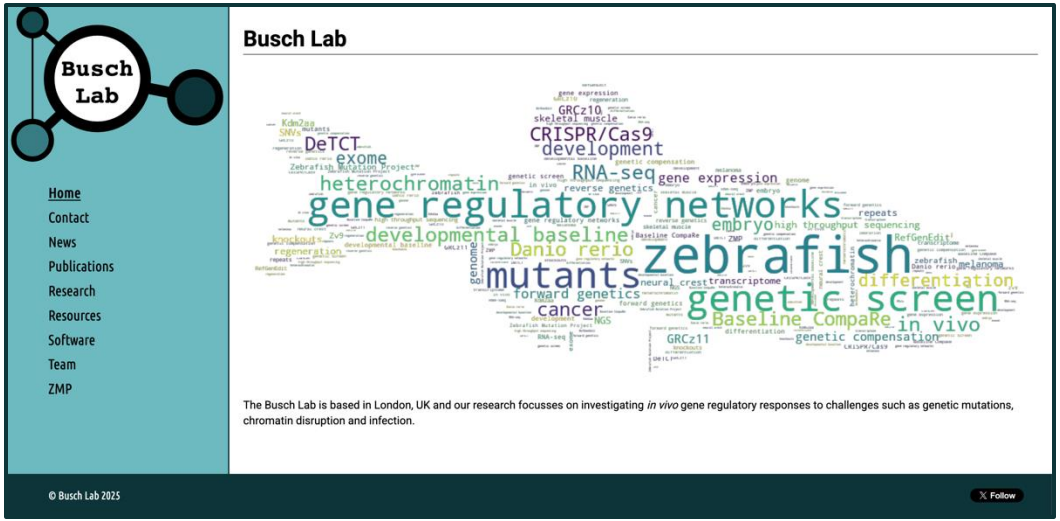
Introduction to RNA-seq and functional interpretation:

Next steps in gene prioritisation

27th Feb 2025

Me

- Ian Sealy
- Busch Lab, QMUL
- Previously at Sanger Institute
- RNA-seq / zebrafish
- Run “*Bioinformatics & Functional Genomics in Zebrafish*” course at EBI

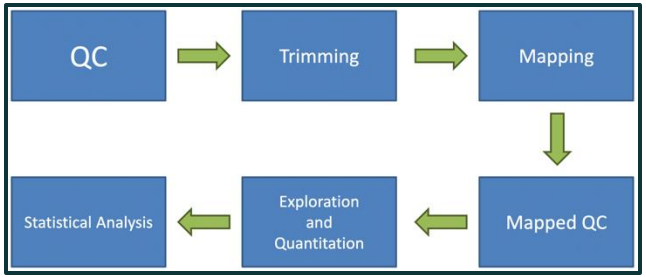


Questions

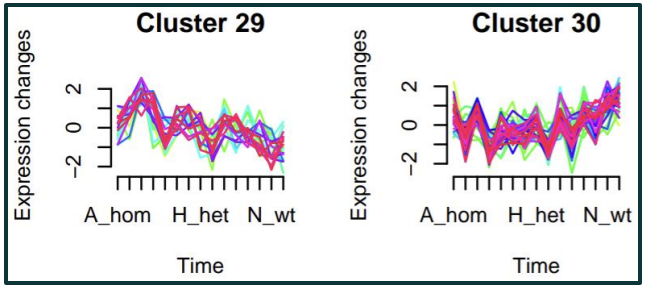
- For urgent questions, just unmute and ask
- If you can wait, then add your question to the Q&A document and I'll answer in a break or later

Gene list of interest

- Starting point for today: **gene list of interest**
- Most likely from RNA-seq differential expression analysis
- But could be a list from any other analysis:
  - Clustering genes with similar expression profiles
  - Microarray analysis
  - Quantitative proteomics
  - Differential methylation analysis
  - etc…



From: Simon Andrews



Unranked or ranked gene list?

- Gene list can be:
  - Unranked (e.g. genes with somatic mutations in cancer sample)
  - Ranked (e.g. sensitivity in a CRISPR screen)
- RNA-seq differential expression analysis produces ranked lists
- Ranked lists are ordered by a score or metric:
  - e.g. adjusted p-value
  - e.g. $\log_2$ fold change
- Ranked lists can also have a threshold applied:
  - e.g. adjusted p-value < 0.05

```
ENSDARG00000043198
ENSDARG00000075229
ENSDARG00000036695
ENSDARG00000092115
ENSDARG00000013076
ENSDARG00000015890
ENSDARG00000060682
ENSDARG00000076241
ENSDARG00000093347
ENSDARG00000098114
```

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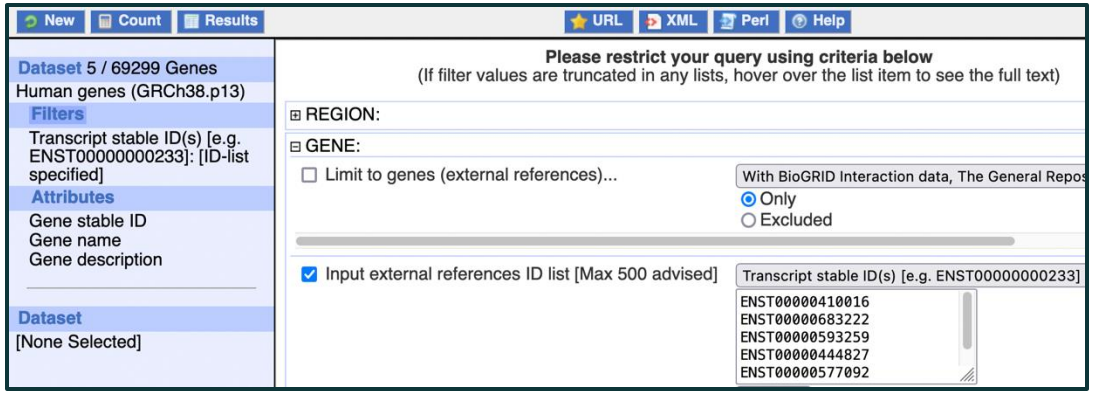
ENSDARG00000075676 0.039
ENSDARG00000104197 0.041
ENSDARG00000004301 0.041
ENSDARG00000079766 0.042
ENSDARG00000030494 0.042
ENSDARG00000116804 0.043
ENSDARG00000100599 0.043
ENSDARG00000104325 0.043
ENSDARG00000111102 0.043
ENSDARG00000022466 0.044
```

“Gene” list of interest

- May not actually be a list of genes
- Could be transcripts or proteins or SNPs, etc…
- Most tools require a list of genes so need to convert
- BioMart is a useful tool for conversions (and other bioinformatics tasks): www.ensembl.org/biomart/martview



What next?

- Have a gene list, but what do you do next?
- How do you relate the gene list to existing knowledge?

Gene	pval	adjp	log2fc
ENSDARG00000041294	4.904002310063973e-37	1.0867269119101765e-32	1.5709251030700861
ENSDARG00000060498	1.1297090308658515e-25	1.2517176061993635e-21	1.5921762041345
ENSDARG00000031683	3.2009883731403506e-25	2.364463411626339e-21	-1.277820860357806
ENSDARG00000077982	5.3336179195843655e-18	2.9548243274497384e-14	0.9349522690823255
ENSDARG00000070480	1.2940060161760502e-17	5.735034663692255e-14	1.0699010828953783
ENSDARG00000007769	4.245003753873642e-17	1.5678213864306653e-13	1.6785196633873156
ENSDARG00000102435	6.025610180317608e-17	1.9075360227976884e-13	1.0539265022132713
ENSDARG00000101482	9.742460938723084e-17	2.6986616800262944e-13	0.9350743176658163
ENSDARG00000034503	2.261103100242347e-16	5.567338300152267e-13	0.6082489350504545

What next?

- Have a gene list, but what do you do next?
- How do you relate the gene list to existing knowledge?
- Add annotation (e.g. BioMart)

Gene	pval	adjp	log2fc	Chr	Start	End	Name	Description
ENSDARG00000041294	4.904002310063973e-37	1.0867269119101765e-32	1.5709251030700861	3	62161184	62169060	noxo1a	NADPH oxidase organizer 1a
ENSDARG00000060498	1.1297090308658515e-25	1.2517176061993635e-21	1.5921762041345	23	30006206	30010042	tnfrsf9a	tumor necrosis factor receptor superfamily, member 9a
ENSDARG00000031683	3.2009883731403506e-25	2.364463411626339e-21	-1.277820860357806	20	46552311	46554440	fosab	v-fos FBJ murine osteosarcoma viral oncogene homolog Ab
ENSDARG00000077982	5.3336179195843655e-18	2.9548243274497384e-14	0.9349522690823255	22	661505	665371	elf3	E74-like factor 3 (ets domain transcription factor, epithelial-specific)
ENSDARG00000070480	1.2940060161760502e-17	5.735034663692255e-14	1.0699010828953783	19	30400372	30404096	agr2	anterior gradient 2
ENSDARG00000007769	4.245003753873642e-17	1.5678213864306653e-13	1.6785196633873156	7	56602521	56606752	sult5a1	sulfotransferase family 5A, member 1
ENSDARG00000102435	6.025610180317608e-17	1.9075360227976884e-13	1.0539265022132713	7	45975537	45976956	plekhf1	pleckstrin homology domain containing, family F (with FYVE domain) member 1
ENSDARG00000101482	9.742460938723084e-17	2.6986616800262944e-13	0.9350743176658163	5	13870340	14004206	hk2	hexokinase 2
ENSDARG00000034503	2.261103100242347e-16	5.567338300152267e-13	0.6082489350504545	2	48309600	48375342	per2	period circadian clock 2

Look up genes in databases

GENE

noxo1a

ID	ZDB-GENE-030131-9700
Name	*NADPH oxidase organizer 1a*
Symbol	*noxo1a* Nomenclature History
Previous Names	*noxo1*, *cb18* (1), *sb:cb18*, *SNX28b* (1), *wu:fd09d09*, *zgc:152911* (1)
Type	protein_coding_gene
Location	Chr: 3 Mapping Details/Browsers
Description	Predicted to have phosphatidylinositol-3-phosphate binding activity and superoxide-generating NADPH oxidase activator activity. Predicted to be involved in superoxide metabolic process. Predicted to localize to NADPH oxidase complex and cytoplasm. Is expressed in EVL; periderm; and pharynx. Orthologous to human NOXO1 (NADPH oxidase organizer 1).
Genome Resources	Alliance (1), Gene:572245 (1), Ensembl(GRCz11):ENSDARG00000041294 (3)
Note	*None*
Comparative Information	

Look up genes in databases

GENE

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GENE

NOXO1

Species	*Homo sapiens*
Symbol	NOXO1
Name	NADPH oxidase organizer 1
Synonyms	MGC20258 NADPH oxidase regulatory protein Show All 12
Biotype	protein coding gene
Automated Description	Enables enzyme binding activity. Involved in extracellular matrix disassembly. Part of NADPH oxidase complex.
RGD Description	This gene encodes an NADPH oxidase (NOX) organizer, which positively regulates NOX1 and NOX3. The protein contains a PX domain and two SH3 domains. Alternatively spliced transcript variants encoding multiple isoforms have been observed for this gene. [provided by RefSeq, Jun 2012]
Cross References	ENSEMBL:ENSG00000196408 NCBI_Gene:124056 Show All 4
Additional Information	Literature

Look up genes in databases

GENE

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Additional Information Literature

Summaries for NOXO1 Gene

Entrez Gene Summary for NOXO1 Gene

This gene encodes an NADPH oxidase (NOX) organizer, which positively regulates NOX1 and NOX3. The protein contains a PX domain and two SH3 domains. Alternatively spliced transcript variants encoding multiple isoforms have been observed for this gene. [provided by RefSeq, Jun 2012]

GeneCards Summary for NOXO1 Gene

NOXO1 (NADPH Oxidase Organizer 1) is a Protein Coding gene. Diseases associated with NOXO1 include Lung Mucoepidermoid Carcinoma and Phagocyte Bactericidal Dysfunction. Among its related pathways are Signaling by Rho GTPases and Disease. Gene Ontology (GO) annotations related to this gene include *identical protein binding* and *phospholipid binding*. An important paralog of this gene is SH3PXD2A.

UniProtKB/Swiss-Prot Summary for NOXO1 Gene

Constitutively potentiates the superoxide-generating activity of NOX1 and NOX3 and is required for the biogenesis of otoconia/otolith, which are crystalline structures of the inner ear involved in the perception of gravity. Isoform 3 is more potent than isoform 1 in activating NOX3. Together with NOXA1, may also substitute to NCF1/p47phox and NCF2/p67phox in supporting the phagocyte NOX2/gp91phox superoxide-generating activity. (NOXO1_HUMAN,Q8NFA2)

Gene Wiki entry for NOXO1 Gene

Additional gene information for NOXO1 Gene

HGNC (19404) NCBI Entrez Gene (124056) Ensembl (ENSG00000196408) OMIM® (611256) UniProtKB/Swiss-Prot (Q8NFA2)
Open Targets Platform(ENSG00000196408)

Alliance of Genome Resources

Look up genes in databases

GENE

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Synonyms

Biotype

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Additional Information

Summaries for NOXO1 Gene

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Gene Wiki entr

Additional gene

HGNC (19404)

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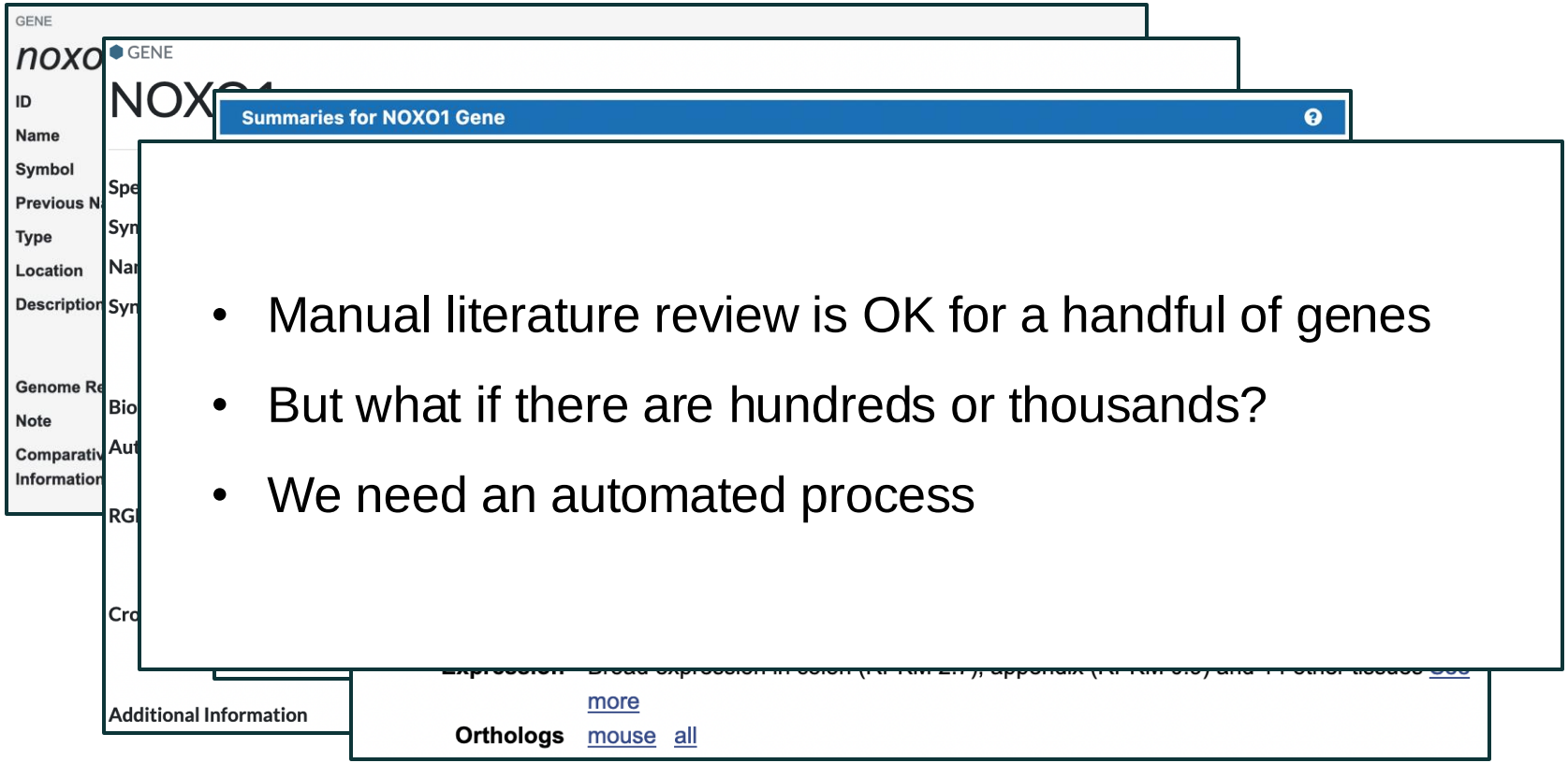
Official Symbol	NOXO1 provided by HGNC
Official Full Name	NADPH oxidase organizer 1 provided by HGNC
Primary source	HGNC:HGNC:19404
See related	Ensembl:ENSG00000196408 MIM:611256; AllianceGenome:HGNC:19404
Gene type	protein coding
RefSeq status	REVIEWED
Organism	Homo sapiens
Lineage	Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini; Catarrhini; Hominidae; Homo
Also known as	SNX28; P41NOX; P41NOXA; P41NOXB; P41NOXC; SH3PXD5
Summary	This gene encodes an NADPH oxidase (NOX) organizer, which positively regulates NOX1 and NOX3. The protein contains a PX domain and two SH3 domains. Alternatively spliced transcript variants encoding multiple isoforms have been observed for this gene. [provided by RefSeq, Jun 2012]
Expression	Broad expression in colon (RPKM 2.7), appendix (RPKM 0.9) and 14 other tissues See more
Orthologs	mouse all

Look up genes in databases



- Manual literature review is OK for a handful of genes
- But what if there are hundreds or thousands?
- We need an automated process

Functional enrichment analysis

- **Functional enrichment analysis** (or over-representation) systematically relates your data to existing knowledge
- Can help you to:
  - Gain biological insight
  - Generate new hypotheses
  - Validate your experiment

Functional gene sets

- Existing knowledge is organised into **functional gene sets** in a standardised way, using data from previous experiments
- A functional gene set is a group of genes with a common biological relationship (e.g. annotated to same biological process or involved in same pathway)
- e.g. circadian rhythm:

Gene Product	Symbol	Qualifier	GO Term	Evidence	Reference	Assigned By	Name
UniProtKB:A0A024QZG3	ATF5	involved_in	GO:0007623 P circadian rhythm	ECO:0000265 IEA	GO_REF:0000107	Ensembl	BZIP domain-containing protein
UniProtKB:A0A024QZQ1	SIRT1	involved_in	GO:0007623 P circadian rhythm	ECO:0000265 IEA	GO_REF:0000107	Ensembl	Deacetylase sirtuin-type domain-containing protein
UniProtKB:A0A024R230	NTRK2	involved_in	GO:0007623 P circadian rhythm	ECO:0000265 IEA	GO_REF:0000107	Ensembl	Tyrosine-protein kinase receptor
UniProtKB:A0A024R241	NFIL3	involved_in	GO:0007623 P circadian rhythm	ECO:0000256 IEA	GO_REF:0000002	InterPro	Nuclear factor interleukin-3-regulated protein

Functional annotation

- Functional annotation is created and maintained by many dedicated databases and projects, e.g.
  - Gene Ontology (GO)
  - Reactome
  - KEGG
  - TRANSFAC

Gene Ontology

Current release 2025-02-06: 40,267 GO terms | 7,905,607 annotations
1,565,873 gene products | 5,443 species (see statistics)

- GO is largest source of gene functional annotation
- Structured, controlled vocabulary of terms (and therefore gene sets)
- Manually annotated by a large consortium
- Data come from experimental and computational analyses

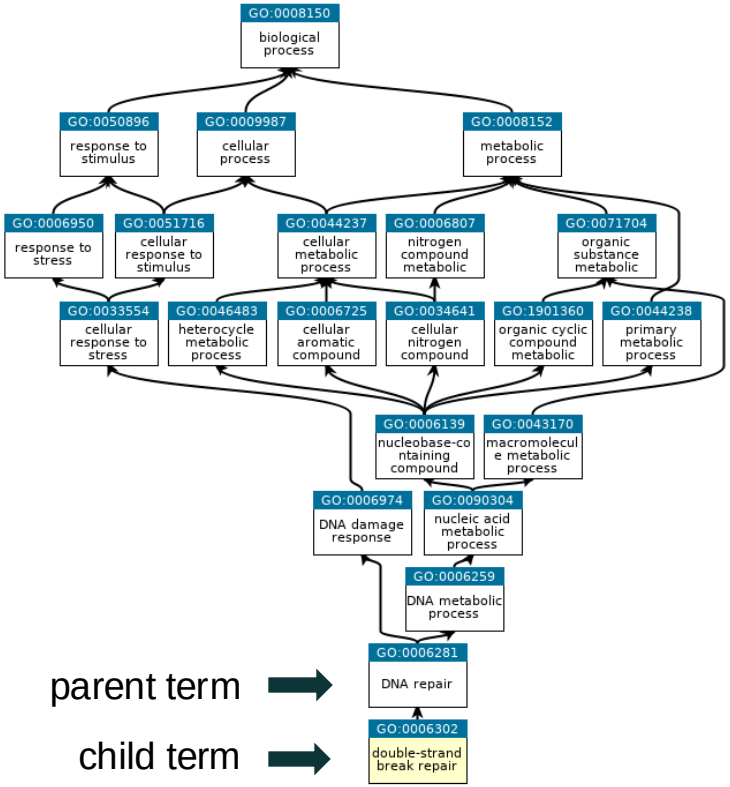
GO ontologies

- Actually three separate ontologies:
  - **Molecular Function** – molecular level activities performed by gene products, e.g. *transporter activity* (broad) or *Toll-like receptor binding* (specific)
  - **Cellular Component** – the cellular location where a function is performed, e.g. *ribosome*
  - **Biological Process** – larger processes accomplished by multiple molecular activities, e.g. *DNA repair* (broad) or *pyrimidine nucleobase biosynthetic process* (specific)
- Generally, in functional enrichment analysis, “biological process” is most useful

GO hierarchy

more genes

fewer genes



parent term

child term

root term

broad terms

specific terms

BRCA2 example

Gene: BRCA2 ENSG00000139618

Description	BRCA2 DNA repair associated [Source:HGNC Symbol;Acc:HGNC:1101]
Gene Synonyms	BRCC2, FACD, FAD, FAD1, FANCD, FANCD1, XRCC11
Location	Chromosome 13: 32,315,086-32,400,268 forward strand. GRCh38:CM000675.2
About this gene	This gene has 15 transcripts (splice variants), 173 orthologues and is associated with 120 phenotypes.
Transcripts	Show transcript table

GO: Molecular function

Show/hide columns (3 hidden) Filter

Accession	Term	Evidence	Annotation source
GO:0002020	protease binding	IPI	UniProt
GO:0003677	DNA binding	IEA	UniProt
GO:0003697	single-stranded DNA binding	IDA	UniProt
GO:0005515	protein binding	IPI	IntAct
GO:0008022	protein C-terminus binding	IDA	MGI
GO:0010484	H3 histone acetyltransferase activity	IDA	UniProt
GO:0010485	H4 histone acetyltransferase activity	IDA	UniProt
GO:0042802	identical protein binding	IPI	IntAct
GO:0043015	gamma-tubulin binding	IPI	UniProt

BRCA2 example

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GO:0042802	identical protein binding	IPI	IntAct
GO:0043015	gamma-tubulin binding	IPI	UniProt

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Description	BRCA2 DNA repair associated [Source:HGNC Symbol;Acc:HGNC:1101]
Gene Synonyms	BRCC2, FACD, FAD, FAD1, FANCD, FANCD1, XRCC11
Location	Chromosome 13: 32,315,086-32,400,268 forward strand. GRCh38:CM000675.2
About this gene	This gene has 15 transcripts (splice variants), 173 orthologues and is associated with 120 phenotypes.
Transcripts	Show transcript table

GO: Cellular component

Show All entries Show/hide columns (3 hidden) Filter

Accession	Term	Evidence	Annotation source
GO:0000152	nuclear ubiquitin ligase complex	IDA	ComplexPortal
GO:0000781	chromosome, telomeric region	IDA	BHF-UCL
GO:0000800	lateral element	IDA	MGI
GO:0005634	nucleus	IDA, IEA	UniProt
GO:0005654	nucleoplasm	IDA	HPA
GO:0005694	chromosome	IEA	Ensembl
GO:0005737	cytoplasm	IEA	UniProt
GO:0005813	centrosome	IDA	UniProt
GO:0005815	microtubule organizing center	IEA	UniProt
GO:0005829	cytosol	IDA	HPA
GO:0005856	cytoskeleton	IEA	UniProt
GO:0030141	secretory granule	IDA	UniProt
GO:0032991	protein-containing complex	IDA	MGI
GO:0033593	BRCA2-MAGE-D1 complex	IDA	UniProt
GO:1990391	DNA repair complex	IPI	ComplexPortal

BRCA2 example

Gene: BRCA2 ENSG00000139618

Description	BRCA2 DNA repair associated [Source:HGNC Symbol;Acc:HGNC:1101]
Gene Synonyms	BRCC2, FACD, FAD, FAD1, FANCD, FANCD1, XRCC11
Location	Chromosome 13: 32,315,086-32,400,268 forward strand. GRCh38:CM000675.2
About this gene	This gene has 15 transcripts (splice variants), 173 orthologues and is associated with 120 phenotypes.
Transcripts	Show transcript table

GO: Biological process

Show All entries | Show/hide columns (3 hidden) | Filter

Accession	Term	Evidence	Annotation source
GO:0000722	telomere maintenance via recombination	IEA	Ensembl
GO:0000724	double-strand break repair via homologous recombination	IEA	
GO:0001556	oocyte maturation	IEA	Ensembl
GO:0001833	inner cell mass cell proliferation	IEA	Ensembl
GO:0006281	DNA repair	IEA	
GO:0006289	nucleotide-excision repair	IMP	UniProt
GO:0006302	double-strand break repair	IMP	UniProt
GO:0006310	DNA recombination	IEA	UniProt
GO:0006355	regulation of DNA-templated transcription	IBA	GO_Central
GO:0006974	cellular response to DNA damage stimulus	IEA	UniProt
GO:0006978	DNA damage response, signal transduction by p53 class mediator resulting in transcription of p21 class mediator	IEA	Ensembl
GO:0007049	cell cycle	IEA	UniProt

GO: Molecular function

Show/hide columns (3 hidden)

Accession	Term
GO:0002020	protease binding
GO:0003677	DNA binding
GO:0003697	single-stranded DNA binding
GO:0005515	protein binding
GO:0008022	protein C-terminus binding
GO:0010484	H3 histone acetyltransferase activity
GO:0010485	H4 histone acetyltransferase activity
GO:0042802	identical protein binding
GO:0043015	gamma-tubulin binding

Evidence	Annotation source
IDA	ComplexPortal
IDA	BHF-UCL
IDA	MGI
IDA, IEA	UniProt
IDA	HPA
IEA	Ensembl
IEA	UniProt
IDA	UniProt
IEA	UniProt
IDA	HPA
IEA	UniProt
IDA	UniProt
IDA	MGI
IDA	UniProt
IPI	ComplexPortal

Functional enrichment analysis

- How do we use all the existing annotation to interpret our gene list?
- Want to identify biological functions that are enriched in our gene list

Testing for functional enrichment

20,000 genes assayed

Gene	adjp
ENSDARG00000041294	1.0867269119101765e-32
ENSDARG00000060498	1.2517176061993635e-21
ENSDARG00000031683	2.364463411626339e-21
ENSDARG00000077982	2.9548243274497384e-14
ENSDARG00000070480	5.735034663692255e-14
ENSDARG00000007769	1.5678213864306653e-13
ENSDARG00000102435	1.9075360227976884e-13
ENSDARG00000101482	2.6986616800262944e-13
ENSDARG00000034503	5.567338300152267e-13
ENSDARG00000013670	2.3318547986985375e-11
ENSDARG00000039490	3.047413661053603e-11
ENSDARG00000004748	3.380766458381055e-11
ENSDARG00000102808	3.652027442583773e-11
ENSDARG00000059294	3.686666857880831e-11
ENSDARG00000058731	7.374599119303466e-11
ENSDARG00000030896	1.0690865837043268e-10
ENSDARG00000117300	3.836221667423099e-10
ENSDARG00000102758	7.675777258160306e-10
ENSDARG00000004754	1.3907777984676073e-9
ENSDARG00000099960	2.0021043563850804e-9
ENSDARG00000056615	2.0321305012048 73e-9
ENSDARG00000073820	3.749723988428957e-9
ENSDARG00000003570	4.027615069700461e-9
ENSDARG00000077799	7.492097636023496e-9
ENSDARG00000053836	7.754409869629003e-9
ENSDARG00000094678	7.81554132440011e-9
ENSDARG00000076914	2.810237900118553 6e-8
ENSDARG00000037421	2.990985153534242e-8
ENSDARG00000014340	3.505316564907291e-8
ENSDARG00000104773	4.106939175430373 5e-8
ENSDARG00000105749	4.106939175430373 5e-8
ENSDARG00000018491	4.275633454932327e-8
ENSDARG00000109648	4.938474248033579 3e-8
ENSDARG00000010231	5.822859503020341e-8
ENSDARG00000039142	7.665381608460826e-8
ENSDARG00000104672	1.250478948653852 7e-7
ENSDARG00000054196	1.356347303797386e-7
ENSDARG00000090548	1.875815969385844 9e-7
ENSDARG00000104919	2.236704678911416 2e-7
ENSDARG00000062788	3.710767234119733 6e-7
ENSDARG00000079227	4.328790910316529 7e-7
ENSDARG00000051888	4.698026165209627 4e-7
ENSDARG00000077169	4.708948570604647 5e-7
ENSDARG00000056196	4.708948570604647 5e-7
ENSDARG00000018283	4.917667288531457e-7
ENSDARG00000105731	5.285812723974355e-7
ENSDARG00000004954	5.526970490118169e-7
ENSDARG00000025903	7.245067792685657e-7
ENSDARG00000025094	7.999474861543812e-7
ENSDARG00000100504	8.037125469772779e-7

Adjusted p-value < 0.05

500 significantly DE genes

Gene	adjp
ENSDARG00000041294	1.0867269119101765e-32
ENSDARG00000060498	1.2517176061993635e-21
ENSDARG00000031683	2.364463411626339e-21
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ENSDARG00000007769	1.5678213864306653e-13
ENSDARG00000102435	1.9075360227976884e-13
ENSDARG00000101482	2.6986616800262944e-13
ENSDARG00000034503	5.567338300152267e-13
ENSDARG00000013670	2.3318547986985375e-11
ENSDARG00000039490	3.047413661053603e-11

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ENSDARG00000070480	5.735034663692255e-14
ENSDARG00000007769	1.5678213864306653e-13
ENSDARG00000102435	1.9075360227976884e-13
ENSDARG00000101482	2.6986616800262944e-13
ENSDARG00000034503	5.567338300152267e-13
ENSDARG00000013670	2.3318547986985375e-11
ENSDARG00000039490	3.047413661053603e-11
ENSDARG00000004748	3.380766458381055e-11
ENSDARG00000102808	3.652027442583773e-11
ENSDARG00000059294	3.686666857880831e-11
ENSDARG00000058731	7.374599119303466e-11
ENSDARG00000030896	1.0690865837043268e-10
ENSDARG00000117300	3.836221667423099e-10
ENSDARG00000102758	7.675777258160306e-10
ENSDARG00000004754	1.3907777984676073e-9
ENSDARG00000099960	2.0021043563850804e-9
ENSDARG00000056615	2.0321305012048 73e-9
ENSDARG00000073820	3.749723988428957e-9
ENSDARG00000003570	4.027615069700461e-9
ENSDARG00000077799	7.492097636023496e-9
ENSDARG00000053836	7.754409869629003e-9
ENSDARG00000094678	7.81554132440011e-9
ENSDARG00000076914	2.810237900118553 6e-8
ENSDARG00000037421	2.990985153534242e-8
ENSDARG00000014340	3.505316564907291e-8
ENSDARG00000104773	4.106939175430373 5e-8
ENSDARG00000105749	4.106939175430373 5e-8
ENSDARG00000018491	4.275633454932327e-8
ENSDARG00000109648	4.938474248033579 3e-8
ENSDARG00000010231	5.822859503020341e-8
ENSDARG00000039142	7.665381608460826e-8
ENSDARG00000104672	1.250478948653852 7e-7
ENSDARG00000054196	1.356347303797386e-7
ENSDARG00000090548	1.875815969385844 9e-7
ENSDARG00000104919	2.236704678911416 2e-7
ENSDARG00000062788	3.710767234119733 6e-7
ENSDARG00000079227	4.328790910316529 7e-7
ENSDARG00000051888	4.698026165209627 4e-7
ENSDARG00000077169	4.708948570604647 5e-7
ENSDARG00000056196	4.708948570604647 5e-7
ENSDARG00000018283	4.917667288531457e-7
ENSDARG00000105731	5.285812723974355e-7
ENSDARG00000004954	5.526970490118169e-7
ENSDARG00000025903	7.245067792685657e-7
ENSDARG00000025094	7.999474861543812e-7
ENSDARG00000100504	8.037125469772779e-7

Adjusted p-value < 0.05

500 significantly DE genes

Gene	adjp
ENSDARG00000041294	1.0867269119101765e-32
ENSDARG00000060498	1.2517176061993635e-21
ENSDARG00000031683	2.364463411626339e-21
ENSDARG00000077982	2.9548243274497384e-14
ENSDARG00000070480	5.735034663692255e-14
ENSDARG00000007769	1.5678213864306653e-13
ENSDARG00000102435	1.9075360227976884e-13
ENSDARG00000101482	2.6986616800262944e-13
ENSDARG00000034503	5.567338300152267e-13
ENSDARG00000013670	2.3318547986985375e-11
ENSDARG00000039490	3.047413661053603e-11

200 genes annotated to DNA repair

200/500 = **40%**

(300 not annotated to DNA repair)

2000 genes annotated to function (e.g. DNA repair)

2000/20000 = **10%**

(18,000 not annotated to DNA repair)

Testing for functional enrichment

20,000 genes assayed

Gene	adjp
ENSDARG00000041294	1.0867269119101765e-32
ENSDARG00000060498	1.2517176061993635e-21
ENSDARG00000031683	2.3644634116263 39e-21
ENSDARG00000077982	2.9548243274497384e-14
ENSDARG00000070480	5.735034663692255e-14
ENSDARG00000007769	1.5678213864306653e-13
ENSDARG00000102435	1.9075360227976884e-13
ENSDARG00000101482	2.6986616800262944e-13
ENSDARG00000034503	5.567338300152267e-13
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ENSDARG00000039490	3.047413661053603e-11
ENSDARG00000004748	3.380766458381055e-11
ENSDARG00000102808	3.652027442583773e-11
ENSDARG00000059294	3.686666857880831e-11
ENSDARG00000058731	7.374599119303466e-11
ENSDARG00000030896	1.069086583704326 8e-10
ENSDARG00000117300	3.836221667423099e-10
ENSDARG00000102758	7.675777258160306e-10
ENSDARG00000004754	1.390777798467607 3e-9
ENSDARG00000099960	2.002104356385080 4e-9
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ENSDARG00000077799	7.492097636023496e-9
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ENSDARG00000037421	2.990985153534242e-8
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ENSDARG00000054196	1.356347303797386e-7
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ENSDARG00000104919	2.236704678911416 2e-7
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ENSDARG00000025903	7.245067792685657e-7
ENSDARG00000025094	7.999474861543812e-7
ENSDARG00000100504	8.037125469772779e-7

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ENSDARG00000070480	5.735034663692255e-14
ENSDARG00000007769	1.5678213864306653e-13
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200/500 = **40%**

(300 not annotated to DNA repair)

2000 genes annotated to function (e.g. DNA repair)

2000/20000 = **10%**

(18,000 not annotated to DNA repair)

Is seeing 200 DNA repair genes significantly differentially expressed more than we would expect by chance?

Testing for functional enrichment

20,000 genes assayed

Gene	adjp
ENSDARG00000041294	1.0867269119101765e-32
ENSDARG00000060498	1.2517176061993635e-21
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ENSDARG00000018283	4.917667288531457e-7
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(300 not annotated to DNA repair)

2000 genes annota
function (e.g. DNA

2000/20000 = **10%**

(18,000 not annota
DNA repair)

	DE	Not DE	**Total**
Annotated to DNA repair	200	1800	2000
Not annotated to DNA repair	300	17700	18000
Total	500	19500	20000

Hypergeometric test

	DE	Not DE	**Total**
Annotated to DNA repair	200	1800	2000
Not annotated to DNA repair	300	17700	18000
Total	500	19500	20000

```
> m  <- 20000 # Total genes
> n  <- 500   # Number of DE genes
> mt <- 2000  # Number of annotated genes
> nt <- 200   # Number of annotated DE genes
> phyper(nt - 1, mt, m - mt, n, lower.tail=FALSE)
[1] 1.65531e-72
```

Use the hypergeometric test to calculate the probability of having 200 or more DE annotated genes when 2000 of the 20,000 total genes are annotated

$$P(\sigma_t \geq n_t) = \sum_{k=n_t}^{min(m_t,n)} \frac{\binom{m_t}{k}\binom{m-m_t}{n-k}}{\binom{m}{n}}$$

Multiple testing correction

- In reality, won't just be doing one test
- Want to test all (or a lot) of the GO terms and other functional gene sets
- Leads to problem of **multiple testing**
- If you test 10,000 GO terms with a significance threshold of < 0.05 then you expect 500 terms to be significant simply by chance
- Need to correct for multiple testing:
  - Bonferroni
  - Benjamini–Hochberg

Bonferroni correction

- Bonferroni is easiest to understand and most conservative
- Simply multiply all p-values by the number of tests (i.e. functional gene sets)
- Get adjusted p-values

GO	pval	adjp
GO:0022008	5.947e-7	5.947e-6
GO:0008038	8.705e-7	8.705e-6
GO:0097367	0.000001	0.000010
GO:0043168	0.000002	0.000020
GO:0010975	0.004917	0.049172
GO:0036211	0.005152	0.051521
GO:0021631	0.020739	0.207394
GO:0065009	0.272362	1.000000
GO:0099545	0.290182	1.000000
GO:1905245	0.496883	1.000000

Benjamini–Hochberg correction

- Benjamini–Hochberg is less conservative and assumes that all tests are statistically independent
- Not true – many functional gene sets overlap:
  - e.g. GO terms are hierarchical so a term's annotations are a subset of their parental annotations
  - e.g. similar pathways can appear in KEGG and WikiPathways
  - e.g. some genes are co-expressed
- Nevertheless, BH is widely and successfully used
- Although Wijesooriya *et al*. (2022) found that 43% of papers surveyed failed to do multiple testing correction: doi.org/10.1371/journal.pcbi.1009935

Background gene set

- Important to choose appropriate background gene set
- Wijesooriya *et al*. (2022) found that only 4% of papers used an appropriate background (although most failed to specify what background was used): doi.org/10.1371/journal.pcbi.1009935
- Best to choose all genes that could have been captured in your experiment
- Examples:
  - All genes
  - All genes with non-zero total read count in DESeq2
  - All genes that pass DESeq2 independent filtering
  - All genes expressed in a particular tissue
  - All genes with annotations

Other methods

- Functional enrichment analysis (or over-representation analysis) is just one method
- Other methods and tests are available, e.g.
  - GSEA (gene set enrichment analysis)
  - Binomial test
- Concentrating on functional enrichment analysis because most widely used and most tools available

Advantages of functional enrichment analysis

- Improves statistical power as you effectively sum up counts from the multiple genes in a functional gene set
- Improves statistical power as there are usually fewer functional annotations than genes, so less multiple testing correction is needed
- Results are easier to interpret because they are familiar concepts like “DNA repair” rather than obscure gene names
- Diverse data (e.g. RNA-seq, proteomics) can be integrated because they map to common terms/pathways
- Results may be more comparable to related data because results are projected to a smaller set of functional annotations

Disadvantages of functional enrichment analysis

- Terms or pathways with few genes are unlikely to ever be enriched
- Hypergeometric test is more likely to identify larger functional gene sets (e.g. pathways with many genes) as significant
- Genes with multiple functions can lead to enrichment of multiple terms/pathways, some of which aren't relevant
- Databases are (obviously) biased towards genes with annotation so unannotated genes (e.g. many non-coding RNA genes) are invisible to functional enrichment analysis

Recommendations based on disadvantages

- For human RNA-seq data, consider excluding functional gene sets with < 10 genes and > 500 genes
- Former are unlikely to ever be significant and latter are too likely to be significant and will often be better represented by other more specific terms/pathways
- Always think about your own experiment:
  - e.g. is apoptosis enrichment expected or a symptom of a problem during sample preparation

Quiz!

- Quiz on Mentimeter (www.menti.com)

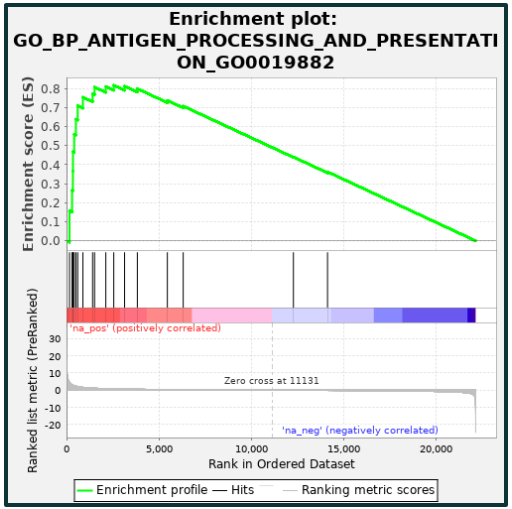
Functional enrichment tools

- Many, many functional enrichment analysis tools exist
- Many are created, published and then never updated
- Best to choose a well used tool
- Using g:Profiler because:
  - Consistently and regularly updated over many years
  - Easy to use
  - Free
  - Well documented
  - Has advanced features, like simultaneous analysis of multiple lists
  - Has web interface but also an API with supported R and Python packages
  - Covers nearly 800 species/strains/varieties

g:Profiler

Other functional enrichment tools

- Other tools are available (and good):
  - Enrichr (maayanlab.cloud/Enrichr/):
    - Web-based
    - Similar to g:Profiler
    - Only human, mouse, fly, yeast, worm and zebrafish
  - GSEA (www.gsea-msigdb.org/gsea/):
    - Desktop software
    - Implements GSEA method
    - Works on whole genome ranked gene lists
    - Looks for gene sets enriched at top or bottom of your ranked list
    - p-values computed by permutating ranked lists



g:Profiler

- g:Profiler uses Ensembl as its primary data source (specifically, BioMart)
- Tracks Ensembl release schedule (every three or four months) but with delay of weeks or months
- Since last month, g:Profiler had been using Ensembl 112, which came out in May last year
- Recommend using Ensembl IDs as input, but not essential

g:Profiler

g:Profiler

News Archives Beta API R client FAQ Docs Contact Cite g:Profiler Services using g:P GMT Helper

g:GOSt Functional profiling	g:Convert Gene ID conversion	g:Orth Orthology search	g:SNPense SNP id to gene name

Query Upload query Upload bed file

Input is whitespace-separated list of genes

Run query random example mixed query example

Options

Organism:

Homo sapiens (Human)

- [x] Highlight driver terms in GO
- [ ] Ordered query
- [ ] Run as multiquery

Advanced options

Data sources

Bring your data (Custom GMT)

g:Profiler – four tools

g:Profiler

News | Archives | Beta | API | R client | FAQ | Docs | Contact | Cite g:Profiler | Services using g:P | GMT Helper

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Run query | random example | mixed query example

Options

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Homo sapiens (Human)

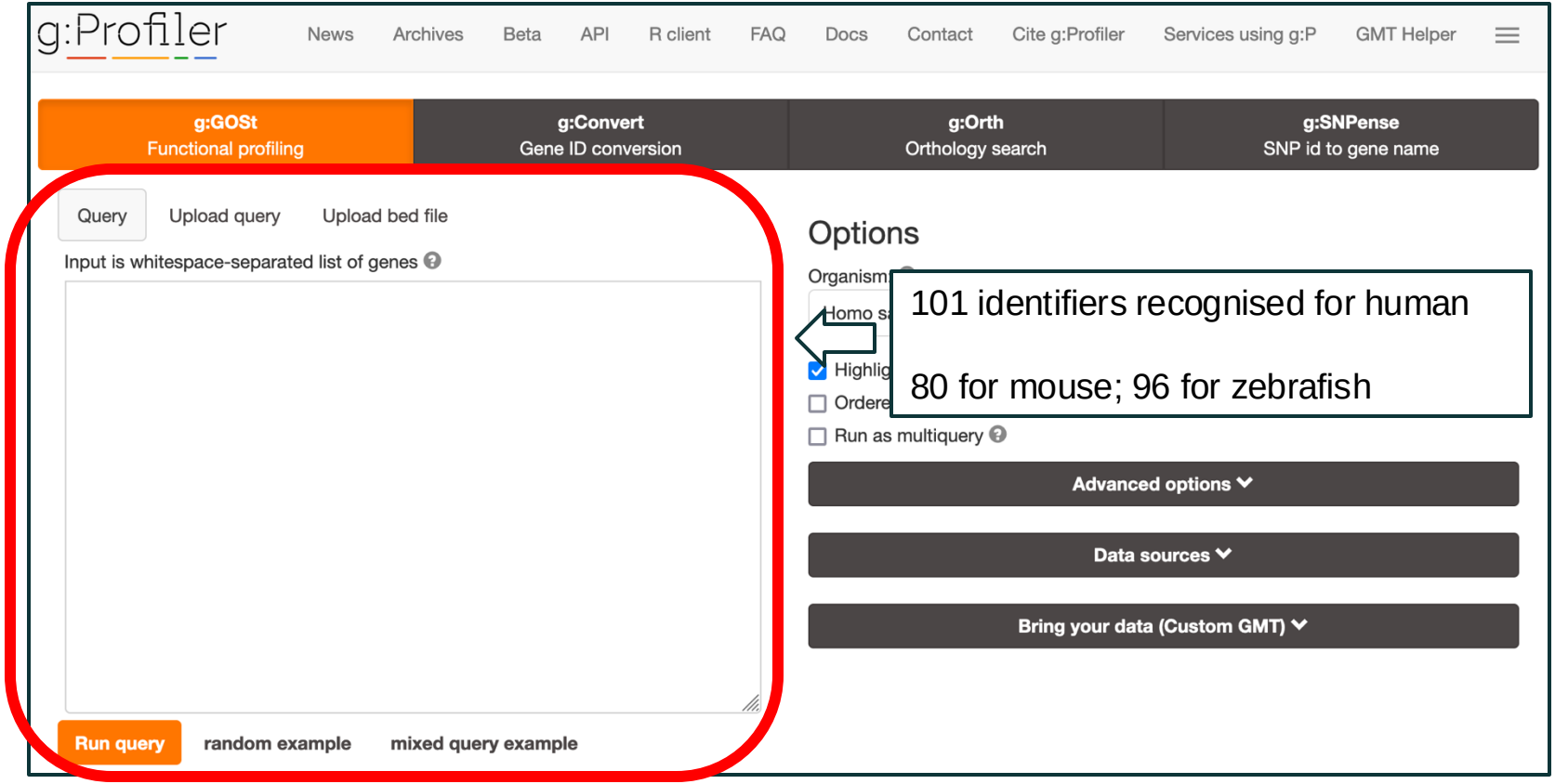
- [x] Highlight driver terms in GO
- [ ] Ordered query
- [ ] Run as multiquery

Advanced options

Data sources

Bring your data (Custom GMT)

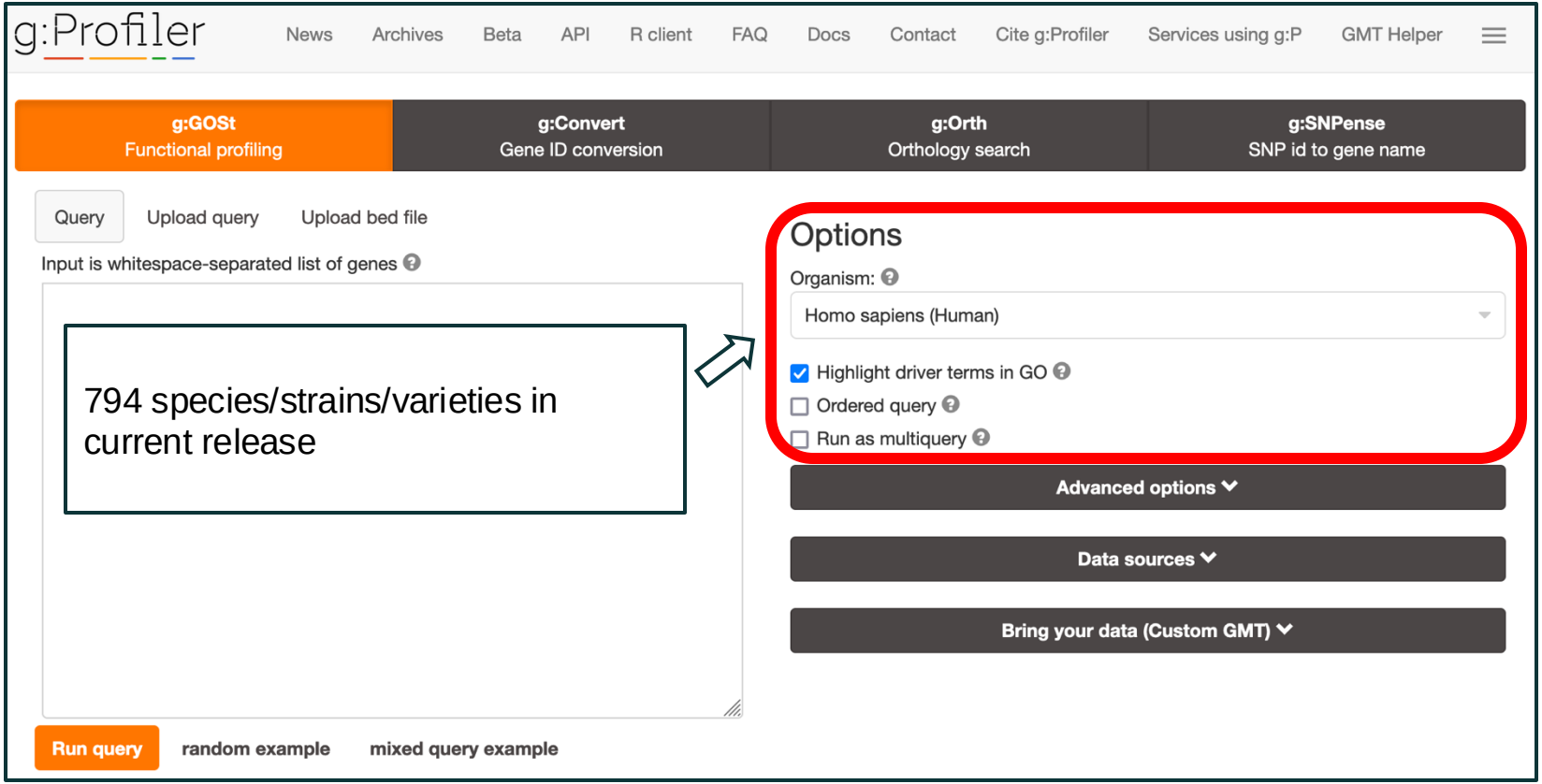
g:Profiler – gene list



101 identifiers recognised for human

80 for mouse; 96 for zebrafish

g:Profiler – options



g:Profiler – advanced options

Query | Upload query | Upload bed file

Input is whitespace-separated list of genes

g:SCS – “Set Counts and Sizes”

Accounts for hierarchical nature of GO

Less conservative than Bonferroni but more conservative than Benjamini-Hochberg

Options

Organism:

Homo sapiens (Human)

- [x] Highlight driver terms in GO
- [ ] Ordered query
- [ ] Run as multiquery

Advanced options

- [ ] All results
- [ ] Measure underrepresentation
- [ ] No evidence codes

Statistical domain scope

Only annotated genes

Significance threshold

g:SCS threshold

User threshold

0.05

Numeric IDs treated as

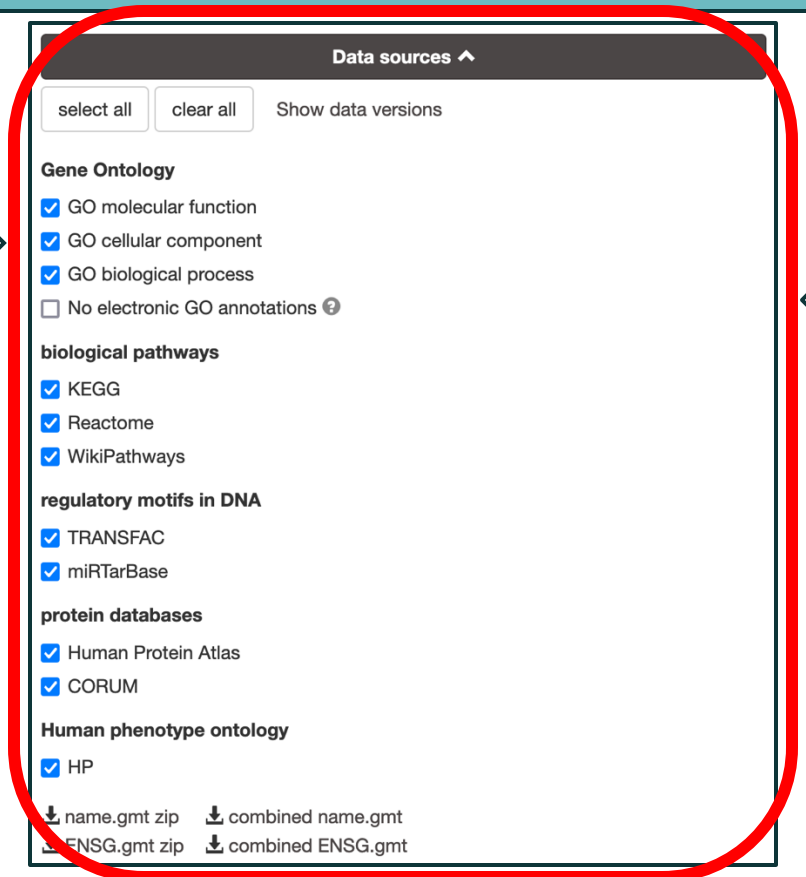
ENTREZGENE_ACC

g:Profiler – data sources

9 data sources

(or 11 if count GO as three separate sources)

All 9 not available for all species

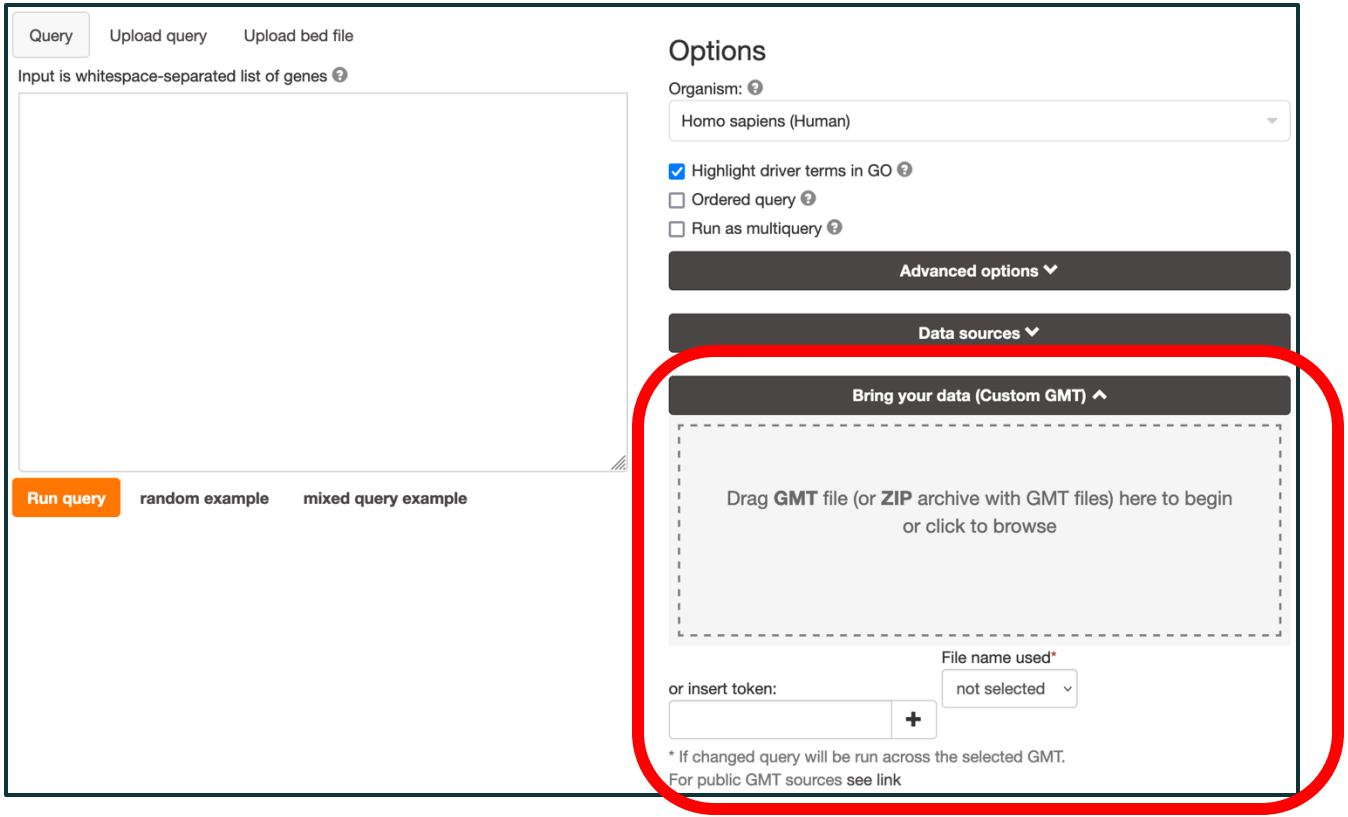


Can exclude GO IEA evidence term (inferred from electronic annotation)

But often as reliable as human annotation (Škunca *et al.* 2012)

Suggest running with and without if using human or model organisms

g:Profiler – bring your data



g:Profiler – documentation

g:Profiler | News | Archives | Beta | API | R client | FAQ | Docs | Contact | Cite g:Profiler | Services using g:P | GMT Helper

g:GOSt Functional profiling	g:Convert Gene ID conversion	g:Orth Orthology search	g:SNPense SNP id to gene name

Welcome to g:Profiler

g:Profiler is a public web server for characterising and manipulating gene lists. g:Profiler has a simple user-friendly web interface with powerful visualisations and is currently available for 400+ species, including mammals, plants, fungi, insects from Ensembl and Ensembl Genomes. g:Profiler is updated approximately in every three months and follows quarterly releases of Ensembl databases. g:Profiler tool set consists of the following tools:

- **g:GOSt** , the core of the g:Profiler, performs statistical enrichment analysis to provide interpretation to user-provided gene lists. The gene lists can be either flat or ordered gene lists. We accept majority of the identifier types, chromosomal regions and term IDs as input. We provide data from multiple sources of functional evidence, including Gene Ontology terms, biological pathways, regulatory motifs of transcription factors and microRNAs, human disease annotations and protein-protein interactions.
- **g:Convert** is a gene identifier conversion tool. It uses information in Ensembl databases to handle hundreds of types of IDs for genes, proteins, transcripts, microarray probesets, etc, for many species, experimental platforms and biological databases. g:Convert is flexible: it accepts a mixed list of IDs and recognises their types automatically. It can also serve as a service to get all genes belonging to a particular functional category.
- **g:Orth** is a tool for mapping homologous genes across related organisms based on Ensembl data. Given a selected target organism, g:Orth retrieves the genes of the target organism that are similar in sequence to the initial genes in the input.
- **g:SNPense** is a tool for mapping human single nucleotide polymorphisms (SNP) to gene names, chromosomal locations and variant consequence terms from Sequence Ontology.

Contents

g:Profiler – archives

g:Profiler

News | Archives | Beta | API | R client | FAQ | Docs | Contact | Cite g:Profiler | Services using g:P | GMT Helper

g:GOSt Functional profiling	g:Convert Gene ID conversion	g:Orth Orthology search	g:SNPense SNP id to gene name

Archives

g:Profiler Archives stores all the past stable versions of g:Profiler, including the associated databases based on various Ensembl and Ensembl Genomes versions. This allows for the reproducibility of results even in case a release of g:Profiler has been retired since running an analysis. The following archived g:Profiler instances are available:

- Ensembl **111**, Ensembl Genomes **58** (database built on 2024-01-25)
- Ensembl **110**, Ensembl Genomes **57** (database built on 2023-09-14)
- Ensembl **109**, Ensembl Genomes **56** (database built on 2023-03-29)
- Ensembl **108**, Ensembl Genomes **55** (database built on 2022-12-28)
- Ensembl **107**, Ensembl Genomes **54** (database built on 2022-09-15)
- Ensembl **106**, Ensembl Genomes **53** (database built on 2022-05-18)
- Ensembl **105**, Ensembl Genomes **52** (database built on 2022-01-03)
- Ensembl **104**, Ensembl Genomes **51** (database built on 2021-05-07)
- Ensembl **103**, Ensembl Genomes **50** (database built on 2021-04-01)
- Ensembl **102**, Ensembl Genomes **49** (database built on 2020-12-15)
- Ensembl **101**, Ensembl Genomes **48** (database built on 2020-10-12)
- Ensembl **100**, Ensembl Genomes **47** (database built on 2020-09-21)
- Ensembl **99**, Ensembl Genomes **46** (database built on 2020-07-22)
- Ensembl **98**, Ensembl Genomes **45** (database built on 2020-03-07)
- Ensembl **97**, Ensembl Genomes **44** (database built on 2019-10-07)
- Ensembl **96**, Ensembl Genomes **43** (database built on 2019-09-10)
- Ensembl **95**, Ensembl Genomes **42** (database built on 2019-05-09)
- Ensembl **94**, Ensembl Genomes **41** (database built on 2018-10-02)

g:Profiler – API and libraries

g:Profiler

News | Archives | Beta | API | R client | FAQ | Docs | Contact | Cite g:Profiler | Services using g:P | GMT Helper

g:GOSt Functional profiling	g:Convert Gene ID conversion	g:Orth Orthology search	g:SNPense SNP id to gene name

g:Profiler client libraries

R client

g:Profiler has an up-to-date R client library `gprofiler2` available from CRAN or conda-forge. For more documentation see the help page

Python client

g:Profiler has an up-to-date python client library `gprofiler-official` available from PyPI or conda-forge. For more documentation see the package description

g:Profiler API

g:Profiler requests are generally made as POST requests with a JSON body and they return JSON output.

Contents

g:Profiler – overview

g:Profiler – detailed results

Overview | **Detailed Results** | GO Context | Query Info

Share: Show query URL | Show short link

Should match... | Term size 1 – 7648

Show only selected | revert selection to driver terms

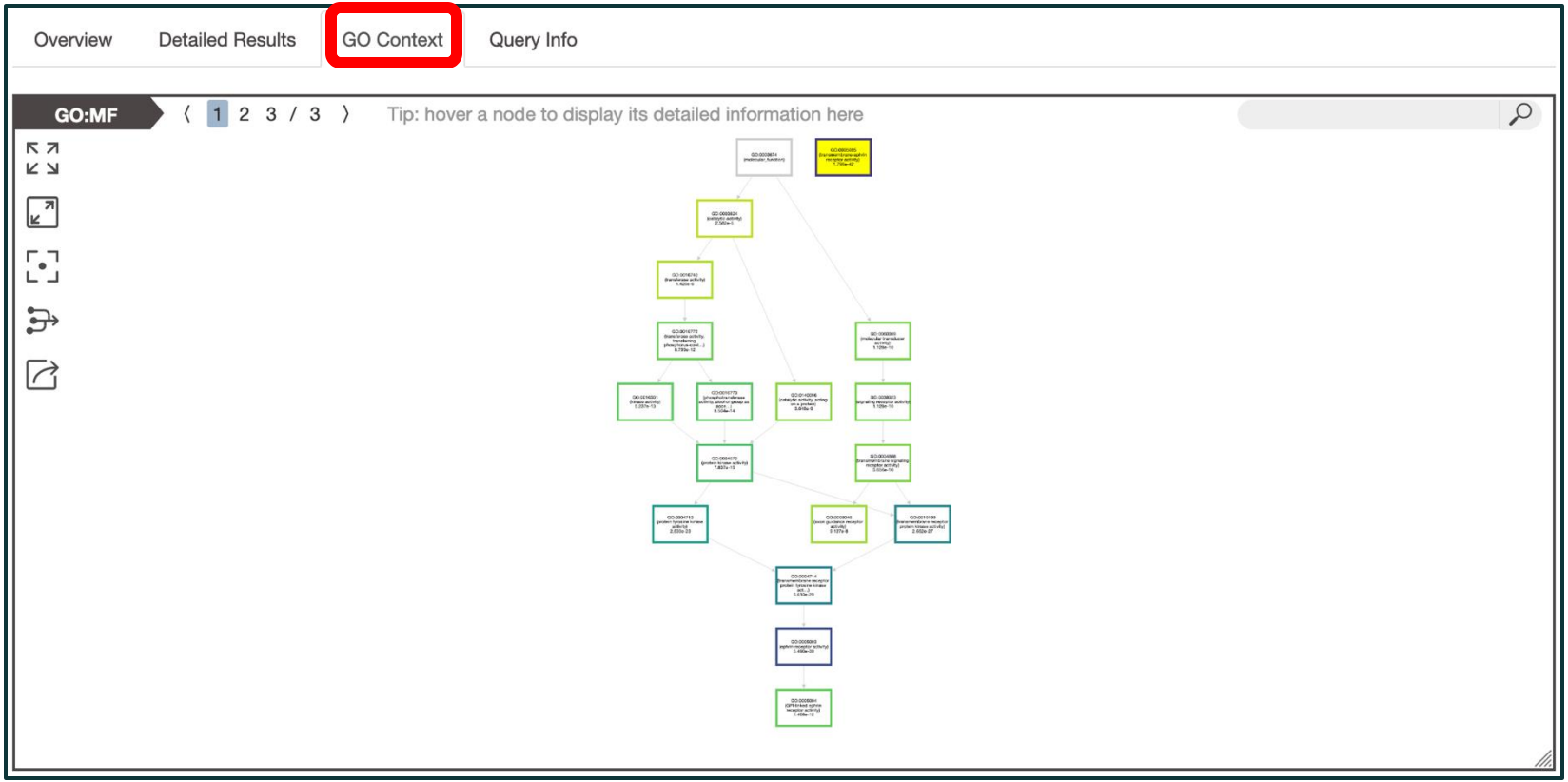
CSV | PNG | GEM

Legend

GO:MF		stats	
Term name	Term ID	p_{adj}	$-\log_{10}(p_{adj})$ (0 – ≤16)
transmembrane-ephrin receptor activity	GO:0005005	1.796×10^{-42}	
ephrin receptor activity	GO:0005003	5.490×10^{-39}	
transmembrane receptor protein tyrosine kinase a...	GO:0004714	6.610×10^{-29}	
transmembrane receptor protein kinase activity	GO:0019199	2.652×10^{-27}	
protein tyrosine kinase activity	GO:0004713	2.530×10^{-23}	
protein kinase activity	GO:0004672	7.837×10^{-15}	
phosphotransferase activity, alcohol group as acce...	GO:0016773	8.504×10^{-14}	
kinase activity	GO:0016301	5.237×10^{-13}	
GPI-linked ephrin receptor activity	GO:0005004	1.408×10^{-12}	
transferase activity, transferring phosphorus-cont...	GO:0016772	8.789×10^{-12}	
molecular transducer activity	GO:0060089	1.128×10^{-10}	
signaling receptor activity	GO:0038023	1.128×10^{-10}	
transmembrane signaling receptor activity	GO:0004888	3.656×10^{-10}	
catalytic activity, acting on a protein	GO:0140096	3.648×10^{-9}	
purine ribonucleoside triphosphate binding	GO:0035639	5.749×10^{-8}	
ATP binding	GO:0005524	7.289×10^{-8}	
axon guidance receptor activity	GO:0008046	9.137×10^{-8}	
purine ribonucleotide binding	GO:0032555	9.257×10^{-8}	
ribonucleotide binding	GO:0032553	1.042×10^{-7}	

Columns: ENSG00000167393, ENSG00000178605, ENSG00000182378, ENSG00000185960, ENSG00000226179, ENSG00000228572, ENSG00000239232, ENSG00000234958, ENSG00000237531, ENSG00000275287, ENSG00000280787, ENSG00000281849, RS17398340, ENSG00000044524, ENSG00000070886, ENSG00000080224, ENSG00000108947, ENSG00000116106, ENSG00000133216, ENSG00000135333, ENSG00000143590

g:Profiler – GO context



g:Profiler – beta

g:Profiler β

News | Archives | Stable | API | R client | FAQ | Docs | Contact | Cite g:Profiler | Services using g:P | GMT Helper

g:GOSt Functional profiling | **g:Convert** Gene ID conversion | **g:Orth** Orthology search | **g:SNPense** SNP id to gene name

Query | Upload query | Upload bed file

Input is whitespace-separated list of genes

Options

Organism:

Homo sapiens (Human)

- [x] Highlight driver terms in GO
- [ ] Ordered query
- [ ] Run as multiquery

Advanced options

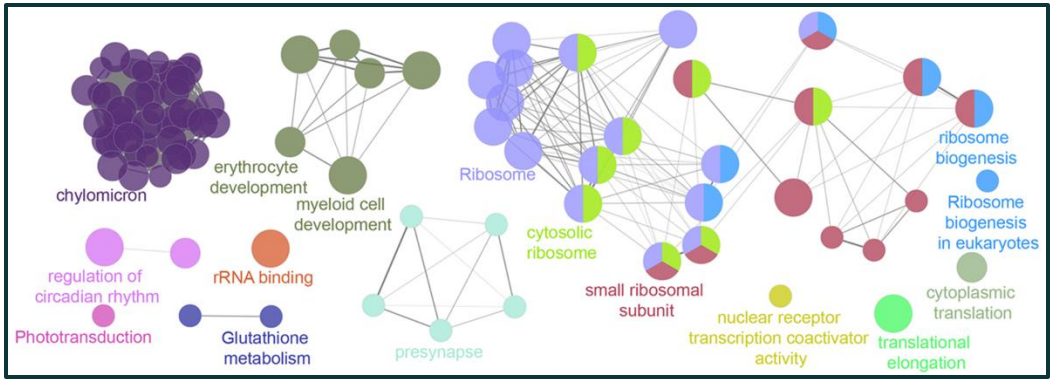
Data sources

Bring your data (Custom GMT)

Run query | **random example** | **mixed query example**

Summarising functional enrichment

- Functional enrichment analysis (hopefully) summarises a gene list into something shorter and more comprehensible
- But what if the list of functional enrichments is also long and/or repetitive?
- The connected components functionality is an attempt to solve that problem
- Other methods:
  - Cytoscape / EnrichmentMap
  - Cytoscape / ClueGO
  - Revigo: http://revigo.irb.hr/



g:Profiler live demo!

- biit.cs.ut.ee/gprofiler/

Exercises (plus data and slides)

- Exercises are available from:
  rnaseq2025.buschlab.org
- Plus data for exercises and these slides
- Everything also available on penelopeCloud