BST280

group6

2025-12-13

1. Load Library

bibliography: references.bib link-citations: true output: pdf_document: default html_document: default editor_options: markdown: wrap: 72 —

Introduction

Breast cancer is currently the most frequently diagnosed malignancy among women globally [@sung2021global]. Among its various subtypes, Estrogen Receptor-positive (ER+) breast cancer is the most prevalent, accounting for approximately 70% of all cases [@giuliano2024treating; @siegel2024cancer]. This high incidence rate underscores the critical need for transcriptomic studies to better understand the molecular drivers of the ER+ phenotype.

Despite clinical advances, understanding the transition from healthy mammary tissue to a malignant ER+ state requires a deep dive into the transcriptome. This analysis integrates high-throughput RNA-sequencing data from two large-scale and well-curated resources:**TCGA (The Cancer Genome Atlas):** Representing the tumoral landscape of ER+ Breast Carcinoma. **GTEx(Genotype-Tissue Expression):** Providing a robust baseline of healthy, non-diseased breast tissue.

Identifying individual tumor-associated genes through differential expression is useful, but it often misses the bigger picture: cancer is fundamentally a systems-level failure of gene regulation. For ER+ breast cancer, the defining features, such as aberrant estrogen signaling and rapid proliferation, which are driven by complex transcription factor networks. By shifting our focus from isolated gene counts to the underlying regulatory architecture, we can better resolve the biological complexities of hormone-dependent tumors.

Objectives: The primary goal of this study is to elucidate the transcriptomic and regulatory landscapes that distinguish ER-positive (ER+) breast carcinoma from healthy mammary tissue. By integrating primary tumor data from TCGA with healthy baseline samples from GTEx, we aim to provide a more robust comparison than traditional cancer-only studies.

Together, these analyses seek to answer a fundamental research question: What specific genes and regulatory networks distinguish ER+ tumor tissues from truly healthy breast tissues (GTEx), and how does this inform our understanding of hormone-driven carcinogenesis?

Method

load data & Preprocessing

The initial phase of our analysis focused on integrating and standardizing transcriptomic data from two primary sources to facilitate a robust comparison between malignant and healthy breast tissues.

```
# 0. Load Library
# install.packages("BiocManager")
# BiocManager::install("SummarizedExperiment")
```

```r
# BiocManager::install("edgeR")

library(dplyr)
library(GenomicRanges)
library(IRanges)
library(S4Vectors)
library(BiocGenerics)
library(matrixStats)
library(ggplot2)
library(enrichplot)
library(gplots)
library(biomaRt)
library(clusterProfiler)
library(msigdbr)
library(SummarizedExperiment)
library(SummarizedExperiment)
library(ggrepel)
library(edgeR)
library(limma)
```

We utilized raw count matrices and metadata from the The Cancer Genome Atlas (TCGA) for tumor samples and the Genotype-Tissue Expression (GTEx) project for healthy control samples. We strictly filtered the TCGA cohort to include only ER-positive samples.

```r
# 1. Load Data
tcga_data <- readRDS(file.path("../data/tcga_brca.rds"))
tcga_counts <- assay(tcga_data)
tcga_meta <- as.data.frame(colData(tcga_data))

write.csv(tcga_counts, file.path("../output/TCGA_BRCA_Counts.csv"), row.names = TRUE)
write.csv(tcga_meta, file.path("../output/TCGA_BRCA_Metadata.csv"), row.names = TRUE)

gtex_data <- readRDS(file.path("../data/gtex_breast.rds"))
gtex_counts <- assay(gtex_data)
gtex_meta <- as.data.frame(colData(gtex_data))

write.csv(gtex_counts, file.path("../output/GTEx_Breast_Counts.csv"), row.names = TRUE)
write.csv(gtex_meta, file.path("../output/GTEx_Breast_Metadata.csv"), row.names = TRUE)
```

To ensure compatibility between the two datasets, we performed gene alignment by identifying the intersection of gene identifiers present in both TCGA and GTEx. Both matrices were subsetted to include only these common genes and then merged into a unified raw counts matrix.

```r
# 2. Filter ER+ samples
grep("estrogen", colnames(tcga_meta), value = TRUE, ignore.case = TRUE)
```

```
## [1] "tcga.xml_breast_carcinoma_estrogen_receptor_status"
## [2] "tcga.xml_positive_finding_estrogen_receptor_other_measurement_scale_text"
## [3] "tcga.xml_metastatic_breast_carcinoma_estrogen_receptor_status"
## [4] "tcga.xml_metastatic_breast_carcinoma_estrogen_receptor_level_cell_percent_category"
```

```r
target_col <- "tcga.xml_breast_carcinoma_estrogen_receptor_status"
table(tcga_meta[[target_col]])
```

```
##
## Indeterminate      Negative      Positive
```

```
##             2           235           802
```

```
er_pos_ids <- which(tcga_meta[[target_col]] == "Positive")
tcga_counts_ER_ONLY <- tcga_counts[, er_pos_ids]
```

```
# 3. Align genes
genes_tcga <- rownames(tcga_counts_ER_ONLY)
genes_gtex <- rownames(gtex_counts)

common_genes <- intersect(genes_tcga, genes_gtex)
print(paste("TCGA:", length(genes_tcga)))
```

```
## [1] "TCGA: 41864"
```

```
print(paste("GTEx:", length(genes_gtex)))
```

```
## [1] "GTEx: 37976"
```

```
print(paste("common:", length(common_genes)))
```

```
## [1] "common: 35168"
```

```
# 4. Subset and order both matrices by common genes
tcga_aligned <- tcga_counts_ER_ONLY[common_genes, ]
gtex_aligned <- gtex_counts[common_genes, ]

final_counts_matrix <- cbind(tcga_aligned, gtex_aligned)
write.csv(final_counts_matrix, file.path("../output/Final_ERpos_Tumor_vs_Normal_Counts.csv"))
print(paste("Dimensions:",nrow(final_counts_matrix),"genes x", ncol(final_counts_matrix), "samples"))
```

```
## [1] "Dimensions: 35168 genes x 1275 samples"
```

To improve statistical power and reduce stochastic noise, we implemented a rigorous "Low-Count Filtering" protocol. We first calculated Counts Per Million (CPM) to normalize for differences in library size across samples. Genes were retained in the final dataset only if they exhibited a expression level of CPM > 1 in at least 25% of the total samples.

This strategy eliminates genes with very low or sporadic expression that could otherwise bias variance estimates during differential expression analysis.

This filtering process significantly refined our dataset, reducing it to approximately half of its original size while preserving high-quality, biologically relevant signals for downstream analysis.

```
# 5. Low Count Filtering
print(nrow(final_counts_matrix))
```

```
## [1] 35168
```

```
library_sizes <- colSums(final_counts_matrix)
cpm_matrix <- t(t(final_counts_matrix) / library_sizes) * 1e6

min_samples <- ncol(final_counts_matrix) / 4
keep <- rowSums(cpm_matrix > 1) >= min_samples

final_counts_filtered <- final_counts_matrix[keep, ]

print(paste("Total genes AFTER filtering:", nrow(final_counts_filtered)))
```

```
## [1] "Total genes AFTER filtering: 20624"
```

```r
print(paste("Removed:", nrow(final_counts_matrix) - nrow(final_counts_filtered), "noisy genes."))
```

```
## [1] "Removed: 14544 noisy genes."
```

```r
write.csv(final_counts_filtered, file.path("../output/Filtered_Final_ERpos_Tumor_vs_Normal_Counts.csv")

dim(final_counts_filtered)
```

```
## [1] 20624  1275
```

Exploratory data analysis (PCA)

PCA

Following the data cleaning step, we performed principal component analysis (PCA) on the normalized expression matrix to explore global transcriptional differences between ER-positive breast tumor samples and normal breast tissues

Before performing PCA, we first preprocessed the RNA-seq data to make the samples suitable for downstream analysis. We normalized the raw RNA-seq counts to counts per million (CPM)using sample-specific library sizes to account for differences in sequencing depth across samples. We then applied a log transformation to the normalized data, adding a constant of one so that genes with zero counts could be included. This transformation reduces the influence of highly expressed genes and helps stabilize variance across genes, allowing PCA to better capture meaningful global expression patterns

```r
# 1. CPM normalization and log transformß
library_sizes <- colSums(final_counts_filtered)

cpm_matrix <- t(t(final_counts_filtered) / library_sizes) * 1e6
logcpm_matrix <- log2(cpm_matrix + 1)
```

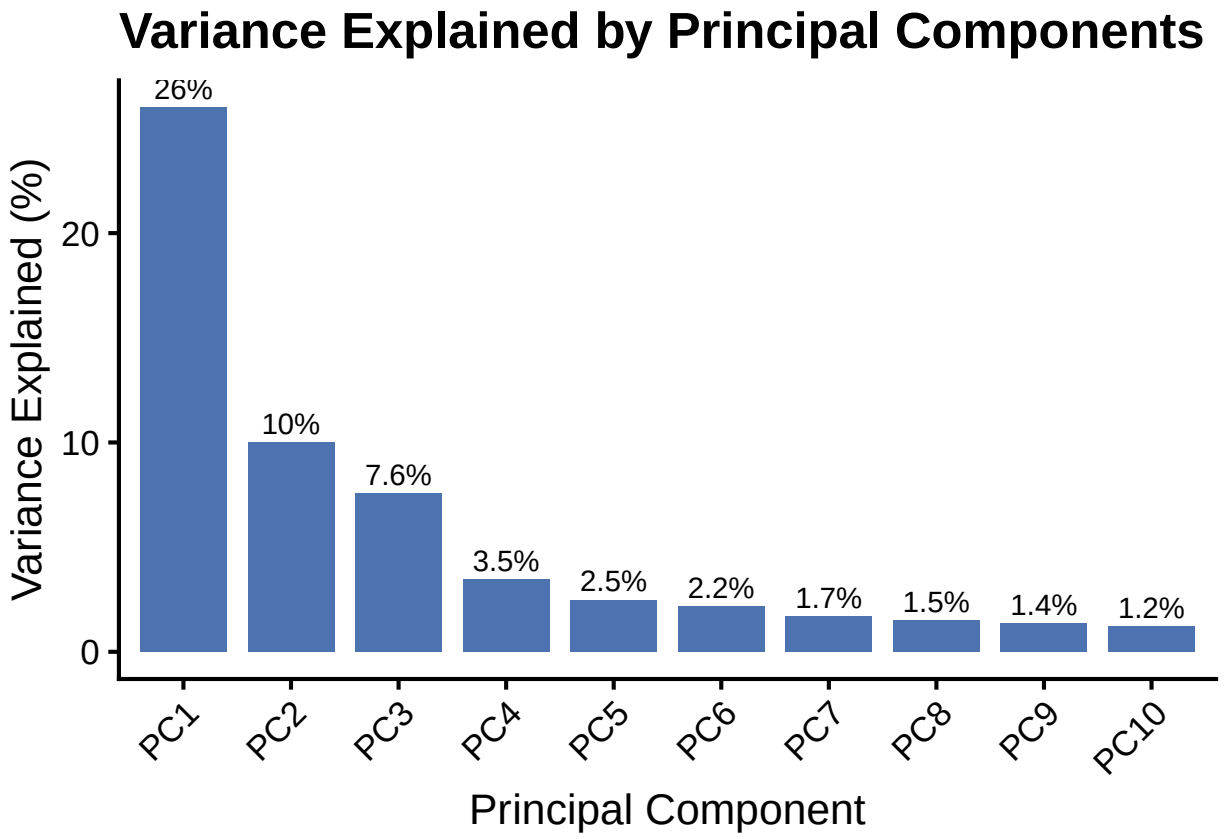
Here the scores for the first two principal components were extracted and combined with sample group labels to facilitate downstream analysis. Group-wise centroids were calculated to summarize the average position of ER-positive tumor and normal samples in the reduced-dimensional space, and the proportion of variance explained by each principal component was computed to quantify their contribution to overall expression variability

```r
# 2. PCA


pca_res <- prcomp(t(logcpm_matrix), scale. = TRUE)

#df
pca_df <- data.frame(
  PC1 = pca_res$x[, 1],
  PC2 = pca_res$x[, 2],
  Group = factor(c(
    rep("ER+ Tumor", ncol(tcga_aligned)),
    rep("Normal", ncol(gtex_aligned))
  ))
)

# centroids
centroids <- aggregate(cbind(PC1, PC2) ~ Group, data = pca_df, mean)


var_exp <- round(100 * summary(pca_res)$importance[2, 1:2], 1)
```

Plot

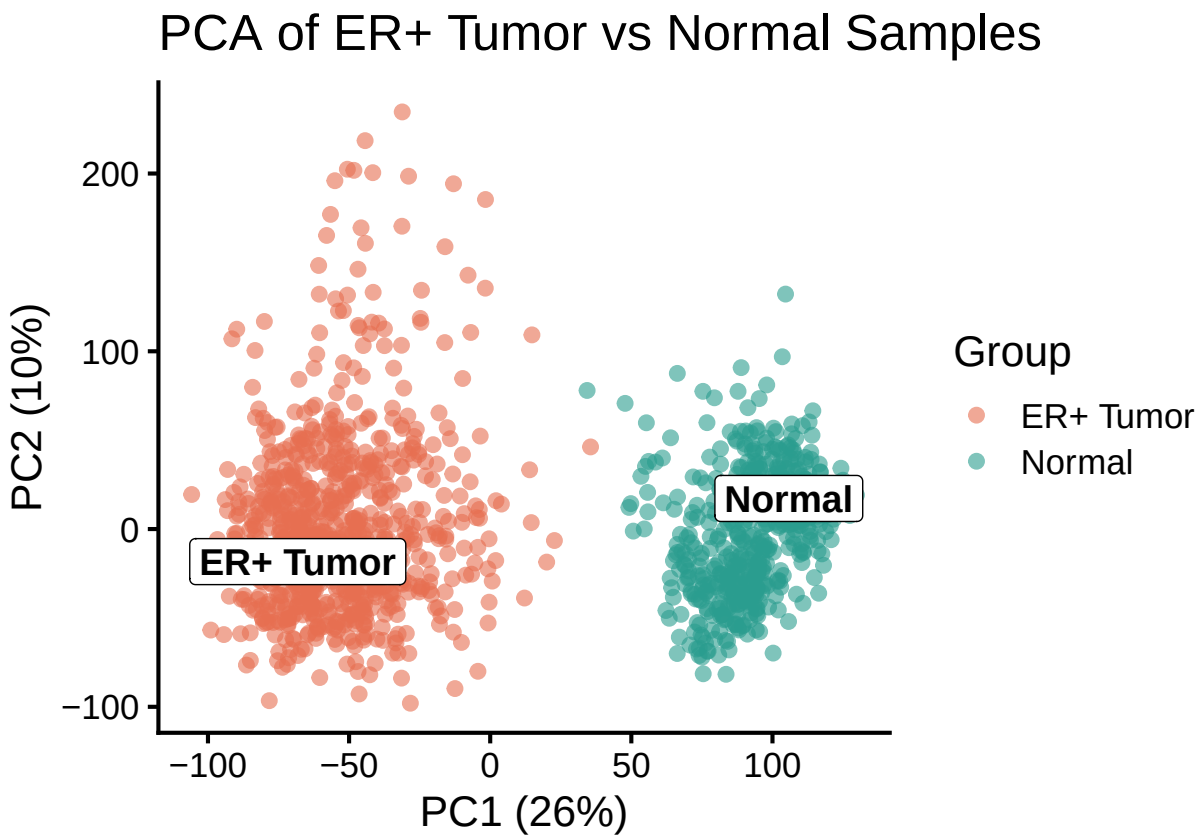
Here are our PCA results. The bar plot summarizes the proportion of total variance in the log-CPM expression matrix explained by each of the top 10 principal components. PC1 captures the largest source of variation in the dataset, explaining 26% of the total variance, while PC2 explains an additional 10%

```
# Plot1

pca_var <- data.frame(
  PC = paste0("PC", seq_along(pca_res$sdev)),
  Variance = (pca_res$sdev^2) / sum(pca_res$sdev^2) * 100
)

# Select top 10 PCs
pca_var_top <- pca_var[1:10, ]

ggplot(pca_var_top, aes(x = reorder(PC, -Variance), y = Variance)) +
  geom_bar(stat = "identity", fill = "#4C72B0", width = 0.8) +
  geom_text(
    aes(label = paste0(round(Variance, 1), "%")),
    vjust = -0.4,
    size = 4
  ) +
  labs(
    title = "Variance Explained by Principal Components",
    x = "Principal Component",
    y = "Variance Explained (%)"
  ) +
  theme_classic(base_size = 16) +
  theme(
    plot.title = element_text(face = "bold"),
    axis.text.x = element_text(angle = 45, hjust = 1)
  )
```



Here is the PCA scatter plot showing samples projected onto the first two principal components, with points colored by biological group. A clear separation between ER-positive tumor samples and normal breast tissues is observed primarily along PC1, indicating that disease status is a major driver of global gene expression variation. In addition, tumor samples display greater dispersion, particularly along PC2, reflecting increased transcriptional heterogeneity in ER-positive tumors compared to normal tissue.

```
# Plot2
ggplot(pca_df, aes(x = PC1, y = PC2, color = Group)) +
  geom_point(alpha = 0.6, size = 2) +
  geom_label_repel(
    data = centroids,
    aes(x = PC1, y = PC2, label = Group),
    inherit.aes = FALSE,
    fontface = "bold",
    size = 5
  ) +
  scale_color_manual(values = c("ER+ Tumor" = "#E76F51", "Normal" = "#2A9D8F")) +
  labs(
    title = "PCA of ER+ Tumor vs Normal Samples",
    x = paste0("PC1 (", var_exp[1], "%)"),
    y = paste0("PC2 (", var_exp[2], "%)")
  ) +
  theme_classic(base_size = 16) +
  theme(legend.position = "right")
```



While PCA reveals clear global differences between ER-positive tumor and normal samples, it does not identify which specific genes are responsible for this separation. Therefore, the next step is differential expression analysis, which allows us to systematically identify genes whose expression levels differ significantly between the two conditions and drive the observed transcriptomic differences.

Differential Expression Analysis

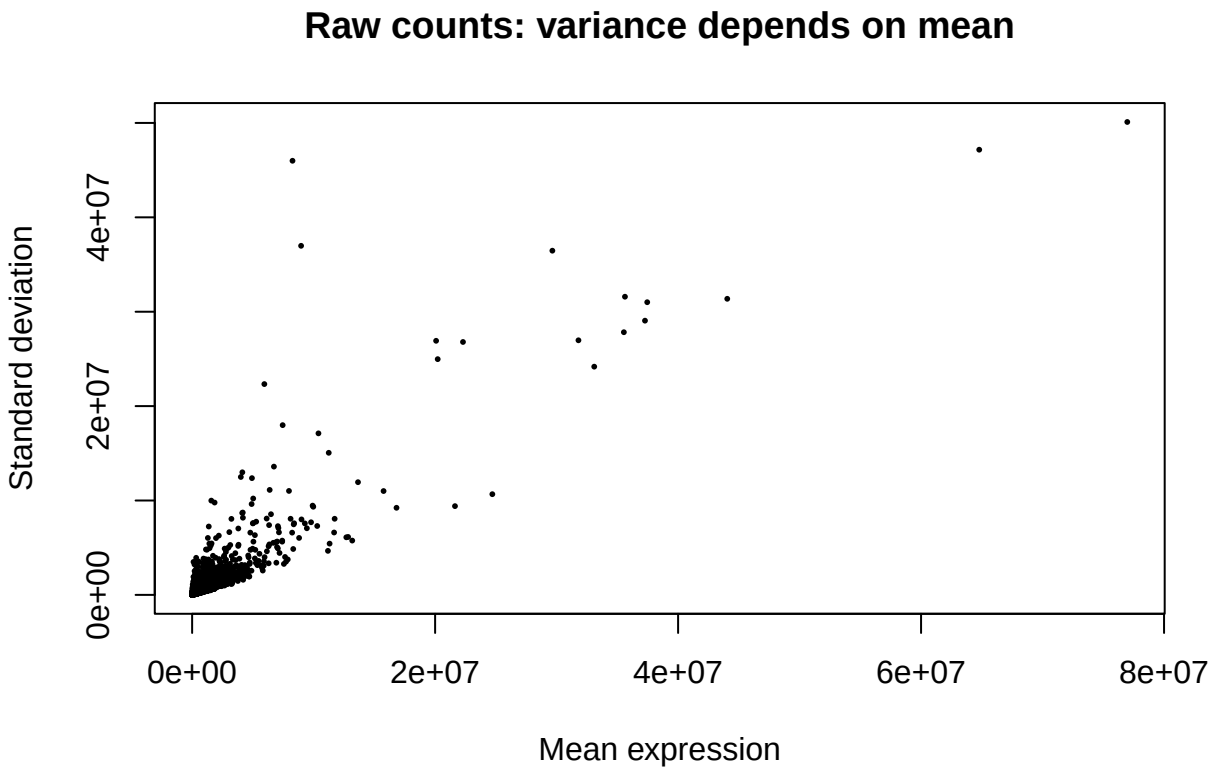
We already remove the genes with low counts in the preprocessing step according to the criteria: dropped if they have very low counts (1cpm) in 25% of the samples. This step reduces noise introduced by genes that are unlikely to be reliably measured or biologically informative and ensures that downstream analyses focus on genes with sufficient expression to support meaningful differential expression and regulatory inference.

To assess the mean–variance relationship in the raw RNA-seq data, we calculated the gene-wise mean expression and standard deviation directly from the filtered count matrix and visualized their relationship.

The resulting plot shows a strong positive association, with genes exhibiting higher average expression also displaying substantially larger variance. This heteroscedasticity violates the constant-variance assumption underlying linear modeling approaches

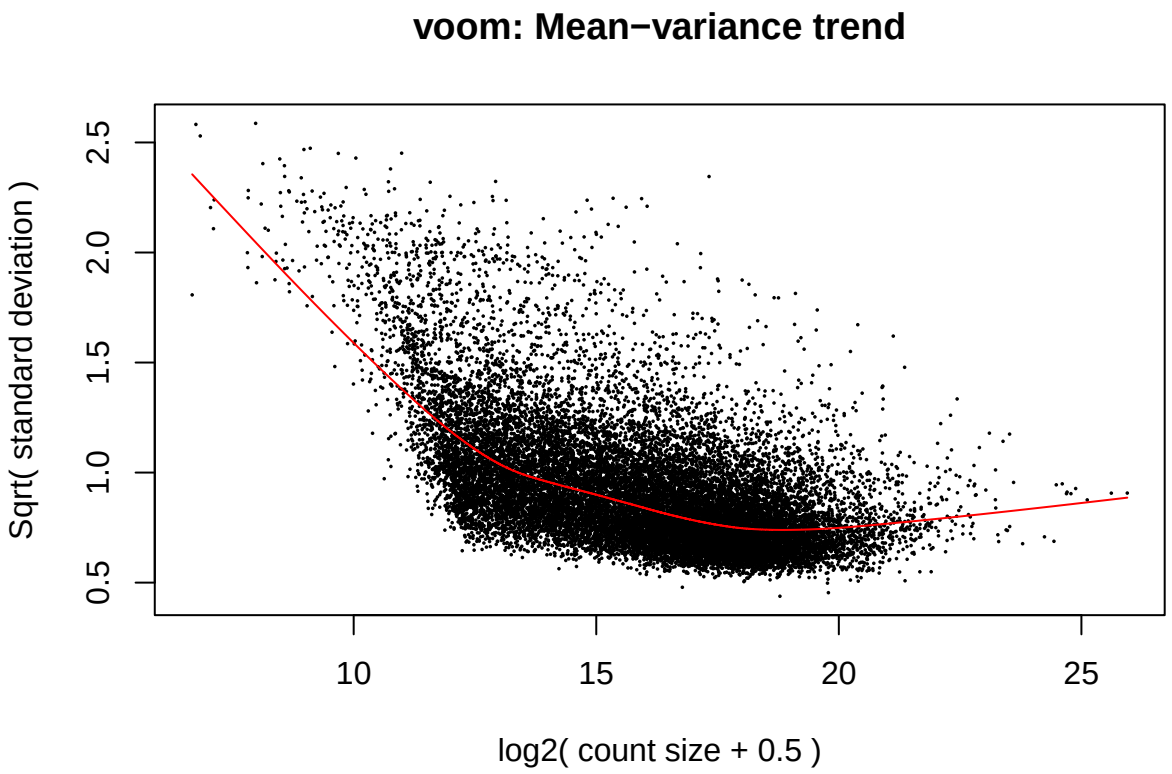
```
# 1. mean-variance relationship
gene_sd   <- apply(final_counts_filtered, 1, sd)
gene_mean <- apply(final_counts_filtered, 1, mean)

plot(
  x = gene_mean,
  y = gene_sd,
  pch = 16,
  cex = 0.4,
  main = "Raw counts: variance depends on mean",
```

```
  xlab = "Mean expression",
  ylab = "Standard deviation"
)
```



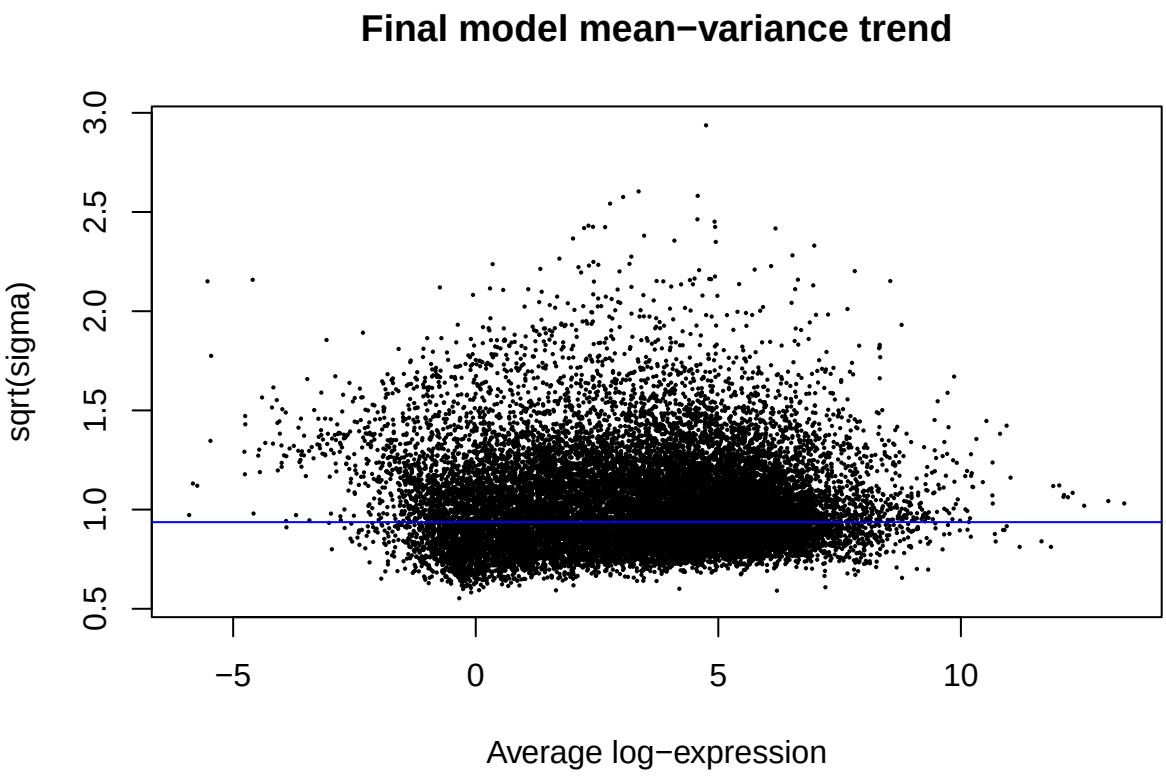
To address this issue and enable valid differential expression analysis, we subsequently applied the voom transformation, which models and corrects the mean–variance relationship, resulting in a smoother and more stable variance structure suitable for linear modeling

```
# 2. Voom Transformation
group <- factor(
  c(
    rep("ER+ Tumor", ncol(tcga_aligned)),
    rep("Normal", ncol(gtex_aligned))
  )
)

design <- model.matrix(~ group)

dge <- DGEList(counts = final_counts_filtered)
dge <- calcNormFactors(dge)

voom_out <- voom(dge, design, plot = TRUE)
```



After applying the voom transformation, we fitted a gene-wise linear model using the limma framework to compare ER-positive tumor and normal samples. Empirical Bayes moderation was then applied to stabilize variance estimates across genes by borrowing information from the full dataset. The resulting diagnostic plot shows that variance is approximately constant across expression levels, indicating that model assumptions are satisfied and that the data are appropriately conditioned for reliable differential expression testing

```
# 3. Linear model + Bayes
fit <- lmFit(voom_out, design)
fit <- eBayes(fit)

plotSA(
  fit,
  main = "Final model mean-variance trend"
)
```



Differential expression results were extracted from the fitted linear model using the topTable function, specifying the contrast between normal and ER-positive tumor samples. For each gene, this step reports the estimated log2 fold change, moderated test statistics, and associated p-values, with multiple-testing correction applied using the Benjamini–Hochberg false discovery rate method. The resulting table provides a ranked list of genes based on statistical evidence for differential expression and serves as the basis for downstream filtering and biological interpretation

```
res <- topTable(fit, coef = "groupNormal", number = Inf, adjust.method = "BH")
head(res)
```

```
##                       logFC    AveExpr          t P.Value adj.P.Val        B
## ENSG00000227034.1  4.227663  0.8805705  112.29845       0         0 1514.070
## ENSG00000231747.1  5.028063  1.2673760  112.11449       0         0 1512.340
## ENSG00000237668.1  4.554055  1.0018745  104.16417       0         0 1427.791
## ENSG00000256338.2 -4.066144  5.2413440 -100.57136       0         0 1388.418
## ENSG00000248124.7  2.898696  2.4879505   99.92621       0         0 1381.101
## ENSG00000242692.1  3.975039 -0.8051933   95.92782       0         0 1333.150
```

Visualization and Enrichment Analysis

Explore the DE results

We first summarized the number of differentially expressed (DE) genes between ER+ tumor (TCGA) and normal breast tissue (GTEx) using a stringent cutoff of FDR < 0.05 and $|\log2 \text{ fold-change}| > 2$. Under this threshold, we identified **3106** DE genes in total.

Because the model coefficient is defined as **log2FC = (Normal − Tumor)**, genes with **negative log2FC** are higher in **ER+ tumor**, while genes with **positive log2FC** are higher in **normal** tissue. Using this convention, **1325 genes** are upregulated in **ER+ tumor** ($\log2FC < -2$) and **1781 genes** are upregulated in **normal** tissue ($\log2FC > 2$).

Overall, this indicates widespread transcriptional differences between ER+ breast tumors and normal breast

tissue, motivating downstream visualization (volcano/MA plots and heatmaps) and pathway-level enrichment analyses to interpret these changes at the level of biological programs.

```
de_all_idx  <- res$adj.P.Val < 0.05 & abs(res$logFC) > 2
n_de_all    <- sum(de_all_idx)

tumor_up_idx  <- res$adj.P.Val < 0.05 & res$logFC < -2
n_tumor_up    <- sum(tumor_up_idx)

normal_up_idx <- res$adj.P.Val < 0.05 & res$logFC >  2
n_normal_up   <- sum(normal_up_idx)

cat("Total DE genes (FDR < 0.05 and |log2FC| > 2):", n_de_all, "\n")
```

```
## Total DE genes (FDR < 0.05 and |log2FC| > 2): 3103
```

```
cat("Up in ER+ Tumor (logFC < -2):", n_tumor_up, "\n")
```

```
## Up in ER+ Tumor (logFC < -2): 1321
```

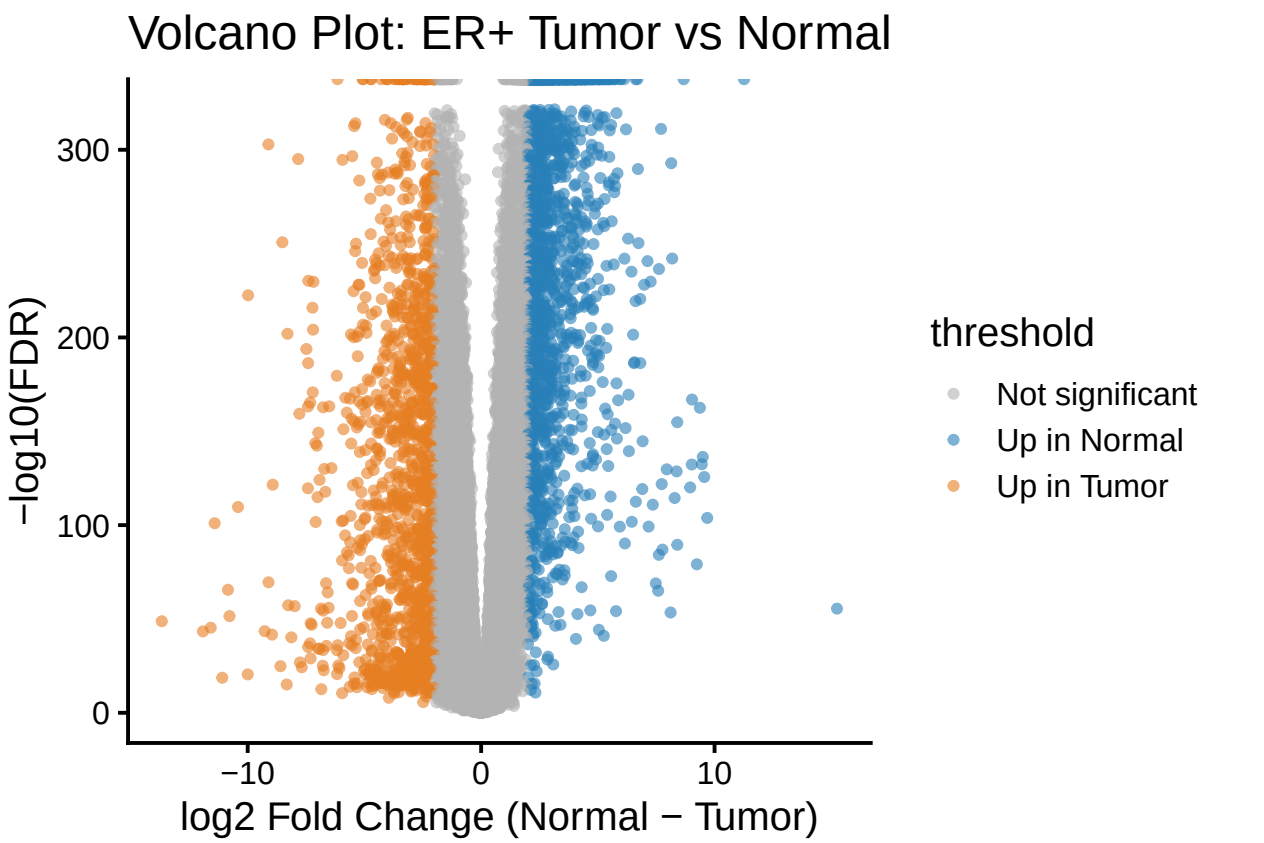
```
cat("Up in Normal (logFC > 2):", n_normal_up, "\n")
```

```
## Up in Normal (logFC > 2): 1782
```

Volcano Plot

We next visualized the DE results using a volcano plot.

```
plot_df <- data.frame(
  logFC = res$logFC,
  negLogFDR = -log10(res$adj.P.Val)
)

plot_df$threshold <- "Not significant"
plot_df$threshold[res$adj.P.Val < 0.05 & res$logFC > 2] <- "Up in Normal"
plot_df$threshold[res$adj.P.Val < 0.05 & res$logFC < -2] <- "Up in Tumor"

ggplot(plot_df, aes(x = logFC, y = negLogFDR, color = threshold)) +
  geom_point(alpha = 0.6, size = 1.2) +
  scale_color_manual(
    values = c(
      "Up in Tumor" = "#E67E22",
      "Up in Normal" = "#2980B9",
      "Not significant" = "grey70"
    )
  ) +
  labs(
    title = "Volcano Plot: ER+ Tumor vs Normal",
    x = "log2 Fold Change (Normal - Tumor)",
    y = "-log10(FDR)"
  ) +
  theme_classic(base_size = 15)
```



The volcano plot summarizes differential expression results by simultaneously displaying effect size and statistical significance. Each point represents one gene, with the x-axis showing the log2 fold change (Normal − Tumor) and the y-axis showing −log10(FDR). Genes farther from zero on the x-axis exhibit larger expression differences, while genes higher on the y-axis are more statistically significant.

Using a cutoff of FDR < 0.05 and $|\log2 \text{ fold-change}| > 2$, genes upregulated in ER+ tumor tissue (log2FC < -2) are highlighted in orange, whereas genes upregulated in normal breast tissue (log2FC > 2) are shown in blue. Genes that do not meet these thresholds are shown in grey.

The plot reveals a large number of highly significant genes on both sides of the distribution, indicating strong transcriptional differences between ER+ breast tumors and normal tissue. Notably, many tumor-upregulated genes exhibit both large effect sizes and extremely low FDR values, consistent with widespread activation of tumor-associated transcriptional programs.

A horizontal band of points is observed at the top of the volcano plot, corresponding to genes with extremely small adjusted p-values. For these genes, the FDR values are numerically close to zero due to very strong statistical evidence, causing −log10(FDR) to approach infinity. This behavior is expected in large RNA-seq datasets with high statistical power and does not affect the interpretation of differential expression patterns.

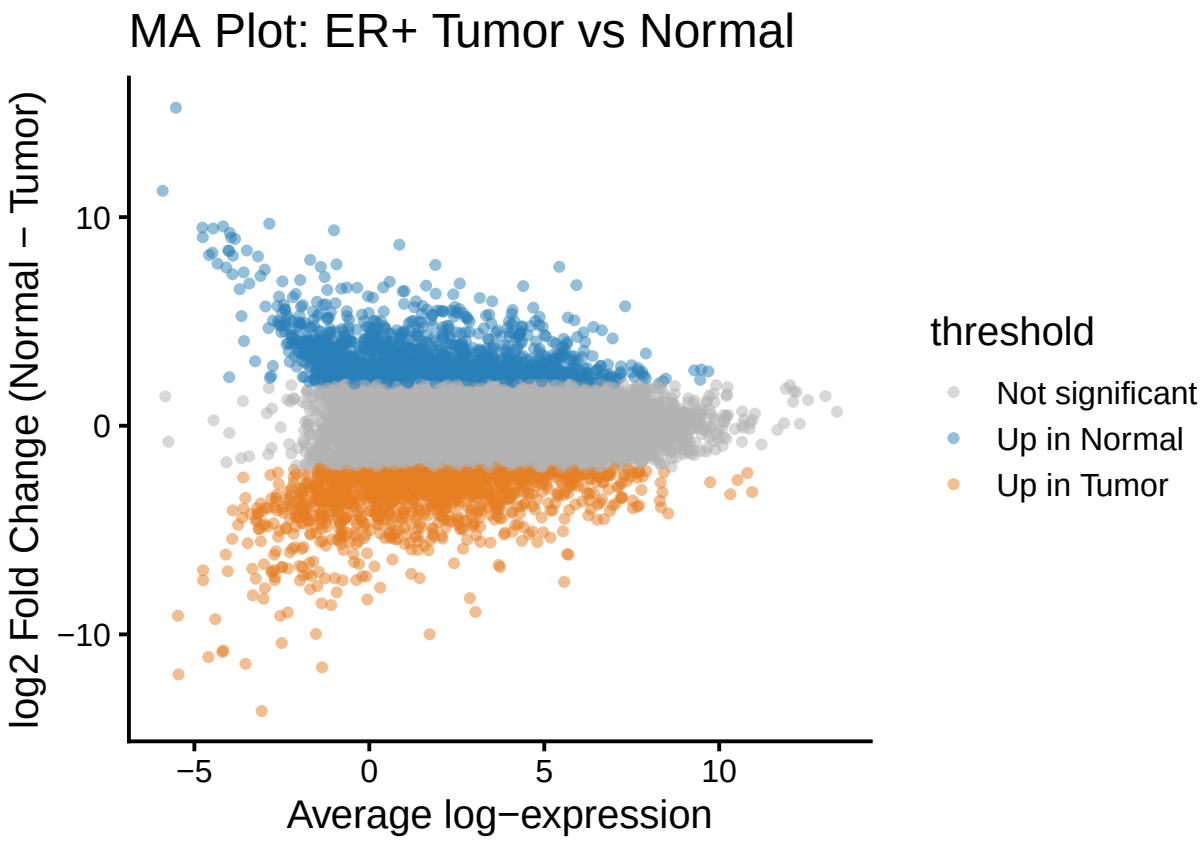
These patterns motivate downstream pathway-level analyses to determine which biological processes drive the observed expression changes.

MA Plot

We additionally used an MA plot to visualize log2 fold-changes as a function of average expression. This plot helps assess whether large fold-changes are concentrated among lowly expressed genes (which can be noisier) and whether the overall distribution of changes is centered around zero.

```
ma_df <- data.frame(
  AveExpr = res$AveExpr,
```

```
  logFC   = res$logFC,
  adjP    = res$adj.P.Val
)

ma_df$threshold <- "Not significant"
ma_df$threshold[ma_df$adjP < 0.05 & ma_df$logFC > 2]  <- "Up in Normal"
ma_df$threshold[ma_df$adjP < 0.05 & ma_df$logFC < -2] <- "Up in Tumor"

ggplot(ma_df, aes(x = AveExpr, y = logFC, color = threshold)) +
  geom_point(alpha = 0.5, size = 1.2) +
  scale_color_manual(
    values = c(
      "Up in Tumor" = "#E67E22",
      "Up in Normal" = "#2980B9",
      "Not significant" = "grey70"
    )
  ) +
  labs(
    title = "MA Plot: ER+ Tumor vs Normal",
    x = "Average log-expression",
    y = "log2 Fold Change (Normal - Tumor)"
  ) +
  theme_classic(base_size = 15)
```



The MA plot displays the relationship between gene expression abundance and differential expression. Each point represents one gene, with the x-axis showing the average log-expression across all samples and the y-axis showing the log2 fold change (Normal − Tumor).

Genes significantly upregulated in normal tissue (FDR < 0.05 and log2FC > 2) are shown in blue, while genes upregulated in ER+ tumor tissue (FDR < 0.05 and log2FC < -2) are shown in orange. Non-significant genes are shown in grey.

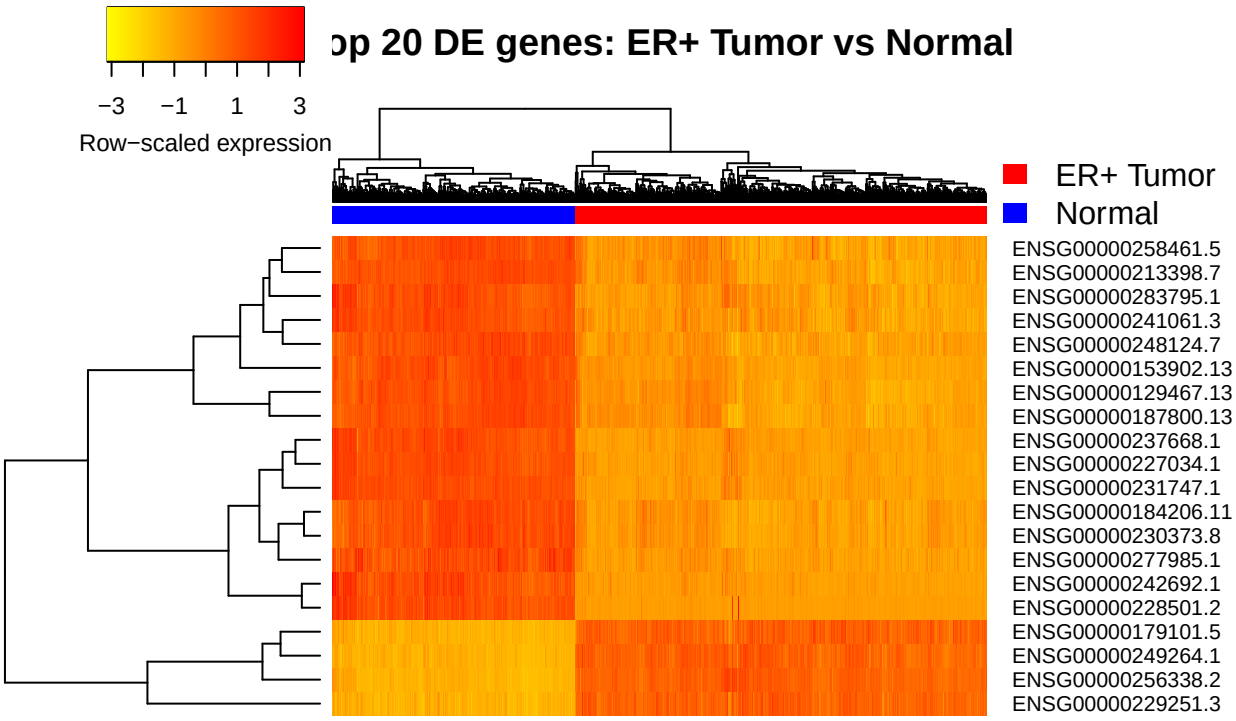
Most genes with low average expression cluster tightly around log2 fold change near zero, reflecting higher variability and lower statistical power at low expression levels. In contrast, highly expressed genes show more stable fold-change estimates, with many exhibiting consistent upregulation in either tumor or normal tissue. The approximately symmetric distribution of fold changes around zero indicates balanced transcriptional shifts between the two conditions rather than a global expression bias.

Overall, the MA plot confirms that the observed differential expression patterns are not driven solely by low-abundance genes and supports the robustness of the differential expression analysis.

Heatmap

To further examine whether the most significant DE genes show consistent expression patterns across samples, we plotted a heatmap of the top 20 genes ranked by adjusted p-value.

```
res_ordered <- res[order(res$adj.P.Val), ]
top_n <- 20
top_genes <- rownames(res_ordered)[1:top_n]

mat <- logcpm_matrix[top_genes, ]
cols_group <- c(
  rep("ER+ Tumor", ncol(tcga_aligned)),
  rep("Normal",    ncol(gtex_aligned))
)
heatmapColColors <- c("ER+ Tumor" = "red", "Normal" = "blue")[cols_group]
heatmapCols <- colorRampPalette(c("yellow", "red"))(250)

heatmap.2(
  mat,
  scale = "row",
  trace = "none",
  ColSideColors = heatmapColColors,
  col = heatmapCols,
  labCol = FALSE,
  margins = c(5, 10),
  density.info = "none",
  key.xlab = "Row-scaled expression",
  key.title = NA,
  main = "Top 20 DE genes: ER+ Tumor vs Normal"
)

legend(
  "topright",
  legend = c("ER+ Tumor", "Normal"),
  inset = c(-0.07, 0),
  fill   = c("red", "blue"),
  border = NA,
  bty = "n",
  xpd = TRUE
)
```



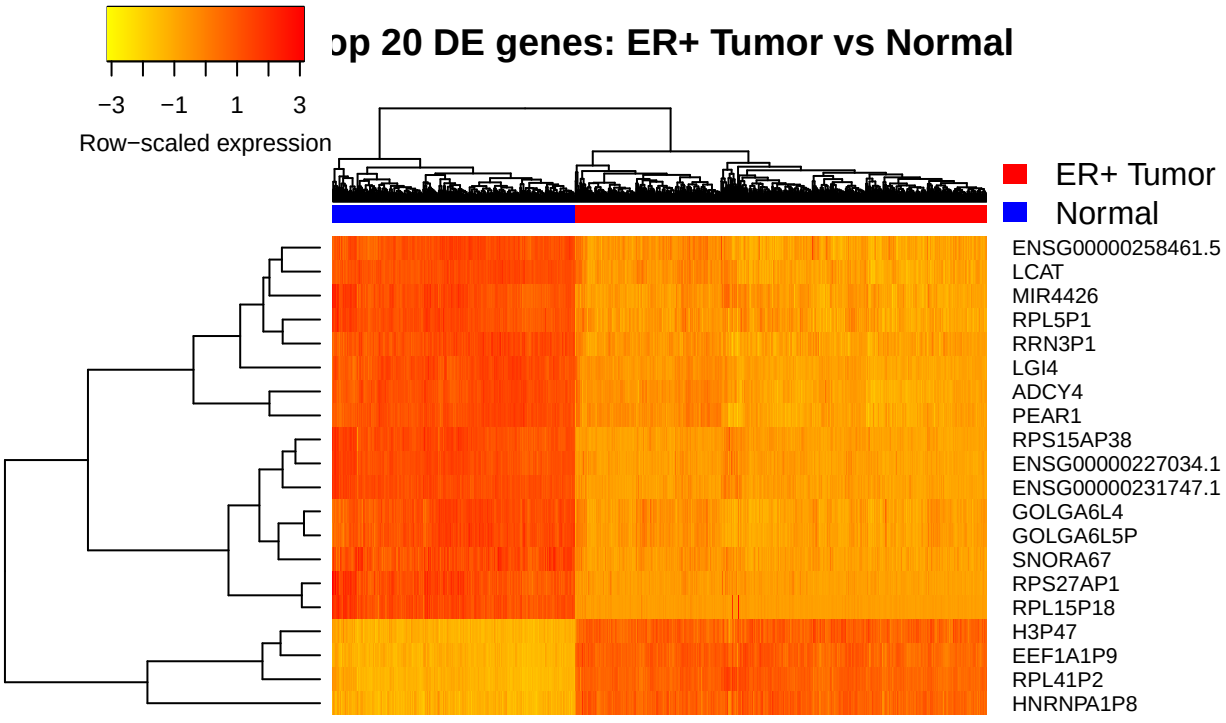
Genes were ranked by adjusted p-value, and the top 20 were selected without applying an additional fold-change filter. Expression values were logCPM-transformed and scaled by row so that colors represent relative expression differences across samples for each gene.

Despite gene labels initially being Ensembl gene IDs, the heatmap reveals a clear separation between ER+ tumor and normal breast samples. Samples largely cluster by condition, indicating that the most statistically significant differentially expressed genes consistently distinguish tumor from normal tissue.

Across these genes, expression patterns are highly coordinated. Many genes show elevated expression in ER+ tumor samples with lower expression in normal samples, while others display the opposite pattern. This consistent structure suggests that the observed differential expression reflects underlying biological differences rather than random variation.

```
top_genes_stripped <- sub("\\..*", "", top_genes)
ensembl <- useMart("ensembl", dataset = "hsapiens_gene_ensembl")
annot <- getBM(
  attributes = c("ensembl_gene_id", "hgnc_symbol"),
  filters    = "ensembl_gene_id",
  values     = top_genes_stripped,
  mart       = ensembl
)

symbols <- annot$hgnc_symbol[match(top_genes_stripped, annot$ensembl_gene_id)]
symbols[symbols == "" | is.na(symbols)] <- top_genes[symbols == "" | is.na(symbols)]

rownames(mat) <- symbols

heatmapCols <- colorRampPalette(c("yellow", "red"))(250)

heatmap.2(
  mat,
  scale = "row",
```

```
  trace = "none",
  ColSideColors = heatmapColColors,
  col = heatmapCols,
  labCol = FALSE,
  margins = c(5, 10),
  density.info = "none",
  key.xlab = "Row-scaled expression",
  key.title = NA,
  main = "Top 20 DE genes: ER+ Tumor vs Normal"
)

legend(
  "topright",
  inset = c(-0.07, 0),
  legend = c("ER+ Tumor", "Normal"),
  fill   = c("red", "blue"),
  border = NA,
  bty = "n",
  xpd = TRUE
)
```



To improve interpretability, Ensembl gene IDs were mapped to HGNC gene symbols using the biomaRt database. Version suffixes were removed from Ensembl IDs prior to annotation to ensure proper matching. For genes without an available HGNC symbol, the original Ensembl IDs were retained.

Replacing Ensembl IDs with gene symbols does not alter the expression data or clustering structure of the heatmap; rather, it facilitates biological interpretation by allowing known genes and pathways to be more readily recognized. The persistence of distinct tumor–normal expression patterns after annotation confirms that the observed structure is driven by expression differences and not by labeling artifacts.

Overall, the heatmap shows that these top DE genes separate ER+ tumor and normal samples into distinct clusters, indicating that the strongest DE signals are coherent across many samples rather than driven

by a small subset of outliers and motivating subsequent pathway-level enrichment analyses to identify the biological programs underlying these gene-level differences.

Hallmark Gene Set Enrichment Analysis (GSEA)

We first prepare gene identifiers for downstream Hallmark GSEA. The differential expression results `res` are indexed by Ensembl gene IDs (e.g., `ENSG00000...`), often including a version suffix (e.g., `.5`). Because many enrichment tools and gene set databases (including MSigDB collections accessed via `msigdbr`) commonly use Entrez gene IDs, we first map Ensembl IDs to Entrez IDs.

Specifically, we remove the version suffix from Ensembl IDs (`sub("\\..*", "", ...)`) to ensure stable matching. We then query the Ensembl BioMart database (`biomaRt`) for an annotation table linking each Ensembl gene ID to its corresponding Entrez gene ID. The resulting mapping (`annot`) will be used to convert our ranked gene list into Entrez space before running Hallmark GSEA.

```
ensembl_ids <- sub("\\..*", "", rownames(res))
ensembl <- useMart("ensembl", dataset = "hsapiens_gene_ensembl")

annot <- getBM(
  attributes = c("ensembl_gene_id", "entrezgene_id"),
  filters    = "ensembl_gene_id",
  values     = ensembl_ids,
  mart       = ensembl
)

head(annot)
```

```
##   ensembl_gene_id entrezgene_id
## 1 ENSG00000000005         64102
## 2 ENSG00000000938          2268
## 3 ENSG00000001630          1595
## 4 ENSG00000002016          5893
## 5 ENSG00000002834          3927
## 6 ENSG00000003137         56603
```

Then we construct the **ranked gene list** required for preranked GSEA.\ 1) We start from the limma output table `res` and create a version-stripped Ensembl ID column (`ensembl_nover`) so it matches the BioMart annotation keys.\ 2) We then merge the differential expression results with the Ensembl→Entrez mapping table `annot`, and remove genes without an Entrez ID (`NA`), since Hallmark gene sets are indexed by Entrez IDs.\ 3) Because multiple Ensembl IDs can map to the same Entrez gene ID, we collapse duplicates by keeping one representative row per Entrez ID. Here we keep the entry with the largest absolute t-statistic (i.e., strongest evidence of differential expression), using: - `arrange(entrezgene_id, desc(abs(t)))` to put the strongest row first within each Entrez ID, and - `distinct(entrezgene_id, .keep_all = TRUE)` to keep only that top row.\ 4) Finally, we create the preranked vector `gene_list`, where: - the values are the limma t-statistics (`res_gsea$t`) representing signed strength of differential expression, and - the names are the corresponding Entrez gene IDs.\ We sort this vector in decreasing order so that genes most positively associated with the contrast appear at the top, which is the expected input format for GSEA.\

Note: the sign of the t-statistic follows the model contrast used in `topTable()`. In our analysis we extracted `coef = "groupNormal"`, so positive t (and positive logFC) indicates higher expression in Normal relative to ER+ Tumor, while negative values indicate higher expression in ER+ Tumor.

```
res_gsea <- res
res_gsea$ensembl_nover <- sub("\\..*", "", rownames(res_gsea))

res_gsea <- merge(
  res_gsea,
```

```
  annot,
  by.x = "ensembl_nover",
  by.y = "ensembl_gene_id"
)

res_gsea <- res_gsea[!is.na(res_gsea$entrezgene_id), ]

res_gsea <- res_gsea %>%
  arrange(entrezgene_id, desc(abs(t))) %>%
  distinct(entrezgene_id, .keep_all = TRUE)

gene_list <- res_gsea$t
names(gene_list) <- res_gsea$entrezgene_id

gene_list <- sort(gene_list, decreasing = TRUE)
```

To interpret differential expression results at the pathway level, we performed Gene Set Enrichment Analysis (GSEA) using the MSigDB Hallmark gene sets. Unlike over-representation analysis that depends on an arbitrary DE cutoff, GSEA uses the entire ranked gene list (here ranked by the limma moderated t-statistic) to test whether genes from a pathway tend to accumulate near the top or bottom of the ranked list. We used the msigdbr package to retrieve Hallmark gene sets and ran clusterProfiler::GSEA with FDR control to identify pathways systematically enriched toward either the ER+ tumor side or the normal side.

```
m_df <- msigdbr(species = "Homo sapiens", category = "H")

hallmark_term2gene <- m_df %>%
  dplyr::select(gs_name, entrez_gene) %>%
  distinct()

set.seed(42)
gsea_hallmark <- GSEA(
  geneList     = gene_list,
  TERM2GENE    = hallmark_term2gene,
  pvalueCutoff = 0.05,
  verbose      = FALSE
)

gsea_hallmark_res <- as.data.frame(gsea_hallmark)
head(gsea_hallmark_res)
```

```
##                                                 ID               Description
## HALLMARK_E2F_TARGETS           HALLMARK_E2F_TARGETS      HALLMARK_E2F_TARGETS
## HALLMARK_G2M_CHECKPOINT     HALLMARK_G2M_CHECKPOINT   HALLMARK_G2M_CHECKPOINT
## HALLMARK_MYC_TARGETS_V1     HALLMARK_MYC_TARGETS_V1   HALLMARK_MYC_TARGETS_V1
## HALLMARK_MTORC1_SIGNALING HALLMARK_MTORC1_SIGNALING HALLMARK_MTORC1_SIGNALING
## HALLMARK_MYOGENESIS             HALLMARK_MYOGENESIS       HALLMARK_MYOGENESIS
## HALLMARK_GLYCOLYSIS             HALLMARK_GLYCOLYSIS       HALLMARK_GLYCOLYSIS
##                           setSize enrichmentScore       NES       pvalue
## HALLMARK_E2F_TARGETS          200      -0.5998196 -3.214358 1.000000e-10
## HALLMARK_G2M_CHECKPOINT       199      -0.6003452 -3.214010 1.000000e-10
## HALLMARK_MYC_TARGETS_V1       200      -0.5444398 -2.917584 1.000000e-10
## HALLMARK_MTORC1_SIGNALING     200      -0.4635249 -2.483972 1.000000e-10
## HALLMARK_MYOGENESIS           179       0.5024520  2.290217 1.000000e-10
## HALLMARK_GLYCOLYSIS           187      -0.3657452 -1.964081 2.852625e-08
```

```
##                              p.adjust       qvalue rank
## HALLMARK_E2F_TARGETS      1.000000e-09 4.842105e-10 3354
## HALLMARK_G2M_CHECKPOINT   1.000000e-09 4.842105e-10 2853
## HALLMARK_MYC_TARGETS_V1   1.000000e-09 4.842105e-10 3399
## HALLMARK_MTORC1_SIGNALING 1.000000e-09 4.842105e-10 3810
## HALLMARK_MYOGENESIS       1.000000e-09 4.842105e-10 3463
## HALLMARK_GLYCOLYSIS       2.377188e-07 1.151059e-07 3311
##                                        leading_edge
## HALLMARK_E2F_TARGETS      tags=64%, list=21%, signal=51%
## HALLMARK_G2M_CHECKPOINT   tags=60%, list=18%, signal=50%
## HALLMARK_MYC_TARGETS_V1   tags=51%, list=21%, signal=41%
## HALLMARK_MTORC1_SIGNALING tags=52%, list=24%, signal=41%
## HALLMARK_MYOGENESIS       tags=46%, list=22%, signal=37%
## HALLMARK_GLYCOLYSIS       tags=38%, list=21%, signal=30%
##
## HALLMARK_E2F_TARGETS      6117/83461/7037/5395/1786/6426/5889/1965/10248/7884/5983/5425/9738/23649/73
## HALLMARK_G2M_CHECKPOINT                                                                 1871/23649/6558/1810/73
## HALLMARK_MYC_TARGETS_V1
## HALLMARK_MTORC1_SIGNALING
## HALLMARK_MYOGENESIS
## HALLMARK_GLYCOLYSIS
```

Each row in the GSEA result corresponds to one Hallmark pathway. setSize is the number of genes from that pathway that overlap with our ranked gene list. The key statistic is the normalized enrichment score (NES), which summarizes the strength and direction of enrichment. Statistical significance is assessed after multiple-testing correction using p.adjust (BH-FDR). The core_enrichment column lists the "leading-edge" genes (Entrez IDs) that contribute most to the enrichment signal for each pathway and can be mapped back to gene symbols for follow-up interpretation.

After performing Hallmark GSEA, we further categorized significantly enriched pathways based on the direction of enrichment. Because the ranked gene list was ordered by decreasing t-statistics for the (Normal − Tumor) contrast, pathways with negative normalized enrichment scores (NES < 0) are enriched toward genes upregulated in ER+ tumors, whereas pathways with positive NES (NES > 0) are enriched toward genes upregulated in normal breast tissue. We therefore separated Hallmark pathways into tumor-enriched and normal-enriched groups and ranked them by adjusted p-values to highlight the most strongly associated biological programs.

```r
gsea_hallmark_res <- as.data.frame(gsea_hallmark)

# Tumor enriched (NES < 0)
tumor_top <- gsea_hallmark_res[gsea_hallmark_res$NES < 0, ]
tumor_top <- tumor_top[order(tumor_top$p.adjust), ]
head(tumor_top[, c("ID","NES","p.adjust")], 10)
```

```
##                                                                    ID       NES
## HALLMARK_E2F_TARGETS                                HALLMARK_E2F_TARGETS -3.214358
## HALLMARK_G2M_CHECKPOINT                          HALLMARK_G2M_CHECKPOINT -3.214010
## HALLMARK_MYC_TARGETS_V1                          HALLMARK_MYC_TARGETS_V1 -2.917584
## HALLMARK_MTORC1_SIGNALING                      HALLMARK_MTORC1_SIGNALING -2.483972
## HALLMARK_GLYCOLYSIS                                HALLMARK_GLYCOLYSIS -1.964081
## HALLMARK_ESTROGEN_RESPONSE_LATE       HALLMARK_ESTROGEN_RESPONSE_LATE -1.937153
## HALLMARK_OXIDATIVE_PHOSPHORYLATION HALLMARK_OXIDATIVE_PHOSPHORYLATION -1.878850
## HALLMARK_ESTROGEN_RESPONSE_EARLY     HALLMARK_ESTROGEN_RESPONSE_EARLY -1.837486
## HALLMARK_PROTEIN_SECRETION                 HALLMARK_PROTEIN_SECRETION -2.020742
## HALLMARK_UNFOLDED_PROTEIN_RESPONSE HALLMARK_UNFOLDED_PROTEIN_RESPONSE -1.850877
##                                       p.adjust
```

```
## HALLMARK_E2F_TARGETS               1.000000e-09
## HALLMARK_G2M_CHECKPOINT            1.000000e-09
## HALLMARK_MYC_TARGETS_V1            1.000000e-09
## HALLMARK_MTORC1_SIGNALING          1.000000e-09
## HALLMARK_GLYCOLYSIS                2.377188e-07
## HALLMARK_ESTROGEN_RESPONSE_LATE    3.777979e-07
## HALLMARK_OXIDATIVE_PHOSPHORYLATION 1.091702e-06
## HALLMARK_ESTROGEN_RESPONSE_EARLY   1.386510e-06
## HALLMARK_PROTEIN_SECRETION         1.476961e-05
## HALLMARK_UNFOLDED_PROTEIN_RESPONSE 7.145981e-05
```

```
# Normal enriched (NES > 0)
normal_top <- gsea_hallmark_res[gsea_hallmark_res$NES > 0, ]
normal_top <- normal_top[order(normal_top$p.adjust), ]
head(normal_top[, c("ID","NES","p.adjust")], 10)
```

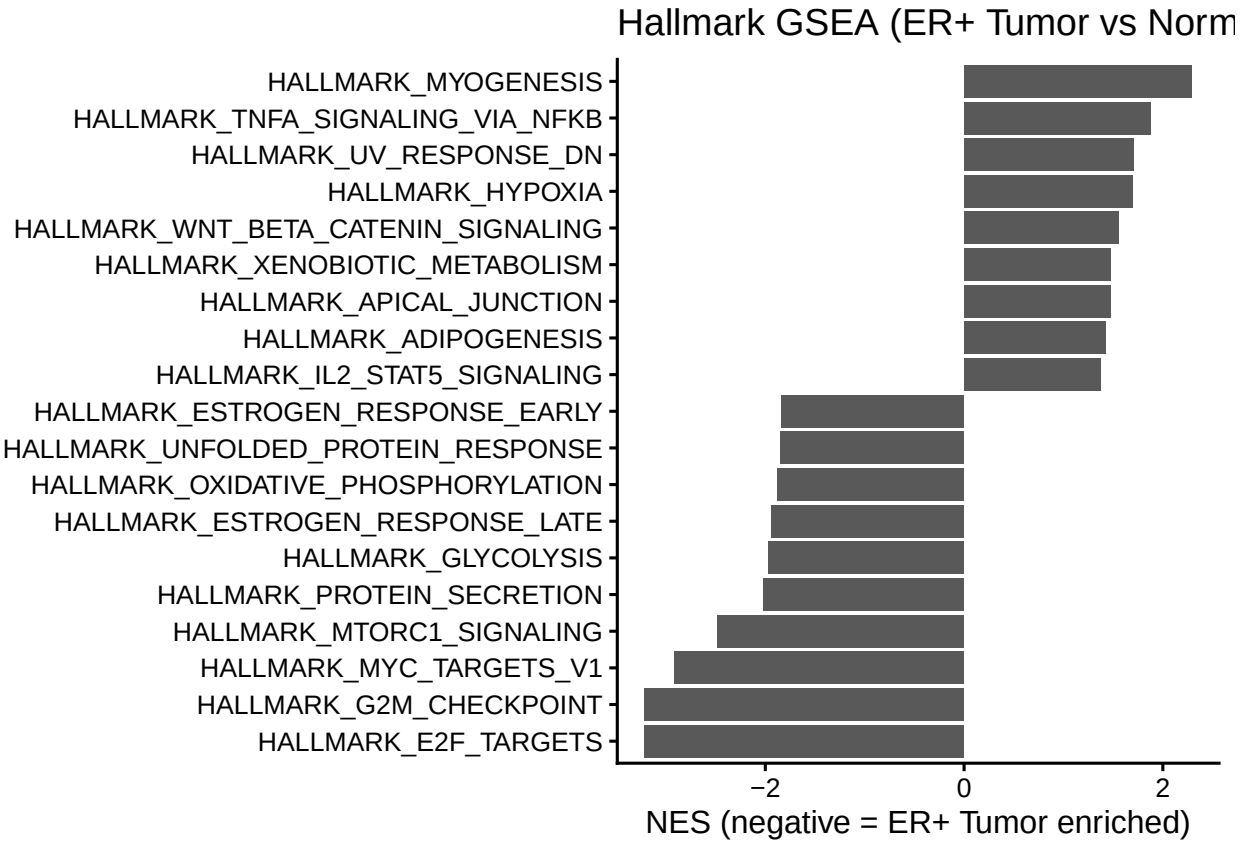
```
##                                                                      ID
## HALLMARK_MYOGENESIS                                 HALLMARK_MYOGENESIS
## HALLMARK_TNFA_SIGNALING_VIA_NFKB       HALLMARK_TNFA_SIGNALING_VIA_NFKB
## HALLMARK_HYPOXIA                                       HALLMARK_HYPOXIA
## HALLMARK_UV_RESPONSE_DN                         HALLMARK_UV_RESPONSE_DN
## HALLMARK_APICAL_JUNCTION                       HALLMARK_APICAL_JUNCTION
## HALLMARK_XENOBIOTIC_METABOLISM           HALLMARK_XENOBIOTIC_METABOLISM
## HALLMARK_ADIPOGENESIS                             HALLMARK_ADIPOGENESIS
## HALLMARK_WNT_BETA_CATENIN_SIGNALING HALLMARK_WNT_BETA_CATENIN_SIGNALING
## HALLMARK_IL2_STAT5_SIGNALING               HALLMARK_IL2_STAT5_SIGNALING
##                                         NES     p.adjust
## HALLMARK_MYOGENESIS                2.290217 1.000000e-09
## HALLMARK_TNFA_SIGNALING_VIA_NFKB   1.875169 4.913077e-06
## HALLMARK_HYPOXIA                   1.698323 3.493510e-04
## HALLMARK_UV_RESPONSE_DN            1.701392 1.072957e-03
## HALLMARK_APICAL_JUNCTION           1.477273 1.222806e-02
## HALLMARK_XENOBIOTIC_METABOLISM     1.477955 1.782964e-02
## HALLMARK_ADIPOGENESIS              1.421828 3.323623e-02
## HALLMARK_WNT_BETA_CATENIN_SIGNALING 1.554568 3.630698e-02
## HALLMARK_IL2_STAT5_SIGNALING       1.370017 3.630698e-02
```

To summarize and compare the most prominent biological programs associated with ER+ tumors and normal breast tissue, we visualized the top Hallmark gene sets identified by Gene Set Enrichment Analysis (GSEA).

Specifically, we selected the top enriched Hallmark pathways on each side-those with the most negative normalized enrichment scores (NES), indicating enrichment in ER+ tumor samples, and those with the most positive NES, indicating enrichment in normal tissue. These pathways were ranked by adjusted p-value, and the top ten from each group were displayed to highlight dominant, condition-specific transcriptional programs.

```
top_show <- rbind(
  head(tumor_top, 10),
  head(normal_top, 10)
)

top_show$ID <- factor(top_show$ID, levels = top_show$ID[order(top_show$NES)])

ggplot(top_show, aes(x = ID, y = NES)) +
  geom_col() +
  coord_flip() +
```

```
  labs(title = "Hallmark GSEA (ER+ Tumor vs Normal)",
       x = NULL, y = "NES (negative = ER+ Tumor enriched)") +
  theme_classic(base_size = 12)
```



The bar plot illustrates a clear functional contrast between ER+ tumors and normal breast tissue at the pathway level. Hallmark gene sets with strongly negative NES values are enriched in ER+ tumors, whereas those with positive NES values are enriched in normal samples.

ER+ tumors show strong enrichment of cell cycle–related and proliferative pathways, including G2M checkpoint, E2F targets, and MYC targets, indicating increased cell division and dysregulated growth control. In addition, pathways such as mTORC1 signaling, glycolysis, and oxidative phosphorylation suggest metabolic reprogramming that supports rapid proliferation and biosynthetic demand in tumor cells. The enrichment of estrogen response (early and late) pathways further reflects the hormone-driven nature of ER+ breast cancer.

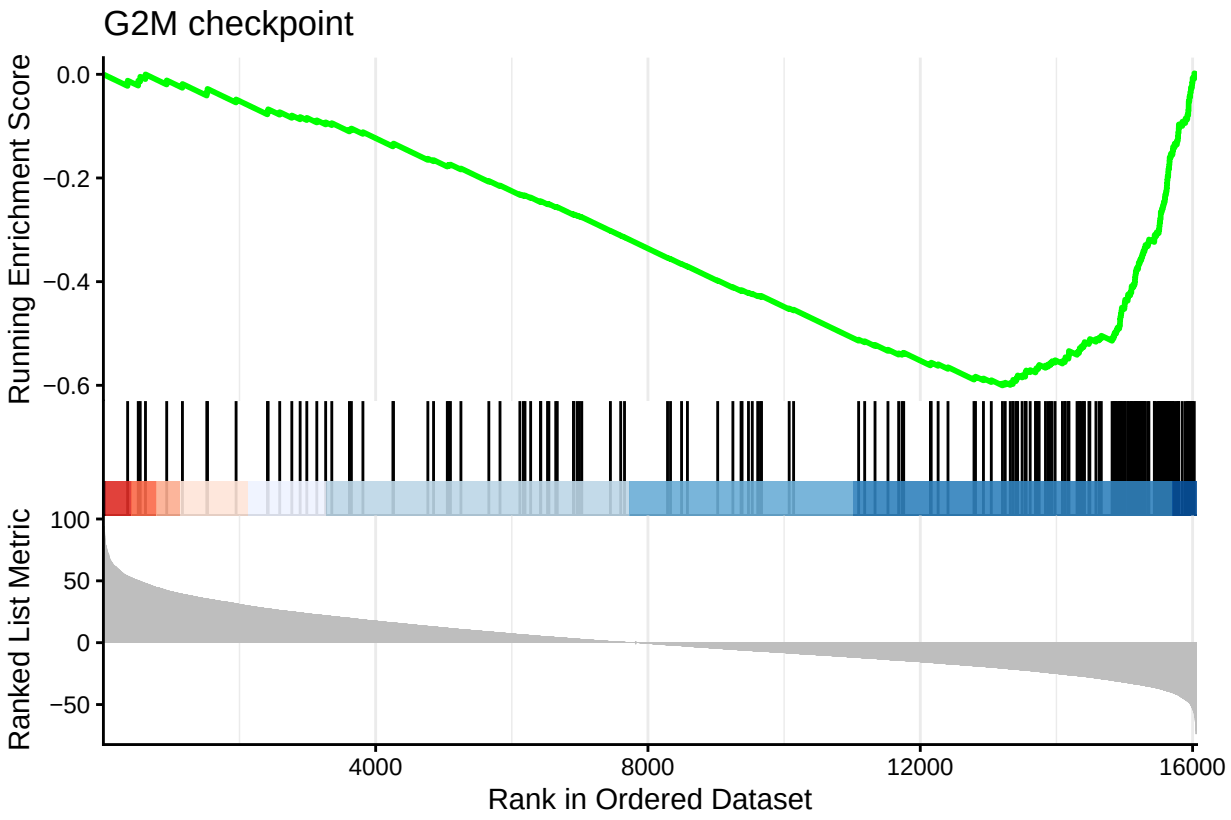
In contrast, normal breast tissue is enriched for pathways related to tissue structure and homeostasis, including myogenesis, apical junction, and adipogenesis, which are consistent with normal differentiation and cellular organization. Enrichment of TNF signaling via NF- B, hypoxia, and immune-related signaling suggests intact stress-response and immune surveillance mechanisms in non-tumor tissue.

Overall, these results demonstrate that ER+ tumors are characterized by heightened proliferative, metabolic, and hormone-responsive programs, while normal tissue exhibits pathways associated with structural integrity, differentiation, and physiological signaling. This pathway-level analysis complements the gene-level differential expression results and highlights coordinated biological processes underlying tumor–normal differences.

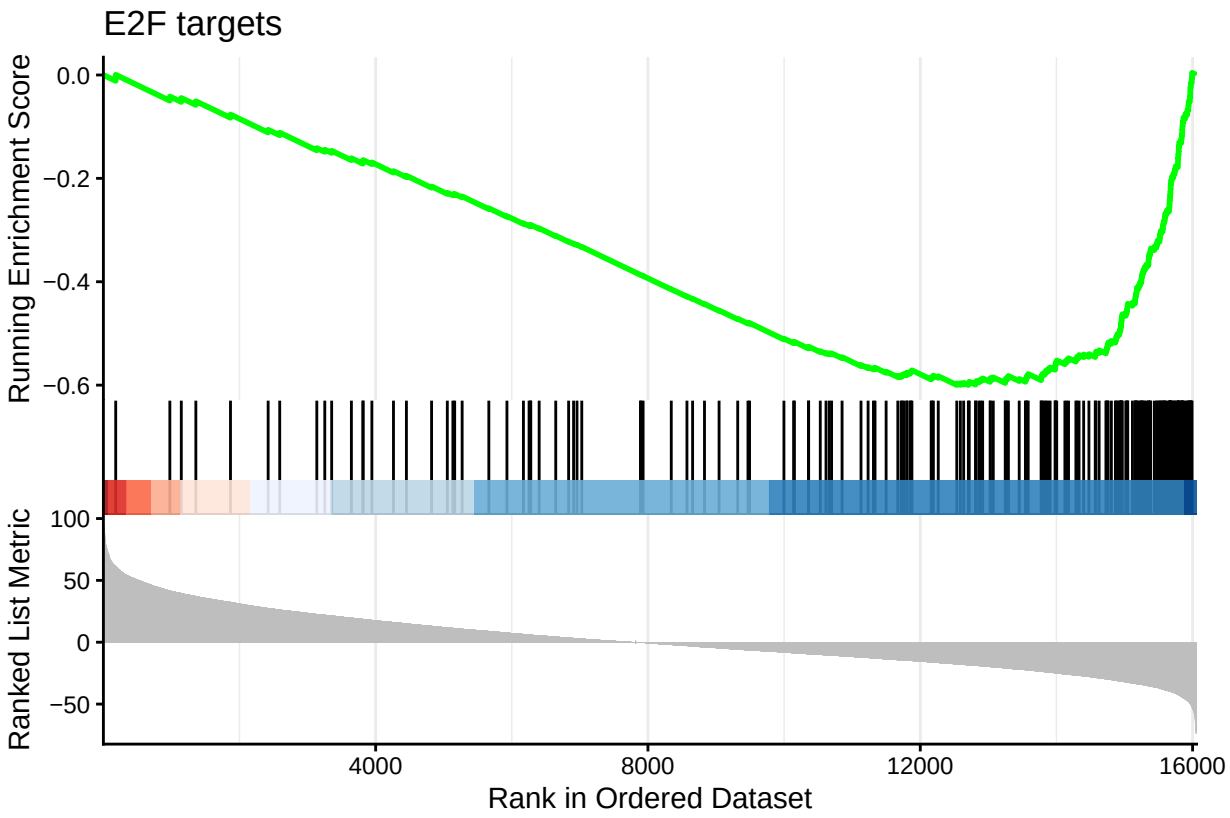
We also visualize each GSEA enrichment plot that shows how genes from a specific Hallmark gene set are distributed along the ranked gene list. The green curve represents the running enrichment score (ES), black vertical bars indicate the positions of genes from the gene set in the ranked list, and the bottom panel shows the ranking metric used to order genes. A negative normalized enrichment score (NES) indicates enrichment

toward the ER+ tumor end of the ranked list, while a positive NES indicates enrichment toward the normal tissue end.

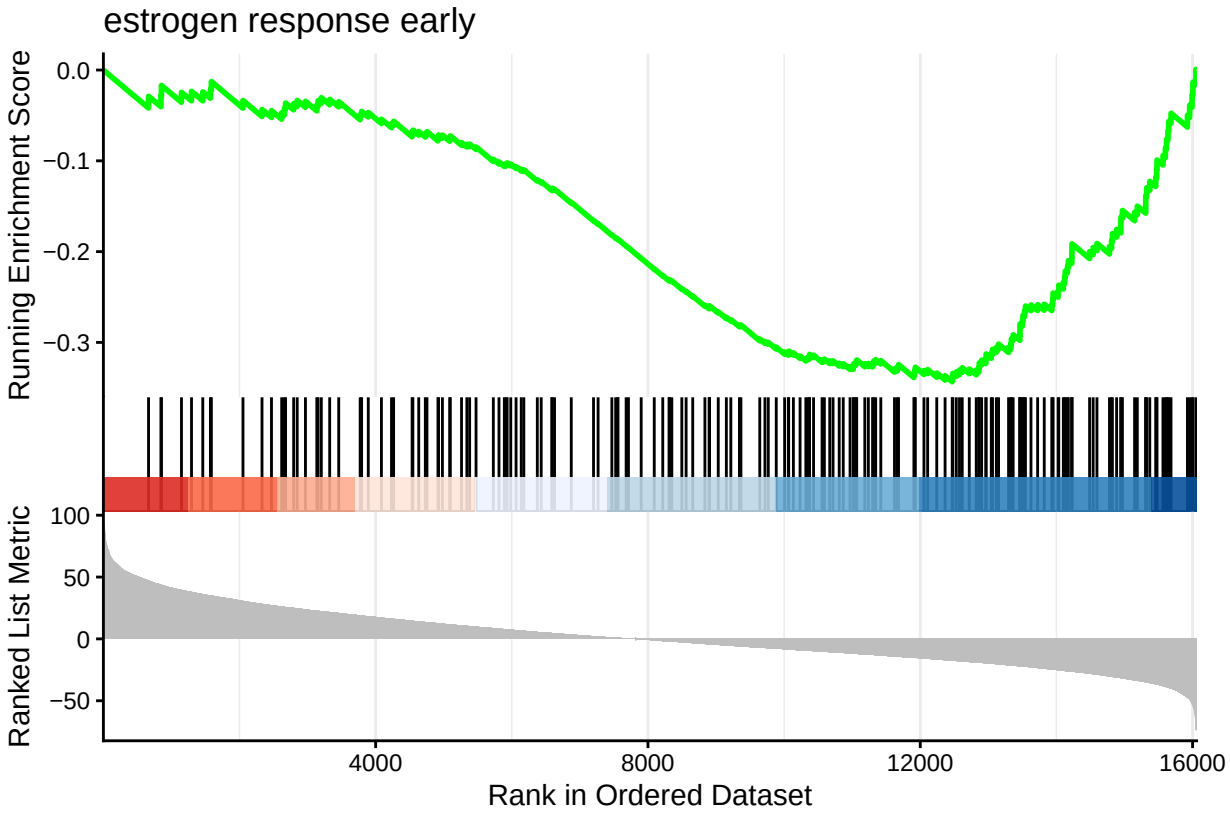
```
gseaplot2(gsea_hallmark, "HALLMARK_G2M_CHECKPOINT", title ="G2M checkpoint" )
```




```
gseaplot2(gsea_hallmark, "HALLMARK_E2F_TARGETS", title = "E2F targets")
```




```
gseaplot2(gsea_hallmark, "HALLMARK_ESTROGEN_RESPONSE_EARLY", title = "estrogen response early")
```



HALLMARK_G2M_CHECKPOINT The G2M checkpoint gene set shows strong negative enrichment (NES < 0), indicating that G2M-related genes are concentrated toward the ER+ tumor end of the ranked gene list. The running enrichment score reaches its most negative value near the tumor-associated genes, with a high density of gene set members appearing late in the ranked list.

Biologically, this suggests enhanced cell cycle progression and increased proliferative activity in ER+ tumor samples. Dysregulation of the G2/M checkpoint is a hallmark of cancer, reflecting uncontrolled cell division and genomic instability, which are consistent with aggressive tumor growth.

HALLMARK_E2F_TARGETS The E2F target gene set also exhibits strong negative enrichment, with most E2F-regulated genes clustered toward the ER+ tumor end of the ranked list. The smooth and sustained decrease in the running enrichment score indicates coordinated upregulation of E2F target genes in tumor samples.

E2F transcription factors play a central role in regulating genes required for DNA replication and cell cycle progression. Enrichment of E2F targets in ER+ tumors suggests activation of proliferative transcriptional programs, reinforcing the observation that tumor samples are characterized by heightened cell cycle activity.

HALLMARK_ESTROGEN_RESPONSE_EARLY The estrogen response early gene set shows significant negative enrichment, indicating that estrogen-responsive genes are preferentially upregulated in ER+ tumor samples. Gene set members are broadly distributed but skewed toward the tumor-associated portion of the ranked list, producing a negative enrichment score.

This pattern is biologically consistent with the estrogen receptor–positive (ER+) subtype of breast cancer, where estrogen signaling is a primary driver of tumor growth. Enrichment of early estrogen response genes reflects active hormone-dependent transcriptional regulation in ER+ tumors.

PANDA network analysis

Prepare expression datasets for PANDA network analysis

Current expression data is using version-stripped Ensembl gene names. To match gene names in the motif-TF and protein-protein interaction files, we need to remove the versions.

```
# Remove Ensembl gene name versions
rownames(logcpm_matrix) <- sub("\\..*$", "", rownames(logcpm_matrix))
```

We will construct separate networks for cancer (ER+) data from TCGA-BRCA and normal breast tissue data from GTEx. To achieve this goal, we outputed two expression datasets that can be used by PANDA analysis directly.

```
# Separate cancer (ER+) and normal sets (filtered & normalized)

n_tcga <- ncol(tcga_aligned)
n_gtex <- ncol(gtex_aligned)

sample_group <- factor(
  c(
    rep("Tumor",  n_tcga),
    rep("Normal", n_gtex)
  )
)

table(sample_group)
```

```
## sample_group
## Normal  Tumor
##    473    802
```

```r
expr_tumor <- logcpm_matrix[, sample_group == "Tumor"]
expr_normal <- logcpm_matrix[, sample_group == "Normal"]

dim(expr_tumor)
```

```
## [1] 20624   802
```

```r
dim(expr_normal)
```

```
## [1] 20624   473
```

```r
write.table(expr_tumor, file = "../output/tcga_brca_expr_ERpos_tumor_filtered_logcpm.txt",
  sep = "\t", quote = FALSE, row.names = TRUE, col.names = NA)

write.table(expr_normal, file = "../output/gtex_breast_expr_normal_filtered_logcpm.txt",
  sep = "\t", quote = FALSE, row.names = TRUE, col.names = NA)
```

Implement PANDA Analysis

To map how gene regulation shifts in ER+ breast cancer, we constructed two condition-specific PANDA regulatory networks: one from normal breast tissue and one from ER+ tumor tissue. PANDA integrates gene expression, transcription factor binding motifs, and protein–protein interaction information to infer regulatory edge weights between transcription factors and their target genes.

Below are the sample codes that we planed to run for PANDA network analysis. However, considering the computational cost of building huge PANDA networks, we ran PANDA in high-performance environment command line accessed from HUIT Open OnDemand platform.

Below is the R code for data preprocessing and running PANDA. Since running PANDA locally is very constricted by computation power, in the R version we selected the top 10,000 genes that explained the most variance. In the following regulatory network analysis, we used the output from the HUIT for completeness of dataset.

Below is the R code for preprocessing data for PANDA. Due to the constraint in computation power, we selected the top 10,000 genes that can explain the most variance. In the subsequent analysis, we used the output from the complete dataset run in HUIT.

```r
#install and load necessary packages

# install.packages("devtools")
# devtools::install_github("netZoo/netZooR", build_vignettes = FALSE)
# eNamespace("BiocManager", quietly = TRUE)
#    install.packages("BiocManager",repos = "http://cran.us.r-project.org")
# BiocManager::install("fgsea")
#install.packages("ggplot2")
#install.packages("reshape2")
#install.packages("visNetwork")

suppressPackageStartupMessages({
  library(devtools)
  library(tibble)
  library(netZooR)
  library(fgsea)
  library(ggplot2)
  library(reshape2)
  library('visNetwork')
  library(AnnotationDbi)
```

```
[yow386@general-dy-general-cr-1 data]$ netzoopy panda
o ~/output_panda_brca_ERpos_tumor_997TFs.txt
/shared/spack/opt/spack/linux-skylake_avx512/minicond
 is deprecated as an API. See https://setuptools.pypa
 pin to Setuptools<81.
  import pkg_resources
NEW: We changed the default behavior of save_tmp (now
NEW: We changed the default beavior of save_panda_res
Input data:
Expression: tcga_brca_expr_ERpos_tumor_filtered_logcp
Motif data: motif_997TFs_ensembl.txt
PPI data: ppi_997TFs.txt
Start Panda run ...
Loading motif data ...
  Elapsed time: 2.82 sec.
Loading expression data ...
  Elapsed time: 2.99 sec.
Loading PPI data ...
Number of PPIs: 138479
  Elapsed time: 0.05 sec.
Calculating coexpression network ...
  Elapsed time: 7.00 sec.
Creating motif network ...
  Elapsed time: 3.63 sec.
Creating PPI network ...
  Elapsed time: 0.12 sec.
intersection motif_997TFs_ensembl.txt tcga_brca_expr_
Normalizing networks ...
  Elapsed time: 32.39 sec.
Running PANDA algorithm ...
Computing panda on CPU
```

```
step: 15, hamming: 0.1161990837316757
step: 16, hamming: 0.0986867488497143
step: 17, hamming: 0.083543091623639
step: 18, hamming: 0.0705179301215489
step: 19, hamming: 0.059367503062477
step: 20, hamming: 0.0498614977632214
step: 21, hamming: 0.0417871778839678
step: 22, hamming: 0.034951417523332
step: 23, hamming: 0.0291812683577809
step: 24, hamming: 0.0243235517727128
step: 25, hamming: 0.0202438194855521
step: 26, hamming: 0.0168249636587138
step: 27, hamming: 0.0139656385066605
step: 28, hamming: 0.0115786256897337
step: 29, hamming: 0.0095892289474715
step: 30, hamming: 0.0079337490567832
step: 31, hamming: 0.006558070473734
step: 32, hamming: 0.0054163740374587
step: 33, hamming: 0.0044699829756315
step: 34, hamming: 0.0036863387948721
step: 35, hamming: 0.0030381013022489
step: 36, hamming: 0.0025023638540751
step: 37, hamming: 0.0020599737412093
step: 38, hamming: 0.0016949469139348
step: 39, hamming: 0.0013939665120736
step: 40, hamming: 0.0011459550897792
step: 41, hamming: 0.0009417111663320
Running panda took: 1093.77 seconds!
Saving PANDA network to /shared/home/yow386/output_pa
WARNING: panda is now saved with the column names.
Use old_compatible=True to save the panda results as
```

```
[yow386@general-dy-general-cr-1 data]$ netzoopy panda
/output_panda_brca_ERpos_tumor_997TFs.txt
/shared/spack/opt/spack/linux-skylake_avx512/minicond
 is deprecated as an API. See https://setuptools.pypa
 pin to Setuptools<81.
  import pkg_resources
NEW: We changed the default behavior of save_tmp (now
NEW: We changed the default beavior of save_panda_res
Input data:
Expression: gtex_breast_expr_normal_filtered_logcpm.t
Motif data: motif_997TFs_ensembl.txt
PPI data: ppi_997TFs.txt
Start Panda run ...
Loading motif data ...
  Elapsed time: 2.73 sec.
Loading expression data ...
  Elapsed time: 1.76 sec.
Loading PPI data ...
Number of PPIs: 138479
  Elapsed time: 0.05 sec.
Calculating coexpression network ...
  Elapsed time: 5.58 sec.
Creating motif network ...
  Elapsed time: 3.54 sec.
Creating PPI network ...
  Elapsed time: 0.12 sec.
intersection motif_997TFs_ensembl.txt gtex_breast_exp
Normalizing networks ...
  Elapsed time: 32.37 sec.
Running PANDA algorithm ...
Computing panda on CPU
```

```
step: 15, hamming: 0.0745591295450893 8
step: 16, hamming: 0.0631581053612048 9
step: 17, hamming: 0.0533418187598295 8
step: 18, hamming: 0.0449303421609152 45
step: 19, hamming: 0.0377531399093172 3
step: 20, hamming: 0.0316521849572381 4
step: 21, hamming: 0.0264835743025606 36
step: 22, hamming: 0.0221180889345548 44
step: 23, hamming: 0.0184410093481340 85
step: 24, hamming: 0.0153514392231111 96
step: 25, hamming: 0.0127613409461475 83
step: 26, hamming: 0.0105944104299321 35
step: 27, hamming: 0.0087848964847614 73
step: 28, hamming: 0.007276426165111 4
step: 29, hamming: 0.0060208814808093 74
step: 30, hamming: 0.0049773512547439 95
step: 31, hamming: 0.0041111732652126 51
step: 32, hamming: 0.0033930718025750 99
step: 33, hamming: 0.0027983902839192 284
step: 34, hamming: 0.0023064154665057 55
step: 35, hamming: 0.0018997869948302 664
step: 36, hamming: 0.0015639852292183 864
step: 37, hamming: 0.0012868896264607 561
step: 38, hamming: 0.0010583999876345 803
step: 39, hamming: 0.0008701130752697 307
Running panda took: 1038.57 seconds!
Saving PANDA network to /shared/home/yow386/output_par
WARNING: panda is now saved with the column names.
Use old_compatible=True to save the panda results as p
  Elapsed time: 57.00 sec.
```


Figure 4: PANDA Analyis for Normal Sampled Implemented on HUIT OnDemand Command (Continued)

```
  library(org.Hs.eg.db)
})
```

```
# load ppi and motif data
ppi <- read.table("../data/ppi_997TFs.txt")
motif <- read.table("../data/motif_997TFs_ensembl.txt")

expr_normal <- as.data.frame(expr_normal)
expr_tumor <- as.data.frame(expr_tumor)

#select the top genes with most variance
gene_vars_normal <- apply(expr_normal, 1, var)
gene_vars_tumor <- apply(expr_tumor, 1, var)
n_genes <- 10000

top_genes_normal <- names(sort(gene_vars_normal, decreasing = TRUE))[1:n_genes]
top_genes_tumor <- names(sort(gene_vars_tumor, decreasing = TRUE))[1:n_genes]

expr_normal_top <- expr_normal[top_genes_normal, ]
expr_tumor_top <- expr_tumor[top_genes_tumor, ]
```

```
#mapping ensemble names to gene names
ens_ids_normal <- rownames(expr_normal_top)

mapping_normal <- mapIds(org.Hs.eg.db,
                  keys = ens_ids_normal,
                  column = "SYMBOL",
                  keytype = "ENSEMBL",
                  multiVals = "first")

expr_normal_top$Symbol <- mapping_normal

expr_clean_normal <- expr_normal_top[!is.na(expr_normal_top$Symbol), ]

expr_clean_normal <- expr_clean_normal[!duplicated(expr_clean_normal$Symbol), ]

rownames(expr_clean_normal) <- expr_clean_normal$Symbol
expr_clean_normal$Symbol <- NULL

ens_ids_tumor <- rownames(expr_tumor_top)

mapping_tumor <- mapIds(org.Hs.eg.db,
                  keys = ens_ids_tumor,
                  column = "SYMBOL",
                  keytype = "ENSEMBL",
                  multiVals = "first")

expr_tumor_top$Symbol <- mapping_tumor

expr_clean_tumor <- expr_tumor_top[!is.na(expr_tumor_top$Symbol), ]

expr_clean_tumor <- expr_clean_tumor[!duplicated(expr_clean_tumor$Symbol), ]

rownames(expr_clean_tumor) <- expr_clean_tumor$Symbol
```

```r
expr_clean_tumor$Symbol <- NULL
```

```r
#select TFs of interest in motifs and ppis
genes_use <- intersect(
  rownames(expr_clean_normal),
  rownames(expr_clean_tumor)
)


motif$gene_symbol <- mapIds(
  org.Hs.eg.db,
  keys = motif$V2,
  keytype = "ENSEMBL",
  column = "SYMBOL",
  multiVals = "first"
)

motif_sym <- motif[!is.na(motif$gene_symbol), ]
motif_sym <- motif_sym[, c("V1", "gene_symbol", "V3")]

motif_use_normal <- motif[
  motif$gene_symbol %in% genes_use &
  motif$V1 %in% rownames(expr_clean_normal),
]

motif_use_normal <- data.frame(
  tf     = motif_use_normal$V1,
  gene   = motif_use_normal$gene_symbol,  # ← SYMBOL
  weight = motif_use_normal$V3
)

tfs_use_normal <- unique(motif_use_normal$tf)

ppi_use_normal <- ppi[
  ppi$V1 %in% tfs_use_normal &
  ppi$V2 %in% tfs_use_normal,
]

motif_use_tumor <- motif[
  motif$gene_symbol %in% genes_use &
  motif$V1 %in% rownames(expr_clean_tumor),
]

motif_use_tumor <- data.frame(
  tf     = motif_use_tumor$V1,
  gene   = motif_use_tumor$gene_symbol,  # ← SYMBOL
  weight = motif_use_tumor$V3
)

tfs_use_tumor <- unique(motif_use_tumor$tf)

ppi_use_tumor <- ppi[
  ppi$V1 %in% tfs_use_tumor &
  ppi$V2 %in% tfs_use_tumor,
```

```
]
```

```
#pandaNormal <- panda(motif_use_normal, expr_clean_normal, ppi_use_normal, mode="intersection")
#pandaTumor <- panda(motif_use_tumor, expr_clean_tumor, ppi_use_tumor, mode="intersection")
```

Visualizing PANDA output networks through Cytoscape

Then, we want to visualize the output networks through Cytoscape for more information. We would combine the two output tables and select top differential edges.

By subtracting the normal network from the tumor network, we computed a differential force for each TF–gene interaction, which represents how much regulatory influence is gained or lost in the tumor state. Positive values indicate interactions that are strengthened or newly gained in tumors, while negative values represent regulatory relationships that are weakened or lost.

To focus on the most biologically meaningful changes and reduce background noise, we restricted the visualization to the top 1,500 differential edges with the largest absolute force differences.

For current network visualization constructed with 1500 top differential edges, there are only 5 edges with negative edge weight differences. Hence, The strongest regulatory rewiring in ER+ BRCA is dominated by gained tumor-specific TF–gene interactions while loss of regulation is present but occurs at lower magnitude and is therefore underrepresented among the highest-ranked edges.

```
# Load output data
panda_tumor  <- read.delim("../output/output_panda_brca_ERpos_tumor_997TFs.txt",
                           stringsAsFactors = FALSE, sep = "")

panda_normal <- read.delim("../output/output_panda_breast_normal_997TFs.txt",
                           stringsAsFactors = FALSE, sep = "")

colnames(panda_tumor)  <- c("TF", "Gene", "Motif", "Force_tumor")
colnames(panda_normal) <- c("TF", "Gene", "Motif", "Force_normal")


# Merge the two output network data by TFs and genes
panda_merged <- inner_join(
  panda_tumor[, c("TF", "Gene", "Motif", "Force_tumor")],
  panda_normal[, c("TF", "Gene", "Force_normal")],
  by = c("TF", "Gene")
)
dim(panda_merged)
```

```
## [1] 20407593        5
```

```
# Convert motif to 0/1 (currently 0.0/1.0)
panda_merged$Motif <- as.integer(panda_merged$Motif)

# Compute the force differences between tumor and normal networks for each TF-gene pair
panda_merged <- panda_merged %>%
  mutate(Diff_Force = Force_tumor - Force_normal)


# Keep top 1500 differential force edges for clearer visualization and include some edges with negative
top_n <- 1500

panda_diff <- panda_merged %>%
  arrange(desc(abs(Diff_Force))) %>%
```

```
  slice(1:top_n)

sum(panda_diff$Diff_Force < 0, na.rm = TRUE)
```

```
## [1] 5
```

```
# Export the data for Cytoscape input
write.table(panda_diff, file = "../output/panda_ERpos_tumor_vs_normal_edges.txt", sep = "\t", quote = F
```

Here is the visualization generated by Cytoscape:

In this image, we represented TFs by yellow triangles and genes by blue ellipse. The size of the nodes depended on their degrees (the connection counts). The edges are red for positive differences between the regulatory weights between ER+ tumor and normal samples and green for negative differences. The width of the edges also showed the magnitude of the differences.

At the global level, this network exhibits a highly centralized, hub-dominated structure. One of the most interesting features is the overwhelming dominance of red edges, indicating that the majority of large-magnitude changes correspond to gains of regulatory interactions in the tumor. This suggests that ER+ tumors are characterized not simply by the loss of normal regulation, but by the acquisition of extensive new regulatory programs.

Rather than being evenly distributed across transcription factors, these gains are concentrated in a small subset of regulators. When we zoom into the network, several transcription factors emerge as clear "master hubs," most notably ZNF235 and ZNF287, which acquire hundreds of new target genes in the tumor network. Other zinc finger transcription factors, including ZNF418, ZNF487, ZNF879, and ZFP28, also show extensive rewiring.

This pattern indicates that tumor-associated regulatory changes are highly structured. A limited number of transcription factors account for a disproportionate fraction of the regulatory rewiring observed in the expression data. In other words, the tumor network is not randomly altered, but reorganized around a small set of dominant regulators.

In this visualization, the large number of edges and nodes (317) result in a network with substantial overlapping. To simplify this, we construct separate network images for gained and lost regulation by selecting the strongest positive and negative differential TF–gene interactions between ER+ breast tumors and normal breast tissue.

Separate Visualization for Gained and Lost Regulartory Interactions

We would choose the top 500 positive and top 100 negative differential TF-gene egdes. Equalizing the number of positive and negative edges would require including weaker regulatory losses while excluding stronger gains, which would bias the network away from the most biologically significant interactions.

```
n_pos <- 500
n_neg <- 100

panda_pos <- panda_merged %>%
  filter(Diff_Force > 0) %>%
  arrange(desc(Diff_Force)) %>%
  slice_head(n = n_pos)

write.table(panda_pos, file = "../output/panda_ERpos_tumor_vs_normal_toppos_edges.txt",
            sep = "\t", row.names = FALSE, quote = FALSE)

panda_neg <- panda_merged %>%
  filter(Diff_Force < 0) %>%
  arrange(Diff_Force) %>%
```

```
  slice_head(n = n_neg)

write.table(panda_neg, file = "../output/panda_ERpos_tumor_vs_normal_topneg_edges.txt",
            sep = "\t", row.names = FALSE, quote = FALSE)
```

Here, we show the Top 500 positive differential edges, representing regulatory interactions that are significantly strengthened or newly formed in ER+ tumors. This network highlights the regulatory architecture that the tumor actively builds to drive proliferation. Similar to the global network, this gain network is dominated by a small number of transcription factor hubs, most notably ZNF235, ZNF287, ZNF418, and PRDM6. These TFs acquire large numbers of new targets, suggesting that they play central roles in establishing tumor-specific transcriptional programs.

Many target genes are co-regulated by multiple hub transcription factors, producing a dense, highly interconnected structure. This suggests coordinated regulatory control rather than independent, one-off TF–gene interactions. From a biological perspective, such coordination is consistent with processes like sustained proliferation, altered differentiation, and large-scale transcriptional reprogramming that are hallmarks of tumor progression.

Now, we show the Top 100 negative differential edges, representing regulatory interactions that were strong in normal tissue but are weakened or lost in the tumor. In contrast to the gain network, this loss network is noticeably smaller and more fragmented. Fewer transcription factors dominate, and the overall connectivity is reduced. This asymmetry suggests that ER+ tumor progression is driven more by the acquisition of new regulatory relationships than by the wholesale loss of normal regulation.

Biologically, this pattern supports a model in which tumor cells do not simply shut down normal gene regulation, but instead overlay new regulatory programs on top of an existing framework. The loss of specific regulatory edges may reflect suppression of normal differentiation, tissue maintenance, or growth-control pathways, while the gains reflect active oncogenic rewiring.

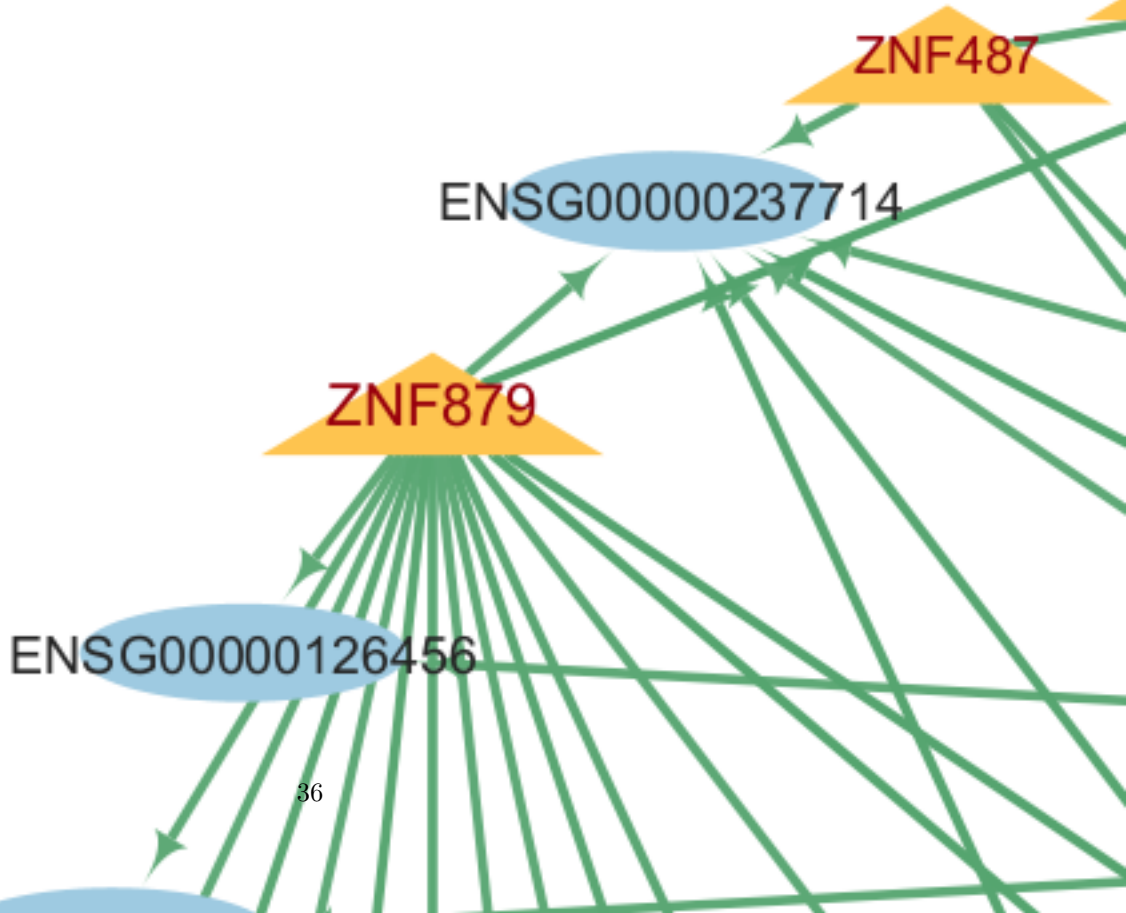
While these transcription factors are visually dominant due to their large number of connections, edge count alone does not tell us what biological processes are being affected. A transcription factor gaining hundreds of targets could be regulating many unrelated genes, or it could be coherently driving specific pathways. Therefore, to move beyond network topology and assess biological relevance, we next examined whether the targets of these gained and lost regulatory interactions are enriched for specific functional pathways and biological processes.

Identifying top TFs from PANDA Analysis

To quantitatively assess transcription factor–level regulatory rewiring, we performed a paired t-test for each of the 997 transcription factors in the PANDA network, comparing regulatory edge weights between ER+ tumor and normal tissue across all target genes for each TF. This paired framework accounts for within-TF variability and tests whether the overall regulatory influence of a TF differs systematically between conditions.

To prioritize biologically meaningful regulators, we ranked transcription factors by their absolute t-statistics rather than by mean differential force alone. This ranking strategy is more robust than relying solely on average changes, as it emphasizes transcription factors whose regulatory shifts are both statistically significant and consistent across their target profiles, while down-weighting TFs driven by a small number of extreme edges or high variability.

```
# Define the function for the Paired T-test:
# The null hypothesis (H0) is: Mean(Force_tumor - Force_normal) = 0
# Rejecting H0 means the TF's regulatory influence has significantly changed.

run_t_test_for_tf <- function(tf_name, data = panda_merged) {
  tf_data <- data %>% filter(TF == tf_name)
```


ZNF487
ENSG00000237714
ZNF879
ENSG00000126456

```
  if (nrow(tf_data) < 2) {
    return(data.frame(TF = tf_name, P_Value = NA, T_Statistic = NA, stringsAsFactors = FALSE))
  }

  test_result <- t.test(tf_data$Force_tumor, tf_data$Force_normal, paired = TRUE)

  return(data.frame(
    TF = tf_name,
    P_Value = test_result$p.value,
    T_Statistic = test_result$statistic,
    Mean_Diff_Force = mean(tf_data$Diff_Force, na.rm = TRUE),
    stringsAsFactors = FALSE
  ))
}


TF_full_summary <- panda_merged %>%
  group_by(TF) %>%
  summarise(
    Mean_Diff_Force = mean(Diff_Force, na.rm = TRUE),
    Sum_Abs_Diff = sum(abs(Diff_Force), na.rm = TRUE),
    Target_Count = n()
  ) %>%
  ungroup()

# Perform the paired t-test on all 997 TFs
all_tfs <- TF_full_summary$TF

print(paste("Starting T-tests for", length(all_tfs), "TFs..."))
```

```
## [1] "Starting T-tests for 997 TFs..."
```

```
t_test_results <- bind_rows(lapply(all_tfs, run_t_test_for_tf))
print("T-tests complete.")
```

```
## [1] "T-tests complete."
```

```
# Rank the results by absolute t-statistics
TF_differential_results <- TF_full_summary %>%
  left_join(
    t_test_results %>% dplyr::select(-Mean_Diff_Force),
    by = "TF"
  ) %>%
  filter(!is.na(P_Value)) %>%
  mutate(
    P_Adj = p.adjust(P_Value, method = "BH"),
    Abs_T_Stat = abs(T_Statistic)
  )


FDR_threshold <- 0.05
top_n_to_show <- 20

Final_TF_List <- TF_differential_results %>%
  filter(P_Adj < FDR_threshold) %>%
```

```
  mutate(
    Direction = case_when(
      Mean_Diff_Force > 0 ~ "GAIN_in_Tumor",
      Mean_Diff_Force < 0 ~ "LOSS_in_Tumor",
      TRUE ~ "Balanced"
    )
  ) %>%
  arrange(desc(Abs_T_Stat)) %>%
  head(top_n_to_show) %>%
  dplyr::select(
    TF,
    Direction,
    Mean_Diff_Force,
    Sum_Abs_Diff,
    Target_Count,
    T_Statistic,
    P_Adj
  )

print(paste(
  "Found",
  nrow(filter(TF_differential_results, P_Adj < FDR_threshold)),
  "significantly differentially targeting TFs (FDR <", FDR_threshold, ")"
))
```

```
## [1] "Found 941 significantly differentially targeting TFs (FDR < 0.05 )"
```

```
print("--- Top 20 TFs Ranked by |T-Statistic| ---")
```

```
## [1] "--- Top 20 TFs Ranked by |T-Statistic| ---"
```

```
knitr::kable(
  Final_TF_List,
  caption = "Top 20 TFs ranked by absolute t-statistic"
)
```

Table 1: Top 20 TFs ranked by absolute t-statistic

TF	Direction	Mean_Diff_Force	Sum_Abs_Diff	Target_Count	T_Statistic	P_Adj
NFYA	LOSS_in_Tumor	-0.2156063	6655.685	20469	-95.70519	0
ZNF565	GAIN_in_Tumor	0.1921460	6335.663	20469	84.87049	0
RFX4	LOSS_in_Tumor	-0.2099665	7941.561	20469	-74.04092	0
ZNF76	LOSS_in_Tumor	-0.1741535	6697.110	20469	-71.77570	0
ZNF282	GAIN_in_Tumor	0.2258337	8649.097	20469	71.41158	0
RFX3	LOSS_in_Tumor	-0.1432716	5678.508	20469	-68.68943	0
NFYB	LOSS_in_Tumor	-0.1567035	6379.545	20469	-64.59448	0
PBX3	LOSS_in_Tumor	-0.2108703	8848.354	20469	-64.52997	0
HSF2	GAIN_in_Tumor	0.1974530	8025.850	20469	63.97523	0
ESRRG	GAIN_in_Tumor	0.1655107	7743.629	20469	56.87692	0
FOXB1	GAIN_in_Tumor	0.2170240	9872.333	20469	56.80521	0
SREBF1	LOSS_in_Tumor	-0.1791768	8659.365	20469	-56.66359	0
RBPJ	GAIN_in_Tumor	0.1739672	8054.406	20469	55.61785	0
TFAP4	GAIN_in_Tumor	0.2334329	11158.219	20469	55.45810	0
ESRRB	GAIN_in_Tumor	0.1180773	5529.669	20469	55.43764	0
TBX20	GAIN_in_Tumor	0.1598025	7478.429	20469	55.35722	0

TF	Direction	Mean_Diff_Force	Sum_Abs_Diff	Target_Count	T_Statistic	P_Adj
HNF4A	GAIN_in_Tumor	0.1888530	8791.356	20469	54.99789	0
NFE2L1	GAIN_in_Tumor	0.1799372	8683.094	20469	54.95653	0
MBNL2	GAIN_in_Tumor	0.2079415	10198.464	20469	53.87090	0
MAFB	GAIN_in_Tumor	0.2366929	11714.047	20469	52.93578	0

Among the top-ranked transcription factors, MAFB, TFAP4, and MBNL2 emerged as particularly interesting These TFs exhibited not only high absolute t-statistics, indicating uniform and statistically reliable differential targeting, but also the largest sums of absolute differential forces, reflecting a substantial overall impact on the regulatory network. This concordance between statistical significance and network-level effect size suggests that these transcription factors act as major drivers of regulatory reprogramming in ER+ tumors.

Notably, TFAP4 has been previously implicated in breast cancer progression, where it promotes tumor growth, cell migration, and invasion and is often regulated alongside renown c-MYC. Its emergence as a top-ranked transcription factor in this analysis provides independent, network-based support for its role as a key oncogenic regulator in ER+ breast cancer.

Finally, to connect these transcription factor–level findings to downstream biological consequences, we extracted the top 100 target genes for each of these highly ranked transcription factors. These target sets were used for subsequent functional and pathway analyses to identify the biological processes most strongly influenced by tumor-specific regulatory rewiring.

```
# TFs chosen for final module analysis (Ranked by reliable T-Stat)
TFs_for_final_modules <- c("TFAP4", "MAFB", "MBNL2")

Final_Gene_Modules <- list()

for (tf in TFs_for_final_modules) {

  module_data <- panda_merged %>%
    filter(TF == tf) %>%
    arrange(desc(abs(Diff_Force)))

  Final_Gene_Modules[[tf]] <- module_data %>%
    head(100) %>%
    dplyr::select(TF, Gene, Diff_Force)

  print(paste("Extracted top 100 differentially rewired targets for:", tf))
}
```

```
## [1] "Extracted top 100 differentially rewired targets for: TFAP4"
## [1] "Extracted top 100 differentially rewired targets for: MAFB"
## [1] "Extracted top 100 differentially rewired targets for: MBNL2"
```

```
Final_Gene_Symbol_Lists <- lapply(Final_Gene_Modules, function(df) unique(df$Gene))
```

Afterwards, we did the regulatory network analysis to have a high-level understanding of the impact of gene changes on pathways in cells.

```
# preprocess data to pass the condition in calcDegree
panda_tumor_df <- as.data.frame(panda_tumor, stringsAsFactors = FALSE)
panda_normal_df <- as.data.frame(panda_normal, stringsAsFactors = FALSE)

panda_tumor_df$Force_tumor <- as.numeric(panda_tumor_df$Force_tumor)
panda_normal_df$Force_normal <- as.numeric(panda_normal_df$Force_normal)
```

```
mat_tumor <- xtabs(
  Force_tumor ~ TF + Gene,
  data = panda_tumor_df
)

mat_normal <- xtabs(
  Force_normal ~ TF + Gene,
  data = panda_normal_df
)

mat_tumor <- as.matrix(mat_tumor)
mat_normal <- as.matrix(mat_normal)
attr(mat_tumor, "class") <- "matrix"
attr(mat_normal, "class") <- "matrix"

# in degree, out degree and difference in degree
tf_out_tumor  <- calcDegree(mat_tumor,  type = "tf")
tf_out_normal <- calcDegree(mat_normal, type = "tf")

gene_in_tumor  <- calcDegree(mat_tumor,  type = "gene")
gene_in_normal <- calcDegree(mat_normal, type = "gene")

gene_in_diff <- calcDegreeDifference(
  mat_tumor,
  mat_normal,
  type = "gene"
)

head(gene_in_diff)
```

```
## ENSG00000000003 ENSG00000000005 ENSG00000000419 ENSG00000000457 ENSG00000000460
##      -14.316040       -6.028988      225.996548      251.549323      153.204478
## ENSG00000000938
##      -90.516532
```

```
# match Ensembl id with gene name
library(org.Hs.eg.db)

gene_symbols <- mapIds(
  org.Hs.eg.db,
  keys = names(gene_in_diff),
  column = "SYMBOL",
  keytype = "ENSEMBL",
  multiVals = "first"
)

gene_in_diff_sym <- gene_in_diff
names(gene_in_diff_sym) <- gene_symbols

#delete entries with unknown gene names and repetitions
gene_in_diff_sym <- gene_in_diff_sym[!is.na(names(gene_in_diff_sym))]
gene_in_diff_sym <- sort(gene_in_diff_sym, decreasing = TRUE)
```

```
df <- data.frame(gene = names(gene_in_diff_sym),
                 score = as.numeric(gene_in_diff_sym),
                 stringsAsFactors = FALSE)

df_unique <- df %>% group_by(gene) %>% summarise(score = score[which.max(abs(score))], .groups="drop")

gene_stats <- df_unique$score
names(gene_stats) <- df_unique$gene
gene_stats <- sort(gene_stats, decreasing = TRUE)
```

We used fgseaMultilevel because it provides more accurate and stable p-value estimates for strongly enriched pathways compared to permutation-based fgsea. By using an adaptive multilevel Monte Carlo approach, fgseaMultilevel avoids the limitations of fixed permutations, particularly when enrichment signals are strong. This makes it more reliable for pathway inference in our network-based GSEA analysis.

```
#actual fgsea analysis
system("curl -O  https://netzoo.s3.us-east-2.amazonaws.com/netZooR/tutorial_datasets/c2.cp.kegg.v7.0.sy
pathways <- gmtPathways("./c2.cp.kegg.v7.0.symbols.gmt")

fgseaMulti <- fgseaMultilevel(pathways, gene_stats,  minSize=15, maxSize=500)

head(fgseaMulti)
```

```
##                                           pathway       pval      padj
##                                            <char>      <num>     <num>
## 1:                          KEGG_ABC_TRANSPORTERS 0.28192771 0.5346905
## 2:                     KEGG_ACUTE_MYELOID_LEUKEMIA 0.66835443 0.8965730
## 3:                          KEGG_ADHERENS_JUNCTION 0.39800995 0.6701188
## 4:            KEGG_ADIPOCYTOKINE_SIGNALING_PATHWAY 0.74295191 0.9502873
## 5: KEGG_ALANINE_ASPARTATE_AND_GLUTAMATE_METABOLISM 0.06341463 0.2552052
## 6:  KEGG_ALDOSTERONE_REGULATED_SODIUM_REABSORPTION 0.31735537 0.5818182
##       log2err         ES        NES  size  leadingEdge
##         <num>      <num>      <num> <int>       <list>
## 1: 0.12563992 -0.2777497 -1.0965625    37 ABCD4, T....
## 2: 0.07627972 -0.2067011 -0.8881328    56 PML, TCF....
## 3: 0.08130273  0.2464453  1.0332797    70 RHOA, PT....
## 4: 0.05070279  0.2070216  0.8452452    60 IRS1, PR....
## 5: 0.22798720  0.4278549  1.4401695    25 GLUL, GF....
## 6: 0.09374654  0.3042102  1.0991939    34 IRS1, PI....
```

```
# Subset to pathways with FDR < 0.05
sig_multi <- fgseaMulti[fgseaMulti$padj < 0.05,]
# Get the top 10 significant pathways enriched for genes having lower targeting in LCLs
sig_multi[order(sig_multi$NES)[1:10],]
```

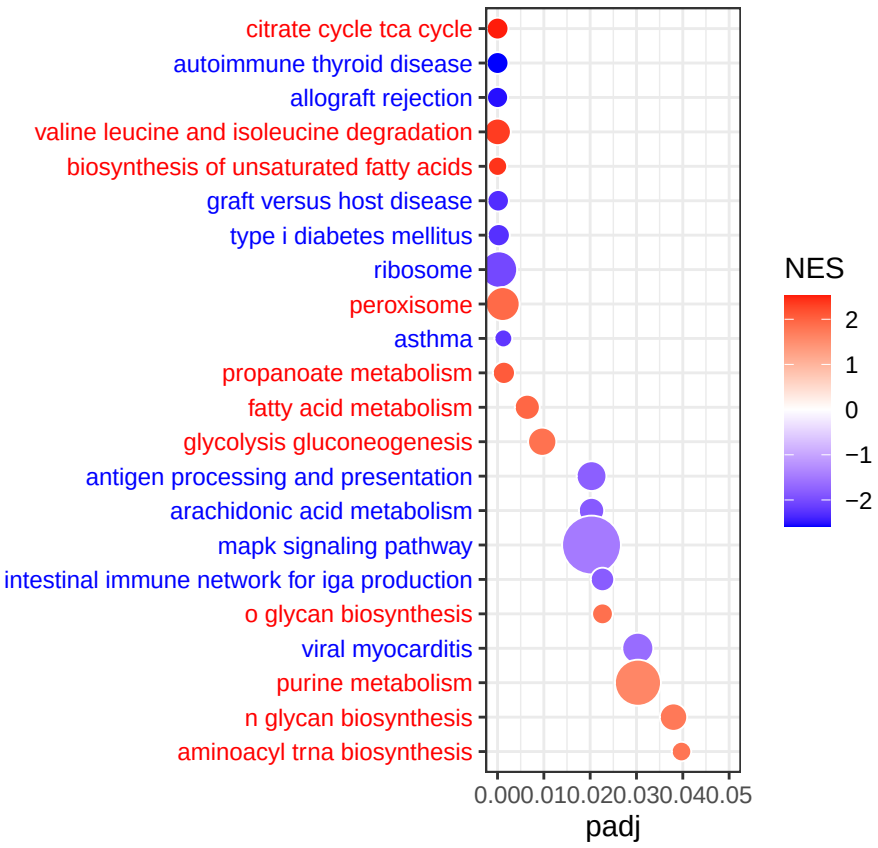
```
##                                             pathway         pval         padj
##                                              <char>        <num>        <num>
##  1:                   KEGG_AUTOIMMUNE_THYROID_DISEASE 5.763759e-08 4.755101e-06
##  2:                         KEGG_ALLOGRAFT_REJECTION 1.067100e-07 5.869047e-06
##  3:                    KEGG_GRAFT_VERSUS_HOST_DISEASE 5.063082e-06 1.392348e-04
##  4:                     KEGG_TYPE_I_DIABETES_MELLITUS 1.124246e-05 2.650008e-04
##  5:                                     KEGG_ASTHMA 7.597895e-05 1.253653e-03
##  6:                                   KEGG_RIBOSOME 1.480209e-05 3.052932e-04
##  7:                  KEGG_ARACHIDONIC_ACID_METABOLISM 1.931914e-03 2.029320e-02
##  8: KEGG_INTESTINAL_IMMUNE_NETWORK_FOR_IGA_PRODUCTION 2.430665e-03 2.265872e-02
```

```
##  9:          KEGG_ANTIGEN_PROCESSING_AND_PRESENTATION 1.967825e-03 2.029320e-02
## 10:                              KEGG_VIRAL_MYOCARDITIS 3.486740e-03 3.027959e-02
##       log2err         ES       NES  size  leadingEdge
##         <num>      <num>     <num> <int>       <list>
##  1: 0.7195128 -0.7136317 -2.575480    28 HLA-E, H....
##  2: 0.7049757 -0.7107184 -2.511680    26 HLA-E, H....
##  3: 0.6105269 -0.6437738 -2.304902    27 HLA-E, H....
##  4: 0.5933255 -0.6222835 -2.273641    29 HLA-E, H....
##  5: 0.5384341 -0.6878345 -2.213256    18 PRG2, HL....
##  6: 0.5933255 -0.4359369 -2.032693    84 RPL36A, ....
##  7: 0.4550599 -0.4643355 -1.837596    39 CYP2E1, ....
##  8: 0.4317077 -0.4799985 -1.818258    33 HLA-DPB1....
##  9: 0.4317077 -0.4168998 -1.782064    55 HLA-E, C....
## 10: 0.4317077 -0.3752329 -1.641571    60 MYH7B, H....
```

This table summarizes the top 10 pathways that are significantly enriched based on gene in-degree differences, with all listed pathways passing multiple-testing correction (adjusted p-values < 0.05). The negative enrichment scores (ES) indicate that genes in these pathways tend to have reduced regulatory in-degree in the tumor condition relative to normal. Notably, immune-related pathways (e.g., autoimmune thyroid disease, allograft rejection, antigen processing and presentation) dominate the results, suggesting widespread suppression or rewiring of immune regulatory programs. The enrichment of ribosome and arachidonic acid metabolism pathways further points to coordinated changes in translational and metabolic regulation associated with the disease state.

```r
dat <- data.frame(fgseaMulti)
# Settings
fdrcut <- 0.05 # FDR cut-off to use as output for significant signatures
dencol_neg <- "blue" # bubble plot color for negative ES
dencol_pos <- "red" # bubble plot color for positive ES
signnamelength <- 4 # set to remove prefix from signature names (2 for "GO", 4 for "KEGG", 8 for "REACT
asp <- 3 # aspect ratio of bubble plot
charcut <- 100 # cut signature name in heatmap to this nr of characters
# Make signature names more readable
a <- as.character(dat$pathway) # 'a' is a great variable name to substitute row names with something mo
for (j in 1:length(a)){
  a[j] <- substr(a[j], signnamelength+2, nchar(a[j]))
}
a <- tolower(a) # convert to lower case (you may want to comment this out, it really depends on what si
for (j in 1:length(a)){
  if(nchar(a[j])>charcut) { a[j] <- paste(substr(a[j], 1, charcut), "...", sep=" ")}
} # cut signature names that have more characters than charcut, and add "..."
a <- gsub("_", " ", a)
dat$NAME <- a
# Determine what signatures to plot (based on FDR cut)
dat2 <- dat[dat[,"padj"]<fdrcut,]
dat2 <- dat2[order(dat2[,"padj"]),]
dat2$signature <- factor(dat2$NAME, rev(as.character(dat2$NAME)))
# Determine what labels to color
sign_neg <- which(dat2[,"NES"]<0)
sign_pos <- which(dat2[,"NES"]>0)
# Color labels
signcol <- rep(NA, length(dat2$signature))
signcol[sign_neg] <- dencol_neg # text color of negative signatures
signcol[sign_pos] <- dencol_pos # text color of positive signatures
signcol <- rev(signcol) # need to revert vector of colors, because ggplot starts plotting these from be
```

```r
# Plot bubble plot
g<-ggplot(dat2, aes(x=padj,y=signature,size=size))
g+geom_point(aes(fill=NES), shape=21, colour="white")+
  theme_bw()+ # white background, needs to be placed before the "signcol" line
  xlim(0,fdrcut)+
  scale_size_area(max_size=10,guide="none")+
  scale_fill_gradient2(low=dencol_neg, high=dencol_pos)+
  theme(axis.text.y = element_text(colour=signcol))+
  theme(aspect.ratio=asp, axis.title.y=element_blank()) # test aspect.ratio
```




```r
# ggsave(
#   filename = "../gsea_dotplot.png",
#   plot = g,
#   width = 7,
#   height = 5,
#   dpi = 300
# )
```

GSEA Enrichment Profile Analysis

The provided dot plot illustrates a distinct transcriptional divergence between metabolic processes and immune-related pathways within the experimental phenotype. The visualization groups pathways by their Normalized Enrichment Score (NES), where red indicates upregulation and blue indicates downregulation, plotted against their adjusted p-value to denote statistical significance. The data reveals a clear bifurcation: the experimental condition is characterized by a robust enhancement of central metabolic and biosynthetic pathways, concomitant with a significant suppression of immune response and translational machinery.

A detailed examination of the upregulated gene sets indicates a comprehensive metabolic reprogramming. There is a statistically significant enrichment in core energy-producing pathways, specifically the citrate cycle (TCA cycle) and glycolysis/gluconeogenesis. This suggests that the cells are undergoing a state of high energy demand or metabolic flux. This energetic shift appears to support specific biosynthetic activities, as evidenced by the positive enrichment of fatty acid metabolism, steroid biosynthesis, and the biosynthesis of unsaturated fatty acids. The concurrent upregulation of peroxisome activity and purine metabolism further corroborates a phenotype geared towards anabolic growth and lipid homeostasis. The presence of aminoacyl tRNA biosynthesis in the upregulated fraction implies that while global translation may be suppressed (as discussed below), the machinery for specific protein synthesis remains active or is being selectively prioritized.

Conversely, the downregulated gene sets present a strong signature of immune suppression and reduced cellular signaling. The most significantly depleted pathways include allograft rejection, autoimmune thyroid disease, and graft-versus-host disease. The downregulation of antigen processing and presentation further suggests a mechanism of immune evasion or a dampening of the adaptive immune response. Structurally, the most prominent negative enrichment is observed in the ribosome pathway, represented by a large dot size indicative of a high gene count. This suggests a broad reduction in ribosomal biogenesis, which is often a cellular response to stress or a regulatory mechanism to conserve energy for the prioritized metabolic tasks identified above. Additionally, the suppression of the MAPK signaling pathway indicates a reduction in specific proliferation or stress-response signaling cascades.

To validate the observed metabolic-immune trade-off, future studies should first corroborate key transcriptomic signatures via quantitative PCR and Western blotting, focusing on rate-limiting metabolic enzymes and antigen-presenting molecules. These molecular findings necessitate functional verification through metabolic profiling (e.g., Seahorse XF analysis) to quantify the energetic shift, coupled with flow cytometry and cytotoxicity assays to assess the physiological impact of the predicted immune evasion. Furthermore, mechanistic interrogation of the suppressed MAPK-ribosome axis using phospho-proteomics is recommended to elucidate the regulatory networks governing this distinct cellular reprogramming.

```r
DE_gene <- read.csv("../data/DE_genes_ERpos_vs_Normal_FDR0.05_logFC2.csv")

overlap <- base::intersect(
  DE_gene$Gene_Symbol,
  names(gene_in_diff_sym)
)

df_overlap <- data.frame(
  gene = overlap,
  reg_change = gene_in_diff_sym[overlap]
) %>%
  left_join(DE_gene, by = c("gene" = "Gene_Symbol")) %>%
  arrange(desc(abs(reg_change)), desc(abs(logFC)))

df_overlap[1:20, ]
```

```
##         gene reg_change      Ensembl_ID     logFC     adj_P_Val
## 1    NPIPB12  -704.6132 ENSG00000169203  2.139955  0.000000e+00
## 2    NPIPB13  -677.6155 ENSG00000198064  2.073642  0.000000e+00
## 3    CENATAC  -594.9147 ENSG00000186166  2.136722  0.000000e+00
## 4       SMTN  -574.8359 ENSG00000183963  2.367936  0.000000e+00
## 5   RPL23AP3  -569.9821 ENSG00000214914 -5.244449 2.270656e-219
## 6     NPIPP1  -563.7164 ENSG00000188599  2.015409  0.000000e+00
## 7       KRT1  -546.8436 ENSG00000167768  4.789499 1.656647e-202
## 8    HSPA8P1   543.6219 ENSG00000234176 -2.621724 9.078379e-301
## 9     REELD1  -539.1628 ENSG00000250673  3.840970 8.746321e-222
## 10    BRI3BP   534.2416 ENSG00000184992 -2.573334  0.000000e+00
## 11      PRG2  -532.8831 ENSG00000186652  2.997246 5.182792e-259
```

```
## 12 LINC00926  -532.4210 ENSG00000247982  2.338162 3.852274e-269
## 13   SLC6A16  -530.7124 ENSG00000063127  3.333162  0.000000e+00
## 14   MASCRNA  -524.5849 ENSG00000274072  2.607494 5.294177e-204
## 15   RPSAP14  -508.4507 ENSG00000233984 -3.307772  0.000000e+00
## 16   CACNA1A  -506.4110 ENSG00000141837  2.038253 1.131551e-229
## 17   HSD17B3  -490.5798 ENSG00000130948  3.722044 1.080134e-256
## 18   FAM193B  -490.0511 ENSG00000146067  2.276971  0.000000e+00
## 19     THRIL   482.7294 ENSG00000280634 -2.889785 7.264420e-203
## 20     NLRP1  -481.1399 ENSG00000091592  3.067477  0.000000e+00
```

This table ranks genes by regulatory change (reg_change) derived from PANDA network analysis, alongside classical differential expression statistics (logFC and adjusted p-value). Importantly, several genes exhibit large regulatory changes despite modest or even opposite expression changes, highlighting regulatory rewiring that would not be captured by expression analysis alone. This discordance supports the value of a network-based approach: genes may become more or less centrally regulated without large shifts in mean expression.

Notably, the top-ranked genes include a mixture of pseudogenes, ribosomal-related genes, cytoskeletal markers, and stress-response genes, many of which have been implicated in cancer biology, either as markers of cellular state (e.g., proliferation, differentiation) or as regulators of transcriptional and post-transcriptional programs. The presence of extremely small adjusted p-values indicates that these expression changes are highly robust, strengthening confidence in their biological relevance when interpreted together with regulatory metrics.

Overall, the observations are consistent with our discovery in the above regulatory network graph, with genes involved in signaling and metabolism pathways ranking high in the list.

Some genes known to contribute to cancer development are discussed below:

HSPA8P1

HSPA8P1 is a pseudogene related to the chaperone HSPA8 (HSC70), which is involved in protein folding, autophagy, and stress responses. Dysregulation of heat shock protein–related pathways is common in ER-positive breast cancer, suggesting a role in tumor stress adaptation and proteostasis.

KRT1

KRT1 encodes a keratin involved in epithelial differentiation. Altered keratin regulation is frequently observed in breast cancer and often reflects changes in tumor differentiation state and epithelial identity, including ER-positive subtypes.

CENATAC

CENATAC is associated with centromere and chromatin organization. Disruption of chromatin and centromere regulation contributes to genomic instability, a hallmark of cancer progression.

SMTN

SMTN encodes smoothelin, a cytoskeletal protein involved in cellular structure. Changes in its regulation may reflect cytoskeletal remodeling relevant to tumor cell plasticity and invasiveness.

REELD1

REELD1 has been implicated in cytoskeletal and cellular organization pathways. Regulatory changes in such genes may contribute to altered cell morphology and tumor progression.

Stepn Gene-Level Deep Dive: TRPS1

A central finding of our integrative approach was the identification of TRPS1 (Trichorhinophalangeal Syndrome 1) as a key regulatory node. By intersecting our DE results with PANDA-inferred targets, we identified TRPS1 as a central repressor and a specific marker of ER-positive identity. In the context of mammary biology, TRPS1 has been identified as a critical, context-dependent regulator of epithelial cell growth and

differentiation [@cornelissen2020]. While our DE analysis identified thousands of candidates, TRPS1 stood out because it directly influences the Estrogen Receptor (ER) signaling landscape [@serandour2018].

TRPS1 has also been found in colon cancer metastasis and osteosarcoma [@Cai2024, @Hong2013], suggesting that its ability in interrupting epithelial cells and forming cartilage tissues may also contribute to cancer metastasis in ER+ BRCA in our case.

Our results show that TRPS1 is significantly upregulated in the ER+ tumor group compared to healthy breast tissue. To validate the biological context, we examined its expression across human tissues via GTEx data by using UCSC.

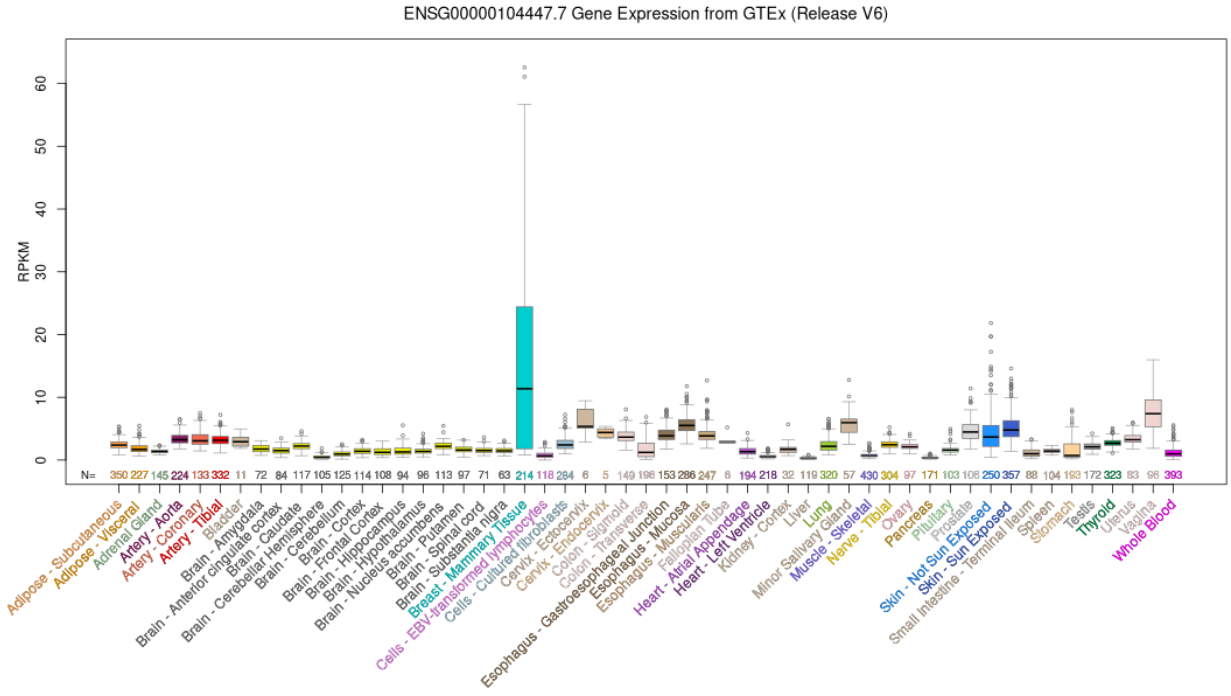


Figure 7: TRPS1 Tissue Specificity

The expression profile confirms that TRPS1 is highly tissue-specific, with its highest levels localized in Breast Mammary Tissue.

This aligns with clinical findings that TRPS1 is a highly sensitive and specific marker for breast carcinoma, serving as a reliable diagnostic tool across various subtypes [@ai2021].

Result

Conclusion

Results & Conclusion in this study, we integrated transcriptomic analysis and regulatorynetwork modeling to characterize molecular differences betweenER-positive breast tumors and normal breast tissue. Exploratory dataanalysis using PCA revealed clear global separation between tumor andnormal samples, indicating that disease status is a dominant driver ofgene expression variation. Differential expression analysis using thelimma–voom framework identified thousands of genes with significantexpression changes, reflecting widespread transcriptional alterationsassociated with tumorigenesis. Pathway-level analysis using HallmarkGSEA further demonstrated that these gene-level changes correspond tocoordinated biological programs, with ER-positive tumors showing strongenrichment of cell cycle, proliferative, metabolic, andestrogen-responsive pathways, while normal tissue exhibited pathwaysrelated to structural organization, differentiation, and immunesignaling.To move beyond expression-level differences, we applied PANDA to

infercondition-specific regulatory networks for ER-positive tumors and normalbreast tissue. Network analysis revealed that regulatory changes arehighly structured and dominated by a small subset of transcriptionfactors whose targeting patterns differ significantly betweenconditions. Paired statistical testing across transcriptionfactor–target interactions identified TFs such as TFAP4, MAFB, and MBNL2 as major drivers of regulatory change. Integrative analysis combining differential expression and PANDA inferred regulatory shifts led us tofocus on TRPS1,which is a lineage-defining transcriptional repressor inER-positive breast cancer. Despite moderate expression changes, TRPS1exhibited substantial changes in regulatory influence, highlighting theimportance of regulatory dynamics beyond differential expression alone. Collectively, these results demonstrate that ER-positive breast canceris characterized not only by widespread transcriptional dysregulationbut also by systematic alterations in transcriptional control,underscoring the value of network-based approaches for understanding breast cancer

Limitations and Future

Direction: This study focused exclusively on ER-positive breast tumors,and therefore the identified regulatory patterns may not generalize to other breast cancer subtypes. Future work could extend this framework to ER-negative tumors to directly compare subtype-specific and shared regulatory mechanisms, as well as incorporate additional layers of regulation such as epigenomic or single-cell data to further refine transcriptional network inference.

References