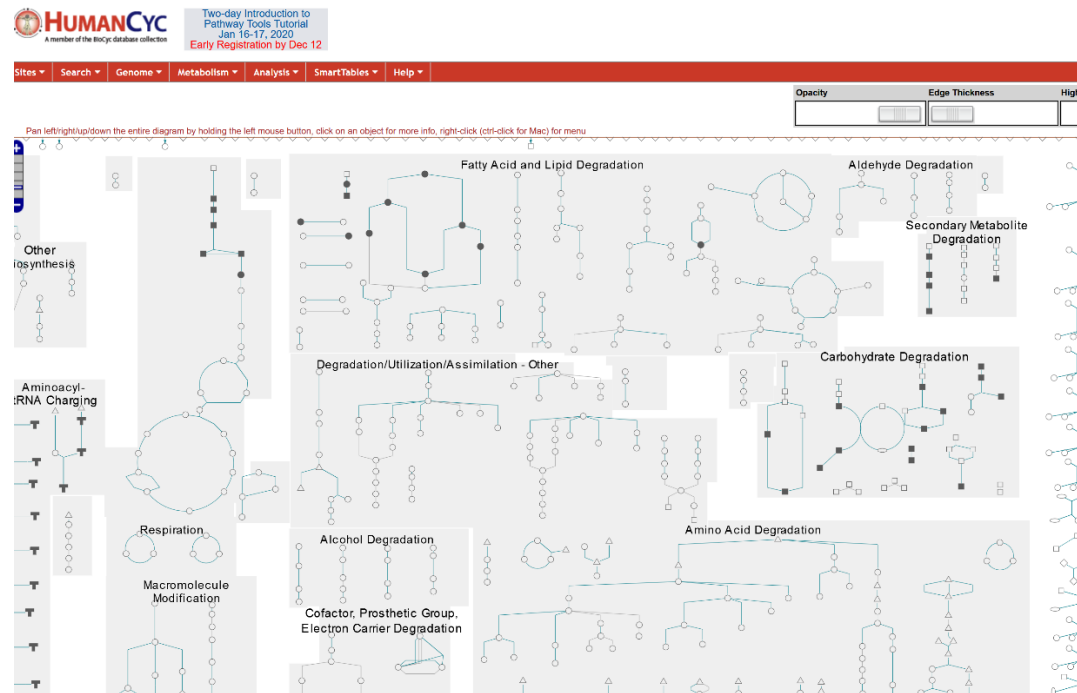


Selected pathways databases

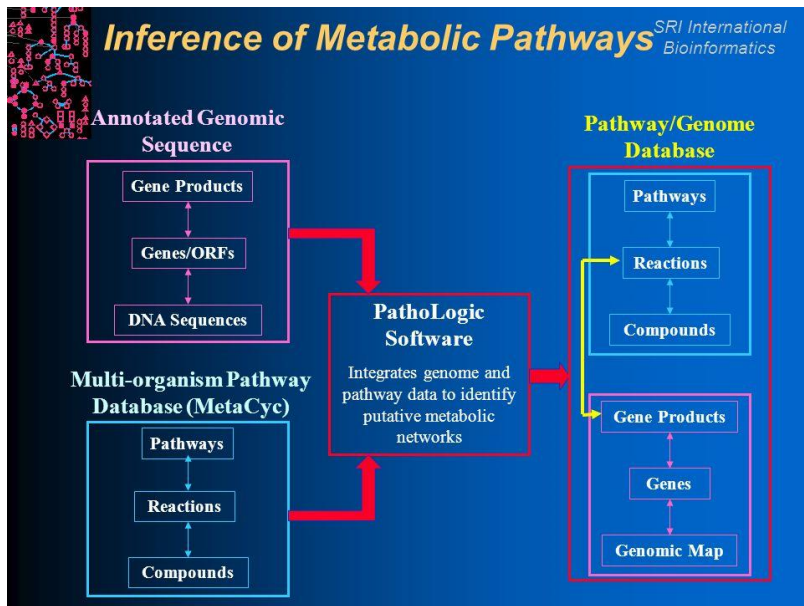
- KEGG (Kyoto Encyclopedia of Genes and Genomes)
- BioCyc and MetaCyc
- Reactome
- BioCarta
- WikiPathways
- PANTHER
- MSigDB
- Pathway Commons

BioCyc is a collection of 14,735 **Pathway/Genome Databases (PGDB)** Computationally predicted metabolic pathways and operons.

Each PGDB describes the genome/metabolic pathways of a single organism



The screenshot shows the HUMANCYC website interface. At the top, there is a navigation bar with links for Sites, Search, Genome, Metabolism, Analysis, SmartTables, and Help. Below the navigation bar, there is a header for a "Two-day Introduction to Pathway Tools Tutorial" held from Jan 16-17, 2020, with "Early Registration by Dec 12". The main content area displays a complex metabolic pathway diagram with various nodes and arrows. The diagram is organized into several panels, including "Fatty Acid and Lipid Degradation", "Aldehyde Degradation", "Secondary Metabolite Degradation", "Carbohydrate Degradation", "Degradation/Utilization/Assimilation - Other", "Amino Acid Degradation", "Alcohol Degradation", "Cofactor, Prosthetic Group, Electron Carrier Degradation", "Respiration", "Macromolecule Modification", "Aminoacyl-tRNA Charging", and "Other biosynthesis". The interface also includes a search bar and a "Pan left/right/up/down the entire diagram by holding the left mouse button, click on an object for more info, right-click (ctrl-click for Mac) for menu" instruction.



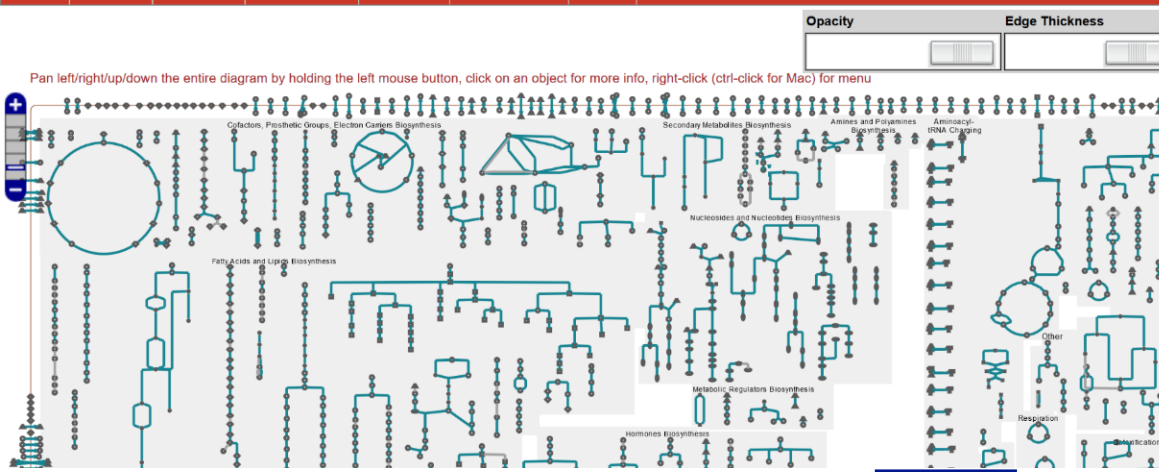
Cellular overview and omics viewer (Metabolic Map Diagram)

Pathway collages: customize and overlay with gene expression data

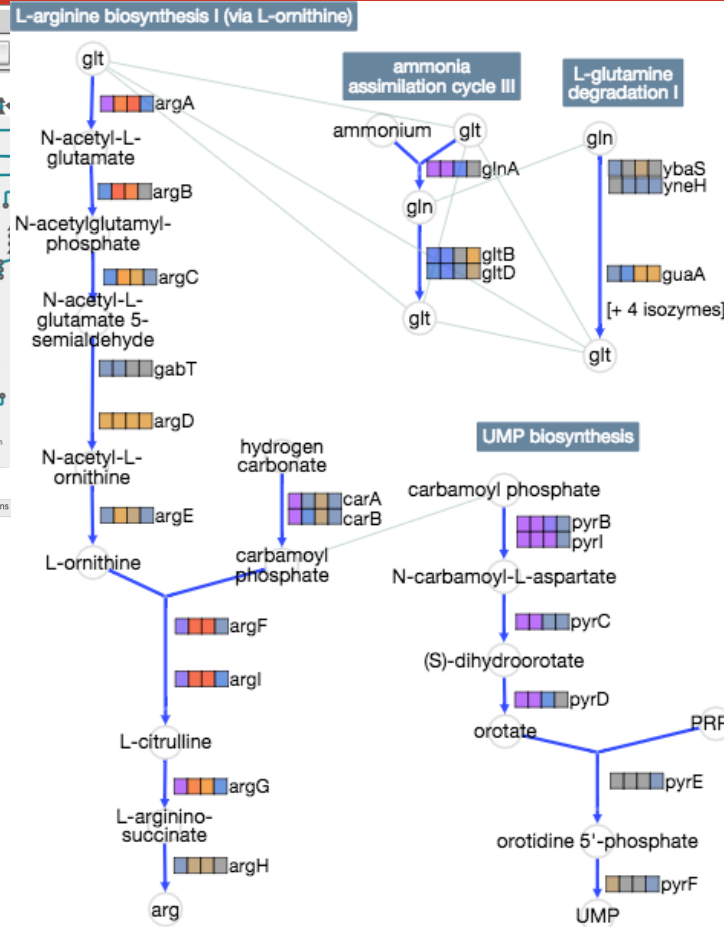
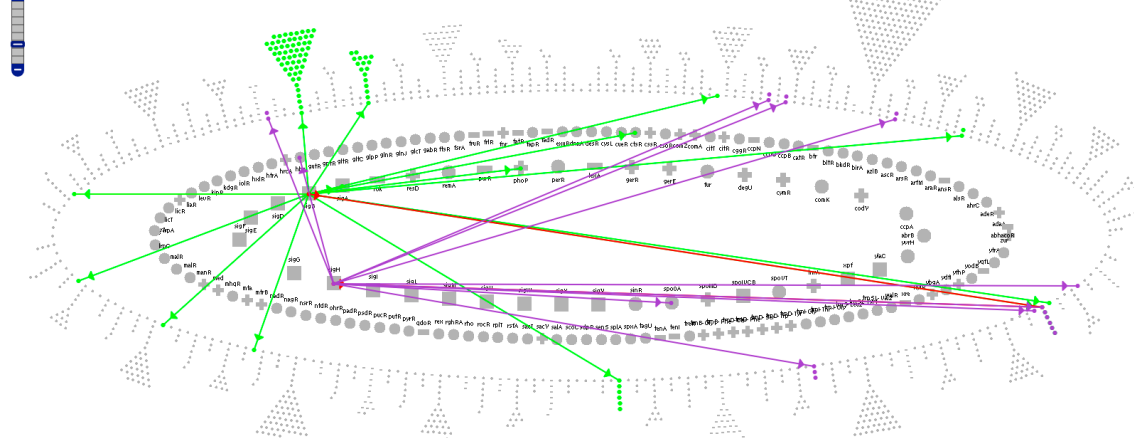


LOGIN | Why Login? | Create New Account
 Enter a gene, protein, metabolite or pathway... Quick Search Gene Search
 Searching *Homo sapiens* change organism database

Sites Search Genome Metabolism Analysis SmartTables Help



Regulatory relationships (for bacteria)



The BioCyc databases have three levels of manual review and updating:

Tier 1: **Intensively** Curated Databases

[EcoCyc](#), [AraCyc](#), [HumanCyc](#), [LeishCyc](#), [YeastCyc](#)

[MetaCyc](#) database is a reference source on metabolic pathways and enzymes from many (2,740) organisms.

Tier 2: Computationally-derived with **moderate** curation

Tier 3: Computationally-derived with **no curation** generated using an automated computational pipeline

Selected pathways databases

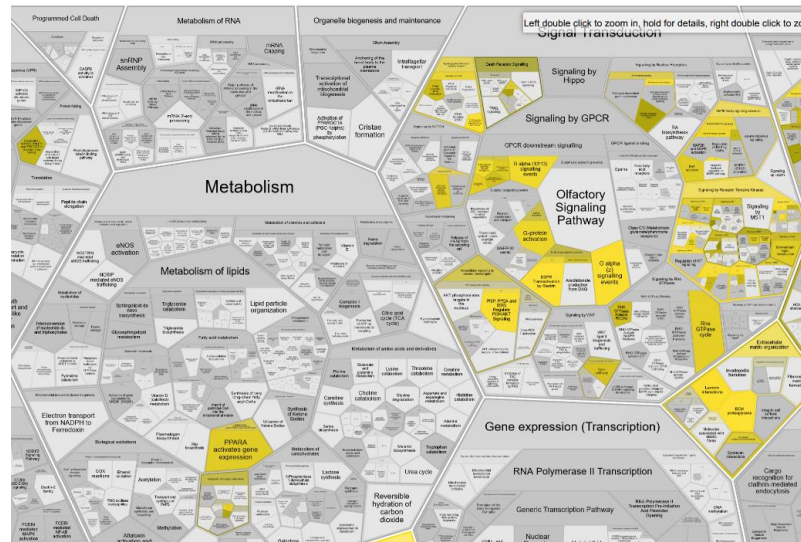
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- **Reactome**
- BioCarta
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- PANTHER
- MSigDB
- Pathway Commons
- STRINGDB

Reactome

<https://reactome.org/>

Open source and open access

- **Rigorous curation** standards – manually curated and peer-reviewed every pathway is traceable to primary literature.
- Pathways include classical intermediary metabolism, signaling, transcriptional regulation, apoptosis and disease.



REACTOME 3.9 Pathways for: Homo sapiens Analysis: Layout:

Event Hierarchy:

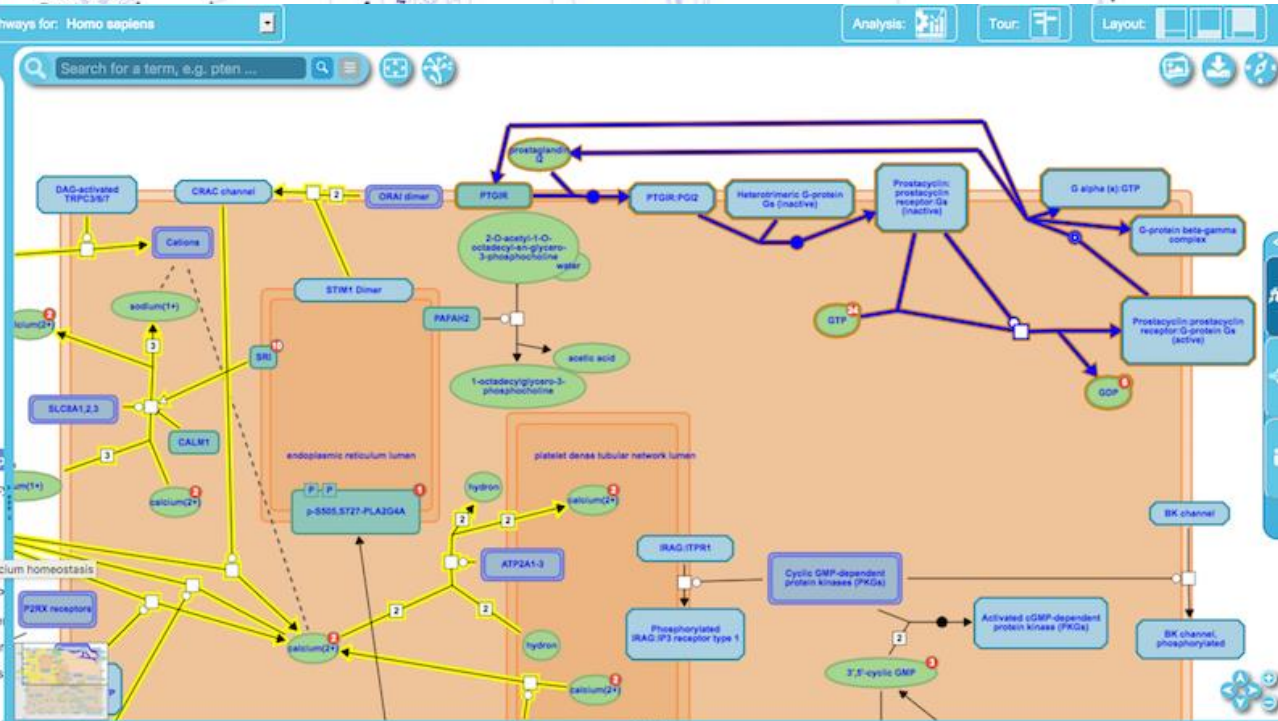
- Cell Cycle
- Cell-Cell communication
- Cellular responses to stress
- Chromatin organization
- Circadian Clock
- Developmental Biology

Reproduction, Immune System, DNA Repair, Chromatin organization, DNA Replication, Cell Cycle, Programmed Cell Death

reactome 3.9 Pathways for: Homo sapiens

Event Hierarchy:

- Cell Cycle
- Cell-Cell communication
- Cellular responses to external stimuli
- Chromatin organization
- Circadian Clock
- Developmental Biology
- Digestion and absorption
- Disease
- DNA Repair
- DNA Replication
- Extracellular matrix organization
- Gene expression (Transcription)
- Hemostasis
- Platelet homeostasis
- Prostacyclin signalling through p2Y12
- Nitric oxide stimulates guanylate cyclase
- Binding of ATP to P2X receptors
- Platelet calcium homeostasis
- Platelet sensitization by Platelet calcium homeostasis
- PAFAH2 hydrolyses PAF to lyso-PAF
- Platelet Adhesion to exposed collagen
- Platelet activation, signaling and aggregation
- Formation of Fibrin Clot (Clotting Cascade)
- Dissolution of Fibrin Clot
- Cell surface interactions at the vascular wall
- Factors involved in megakaryocyte development
- Immune System
- Metabolism
- Metabolism of proteins
- Metabolism of RNA
- Mitophagy
- Muscle contraction
- Neuronal System



Description | **Molecules** | **Structures** | **Expression** | **Analysis** | **Downloads**

Prostacyclin signalling through prostacyclin receptor | Id: R-HSA-392851.2 | Species: Homo sapiens

Summation

Prostacyclin (PGI2) is continuously produced by healthy vascular endothelial cells. It inhibits platelet activation through interaction with the Gs-coupled receptor PTGIR, leading to increased cAMP, a consequent increase in cAMP-dependent protein kinase activity which prevents increases of cytoplasmic [Ca2+] necessary for activation (Wouffe et al. 2001). PGI2 is also an effective vasodilator. These effects oppose the effects of thromboxane (TXA2), another eicosanoid, creating a balance of blood circulation and platelet activation.

▶ Background literature references...

External Identifiers

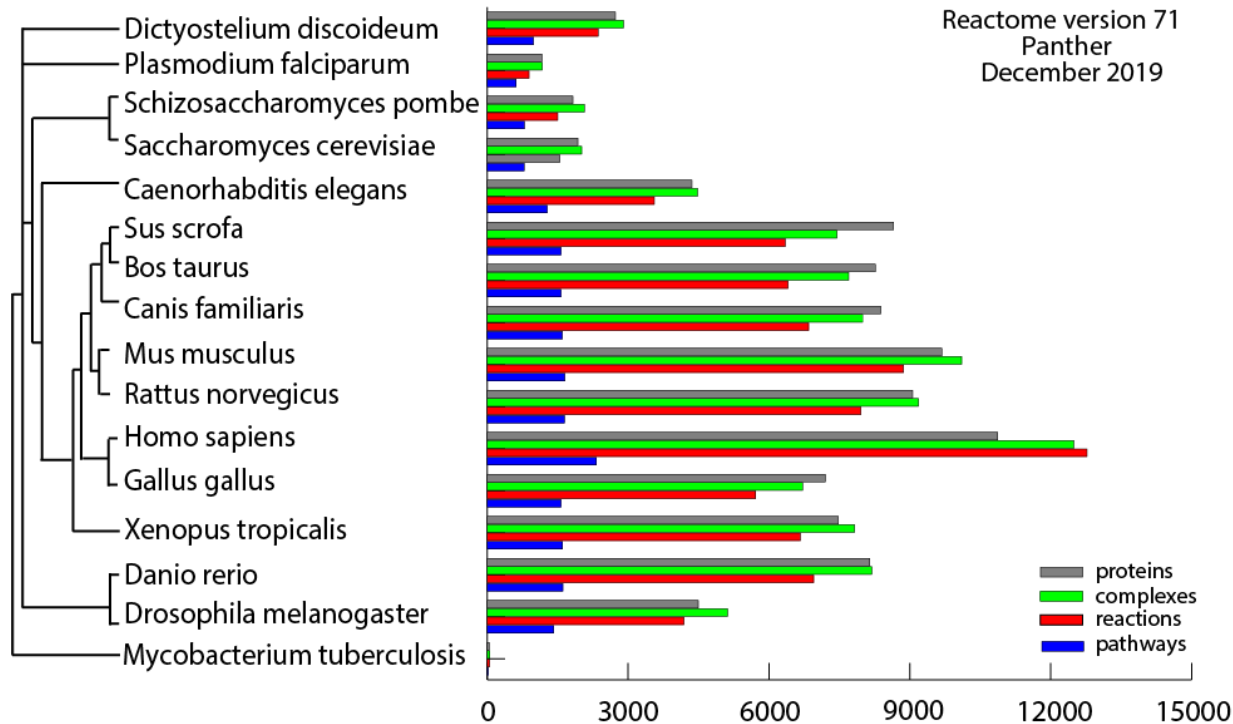
Transition Binding Dissociation Omitted Uncertain

The legend shows five types of external identifiers represented by different symbols and colors:

- Transition:** A green box with a white circle and a vertical line.
- Binding:** A green box with a white circle and a horizontal line.
- Dissociation:** A white circle with a horizontal line.
- Omitted:** A white box with a vertical line.
- Uncertain:** A white box with a question mark.

Reactome

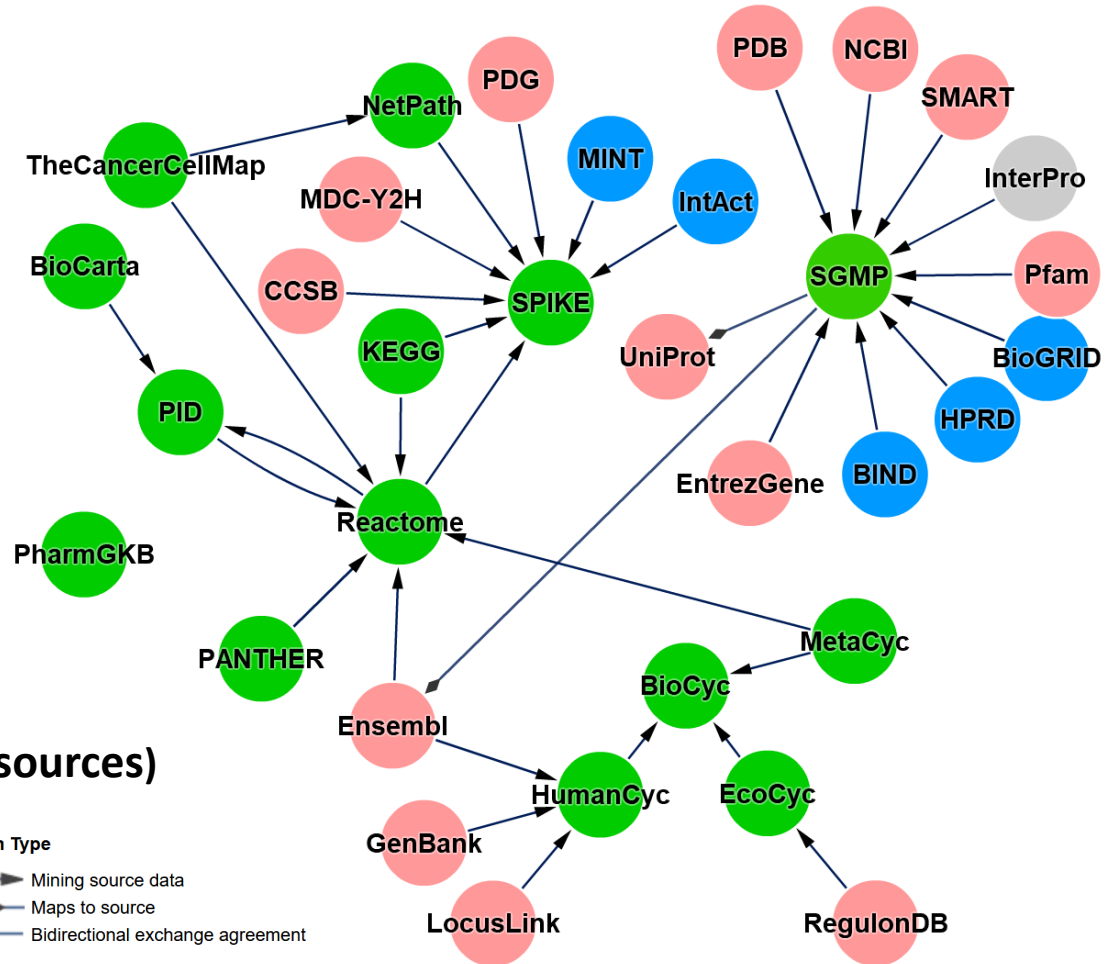
- Find corresponding pathways in other species



- Cross-reference to many other bioinformatics databases
- Provides data visualization tools, calculates gene overrepresentation in pathways

A network of pathways databases

- KEGG (Kyoto Encyclopedia of Genes and Genomes)
- BioCyc and MetaCyc
- Reactome
- BioCarta
- WikiPathways
- PANTHER
- MSigDB
- Pathway Commons
- ...



<http://www.pathguide.org/>

(702 biological pathway related resources)

Resource Type

- Interactions
- Pathways
- Predictive interactions
- Metamining
- Exchange format language
- Unifying efforts
- Not categorized

Interaction Type

- Source → Mining source data
- Source ← Maps to source
- Bidirectional exchange agreement

A plethora of tools are available for pathways analysis

More than 70 available pathway analysis methods in >700 web tools

Cytoscape

DAVID

EnrichmentMap

Enrichr

GeneAnalytics

g:Profiler

GSEA

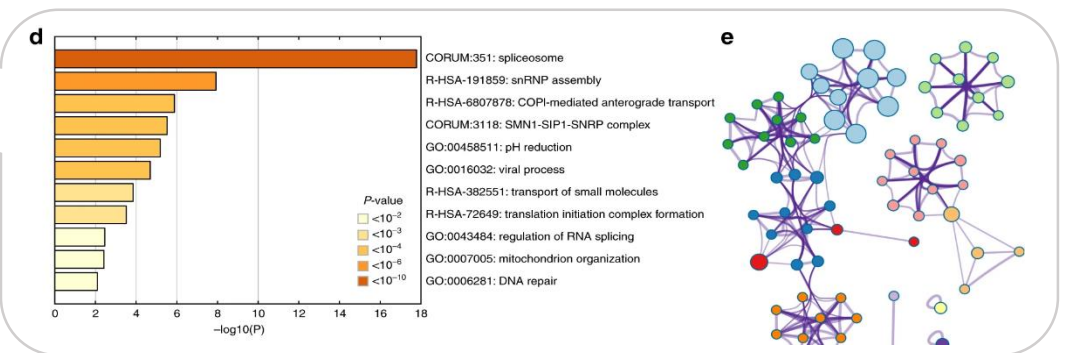
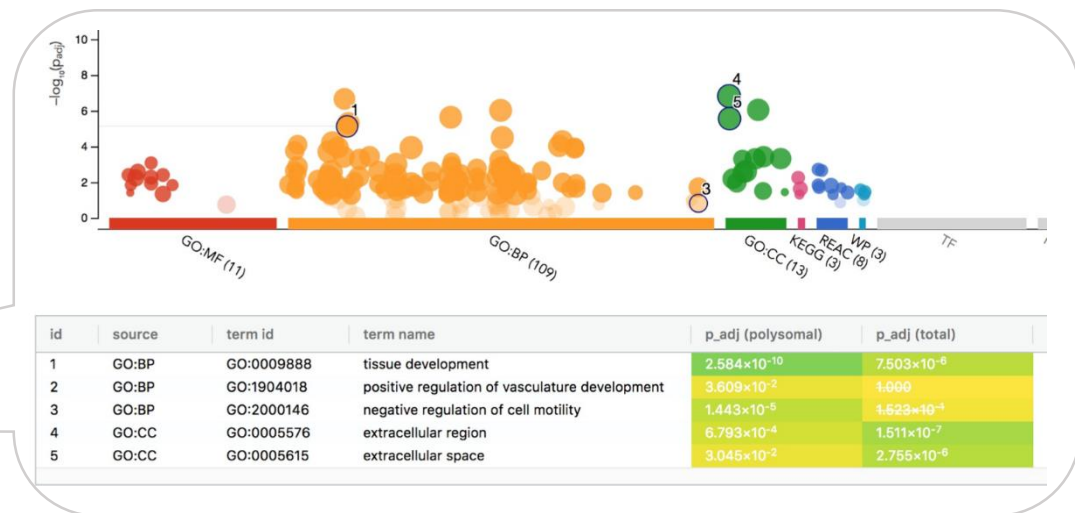
Ingenuity Pathway Analysis (IPA)

Metascape

PANTHER

WebGestalt

...



Navigation

- Protein-Protein Interactions
- Metabolic Pathways
- Signaling Pathways
- Pathway Diagrams
- Transcription Factors / Gene Regulatory Networks
- Protein-Compound Interactions
- Genetic Interaction Networks
- Protein Sequence Focused
- Other

Search

Organisms

Availability

Standards

Analysis

- Statistics
- Database Interactions

Contact

Comments, Questions, Suggestions are Always Welcome!

Complete Listing of All Pathguide Resources

Pathguide contains information about **702** biological pathway related resources and molecular interaction related resources. Click on a link to go to the resource home page or 'Details' for a description page. Databases that are free and those supporting BioPAX, CellML, PSI-MI or SBML standards are respectively indicated.

If you know of a pathway resource that is not listed here, or have other questions or comments, please [send us an e-mail](#).

News

Major new update of Pathguide September 2017
We now have information for over 690 resources!

Major new update of Pathguide August 2013
We now have information about ~550 resources!

Protein-Protein Interactions

Database Name (Order: alphabetically by web popularity)	Full Record	Availability	Standards
2P2Idb - The Protein-Protein Interaction Inhibition Database	Details	Free	
3D-Interologs - 3D-Interologs	Details	Free	
3DID - 3D interacting domains	Details	Free	
ACSN - Atlas of Cancer Signalling Network	Details	Free	BioPAX
ADAN - Prediction of protein-protein interaction of modular domains	Details	X	
AHD2.0 - Arabidopsis Hormone Database 2.0	Details	Free	
AllFuse - Functional Associations of Proteins in Complete Genomes	Details	X	
aMAZE - Protein Function and Biochemical Pathways Project	Details	X	
ANAP - Arabidopsis Network Analysis Pipeline	Details	Free	
ANIA - ANnotation and Integrated Analysis of the 14-3-3 interactome	Details	Free	
AnimalTFDB - Animal Transcription Factor Database	Details	Free	
AntiJen - AntiJen a Kinetic, Thermodynamic and Cellular Database	Details	Free	
APID - Agile Protein Interactomes DataServer	Details	Free	PSI-MI
Araport - Arabidopsis Information Portal	Details	Free	
ARN - The Autophagy Regulatory Network	Details	X	PSI-MI BioPAX SBML

Analysis tools differ from each other by:

Database:

- Supported **organisms** and gene identifiers (gene ID conversion)
- **Source** of pathways information (coverage of functional categories, cell types, disease association, protein properties)
- Level of data **curation** (pre-built pathways, predicted, de-novo networks)

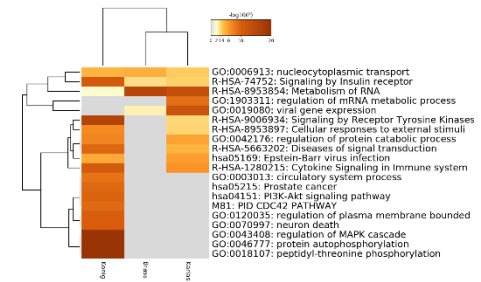
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Tool:

- **Appearance** and user experience (data visualization, input format, export of outputs)
- **Free** for academic or commercial
- **Platform** (Web-based, R package or standalone)



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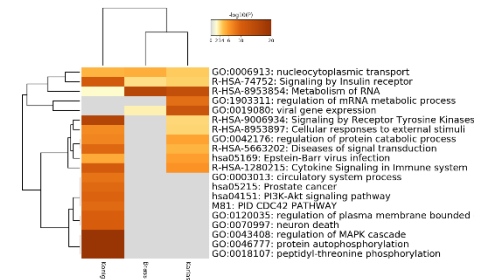
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Method:

- Supports **multiple** gene list comparison?
- **Statistical** methodology for calculating enrichment (control thresholds, FDR, gene background, term size)
- ‘multi-omic’ profiling
- Based on pre-built pathways, or on **de-novo** network construction/prediction

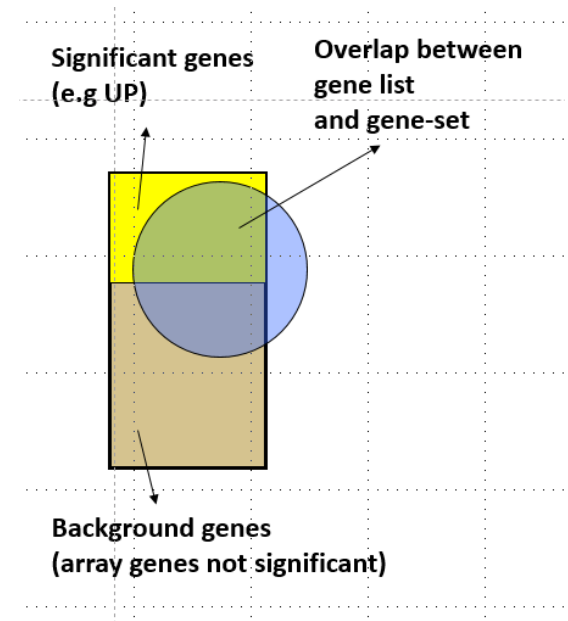


Pathways enrichments analysis

The aim is to give a score to the probability of observing the genes in a pathways just by chance

Scoring methods for **over-representation analysis** vary among tools, but usually use hypergeometric distribution, binomial distribution, chi-square distribution.

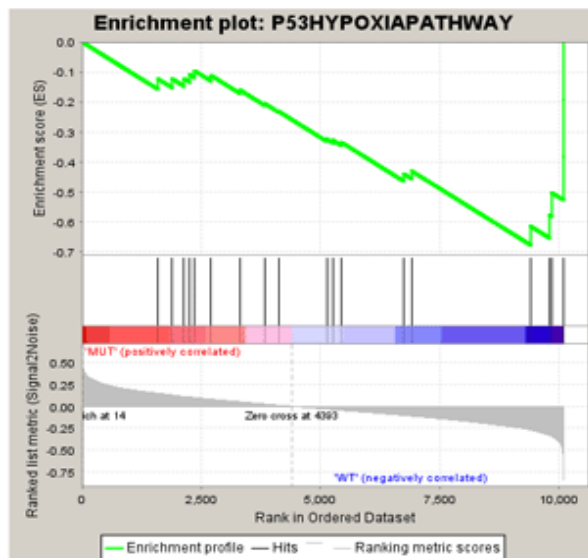
Over-Representation Analysis assumes that each gene is independent of the other genes! (not true in biology)



Pathways enrichments analysis

- Input gene list: using cut-off (arbitrary) methods vs whole gene list methods
- Controlling **thresholds**, FDR, gene background, term size)
- Considering **direction** of the change (up / down regulated)

GSEA



g:Profiler

Advanced options

All results

Measure underrepresentation

Statistical domain scope

Only annotated genes

Significance threshold

Bonferroni correction

User threshold

0.05

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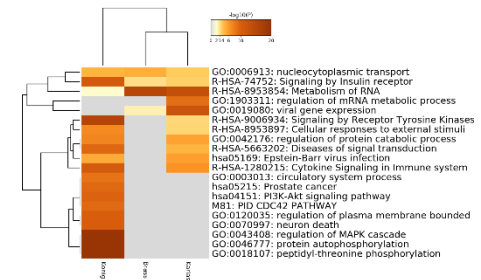
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Description Sample gene list (375 genes)



BioPlanet 2019



Glyoxylate and dicarboxylate metabolism
 Metabolism
 Glycogen biosynthesis
 Amino acid metabolism
 Molybdenum cofactor biosynthesis

WikiPathways 2019 Human



Methionine metabolism leading to Sulphur
 Mitochondrial Gene Expression WP391
 NAD Biosynthesis II (from tryptophan) WP24
 Methylation Pathways WP704
 Vitamin B12 Disorders WP4271

WikiPathways 2019 Mouse



Mitochondrial Gene Expression WP1263
 Amino Acid metabolism WP662
 Methylation WP1247
 Glycogen Metabolism WP317
 Mitochondrial LC-Fatty Acid Beta-Oxidation

KEGG 2019 Human



Glyoxylate and dicarboxylate metabolism
 Tyrosine metabolism
 Tryptophan metabolism
 beta-Alanine metabolism
 Fatty acid biosynthesis

ARCHS4 Kinases Coexp



TRPM7_human_kinase_ARCHS4_coexpression
 AAK1_human_kinase_ARCHS4_coexpression
 CDK17_human_kinase_ARCHS4_coexpression
 ICK_human_kinase_ARCHS4_coexpression
 STK16_human_kinase_ARCHS4_coexpression

KEGG 2019 Mouse



Glyoxylate and dicarboxylate metabolism
 Tyrosine metabolism
 Tryptophan metabolism
 Propanoate metabolism
 Valine, leucine and isoleucine degradation

BioCarta 2016



Visceral Fat Deposits and the Metabolic Syndrome
 RNA polymerase III transcription_Homo sapiens
 SODD/TNFR1 Signaling Pathway_Homo sapiens
 Reversal of Insulin Resistance by Leptin_Homo sapiens
 Regulators of Bone Mineralization_Homo sapiens

Reactome 2016



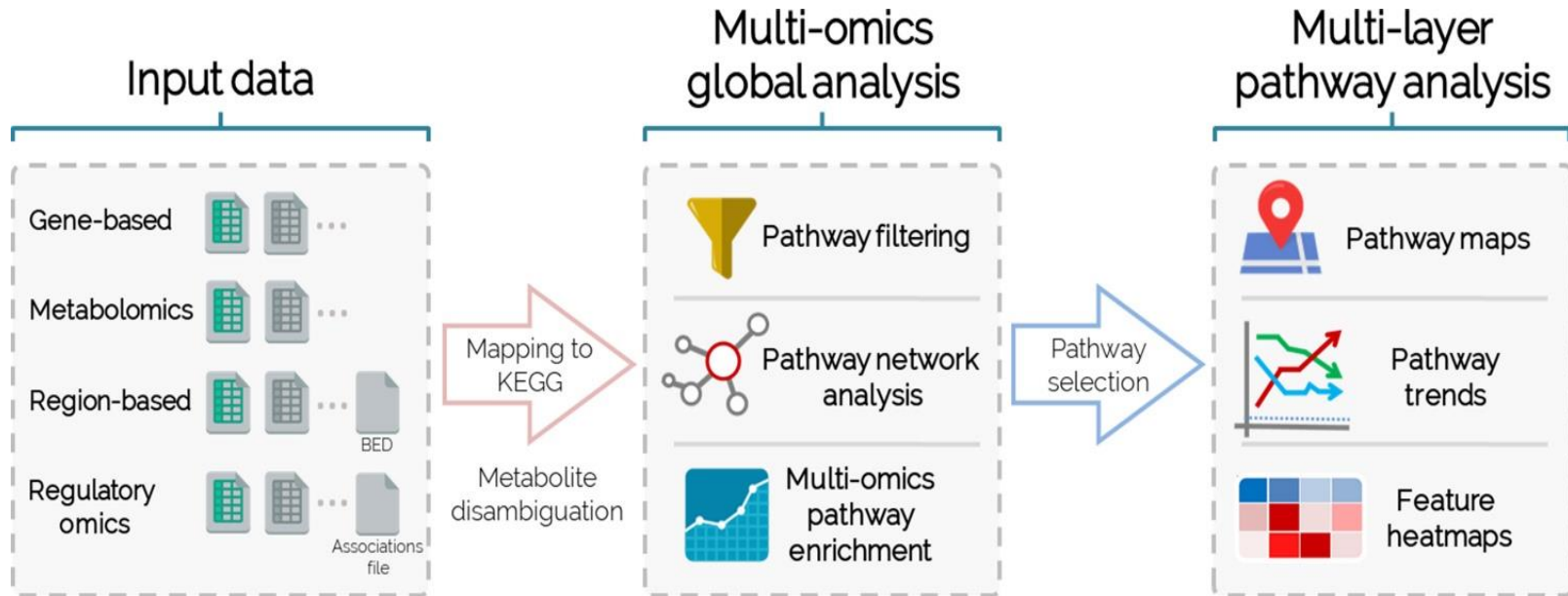
Metabolism of water-soluble vitamins and cofactors
 Glycogen synthesis_Homo sapiens_R-HSA-3525
 Molybdenum cofactor biosynthesis_Homo sapiens
 Gamma carboxylation, hypusine formation and
 Histidine, lysine, phenylalanine, tyrosine, proline

HumanCyc 2016

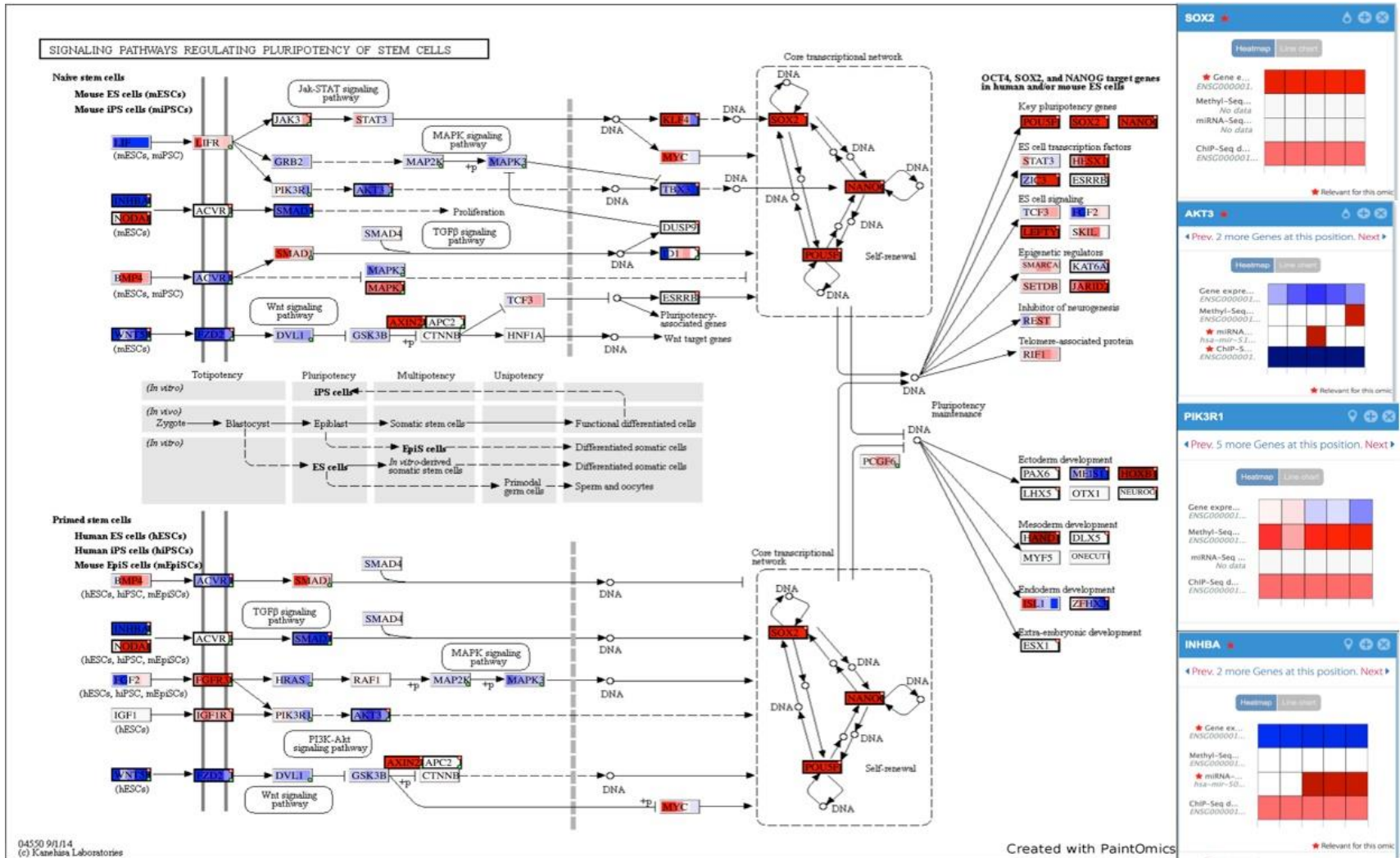


glycogen biosynthesis_Homo sapiens_PWY-510
 tryptophan degradation_Homo sapiens_TRYF00001
 L-kynurenine degradation_Homo sapiens_PWY-510
 tyrosine degradation_Homo sapiens_TYRF00001
 tryptophan degradation to 2-amino-3-carboxy-5-hydroxyindole-3-pyruvate

'Multi-omic' profiling, e.g. using PaintOmics 3:



'Multi-omic' profiling, e.g. using PaintOmics 3:



Signaling pathways regulating pluripotency of stem cells

Classification:

- Cellular Processes
- Cellular community - eukaryotes

	Matched features	p-value
ChIP-Seq data H3K4me3	128 (26)	0.052453
Gene expression	105 (27)	5.1746e-4
Methyl-Seq data	125 (6)	0.998886
miRNA-Seq data	12 (7)	0.502636

Gene expression
 1 major trends in this pathway.



ChIP-Seq data H3K4me3
 2 major trends in this pathway.



miRNA-Seq data
 1 major trends in this pathway.



Methyl-Seq data
 1 major trends in this pathway.



Next steps in Pathway-Based Analysis Tools

Evolving methods for pathways **prediction**, **modeling** networks, or machine learning.

For example:

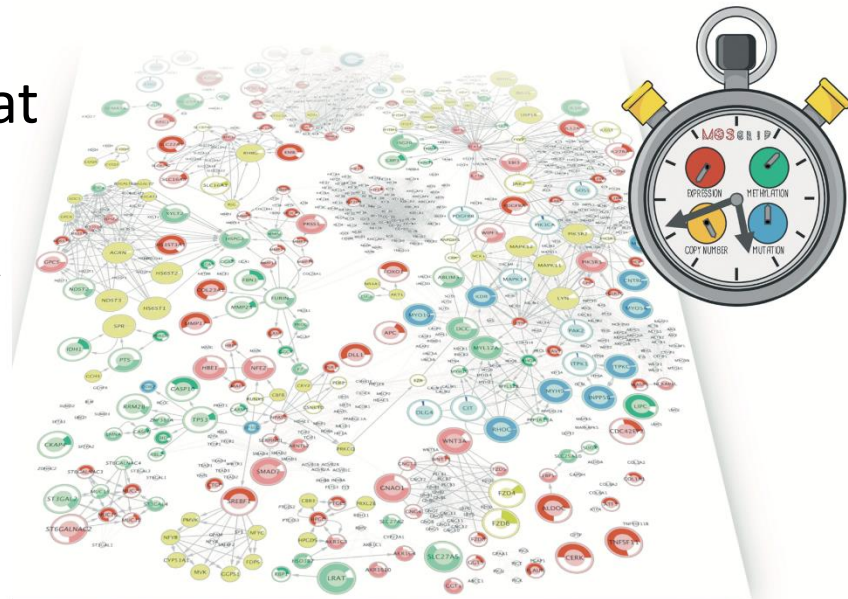
MetaCore and **IPA** use networks to visualize the data integration

MOSClip: Multi-Omic analysis tools that look for survival-associated gene modules in gene expression data, DNA mutations, methylation, and copy number data .

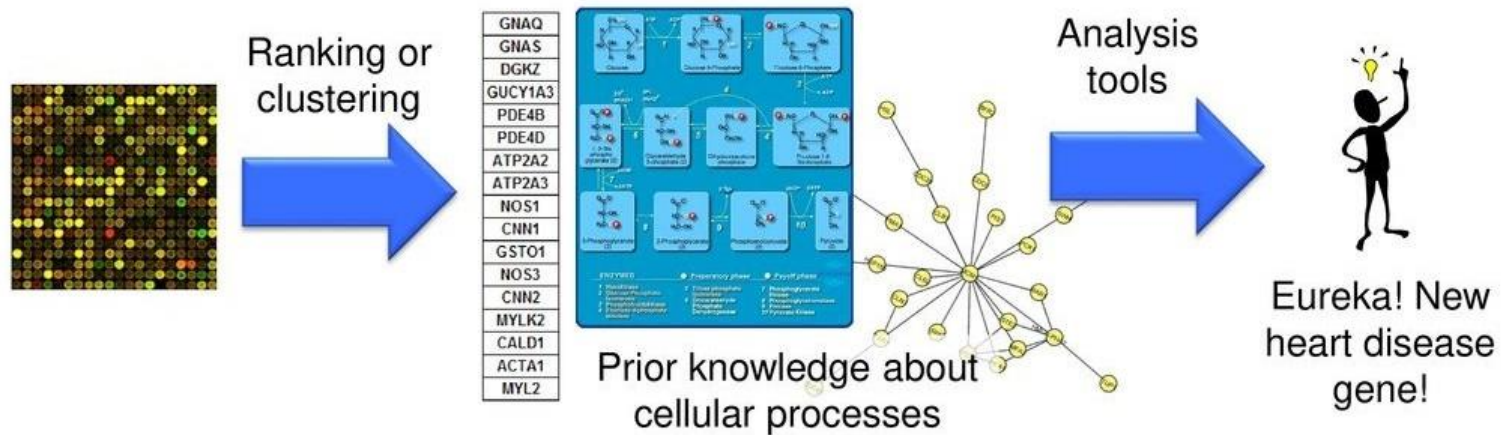
Nucleic Acids Research

VOLUME 47 ISSUE 14 2019

<https://academic.oup.com/nar>



Tips for choosing your pathway analysis tools



What is your biological question?

- Are you looking at a time course?
Comparing Treatment/Control?
- Selecting the gene list input : Upregulated and Downregulated?
Clustered?
 - Separately or Together?
 - Gene list or Ranked list?
- Is the fold change important?

Summary

Pathways analysis gives us the ability to conceptualize the mapping of genotype – phenotype

We discussed what is a pathway diagram
Pros and cons of using pathways analysis

Available resources for pathways analysis and enrichment
How to choose between them?

The field is evolving, with plenty of web tools and analysis approaches
Critically choose your analysis tools and evaluate their results

Tomorrow's exercise is about:

