

Pathway and Gene Set Analysis

Part 1

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The early steps of a microarray study

- Scientific Question (biological)
- Study design (biological/statistical)
- Conducting Experiment (biological)
- Preprocessing/Normalizing Data (statistical)
- Finding differentially expressed genes (statistical)

A data example

- Lee et al (2005) compared adipose tissue (abdominal subcutaneous adipocytes) between obese and lean Pima Indians
- Samples were hybridised on HGu95e-Affymetrix arrays (12639 genes/probe sets)
- Available as GDS1498 on the GEO database
- We selected the male samples only
 - 10 obese vs 9 lean

Y. H. Lee · S. Nair · E. Rousseau · D. B. Allison ·
G. P. Page · P. A. Tataranni · C. Bogardus ·
P. A. Permana

Microarray profiling of isolated abdominal subcutaneous adipocytes from obese vs non-obese Pima Indians: increased expression of inflammation-related genes

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Abstract *Aims/hypothesis:* Obesity increases the risk of developing major diseases such as diabetes and cardiovascular disease. Adipose tissue, particularly adipocytes, may play a major role in the development of obesity and its comorbidities. The aim of this study was to characterise, in adipocytes from obese people, the most differentially expressed genes that might be relevant to the development of obesity. *Methods:* We carried out microarray gene profiling of isolated abdominal subcutaneous adipocytes from 20 non-obese (BMI 25 ± 3 kg/m²) and 19 obese (BMI 55 ± 8 kg/m²) non-diabetic Pima Indians using Affymetrix HG-U95 GeneChip arrays. After data analyses, we measured the transcript levels of selected genes based on their biological functions and chromosomal positions using quantitative real-time PCR. *Results:* The most differentially ex-

pressed genes in adipocytes of obese individuals consisted of 433 upregulated and 244 downregulated genes. Of these, 410 genes could be classified into 20 functional Gene Ontology categories. The analyses indicated that the inflammation/immune response category was over-represented, and that most inflammation-related genes were upregulated in adipocytes of obese subjects. Quantitative real-time PCR confirmed the transcriptional upregulation of representative inflammation-related genes (*CCL2* and *CCL3*) encoding the chemokines monocyte chemoattractant protein-1 and macrophage inflammatory protein 1 α . The differential expression levels of eight positional candidate genes, including inflammation-related *THY1* and *CIQTNF5*, were also confirmed. These genes are located on chromosome 11q22–q24, a region with linkage to obesity in the Pima Indians. *Conclusions/interpretation:* This study provides evidence supporting the active role of mature adipocytes in obesity-related inflammation. It also provides potential candidate genes for susceptibility to obesity.

Electronic supplementary material Supplementary material is available in the online version of this article at <http://dx.doi.org/10.1007/s00125-005-1867-3>.

The “Result”

Probe Set ID	log.ratio	pvalue	adj.p
73554_at	1.4971	0.0000	0.0004
91279_at	0.8667	0.0000	0.0017
74099_at	1.0787	0.0000	0.0104
83118_at	-1.2142	0.0000	0.0139
81647_at	1.0362	0.0000	0.0139
84412_at	1.3124	0.0000	0.0222
90585_at	1.9859	0.0000	0.0258
84618_at	-1.6713	0.0000	0.0258
91790_at	1.7293	0.0000	0.0350
80755_at	1.5238	0.0000	0.0351
85539_at	0.9303	0.0000	0.0351
90749_at	1.7093	0.0000	0.0351
74038_at	-1.6451	0.0000	0.0351
79299_at	1.7156	0.0000	0.0351
72962_at	2.1059	0.0000	0.0351
88719_at	-3.1829	0.0000	0.0351
72943_at	-2.0520	0.0000	0.0351
91797_at	1.4676	0.0000	0.0351
78356_at	2.1140	0.0001	0.0359
90268_at	1.6552	0.0001	0.0421

What happened to the Biology???

Slightly more informative results

Probe Set ID	Gene Symbol	Gene Title	go biological process term	go molecular function term	log.ratio	pvalue	adj.p
73554_at	CCDC80	coiled-coil domain contain	---	---	1.4971	0.0000	0.0004
91279_at	C1QTNF5	C1q and tumor necrosis f	visual perception	embryonic development	0.8667	0.0000	0.0017
74099_at	---	---	---	---	1.0787	0.0000	0.0104
83118_at	RNF125	ring finger protein 125	immune response	modulation of protein binding	-1.2142	0.0000	0.0139
81647_at	---	---	---	---	1.0362	0.0000	0.0139
84412_at	SYNPO2	synaptopodin 2	---	actin binding	1.3124	0.0000	0.0222
90585_at	C15orf59	chromosome 15 open reading frame	---	---	1.9859	0.0000	0.0258
84618_at	C12orf39	chromosome 12 open reading frame	---	---	-1.6713	0.0000	0.0258
91790_at	MYEOV	myeloma overexpressed	---	---	1.7293	0.0000	0.0350
80755_at	MYOF	myoferlin	muscle contraction	protein binding	1.5238	0.0000	0.0351
85539_at	PLEKHH1	pleckstrin homology domain containing	---	binding	0.9303	0.0000	0.0351
90749_at	SERPINB9	serpin peptidase inhibitor, anti-apoptosis	signal transduction	endopeptidase inhibitor activity	1.7093	0.0000	0.0351
74038_at	---	---	---	---	-1.6451	0.0000	0.0351
79299_at	---	---	---	---	1.7156	0.0000	0.0351
72962_at	BCAT1	branched chain aminotransferase	G1/S transition of mitotic cell cycle	catalytic activity	2.1059	0.0000	0.0351
88719_at	C12orf39	chromosome 12 open reading frame	---	---	-3.1829	0.0000	0.0351
72943_at	---	---	---	---	-2.0520	0.0000	0.0351
91797_at	LRRC16A	leucine rich repeat containing	---	---	1.4676	0.0000	0.0351
78356_at	TRDN	triadin	muscle contraction	receptor binding	2.1140	0.0001	0.0359
90268_at	C5orf23	chromosome 5 open reading frame	---	---	1.6552	0.0001	0.0421

If we are lucky, some of the top genes mean something to us

But what if they don't?

And how what are the results for other genes with similar biological functions

How to incorporate biological knowledge

- The type of knowledge we deal with is rather simple:

We know groups/sets of genes that for example

- Belong to the same pathway
- Have a similar function
- Are located on the same chromosome, etc...

- We will assume these groupings to be given, i.e. we will not yet discuss methods used to detect pathways, networks, gene clusters
 - We will later!

What is a pathway?

- No clear definition
 - Wikipedia: “In biochemistry, **metabolic pathways** are series of chemical reactions occurring within a cell. In each pathway, a principal chemical is modified by chemical reactions.”
 - These pathways describe enzymes and metabolites
- But often the word “pathway” is also used to describe gene regulatory networks or protein interaction networks
- In all cases a pathway describes a biological function very specifically

What is a Gene Set?

- Just what it says: a set of genes!
 - All genes involved in a pathway are an example of a Gene Set
 - All genes corresponding to a Gene Ontology term are a Gene Set
 - All genes mentioned in a paper of Smith et al might form a Gene Set
- A Gene Set is a much more general and less specific concept than a pathway
- Still: we will sometimes use two words interchangeably, as the analysis methods are mainly the same

Where Do Gene Sets/Lists Come From?

- Molecular profiling e.g. mRNA, protein
 - Identification → Gene list
 - Quantification → Gene list + values
 - Ranking, Clustering (biostatistics)
- Interactions: Protein interactions, Transcription factor binding sites (ChIP)
- Genetic screen e.g. of knock out library
- Association studies (Genome-wide)
 - Single nucleotide polymorphisms (SNPs)
 - Copy number variants (CNVs)
 -

What is Gene Set/Pathway analysis?

- The aim is to give one number (score, p-value) to a Gene Set/Pathway
 - Are many genes in the pathway differentially expressed (up-regulated/downregulated)
 - Can we give a number (p-value) to the probability of observing these changes just by chance?

Goals

- Pathway and gene set data resources
 - Gene attributes
 - Database resources
 - GO, KeGG, Wikipathways, MsigDB
 - Gene identifiers and issues with mapping
- Differences between pathway analysis tools
 - Self contained vs. competitive tests
 - Cut-off methods vs. global methods
 - Issues with multiple testing

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Gene Attributes

- Functional annotation
 - Biological process, molecular function, cell location
- Chromosome position
- Disease association
- DNA properties
 - TF binding sites, gene structure (intron/exon), SNPs
- Transcript properties
 - Splicing, 3' UTR, microRNA binding sites
- Protein properties
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Database Resources

- Use functional annotation to aggregate genes into pathways/gene sets
- A number of databases are available
 - Different analysis tools link to different databases
 - Too many databases to go into detail on every one
 - Commonly used resources:
 - GO
 - KeGG
 - MsigDB
 - WikiPathways

Pathway and Gene Set data resources

- The Gene Ontology (GO) database
 - <http://www.geneontology.org/>
 - GO offers a relational/hierarchical database
 - Parent nodes: more general terms
 - Child nodes: more specific terms
 - At the end of the hierarchy there are genes/proteins
 - At the top there are 3 parent nodes: biological process, molecular function and cellular component
- Example: we search the database for the term “inflammation”

Term Lineage

[Switch to viewing term parents, siblings and children](#)

▼ Filter tree view ?

Filter Gene Product Counts	View Options	Buttons										
<table border="1"><thead><tr><th>Data source</th><th>Species</th></tr></thead><tbody><tr><td>All</td><td>All</td></tr><tr><td>AspGD</td><td>Anaplasma phagocy...</td></tr><tr><td>CGD</td><td>Arabidopsis thaliana</td></tr><tr><td>dictyBase</td><td>Bacillus anthraci...</td></tr></tbody></table>	Data source	Species	All	All	AspGD	Anaplasma phagocy...	CGD	Arabidopsis thaliana	dictyBase	Bacillus anthraci...	Tree view <input checked="" type="radio"/> Full <input type="radio"/> Compact	<input type="button" value="Set filters"/> <input type="button" value="Remove all filters"/>
Data source	Species											
All	All											
AspGD	Anaplasma phagocy...											
CGD	Arabidopsis thaliana											
dictyBase	Bacillus anthraci...											

- ▣ all : all [377382 gene products]
- ▣ **GO:0008150** : biological_process [270820 gene products]
- ▣ **GO:0050896** : response to stimulus [30457 gene products]
- ▣ **GO:0009605** : response to external stimulus [5585 gene products]
- ▣ **GO:0009611** : response to wounding [2289 gene products]
- ▣ **GO:0006954** : inflammatory response [1173 gene products]
- ▣ **GO:0002526** : acute inflammatory response [427 gene products]
- ▣ **GO:0002532** : production of molecular mediator of acute inflammatory response [44 gene products]
- ▣ **GO:0006950** : response to stress [16147 gene products]
- ▣ **GO:0006952** : defense response [4501 gene products]
- ▣ **GO:0006954** : inflammatory response [1173 gene products]
- ▣ **GO:0002526** : acute inflammatory response [427 gene products]
- ▣ **GO:0002532** : production of molecular mediator of acute inflammatory response [44 gene products]
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- ▣ **GO:0002532** : production of molecular mediator of acute inflammatory response [44 gene products]

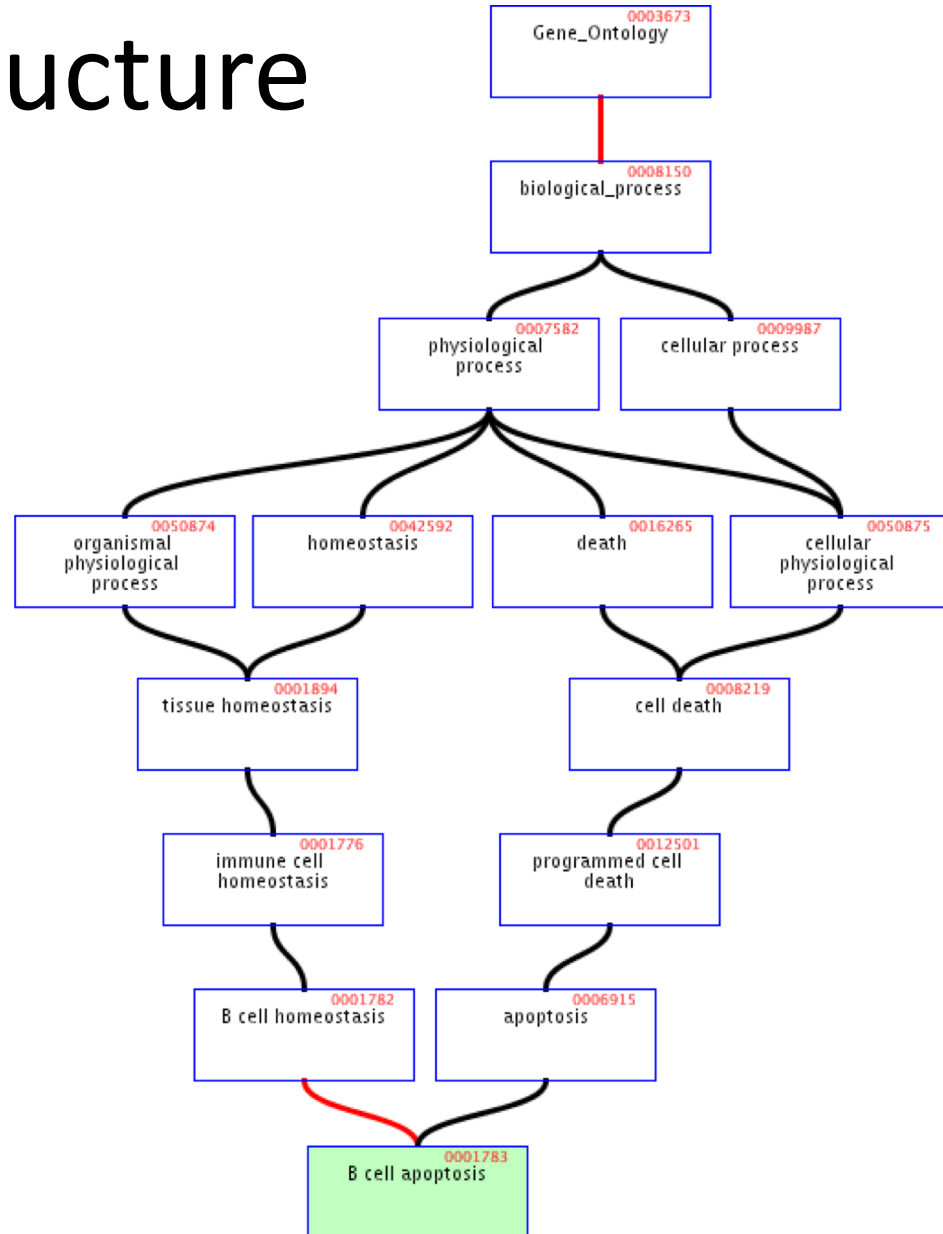
The genes on our array that code for one of the 44 gene products would form the corresponding “inflammation” gene set

What is the Gene Ontology (GO)?

- Set of biological phrases (terms) which are applied to genes:
 - protein kinase
 - apoptosis
 - membrane
- **Ontology: A formal system for describing knowledge**
- Gaudet P., Dessimoz C. (2017) Gene Ontology: Pitfalls, Biases, and Remedies. In: Dessimoz C., Škunca N. (eds) The Gene Ontology Handbook. Methods in Molecular Biology, vol 1446. Humana Press, New York, NY

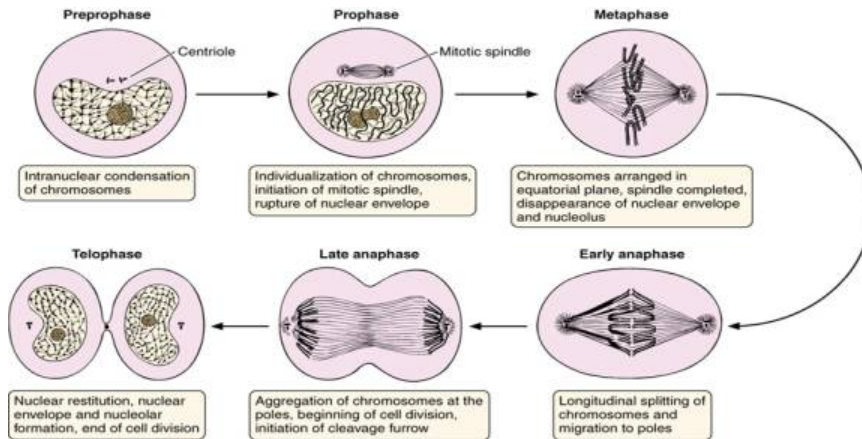
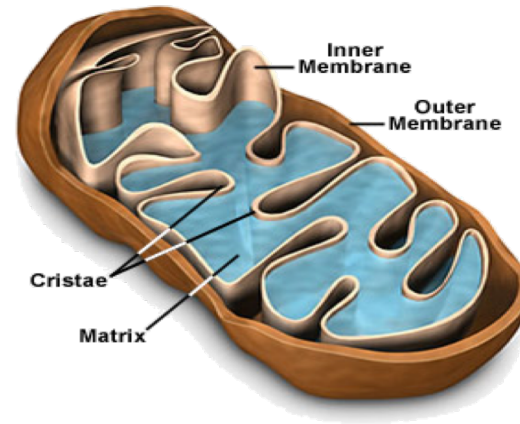
GO Structure

- Terms are related within a hierarchy
 - is-a
 - part-of
- Describes multiple levels of detail of gene function
- Terms can have more than one parent or child



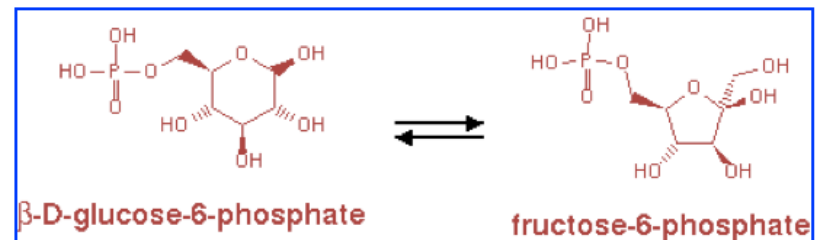
What GO Covers?

- GO terms divided into three aspects:
 - cellular component
 - molecular function
 - biological process



Cell division

glucose-6-phosphate isomerase activity



Terms

- Where do GO terms come from?
 - GO terms are added by editors at EBI and gene annotation database groups
 - Terms added by request
 - Experts help with major development
 - 27734 terms, 98.9% with definitions.
 - 16731 biological_process
 - 2385 cellular_component
 - 8618 molecular_function

Annotations

- Genes are linked, or associated, with GO terms by trained curators at genome databases
 - Known as ‘gene associations’ or GO annotations
 - Multiple annotations per gene
- Some GO annotations created automatically

Annotation Sources

- Manual annotation
 - Created by scientific curators
 - High quality
 - Small number (time-consuming to create)
- Electronic annotation
 - Annotation derived without human validation
 - Computational predictions (accuracy varies)
 - Lower 'quality' than manual codes
- Key point: be aware of annotation origin

Evidence Types

- **ISS:** Inferred from Sequence/Structural Similarity
- **IDA:** Inferred from Direct Assay
- **IPI:** Inferred from Physical Interaction
- **IMP:** Inferred from Mutant Phenotype
- **IGI:** Inferred from Genetic Interaction
- **IEP:** Inferred from Expression Pattern
- **TAS:** Traceable Author Statement
- **NAS:** Non-traceable Author Statement
- **IC:** Inferred by Curator
- **ND:** No Data available



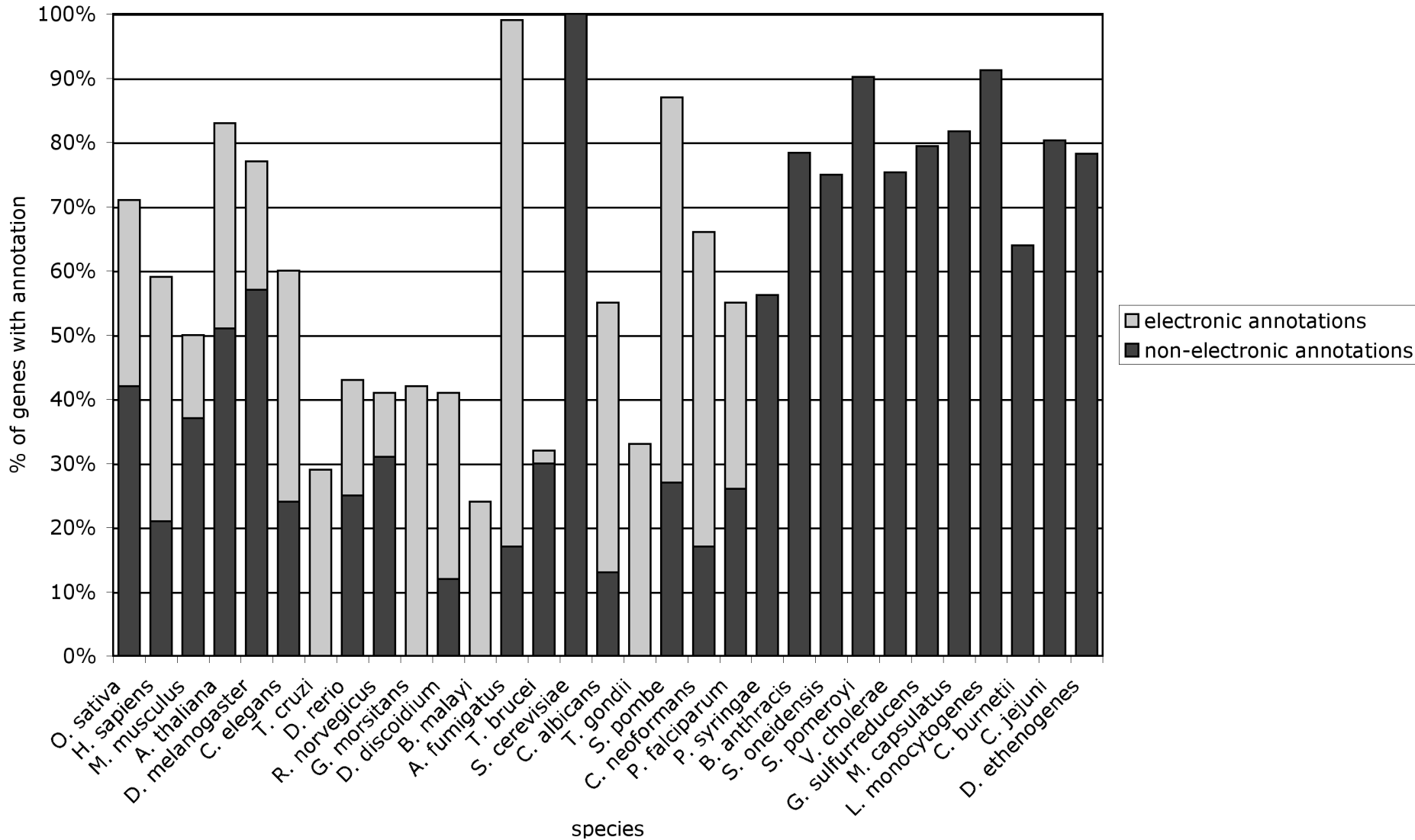
- **IEA:** Inferred from electronic annotation



Species Coverage

- All major eukaryotic model organism species
- Human via GOA group at UniProt
- Several bacterial and parasite species through TIGR and GeneDB at Sanger
- New species annotations in development

Variable Coverage

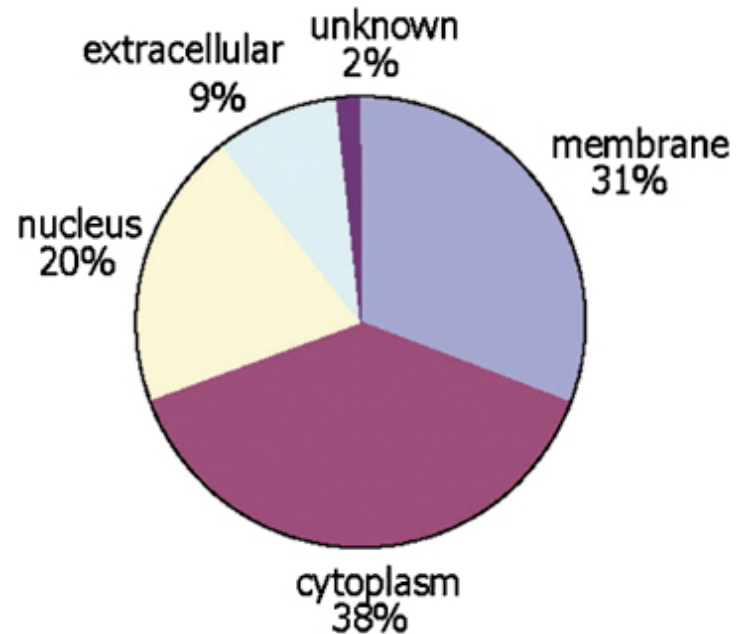


Contributing Databases

- [Berkeley Drosophila Genome Project \(BDGP\)](#)
- [dictyBase](#) (*Dictyostelium discoideum*)
- [FlyBase](#) (*Drosophila melanogaster*)
- [GeneDB](#) ([Schizosaccharomyces pombe](#), *Plasmodium falciparum*, *Leishmania major* and *Trypanosoma brucei*)
- [UniProt Knowledgebase](#) (Swiss-Prot/TrEMBL/PIR-PSD) and [InterPro](#) databases
- [Gramene](#) (grains, including rice, *Oryza*)
- [Mouse Genome Database \(MGD\)](#) and [Gene Expression Database \(GXD\)](#) (*Mus musculus*)
- Rat Genome Database (RGD) (*Rattus norvegicus*)
- [Reactome](#)
- [Saccharomyces Genome Database \(SGD\)](#) (*Saccharomyces cerevisiae*)
- [The Arabidopsis Information Resource \(TAIR\)](#) (*Arabidopsis thaliana*)
- [The Institute for Genomic Research \(TIGR\)](#): databases on several bacterial species
- [WormBase](#) (*Caenorhabditis elegans*)
- [Zebrafish Information Network \(ZFIN\)](#): (*Danio rerio*)

GO Slim Sets

- GO has too many terms for some uses
 - Summaries (e.g. Pie charts)
- GO Slim is an official reduced set of GO terms
 - Generic, plant, yeast



GO Software Tools

- GO resources are freely available to anyone without restriction
 - Includes the ontologies, gene associations and tools developed by GO
- Other groups have used GO to create tools for many purposes
 - <http://www.geneontology.org/GO.tools>

Accessing GO: QuickGO

Search for a GO term:



examples - [apoptosis](#), [GO:0006915](#)

Search for a Protein:



examples - [tropomyosin](#), [P06727](#)

Compare GO terms:



example - [GO:0000122](#), [GO:0000001](#)

Find, view and download [annotation](#)



GO:0006915 apoptosis

A form of programmed cell death induced by external or internal signals that trigger the activity of proteolytic caspases, whose actions disintegrate the cell internally with condensation and subsequent fragmentation of the cell nucleus (blebbing) while the plasma membrane remains intact. Other features include the exposure of phosphatidyl serine on the cell surface.

[Term Information](#)

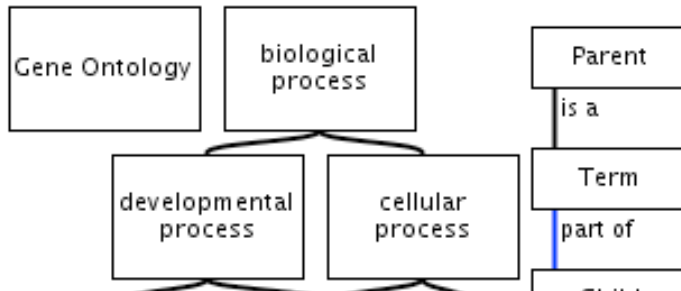
[Ancestor chart](#)

[Ancestor table](#)

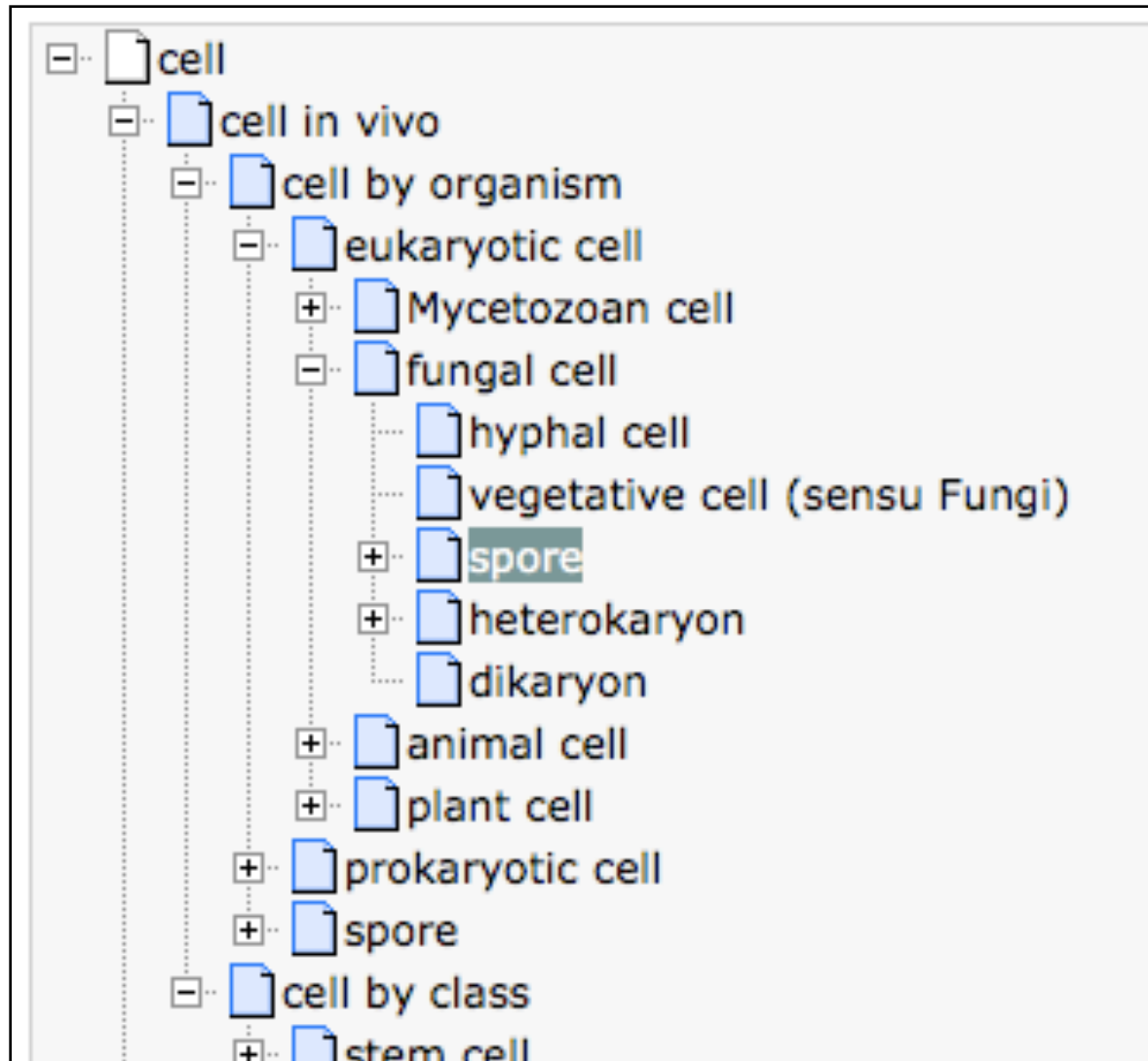
[Child Terms](#)

[Protein Annotation](#)

[Statistics](#)



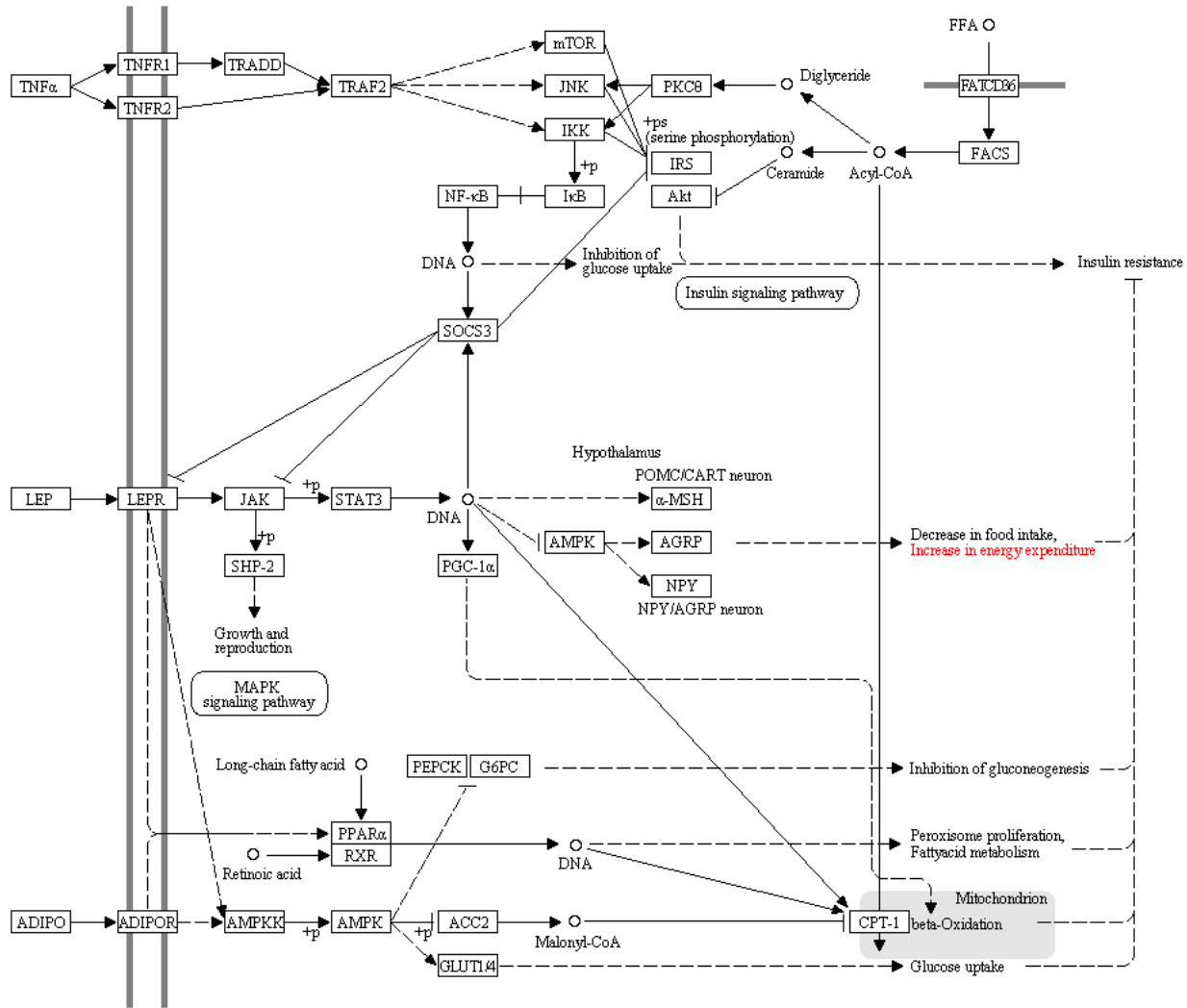
Other Ontologies



KEGG pathway database

- KEGG = Kyoto Encyclopedia of Genes and Genomes
 - <http://www.genome.jp/kegg/pathway.html>
 - The pathway database gives far more detailed information than GO
 - Relationships between genes and gene products
 - But: this detailed information is only available for selected organisms and processes
 - Example: Adipocytokine signaling pathway

ADIPOCYTOKINE SIGNALING PATHWAY



KEGG pathway database

- Clicking on the nodes in the pathway leads to more information on genes/proteins
 - Other pathways the node is involved with
 - Entries in Gene/Protein databases
 - References
 - Sequence information
- Ultimately this allows to find corresponding genes on the microarray and define a Gene Set for the pathway

Wikipathways

- <http://www.wikipathways.org>
- A wikipedia for pathways
 - One can see and download pathways
 - But also edit and contribute pathways
- The project is linked to the GenMAPP and Pathvisio analysis/visualisation tools

MSigDB

- MSigDB = Molecular Signature Database

<http://www.broadinstitute.org/gsea/msigdb>

- Related to the the analysis program GSEA
- MSigDB offers gene sets based on various groupings
 - Pathways
 - GO terms
 - Chromosomal position,...

- ▶ MSigDB Home
- ▶ About Collections
- ▶ Browse Gene Sets
- ▶ Search Gene Sets
- ▶ Annotate Gene Sets
- ▶ View Gene Families
- ▶ Help



MSigDB
Molecular Signatures
Database

Molecular Signatures Database

Overview

The Molecular Signatures Database (MSigDB) is a collection of gene sets for use with GSEA software. From this web site, you can

- ▶ **Search** for gene sets
- ▶ **Browse** gene sets
- ▶ **View annotations** by clicking a gene set name to display its gene set page; for example, [AKTPATHWAY](#)
- ▶ **Download** gene sets
- ▶ **Compute overlaps** between your gene set and other gene sets in MSigDB
- ▶ **Categorize** members of a gene set by gene families
- ▶ **Build an expression signature** of the gene set using a compendium of expression profiles

Registration

Please [register](#) to download the GSEA software and view the MSigDB gene sets. After registering, you can log in at any time using your email address. Registration is free. Its only purpose is to help us track usage for reports to our funding agencies.

Current Version

GSEA/MSigDB web site v2.0 released December 14 2007
MSigDB database v2.5 updated April 7 2008, [Release notes](#).

Collections

The MSigDB gene sets are divided into five major collections:

c1 **positional gene sets** for each human chromosome and each cytogenetic band.

c2 **curated gene sets** from online pathway databases, publications in PubMed, and knowledge of domain experts.

c3 **motif gene sets** based on conserved *cis*-regulatory motifs from a comparative analysis of the human, mouse, rat and dog genomes.

c4 **computational gene sets** defined by expression neighborhoods centered on 380 cancer-associated genes.

c5 **GO gene sets** consist of genes annotated by the same GO terms.

Some Warnings

- In many cases the definition of a pathway/gene set in a database might differ from that of a scientist
- The nodes in pathways are often proteins or metabolites; the activity of the corresponding gene set is not necessarily a good measurement of the activity of the pathway
- There are many more resources out there (BioCarta, BioPax)
- Commercial packages often use their own pathway/gene set definitions (Ingenuity, Metacore, Genomatix,...)
- Genes in a gene set are usually not given by a Probe Set ID, but refer to some gene data base (Entrez IDs, Unigene IDs)
 - Conversion can lead to errors!

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 - Biological process, molecular function, cell location
- Chromosome position
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- DNA properties
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Sources of Gene Attributes

- Ensembl BioMart (eukaryotes)
 - <http://www.ensembl.org>
- Entrez Gene (general)
 - <http://www.ncbi.nlm.nih.gov/sites/entrez?db=gen>
e
- Model organism databases
 - E.g. SGD: <http://www.yeastgenome.org/>
- Many others.....

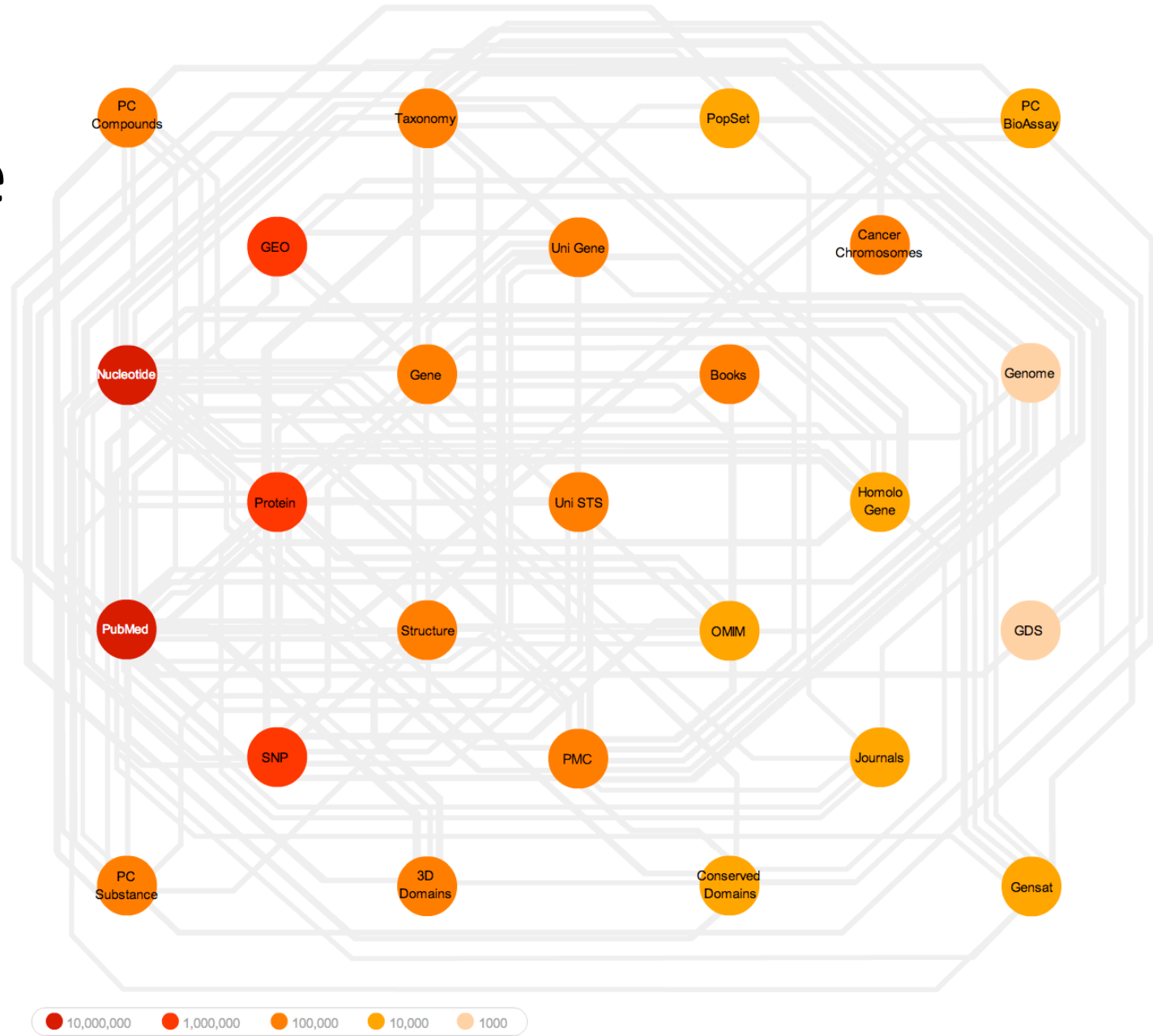
Gene and Protein Identifiers

- Identifiers (IDs) are ideally unique, stable names or numbers that help track database records
 - E.g. Social Insurance Number, Entrez Gene ID 41232
- Gene and protein information stored in many databases
 - → Genes have many IDs
- Records for: Gene, DNA, RNA, Protein
 - Important to recognize the correct record type
 - E.g. Entrez Gene records don't store sequence. They link to DNA regions, RNA transcripts and proteins.

NCBI Database Links

NCBI:
U.S. National Center
for Biotechnology
Information

Part of National
Library of Medicine
(NLM)



http://www.ncbi.nlm.nih.gov/Database/datamodel/data_nodes.swf

Common Identifiers

Gene

Ensembl [ENSG00000139618](#)

Entrez Gene [675](#)

Unigene [Hs.34012](#)

RNA transcript

GenBank [BC026160.1](#)

RefSeq [NM_000059](#)

Ensembl [ENST00000380152](#)

Protein

Ensembl [ENSP00000369497](#)

RefSeq [NP_000050.2](#)

UniProt [BRCA2_HUMAN](#) or

[A1YBP1_HUMAN](#)

IPI [IPI00412408.1](#)

EMBL [AF309413](#)

PDB [1MIU](#)

Species-specific

HUGO HGNC [BRCA2](#)

MGI [MGI:109337](#)

RGD [2219](#)

ZFIN [ZDB-GENE-060510-3](#)

FlyBase [CG9097](#)

WormBase [WBGene00002299](#) or [ZK1067.1](#)

SGD [S000002187](#) or [YDL029W](#)

Annotations

InterPro [IPR015252](#)

OMIM [600185](#)

Pfam [PF09104](#)

Gene Ontology [GO:0000724](#)

SNPs [rs28897757](#)

Experimental Platform

Affymetrix [208368_3p_s_at](#)

Agilent [A_23_P99452](#)

CodeLink [GE60169](#)

Illumina [GI_4502450-S](#)

Red = Recommended

Identifier Mapping

- So many IDs!
 - Mapping (conversion) is a headache
- Four main uses
 - Searching for a favorite gene name
 - Link to related resources
 - Identifier translation
 - E.g. Genes to proteins, Entrez Gene to Affy
 - Unification during dataset merging
 - Equivalent records

ID Mapping Challenges

- Avoid errors: map IDs correctly
- Gene name ambiguity – not a good ID
 - e.g. FLJ92943, LFS1, TRP53, p53
 - Better to use the standard gene symbol: TP53
- Excel error-introduction
 - OCT4 is changed to October-4
- Problems reaching 100% coverage
 - E.g. due to version issues
 - Use multiple sources to increase coverage

Summary

- Databases
- Choice makes a difference
- Not all use the same IDs – watch out 😊

Questions?