RNA-SEQ PROFILING DROSOPHILA CELLS AFTER THE DEPLETION OF A REGULATORY GENE

GROUP 6

Background

IN THE STUDY OF BROOKS ET AL. 2011, THE AUTHORS IDENTIFIED GENES AND PATHWAYS REGULATED BY THE PASILLA GENE (THE DROSOPHILA HOMOLOG OF THE MAMMALIAN SPLICING REGULATORS NOVA-1 AND NOVA-2 PROTEINS) USING RNA-SEQ DATA. THEY DEPLETED THE PASILLA (PS) GENE IN DROSOPHILA MELANOGASTER BY RNA INTERFERENCE (RNAI).

TOTAL RNA WAS THEN ISOLATED AND USED TO PREPARE BOTH SINGLE-END AND PAIRED-END RNA-SEQ LIBRARIES FOR TREATED (PS DEPLETED) AND UNTREATED SAMPLES. THESE LIBRARIES WERE SEQUENCED TO OBTAIN RNA-SEQ READS FOR EACH SAMPLE.

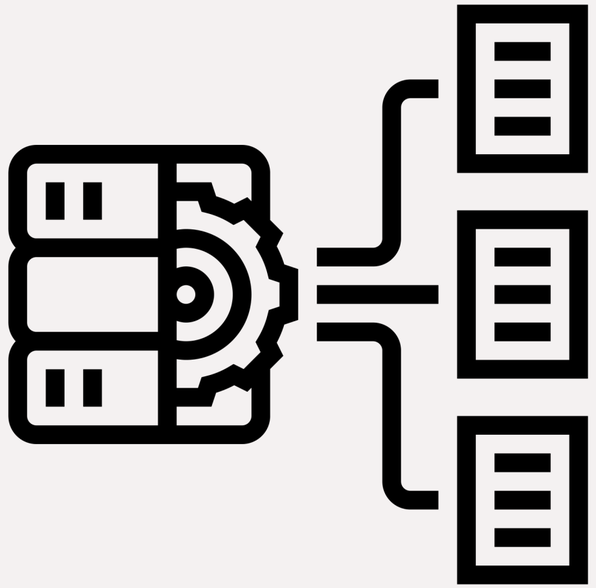
Motivation

OUR INTEREST IN THE PARTICULAR GENE WAS PIQUED WHEN WE READ THE STUDY OF BROOKS ET AL. THE PAPER EXPLORED THE POSSIBILITY OF CONSERVATION OF REGULATORY CODES AND CONCLUDED THAT THE RNA MAP OF PS AND NOVA1/2 IS HIGHLY CONSERVED BETWEEN MAMMALS AND INSECTS.

Datasets

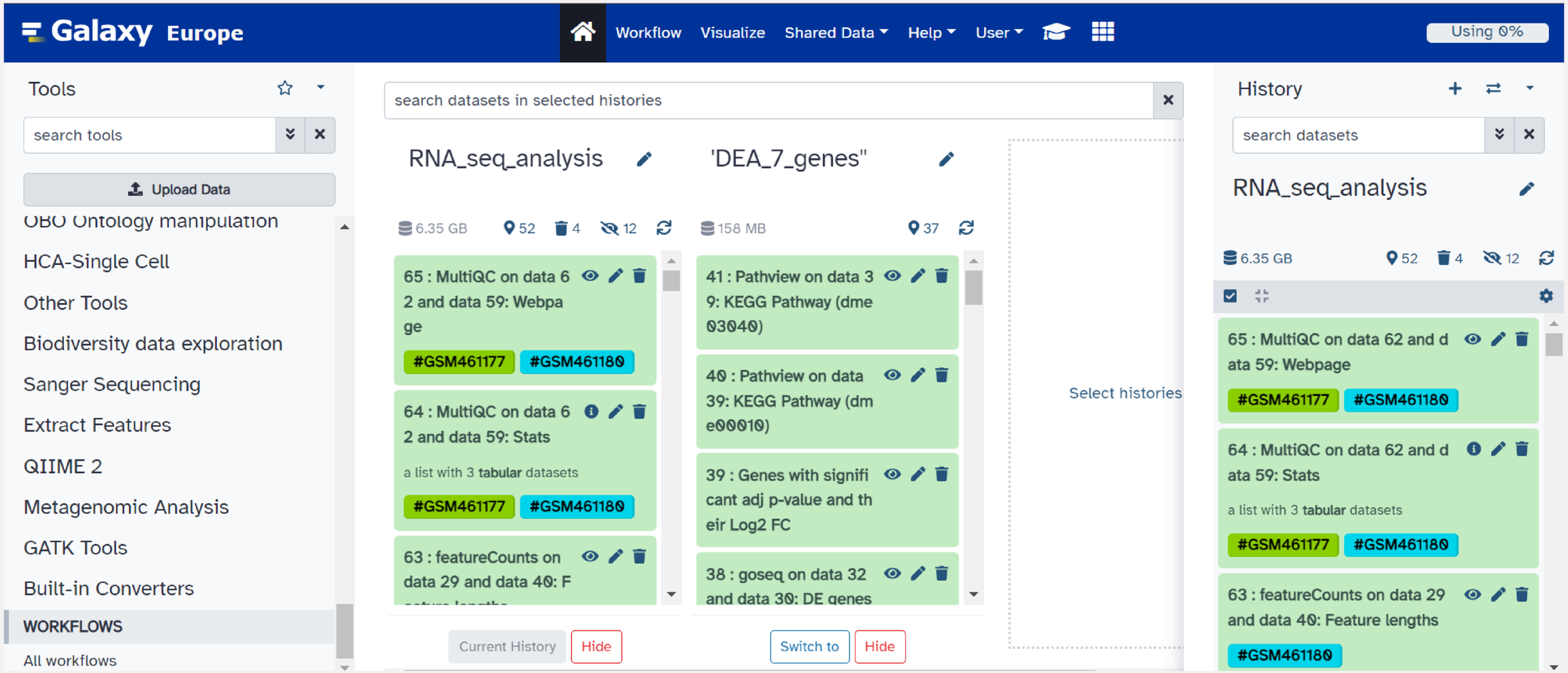
- 4 UNTREATED SAMPLES: GSM461176, GSM461177, GSM461178, GSM461182
- 3 TREATED SAMPLES (PASILLA GENE DEPLETED BY RNAI): GSM461179, GSM461180, GSM461181

EACH SAMPLE CONSTITUTES A SEPARATE BIOLOGICAL REPLICATE OF THE CORRESPONDING CONDITION (TREATED OR UNTREATED). MOREOVER, TWO OF THE TREATED AND TWO OF THE UNTREATED SAMPLES ARE FROM A PAIRED-END SEQUENCING ASSAY, WHILE THE REMAINING SAMPLES ARE FROM A SINGLE-END SEQUENCING EXPERIMENT.



Galaxy

Galaxy | Europe - https://usegalaxy.eu



STEPS

1. Quality Control
2. Mapping
3. Counting no. of reads
4. Differential Gene Expression Analysis
5. Functional enrichment analysis of DE genes
6. Conclusion

STEP 1 – QUALITY CONTROL

⇒ WHY CONTROL QUALITY?

- Ensures accurate and reliable results
- Poor quality data leads to errors and false conclusions
- High-quality data necessary for downstream analysis

⇒ HOW?

FASTQC

Short read data from your current history

MULTIQC

To aggregate the FastQC reports

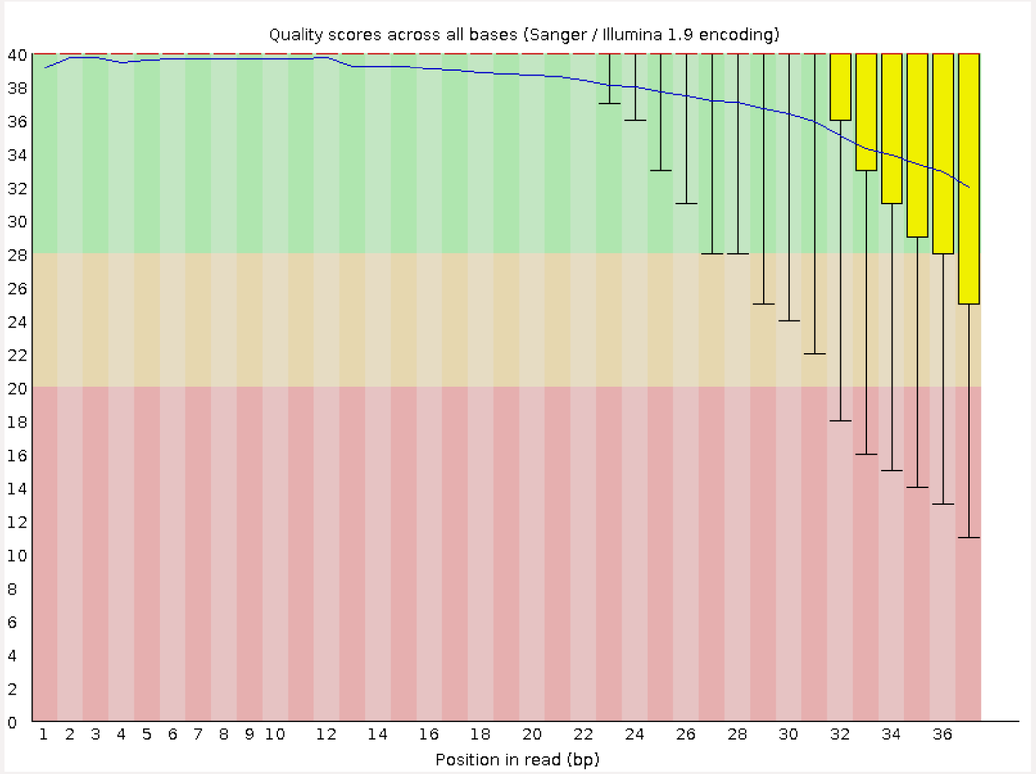
CUTADAPT

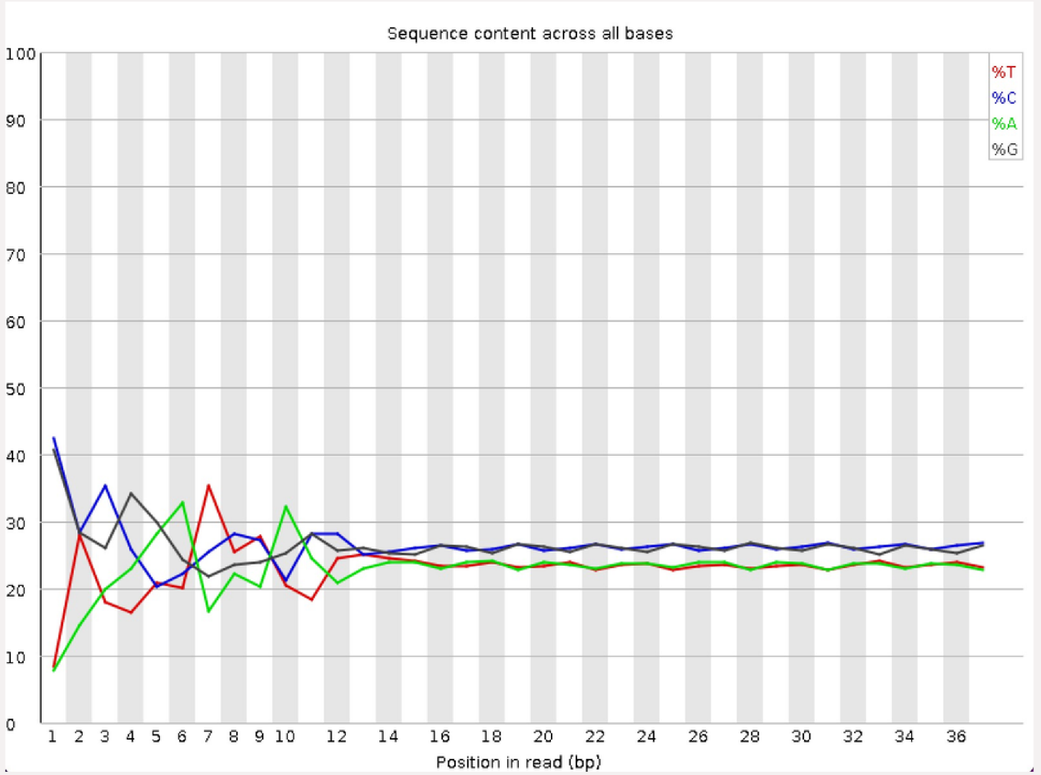
Trim low quality sequences

MULTIQC

To aggregate the Cutadapt reports

⇒ OUTPUTS:–


Quality scores across all bases (Sanger / Illumina 1.9 encoding)
Position in read (bp)


Sequence content across all bases
%T
%C
%A
%G
Position in read (bp)

STEP 2 – MAPPING

⇒ WHAT IS MAPPING?

PROCESS OF ALIGNING THE SEQUENCE READS TO A REFERENCE GENOME

⇒ WHAT IS A REFERENCE GENOME?

A REFERENCE GENOME IS A WELL-ANNOTATED GENOME SEQUENCE THAT SERVES AS A REPRESENTATIVE EXAMPLE FOR A SPECIES OR GROUP OF RELATED SPECIES.

Preparation of reference genome

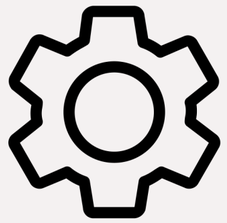
→

Sequence Alignment

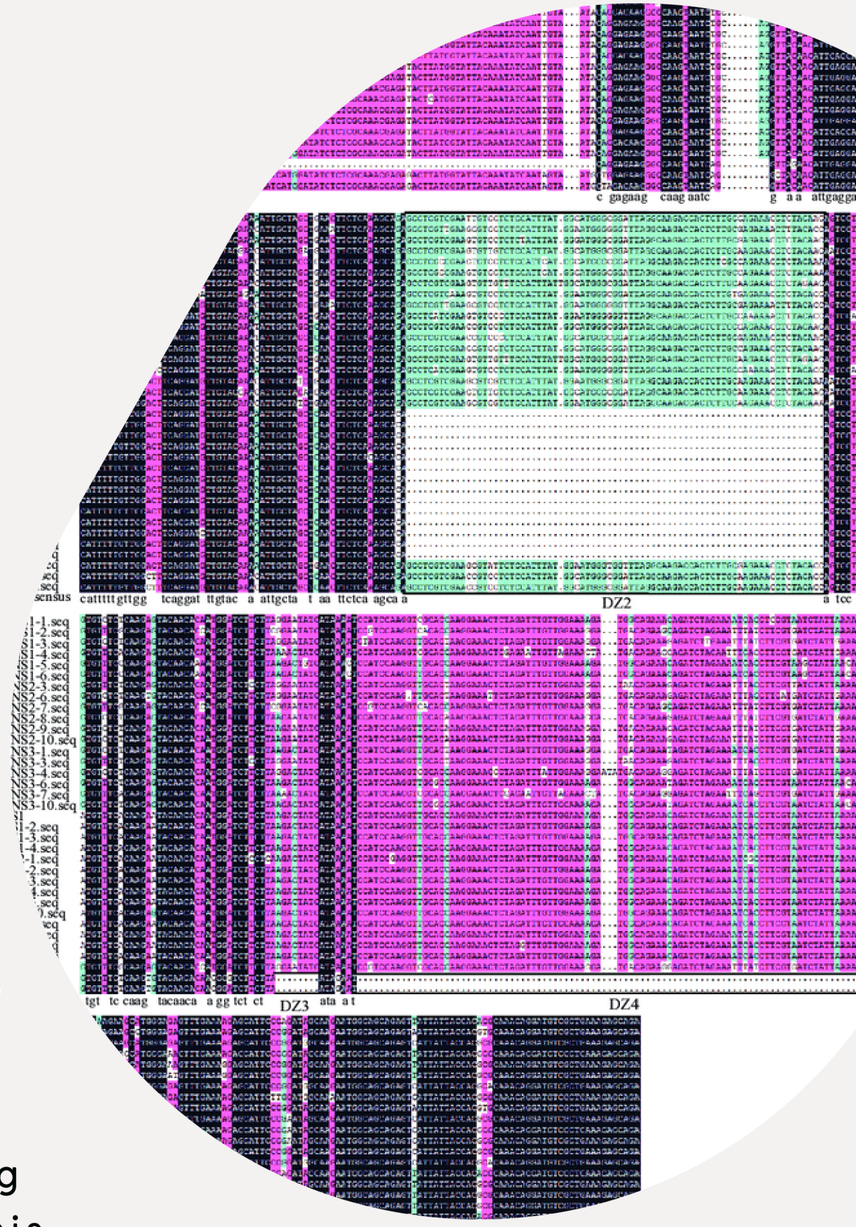
→

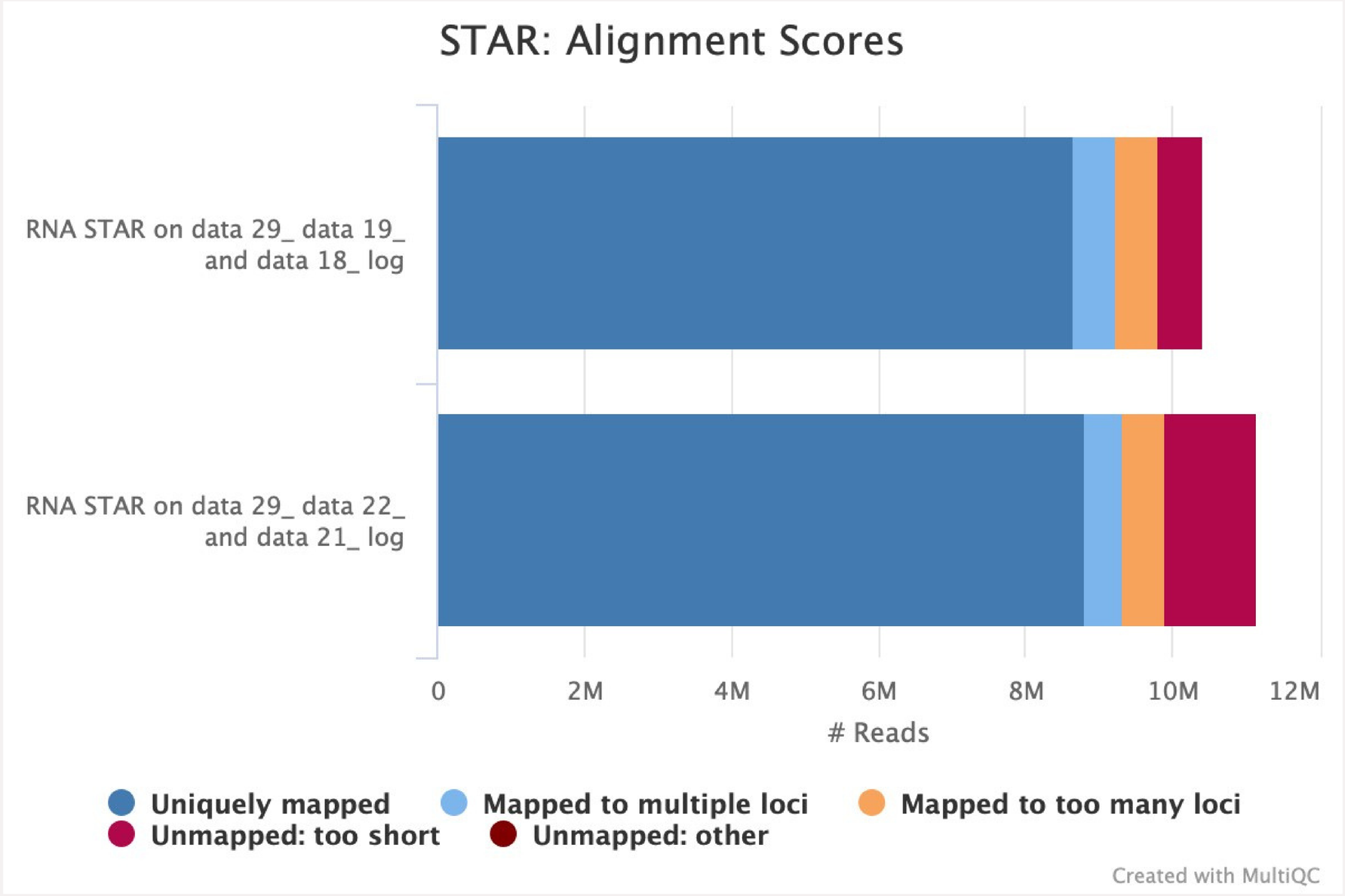
BAM/SAM File

→



Post Processing and Analysis




STAR: Alignment Scores
RNA STAR on data 29_ data 19_ and data 18_ log
RNA STAR on data 29_ data 22_ and data 21_ log
0
2M
4M
6M
8M
10M
12M
Reads
Uniquely mapped
Mapped to multiple loci
Mapped to too many loci
Unmapped: too short
Unmapped: other
Created with MultiQC

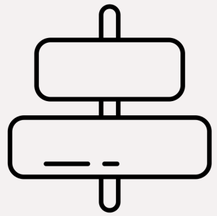
STEP 3 – COUNTING NUMBER OF READS

⇒ WHY?

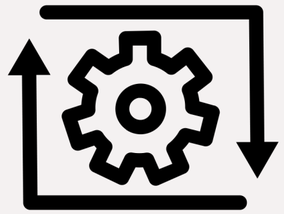
TO COMPARE GENE EXPRESSION BETWEEN CONDITIONS, WE NEED TO QUANTIFY THE NUMBER OF READS PER GENE.

⇒ STRANDEDNESS

- STRANDNESS OF THE LIBRARY MUST BE DETERMINED FOR ACCURATE COUNTING OF READS OVERLAPPING WITH GENOMIC FEATURES.



ALIGN READS EVALUATE COUNTS IN THREE STRANDNESS POSSIBILITIES



DETERMINE LIBRARY STRANDNESS FROM STAR RESULTS

USE STAR TO COUNT READS PER GENE AND GENERATE A TABLE WITH COUNTS MAPPED

STEP 4 – DIFFERENTIAL EXPRESSION ANALYSIS

⇒ OBJECTIVES

- NORMALISE DATASET AND GENERATE A SINGLE FILE WITH ALL SAMPLE DATA
- PLOT PCA AND SAMPLE-TO-SAMPLE DISTANCE
- GET DIFFERENTIALLY EXPRESSED GENES

USES DEseq TO NORMALISE DATA OVER CONVENTIONAL PROCEDURES

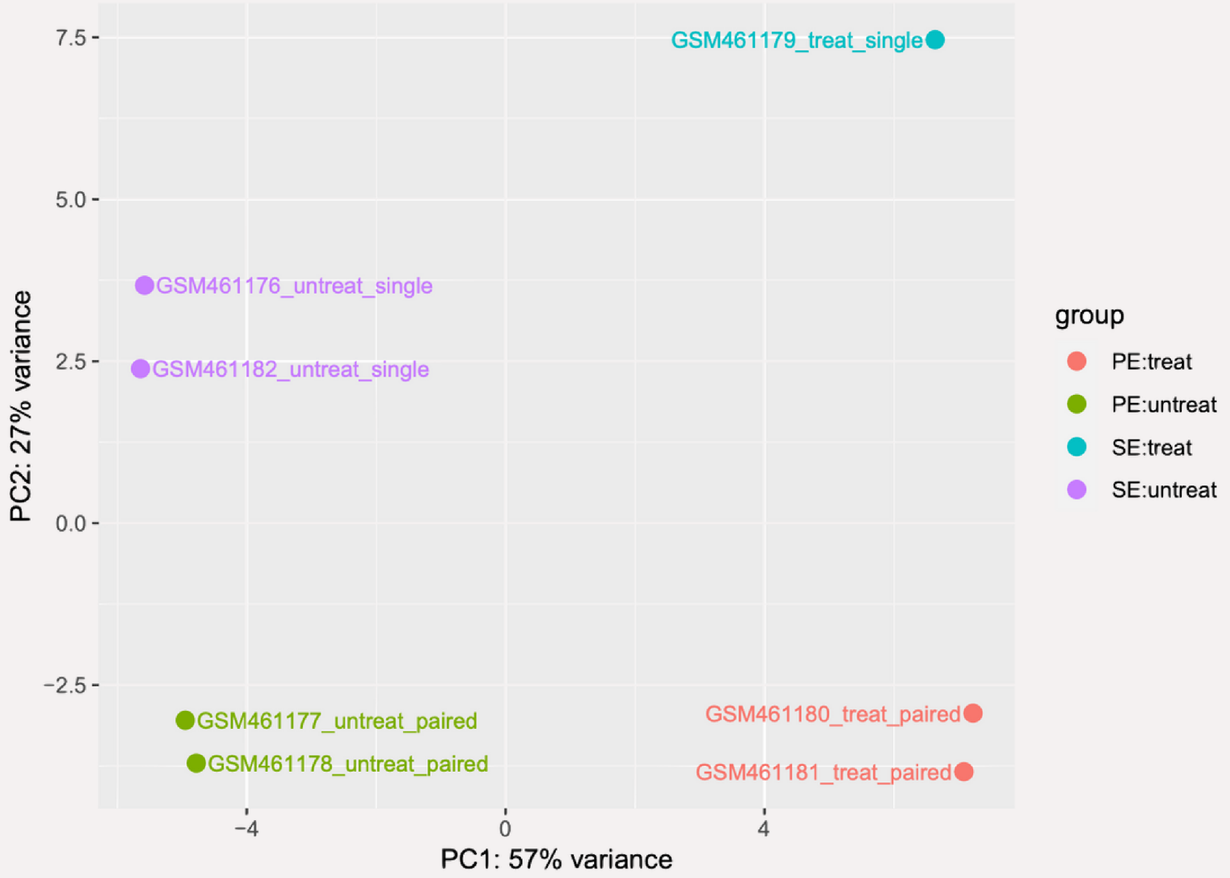
⇒ USES SEQUENCING DEPTH AND NORMALISATION AND LIBRARY COMPOSITION

⇒ RPKM OR TPM DON'T ACCOUNT OF DIFFERENCE IN LIBRARY COMPOSITION

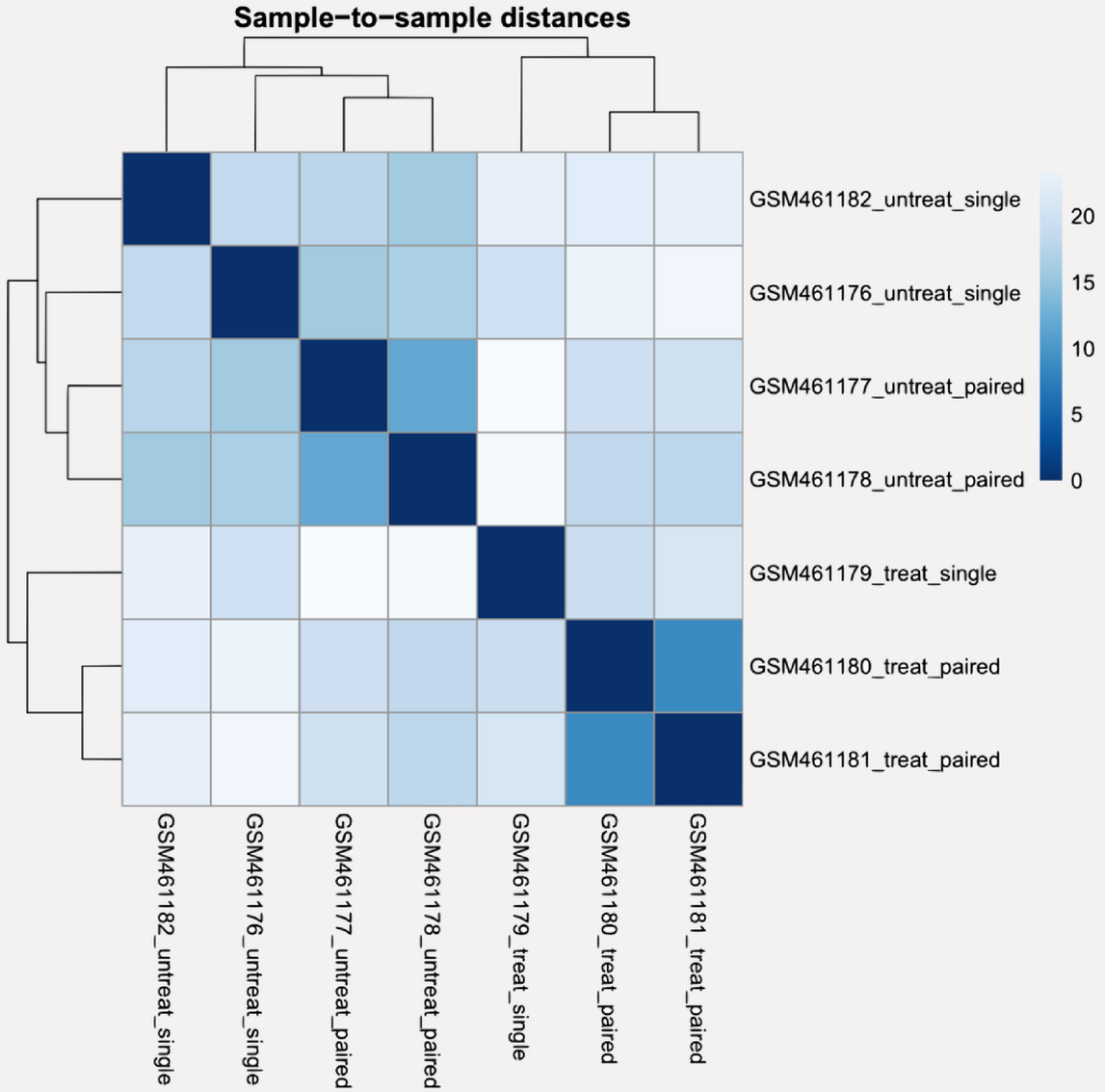
⇒ ABOUT 17000 GENES BETWEEN THE 7 SAMPLES

Column 1	Column 2	Column 3	Column 4	Column 5	Column 6	Column 7
	GSM461176_untreat_single	GSM461177_untreat_paired	GSM461178_untreat_paired	GSM461182_untreat_single	GSM461179_treat_single	GSM461180_treat
FBgn0085804	0	0	0	0	0	
FBgn0267431	2.19988706875227	0	0	0	8.51758304160428	2.24298440
FBgn0039987	0	0	0	0	0	
FBgn0058182	0	0	0	0	0	
FBgn0267430	0	0	0	0	1.54865146210987	1.12149220
FBgn0266747	0	0	0	0	0	
FBgn0086917	1.46659137916818	0	0	0	1.54865146210987	

PRINCIPLE COMPONENT ANALYSIS



DISTANCE BETWEEN SAMPLES

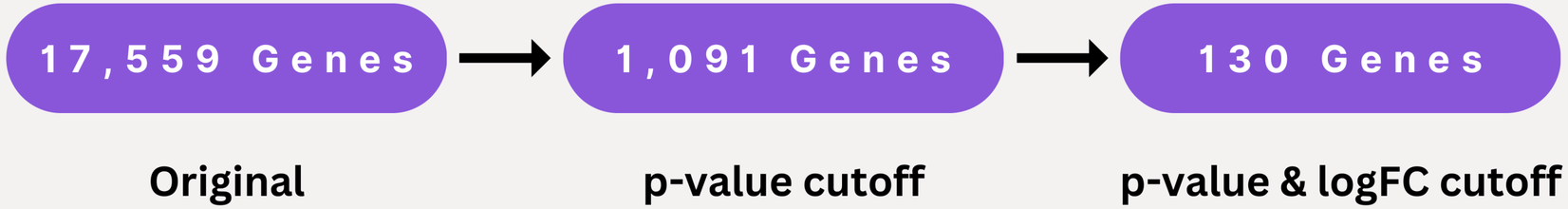


RESULT AND ANNOTATION

⇒ PVALUE ADJUSTED FOR COMPARISON ACROSS GENES

⇒ LOG FOLD CHANGE VALUES USED TO DETERMINED EXPRESSION OF GENES

⇒ SELECT GENES BASED ON PVALUE <0.05 AND ABSOLUTE LOG FC VALUED > 1



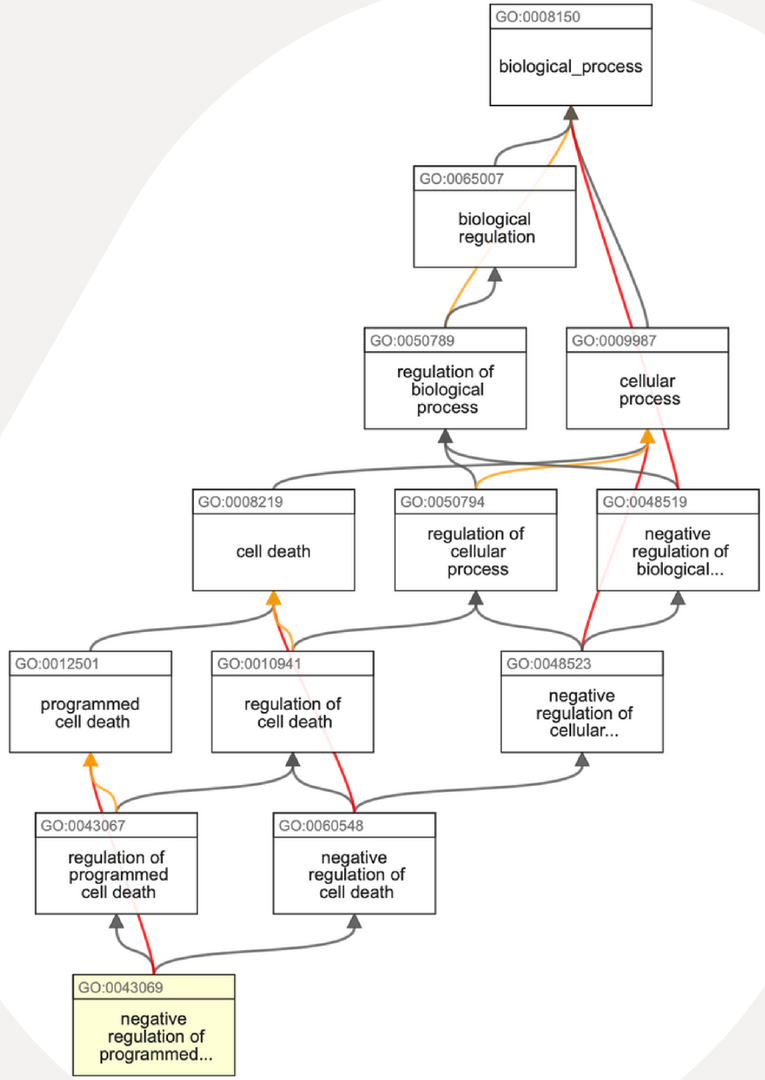
⇒ ADD ATTRIBUTES TO GENES TO MAKE DATA INTUITIVE AND DIGESTIBLE

STEP 5 – FUNCTIONAL ENRICHMENT ANALYSIS OF DE GENES

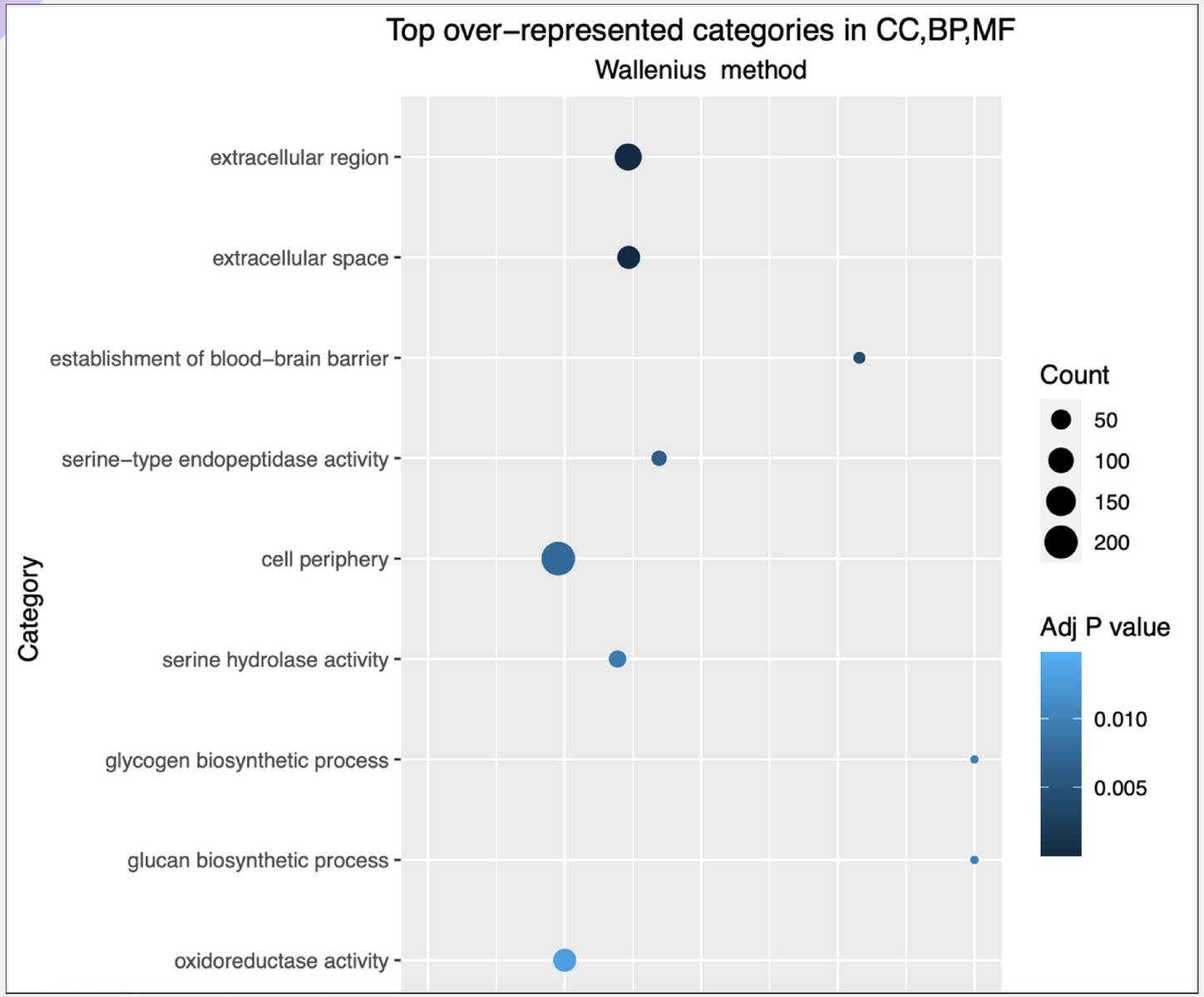
⇉ DE GENE FUNCTIONAL ENRICHMENT ANALYSIS IDENTIFIES OVER-REPRESENTED BIOLOGICAL PATHWAYS, FUNCTIONAL CATEGORIES, OR GENE SETS IN A GIVEN LIST OF GENES OR PROTEINS.

⇉ GO TERMS DESCRIBE GENE FUNCTIONS IN MF, BP, AND CC CATEGORIES, PROVIDING A STANDARDIZED WAY TO DESCRIBE BIOLOGICAL PATHWAYS AND FUNCTIONS.

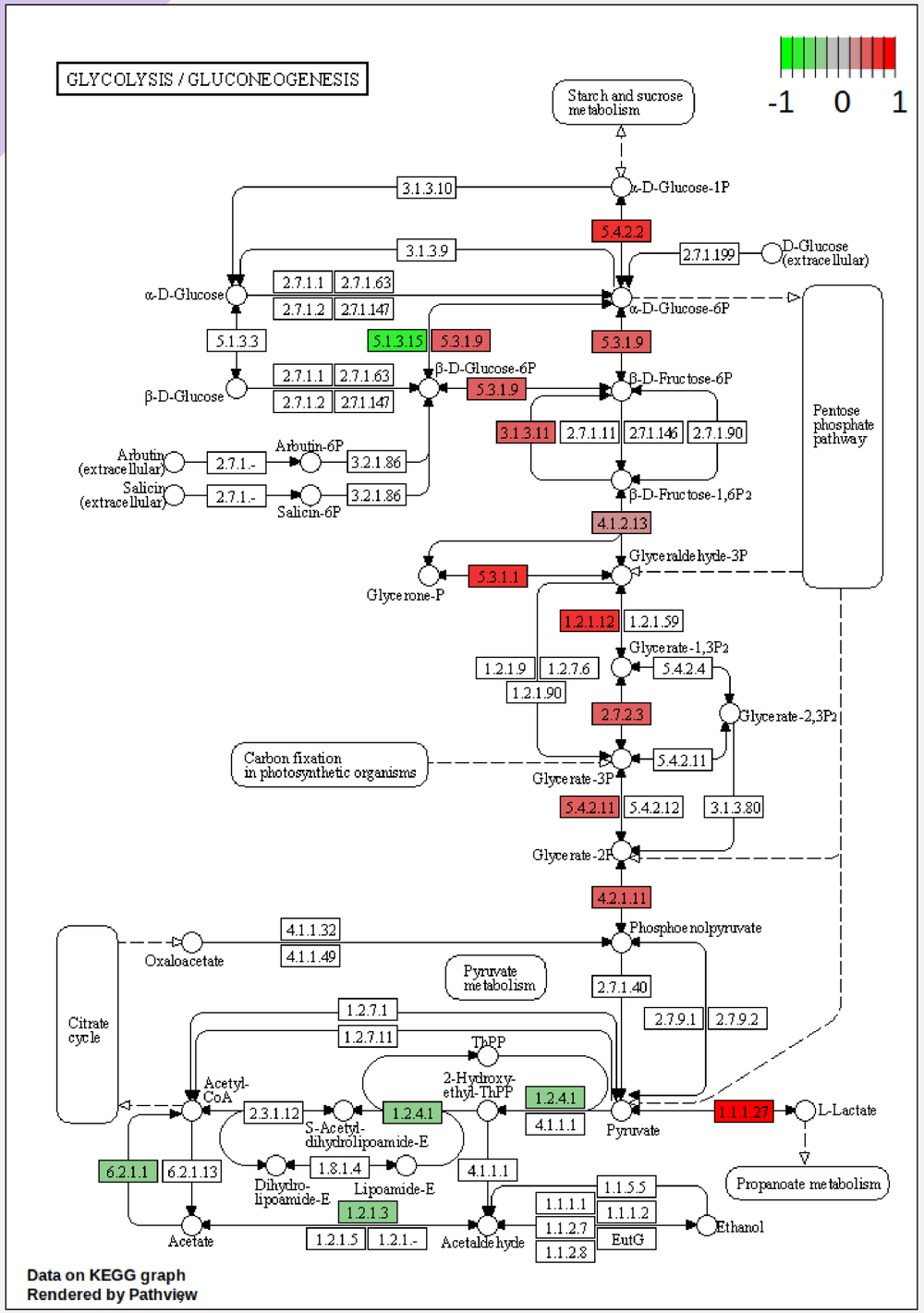
⇉ ENRICHMENT ANALYSIS TYPICALLY INVOLVES TWO MAJOR STEPS: GENE SET ENRICHMENT ANALYSIS AND PATHWAY VISUALIZATION/INTERPRETATION.



ENRICHMENT ANALYSIS RESULTS WITH WALLENIUS METHOD

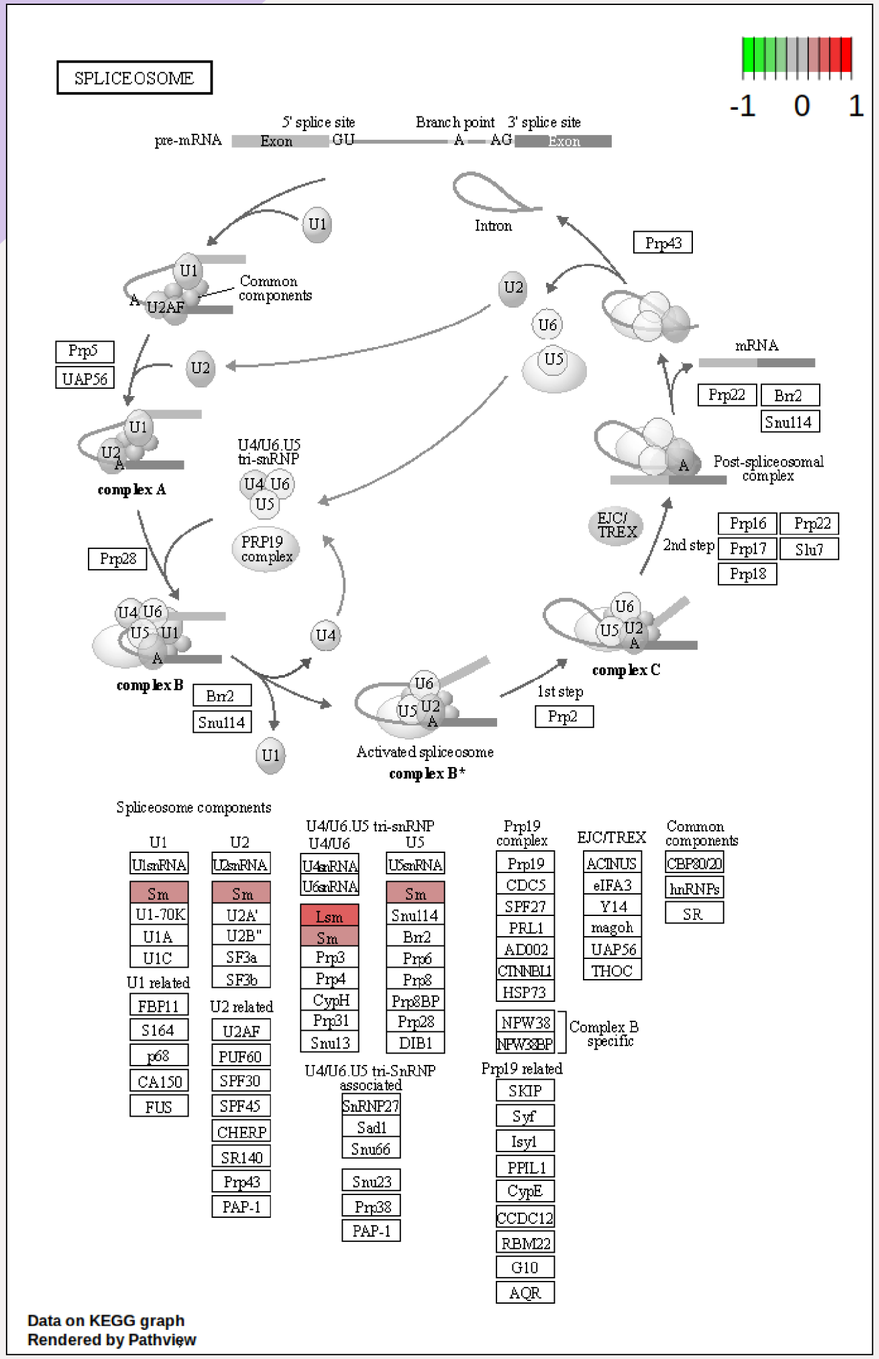


THIS GRAPH SHOWS THE TOP 10 OVER-REPRESENTED CATEGORIES IN CC, BP, AND MF AS IDENTIFIED THROUGH ENRICHMENT ANALYSIS USING THE WALLENIUS METHOD. CC STANDS FOR CELLULAR COMPONENT, BP STANDS FOR BIOLOGICAL PROCESS, AND MF STANDS FOR MOLECULAR FUNCTION. THE WALLENIUS METHOD IS A STATISTICAL METHOD USED TO CORRECT FOR GENE SET SIZE BIAS IN ENRICHMENT ANALYSIS. THE PERCENTAGE OF GENES THAT ARE DIFFERENTIALLY EXPRESSED AND THE ENRICHMENT FACTOR ARE ALSO SHOWN.



KEGG PATHWAY GRAPH

- REVIEWING THE TABLE CAN BE TIME-CONSUMING AND CUMBERSOME.
- TO BETTER VISUALIZE THIS INFORMATION, WE UTILIZED PATHVIEW.
- PATHVIEW IS A TOOL THAT AUTOMATICALLY GENERATES PATHWAY IMAGES WITH ADDED GENE INFORMATION, SUCH AS GENE EXPRESSION DATA.



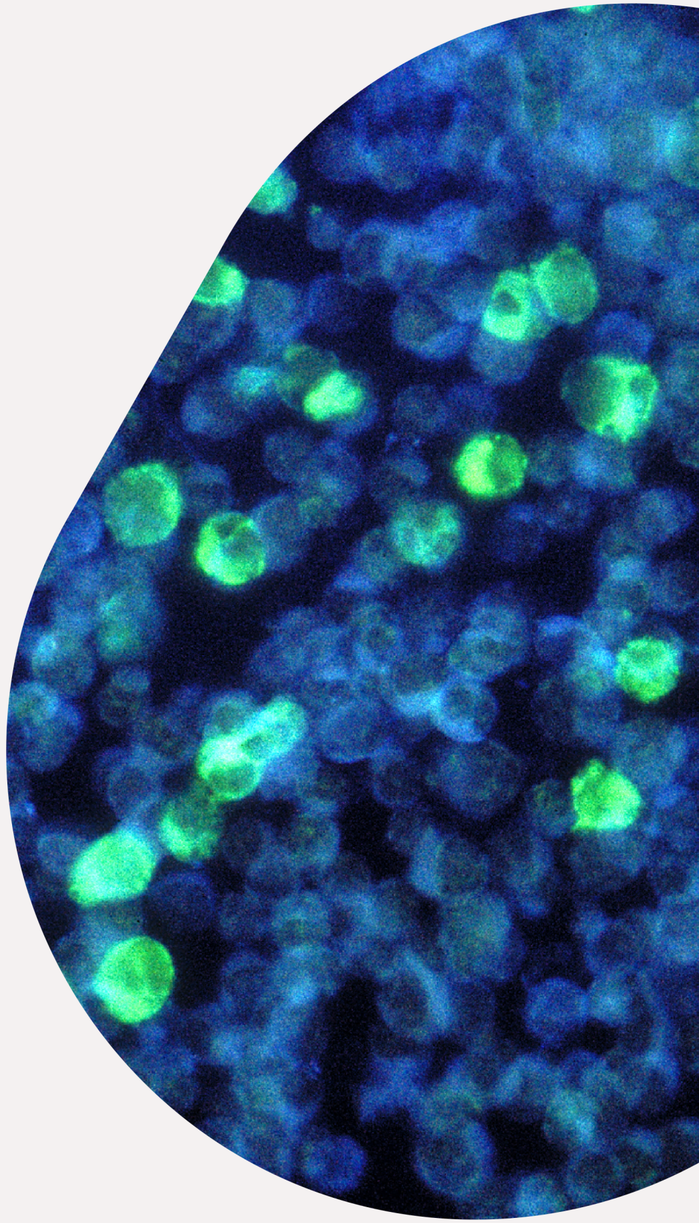
KEGG PATHWAY GRAPH

VISUALIZING THE SPLICEOSOME PATHWAY:-

- KEGG PATHWAY ID: 03040
- INVOLVED IN RNA PROCESSING FOR REMOVAL OF INTRONS AND SPLICING OF EXONS
- NOT SIGNIFICANTLY OVER-REPRESENTED IN ANALYSIS, BUT STILL INVESTIGATED DE GENES IN PATHWAY
- USED PATHVIEW TO GENERATE PLOT WITH GENE NODES COLORED BY LOG2 FOLD CHANGE
- PLOT REVEALED SEVERAL GENES WITH ALTERED EXPRESSION IN PATHWAY, PROVIDING POTENTIAL INSIGHTS INTO TREATMENT'S IMPACT ON BIOLOGICAL PROCESSES.

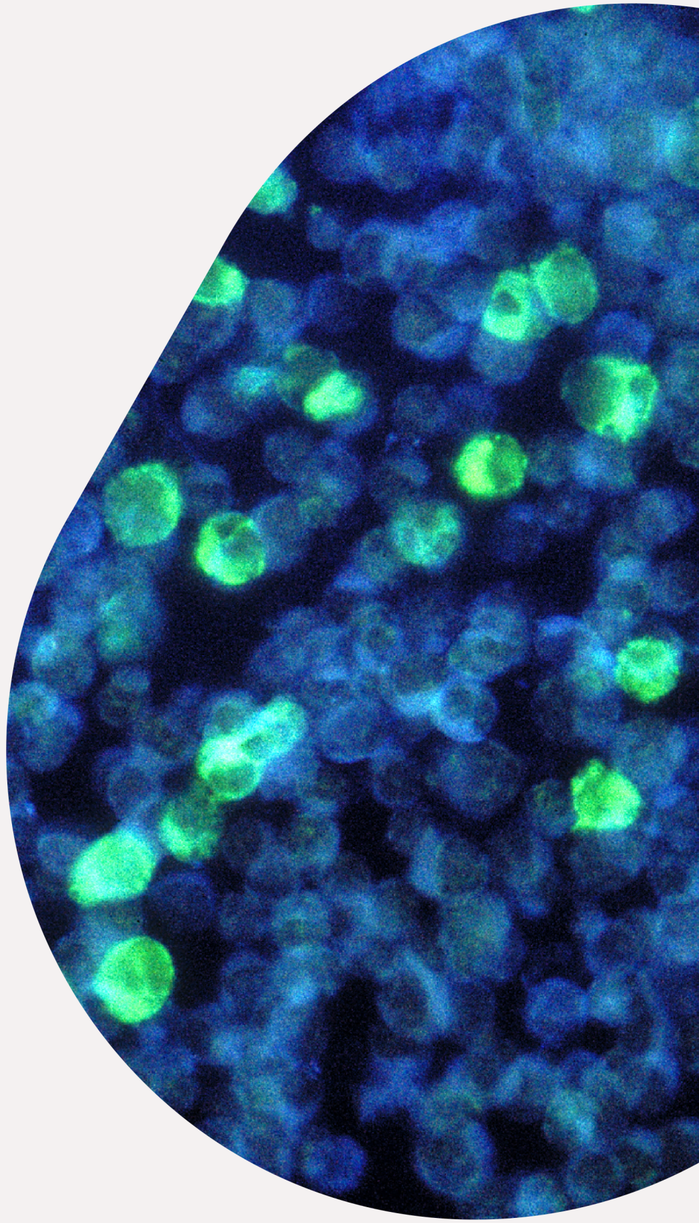
STEP 6 – CONCLUSION

- DEPLETING PASILLA GENE AFFECTS THE SPLICING RATE OF GENE A AND B DIFFERENTLY.
- UPREGULATION OF GENE A AND DOWNREGULATION OF GENE B SUGGEST THAT PASILLA SUPPRESSES AND PROMOTES THEIR SPLICING RATE, RESPECTIVELY.
- PASILLA DEPLETION LEADS TO UNDERREPRESENTATION OF SPLICEOSOME PATHWAY GENES, IMPLYING THAT CORE SPLICEOSOME GENES ARE NOT AFFECTED.
- UNDERREPRESENTATION OF THE SPLICEOSOME PATHWAY DOES NOT MEAN NO CHANGE IN SPLICING EVENTS, AND ANALYZING DIFFERENTIAL SPLICING EVENTS CAN PROVIDE BETTER INSIGHTS.
- PASILLA DEPLETION SIGNIFICANTLY AFFECTS THE EXPRESSION LEVELS OF GENES INVOLVED IN THE GLYCOLYSIS PATHWAY.



CONTD...

- THE OBSERVED DIFFERENTIAL EXPRESSION IN THE GLYCOLYSIS PATHWAY SUGGESTS THAT PASILLA DEPLETION HAS CASCADING EFFECTS ON OTHER GENES, POSSIBLY THROUGH ALTERED ALTERNATIVE SPLICING PATTERNS.
- PASILLA AND NOVA1/2 GENES ARE INVOLVED IN ALTERNATIVE SPLICING REGULATION IN INSECTS AND MAMMALS, RESPECTIVELY.
- THE RNA MAPS OF PASILLA AND NOVA1/2 ARE HIGHLY CONSERVED BETWEEN MAMMALS AND INSECTS, SUGGESTING SIMILAR SPLICING PATTERNS AND CONSERVED ROLES IN BIOLOGICAL PATHWAYS.
- THE STUDY'S RESULTS CAN BE EXTENDED TO STUDYING NOVA GENES IN MAMMALS.



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STAR: ultrafast universal RNA-seq aligner
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Pathview: an R/Bioconductor package for pathway-based data integration and visualization.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3032923/

Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3032923/

MultiQC: summarize analysis results for multiple tools and samples in a single report.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3032923/

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THANK YOU !