Analysis of RNA-Seq experiments

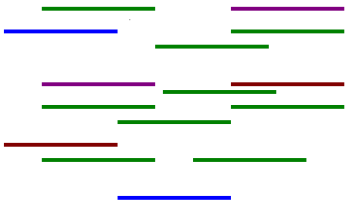
Matthias Lienhard

Seminar RNA bioinformatics

Dec 10th 2014

RNA-Seq Pipeline

Short Reads



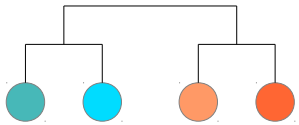
Mapping

Alignment

Quantification

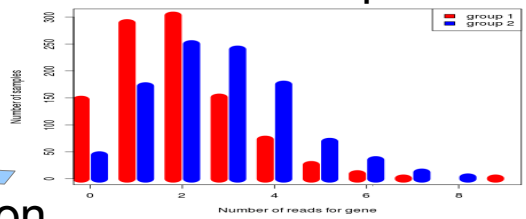
Count Table

	BaP 12h	Ct 12h	...
GENE 1	321	422	12
GENE 2	32	50	20
GENE 3	132466	72921	43223



Exploratory Analysis

Differential Expression



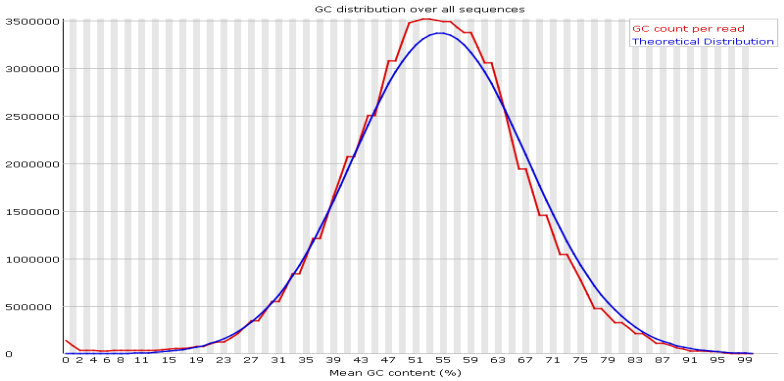
Statistical Test

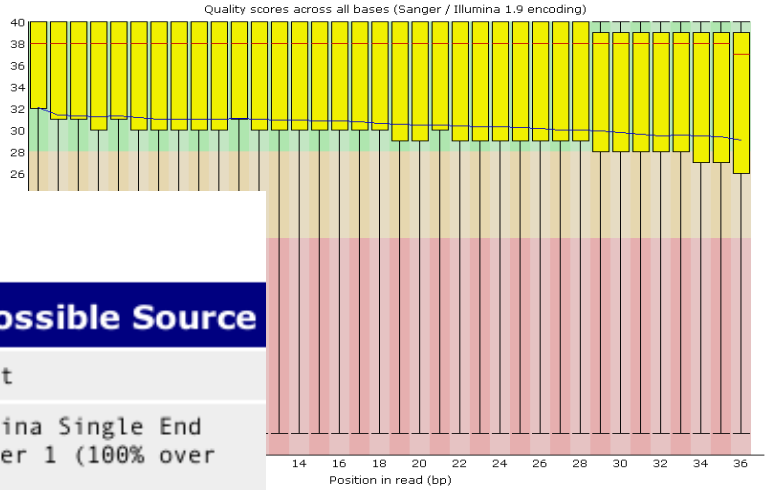
Interpretation

M. Lienhard (2011), “Analysis of RNA-seq Experiments” Master thesis, Freie Universität Berlin.

Quality Control

- Base Call Quality
- PCA artifacts
- Base composition
- Positional base frequency
- Adapter contamination





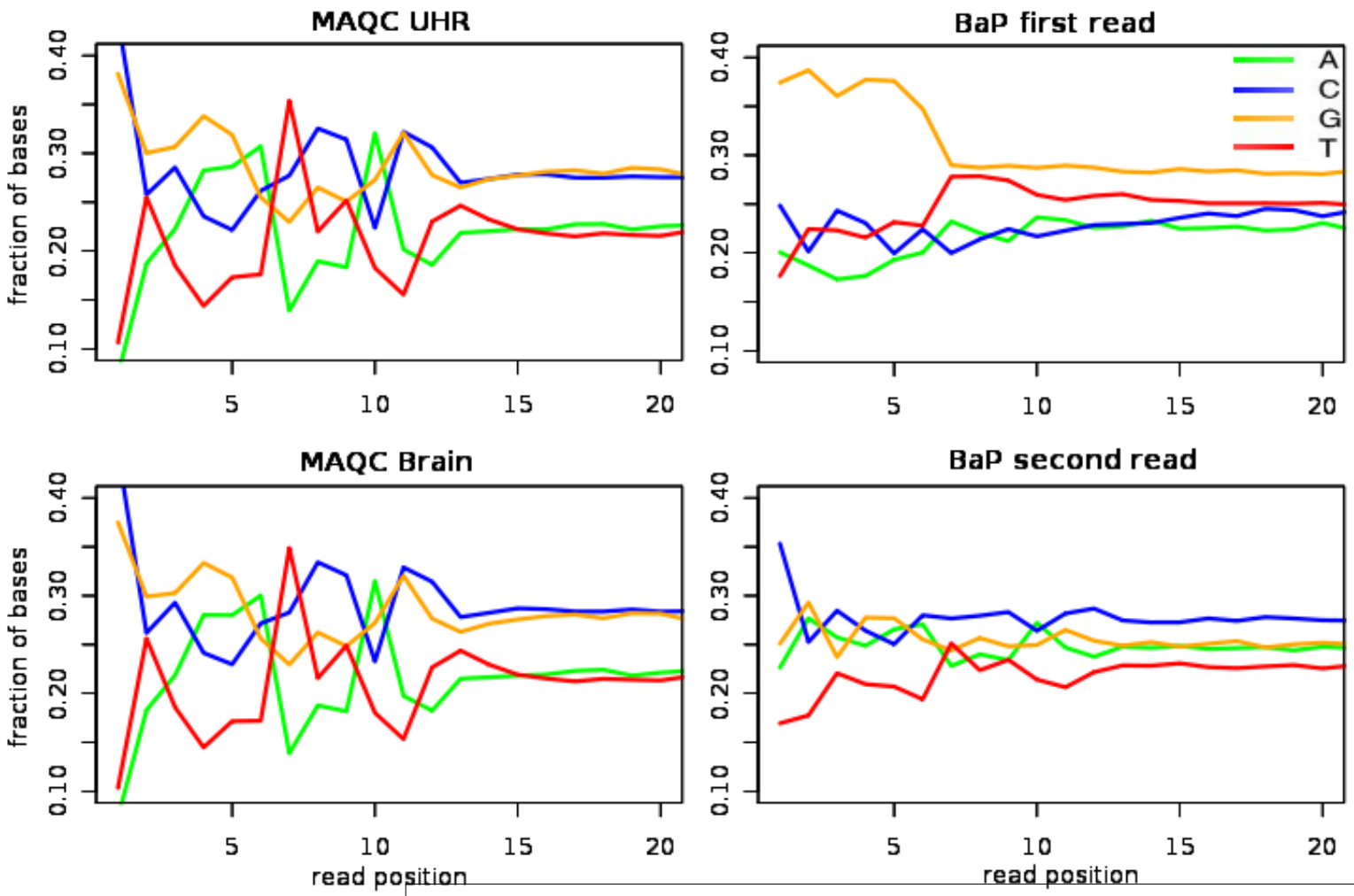
Overrepresented sequences

Sequence	Count	Percentage	Possible Source
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN	119709	0.30700917731983646	No Hit
GATCGGAAGAGCTCGTATGCCGTCTTCTGCTTGAAA	113700	0.29159832144003717	Illumina Single End Adapter 1 (100% over 33bp)

http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

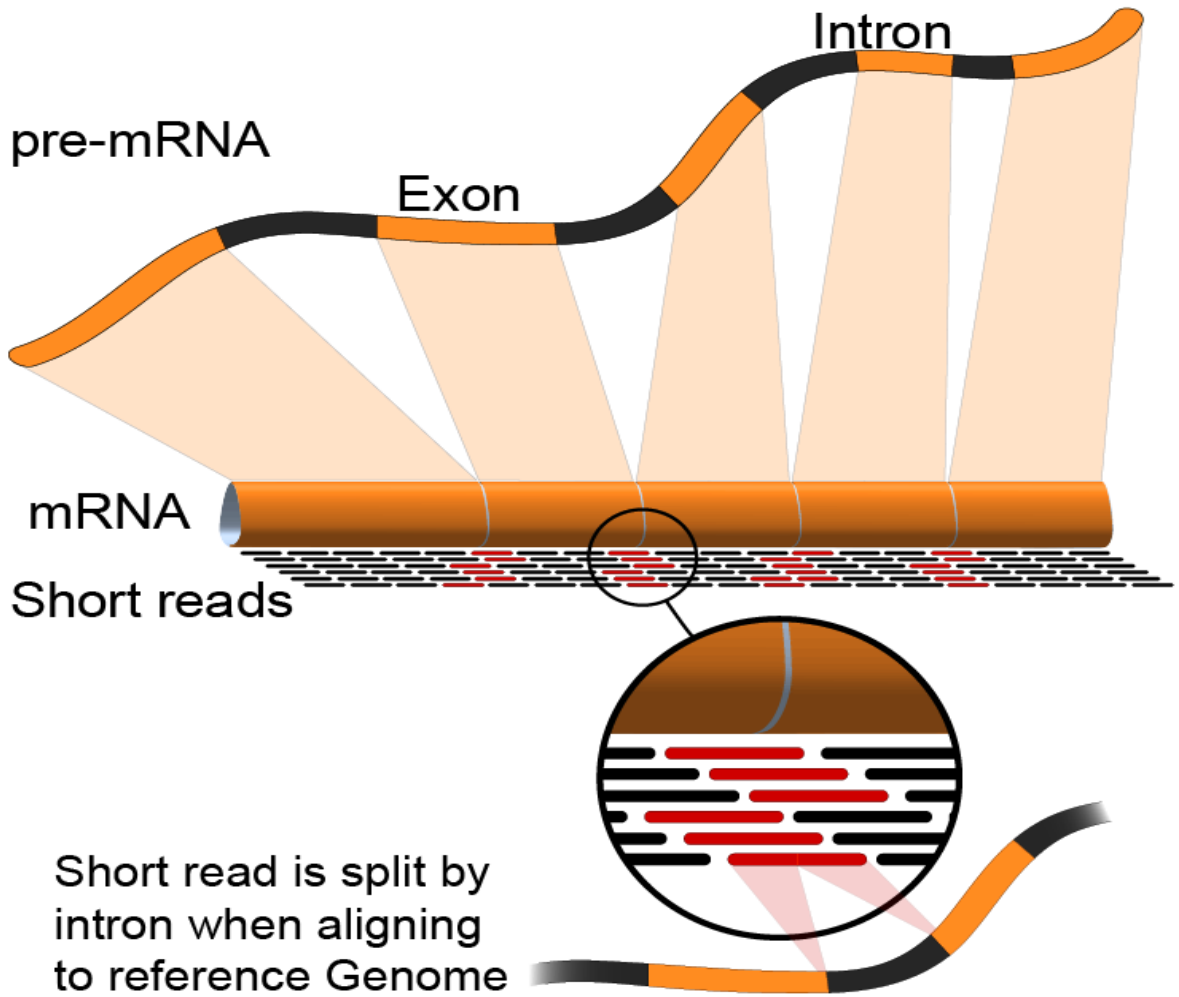
Quality Control

RNA seq has strange base-position patterns



KD Hansen et al. (2010): Biases in Illumina transcriptome sequencing caused by random hexamer priming, NAR

Read Mapping



Tophat

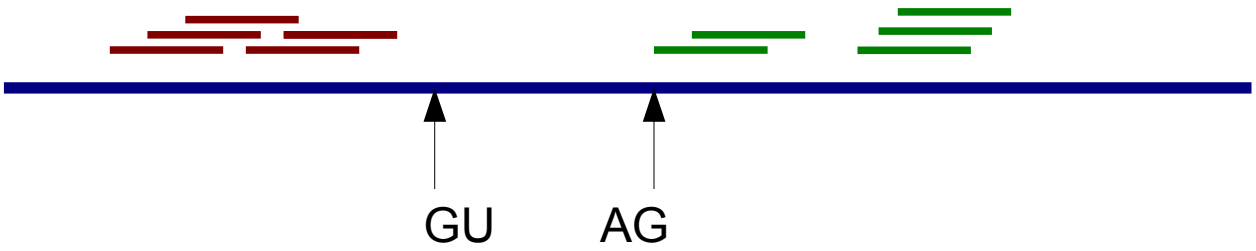
1.Genome alignment

Trapnell, C et al. (2009), TopHat: discovering splice junctions with RNA-Seq; Bioinformatics

Tophat

1. Genome alignment

2. look for canonical splice sites



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Tophat

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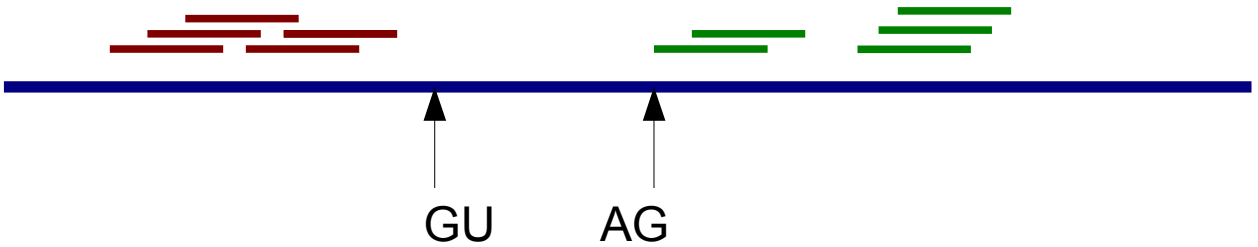

2. look for canonical splice sites

3. add known splice sites from database (eg refseq)

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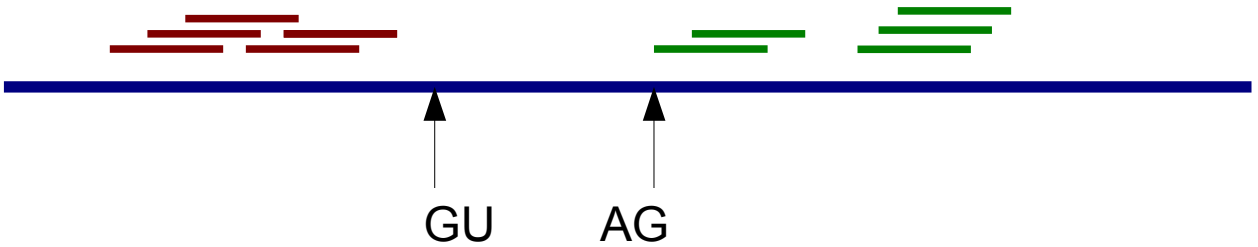

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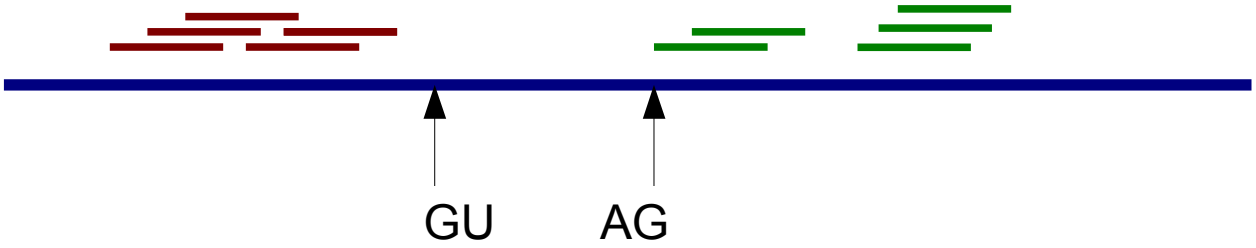

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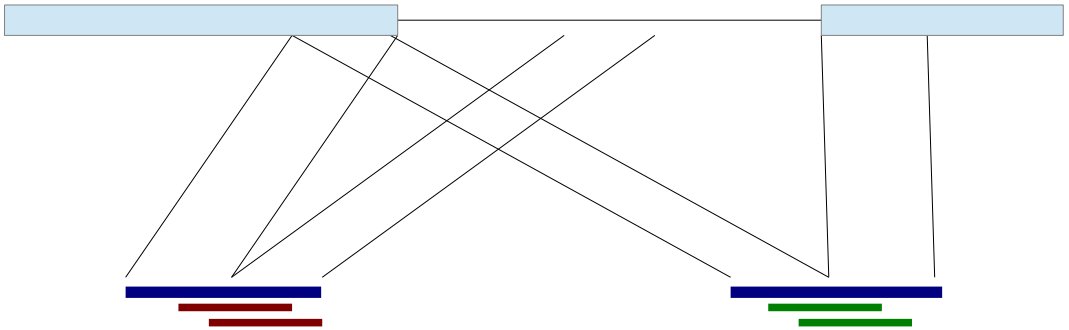
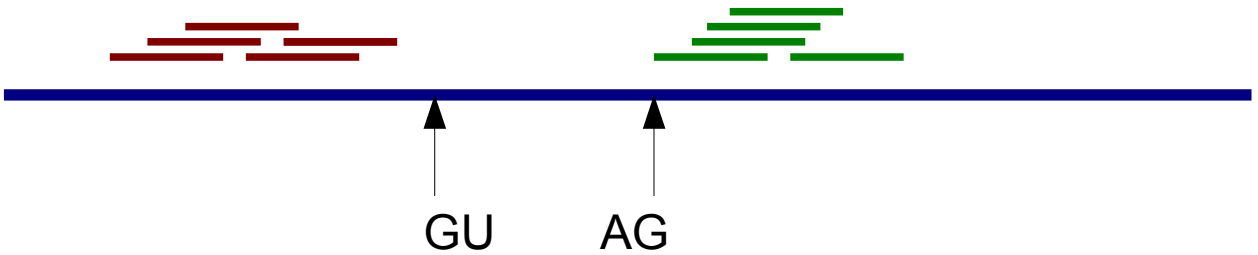


4. assemble sequences at splice sites and map reads

Trapnell, C et al. (2009), TopHat: discovering splice junctions with RNA-Seq; Bioinformatics

Tophat

1. Genome alignment



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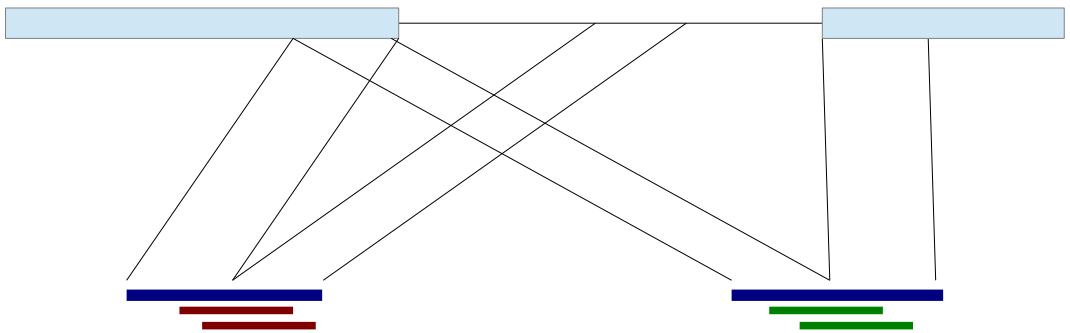
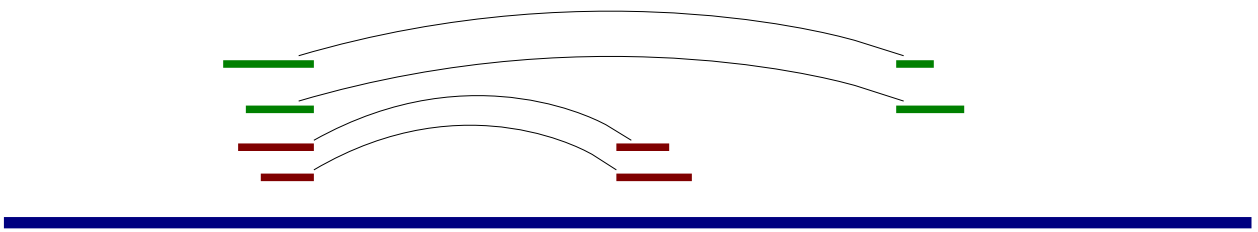


4. assemble sequences at splice sites and map reads

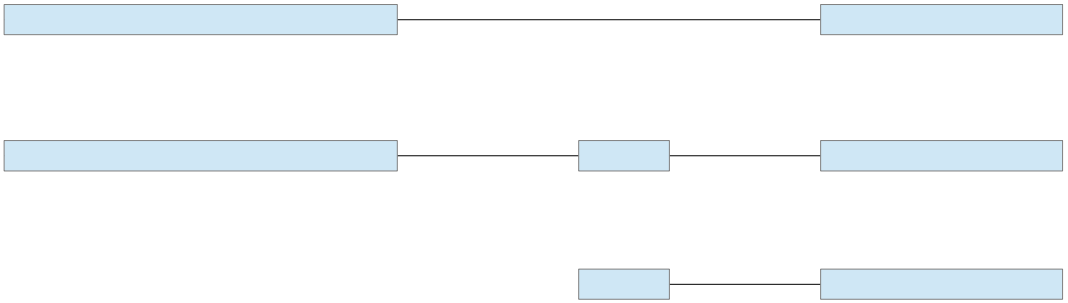
5. Map reads to genome



Trapnell, C et al. (2009), TopHat: discovering splice junctions with RNA-Seq; Bioinformatics

Quantification

- Gene / Isoform / Exon Level
- Reads on boundaries / intronic reads
  - htseq-count strategies
- ambiguous reads



S Anders *et al.*(2014): HTSeq — A Python framework to work with high-throughput sequencing data. Bioinformatics

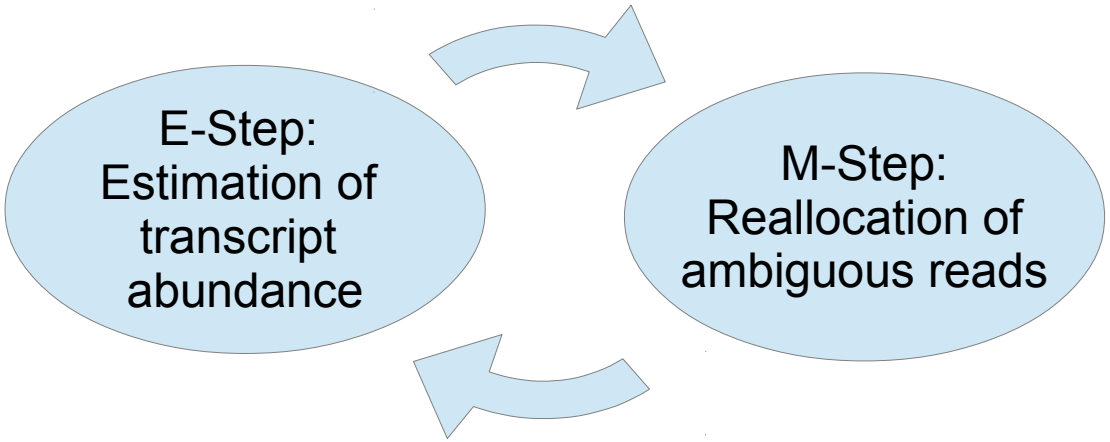
Quantification

Ambiguous reads:

~ 20% gene-level

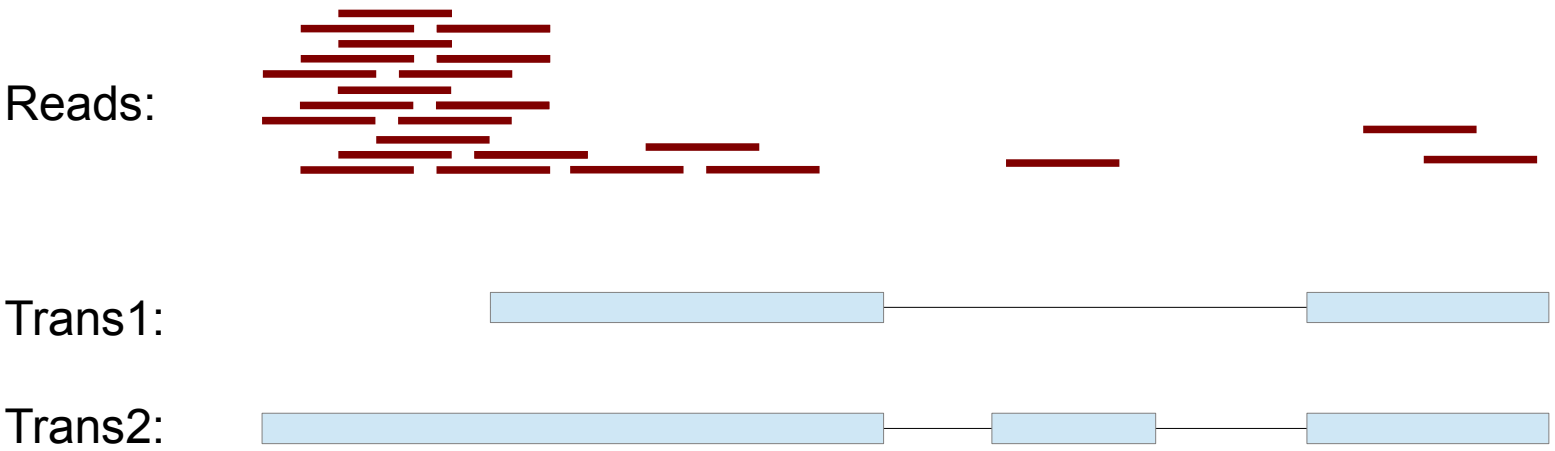
~ 30-90% on isoform level

RSEM multiread assignment EM algorithm:



B Li et al, (2011): RSEM: accurate transcript quantification from RNA-Seq data. BMC Bioinformatics

Isoform Quantification

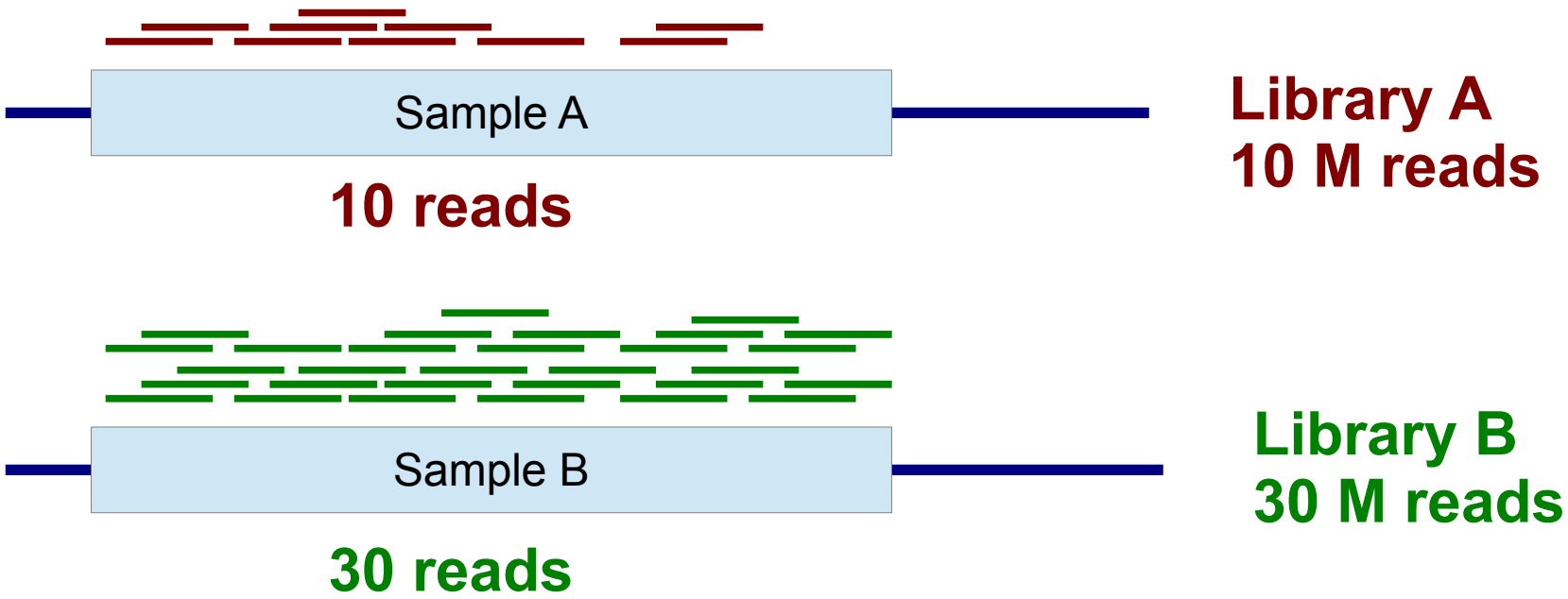


- Problems:
  - Uneven read distribution
  - Imperfect transcript annotation

  → be careful


Sample A
10 reads
Sample B
30 reads

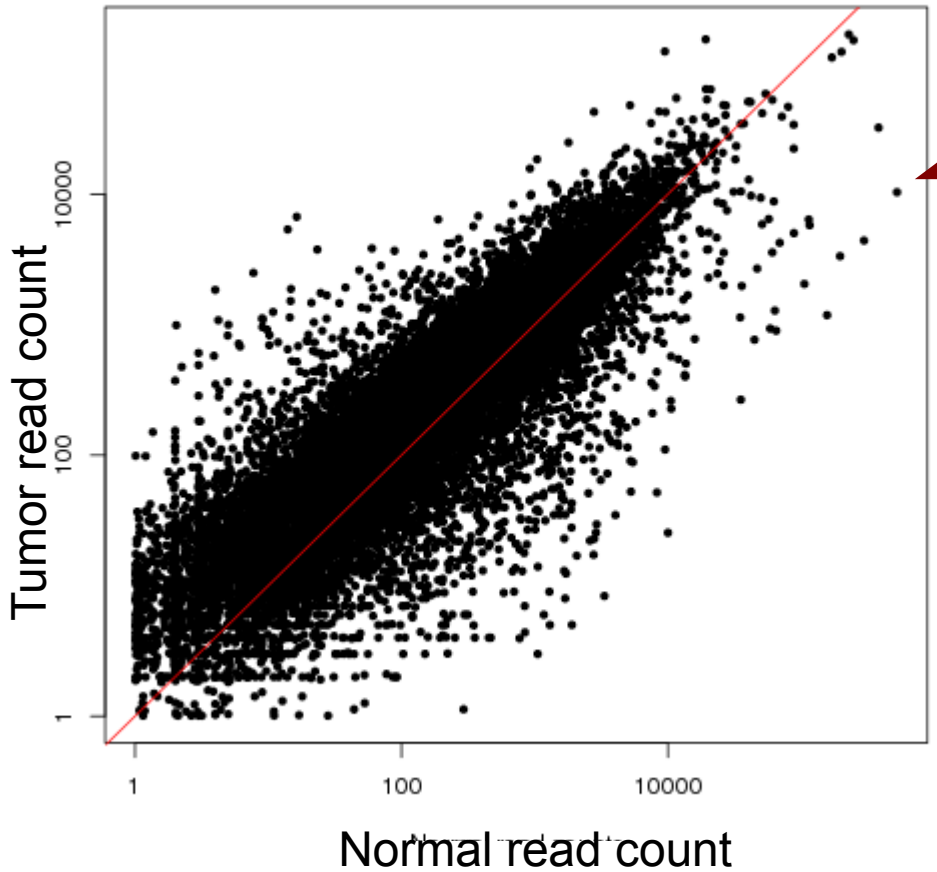
Library Size Normalization



→ Scale Samples to common size

Scaling Library Size Normalization

- Divide by total number
  - Highly expressed genes predominate factor



MYH11
Tumor: 10.371 reads
Normal: 523.926 reads

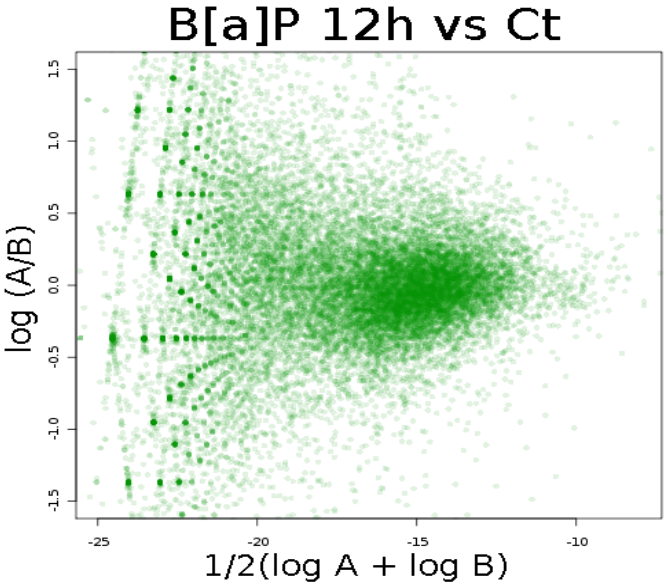
Scaling Library Size Normalization

- Divide by total number
  - Highly expressed genes predominate factor
- Upper Quantile Normalization (DESeq)
  - Median is perturbed by 1 and 2 read genes (noise)
  - 75% quartile usually works

JH Bullard (2010): Evaluation of statistical methods for normalization and differential expression in mRNA-seq experiments. BMC Bioinformatics

Scaling Library Size Normalization

- Divide by total number
  - Highly expressed genes predominate factor
- Upper Quantile Normalization (DESeq)
  - Median is perturbed by 1 and 2 read genes
  - 75% quartile usually works
- TMM: trimmed mean of M Values (edgeR)
  - Idea: center the “main dot cloud” in M vs A plot

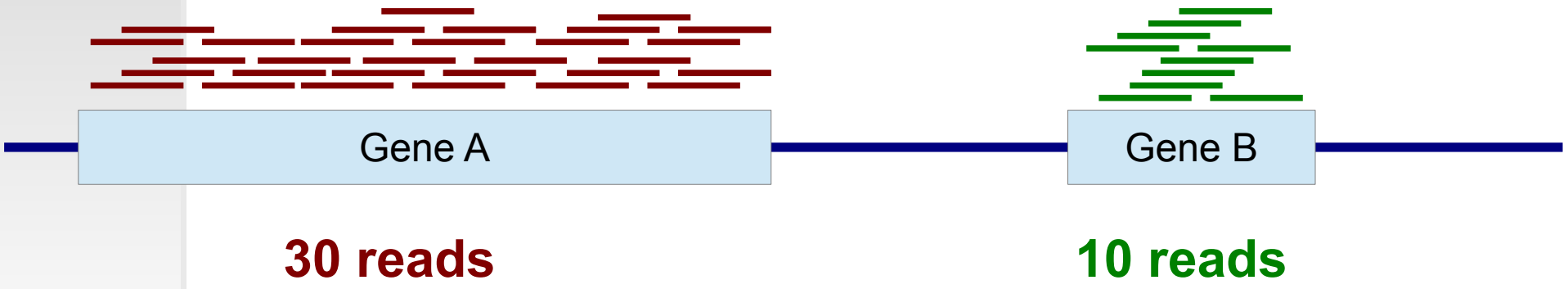


MD Robinson et al.(2010):A scaling normalization method for differential expression analysis of RNA-seq data; Genome Biology

Read length normalization

- Read length normalization
  - Compare different genes

    → Reads per kilobase of RNA per million (rpkm)



Exploratory Analysis

First Check of Quality and Hypotheses:

- How related are the samples?
- Are there distinct groups?
- Are the samples assigned correctly?
- Do we have contamination?
- Do technical differences have effects?
- Do other factors (sex, age, ...) have major influence?

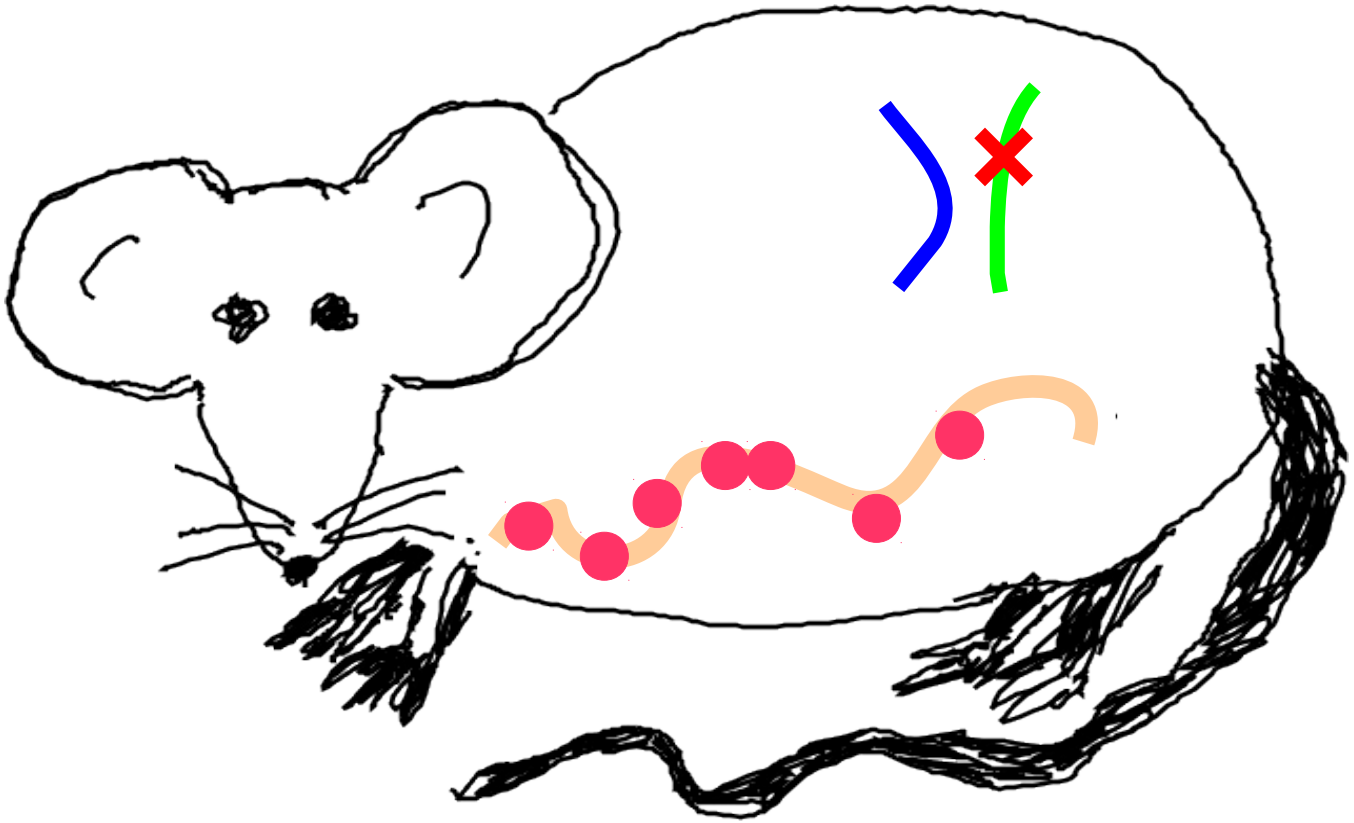
Hierarchical Clustering

Two choices:

- Clustering function
  - Single, complete, avg (UPGMA), Ward, centroid ...
- Distance function
  - Euclidean
  - Correlation based: $d(x,y)=1-cor(x,y)$

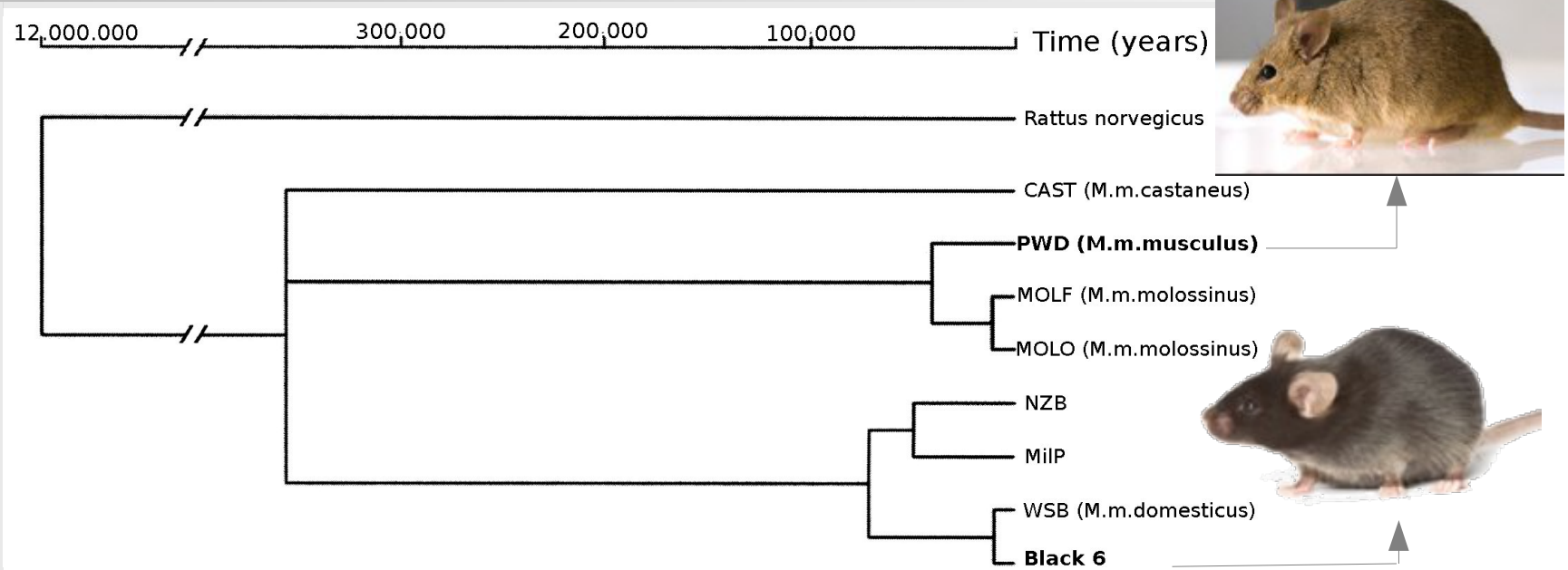
Example: Colon Cancer Mouse Model

APC $^{min/+}$ Mouse



Multiple **I**ntestinal **N**eoplasia

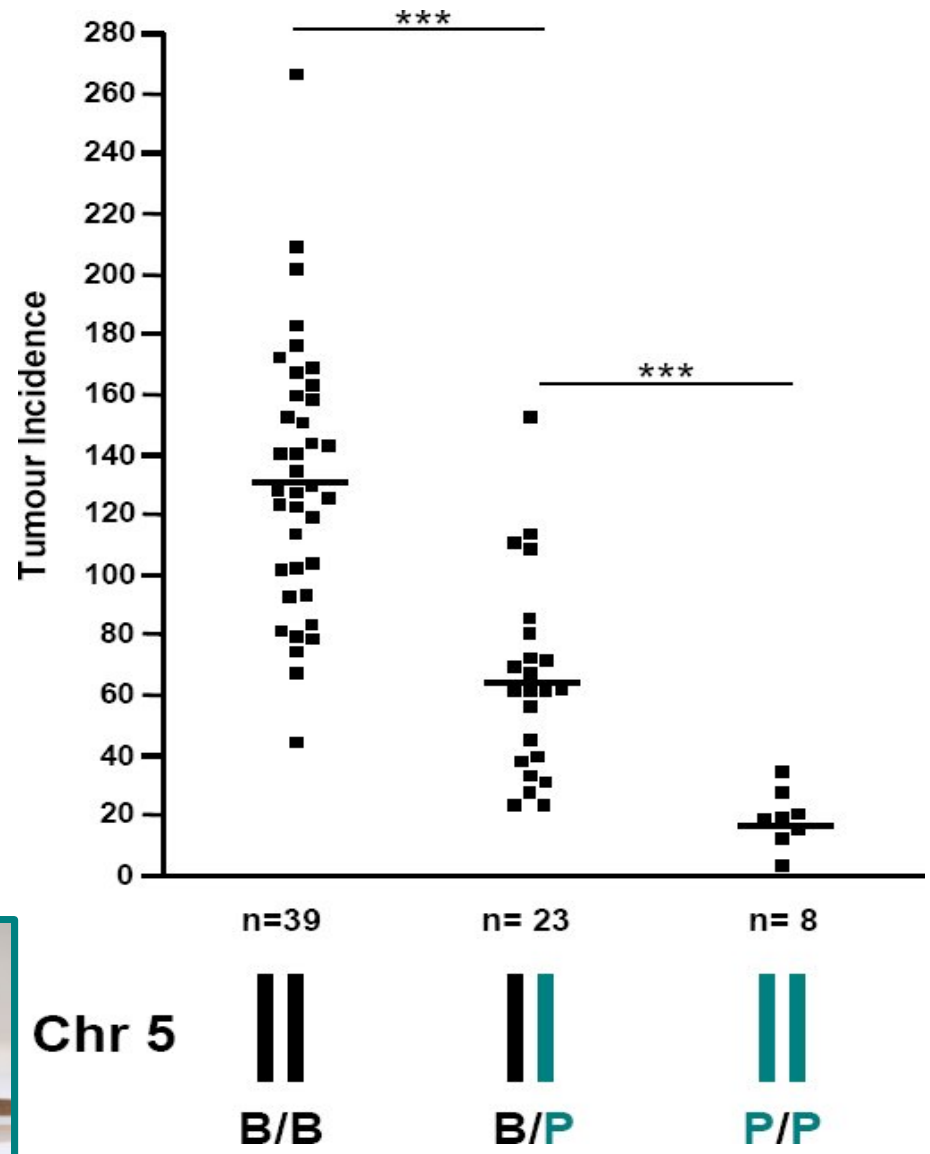
Genetic variation



- Genetically divergent
- High degree of sequence polymorphisms

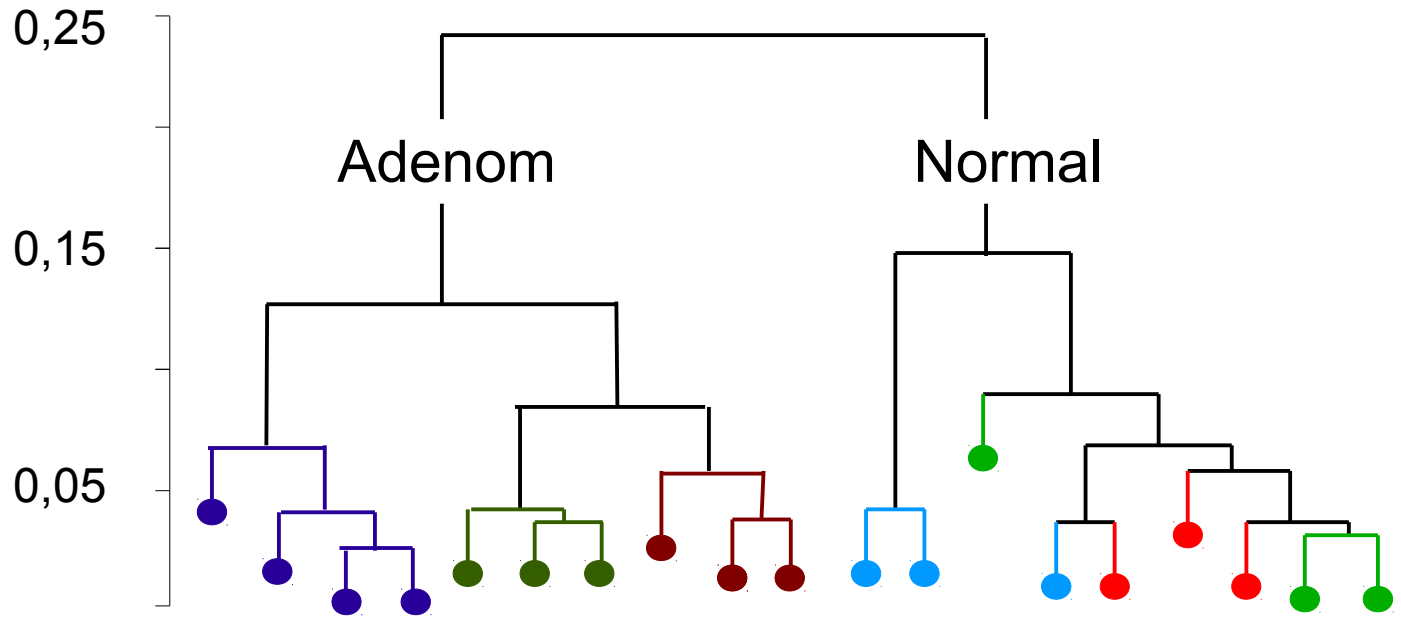
Goios *et al* (2007): mtDNA phylogeny and evolution of laboratory mouse strains, Genome Res.

Genetic Background Matters Chromosome Substitution Stains:



RNA-Seq Clustering

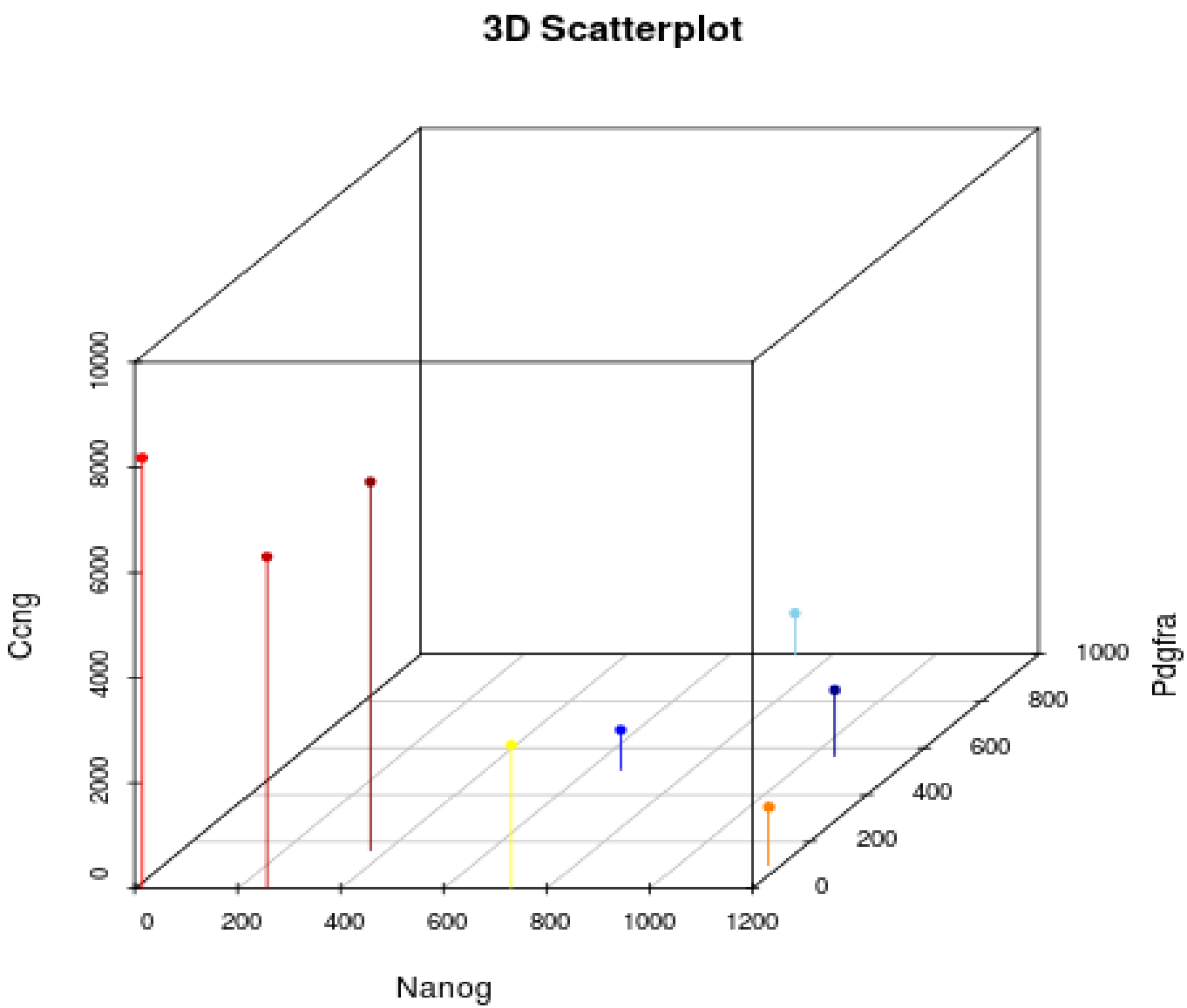
Cluster Dendrogramm w/o chr 5



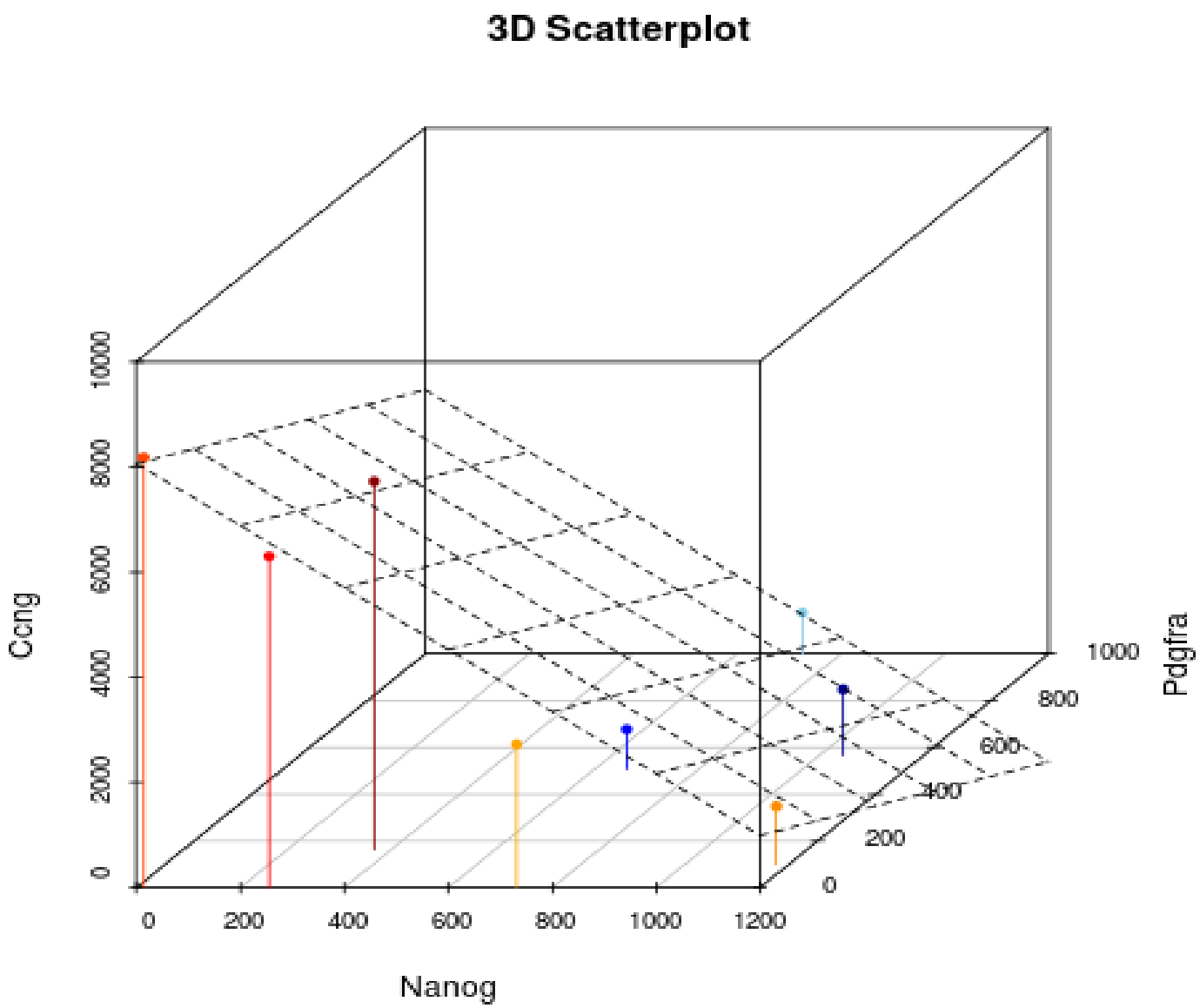
- Black 6
- C5 PWD/B6
- C5 PWD

Data: Tumor Modifiers (unpublished)

PCA


3D Scatterplot
Ccng
0
2000
4000
6000
8000
10000
Nanog
0
200
400
600
800
1000
1200
Pdgfra
0
200
400
600
800
1000

PCA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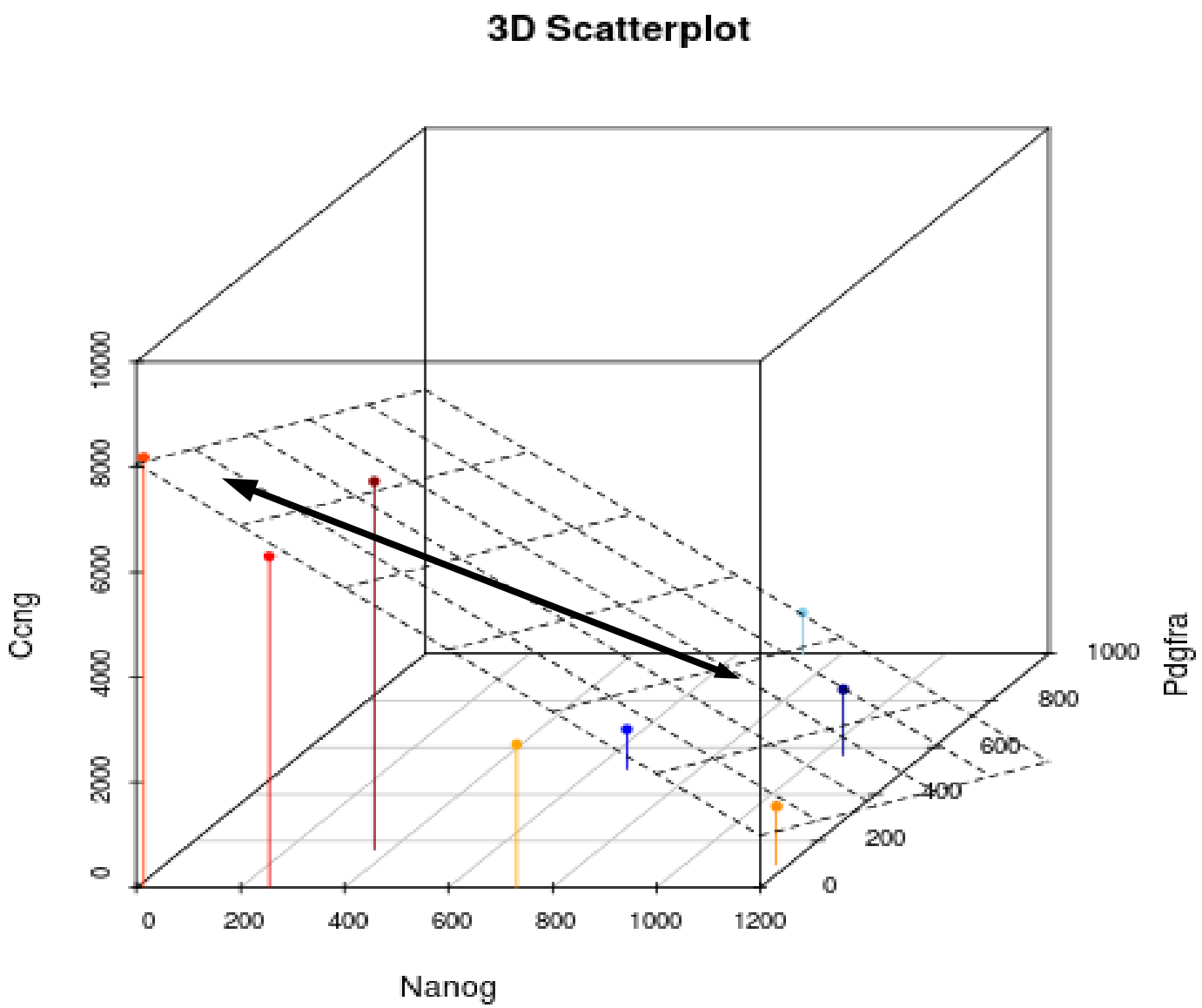
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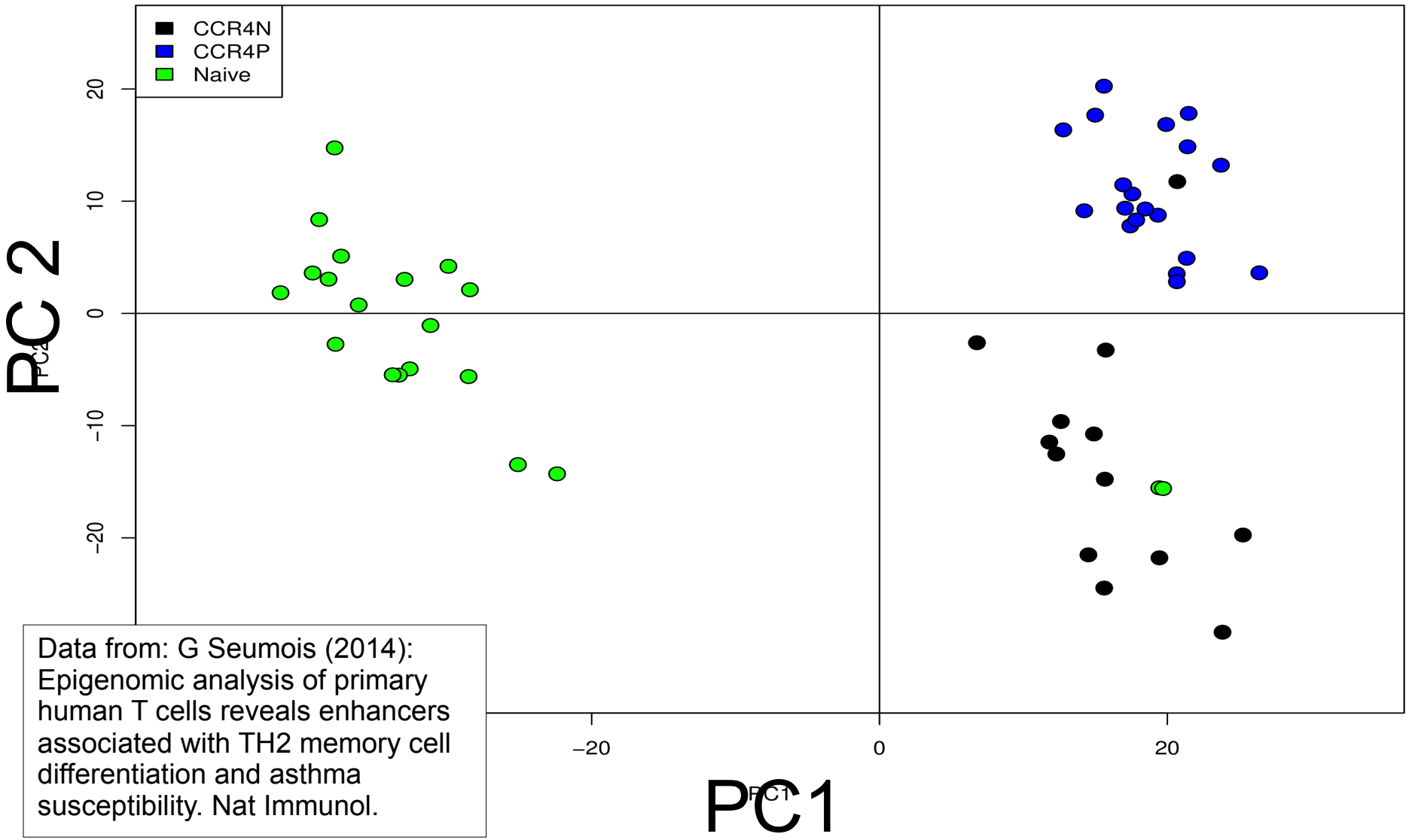
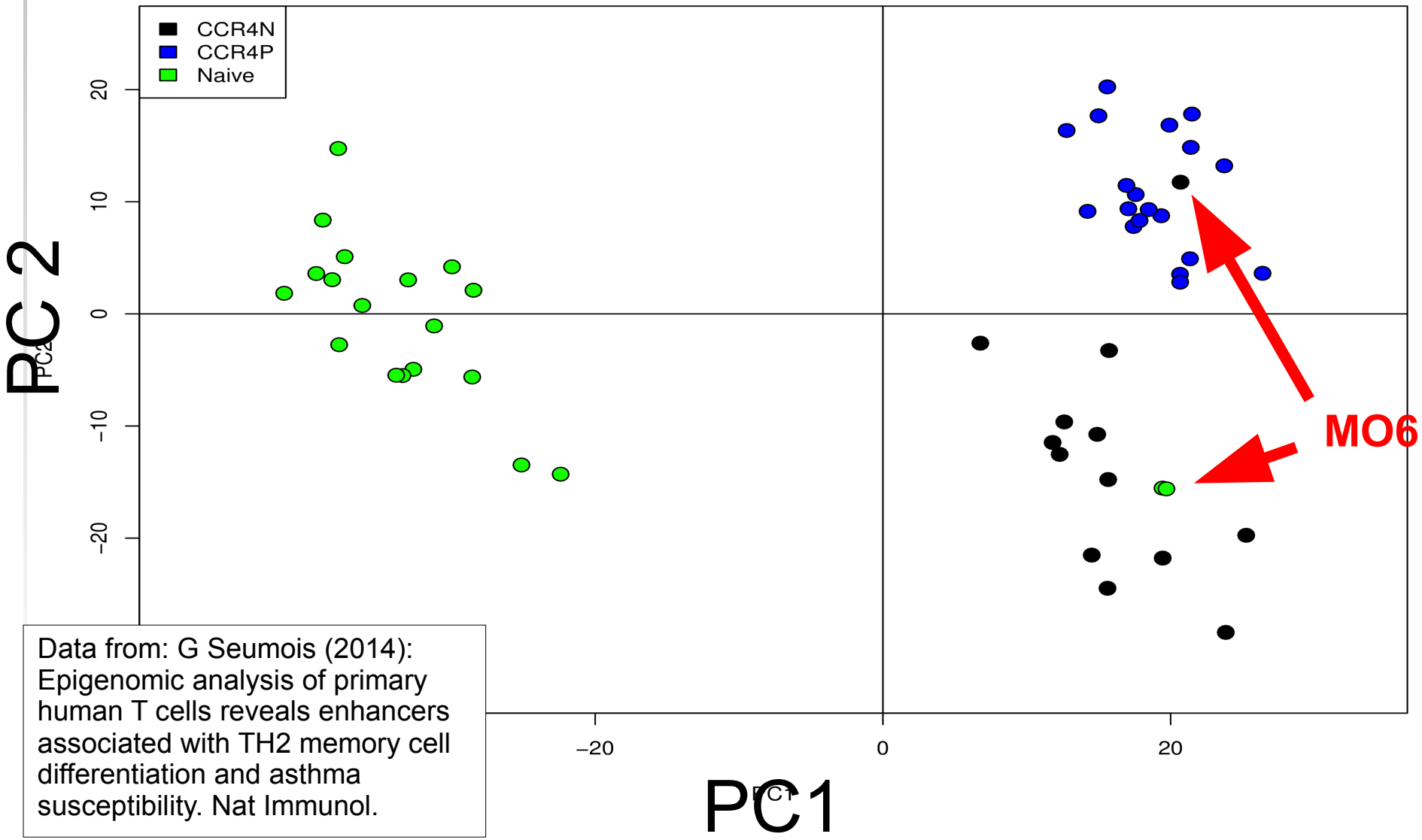


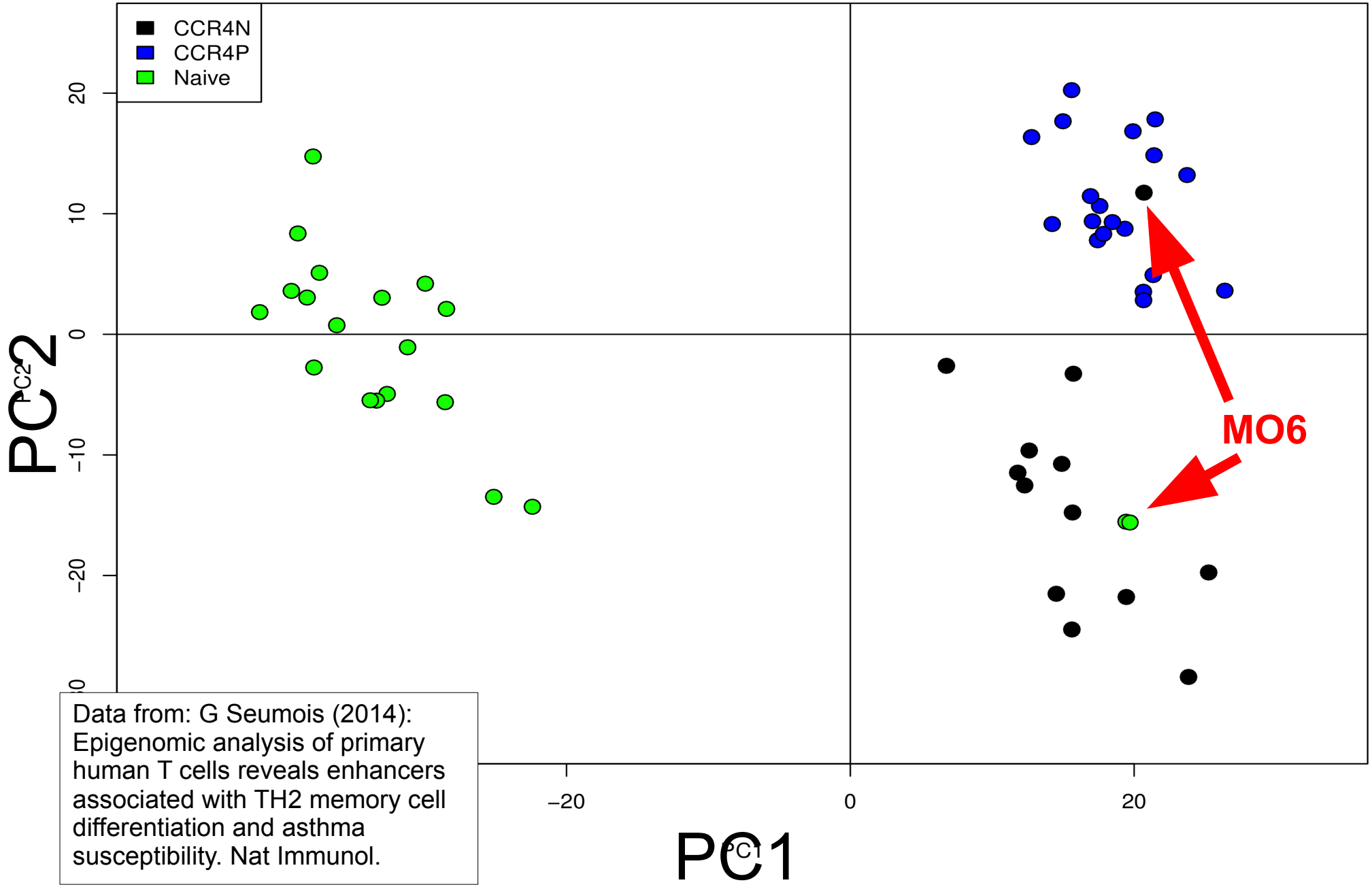
Data from: G Seumois (2014): Epigenomic analysis of primary human T cells reveals enhancers associated with TH2 memory cell differentiation and asthma susceptibility. Nat Immunol.

PCA



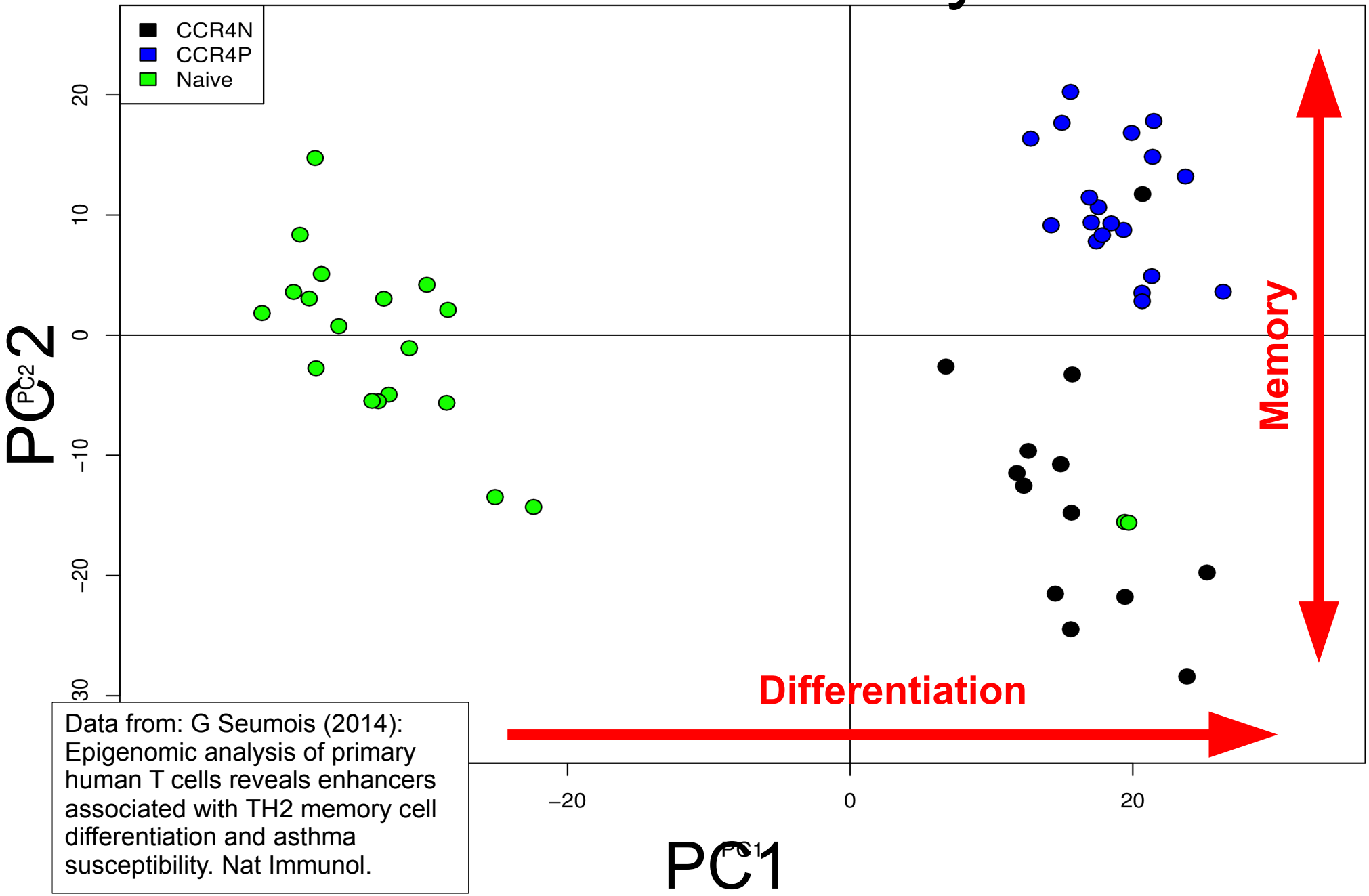
Data from: G Seumois (2014): Epigenomic analysis of primary human T cells reveals enhancers associated with TH2 memory cell differentiation and asthma susceptibility. Nat Immunol.

PCA – sample verification



Data from: G Seumois (2014): Epigenomic analysis of primary human T cells reveals enhancers associated with TH2 memory cell differentiation and asthma susceptibility. Nat Immunol.

PCA – factor analysis



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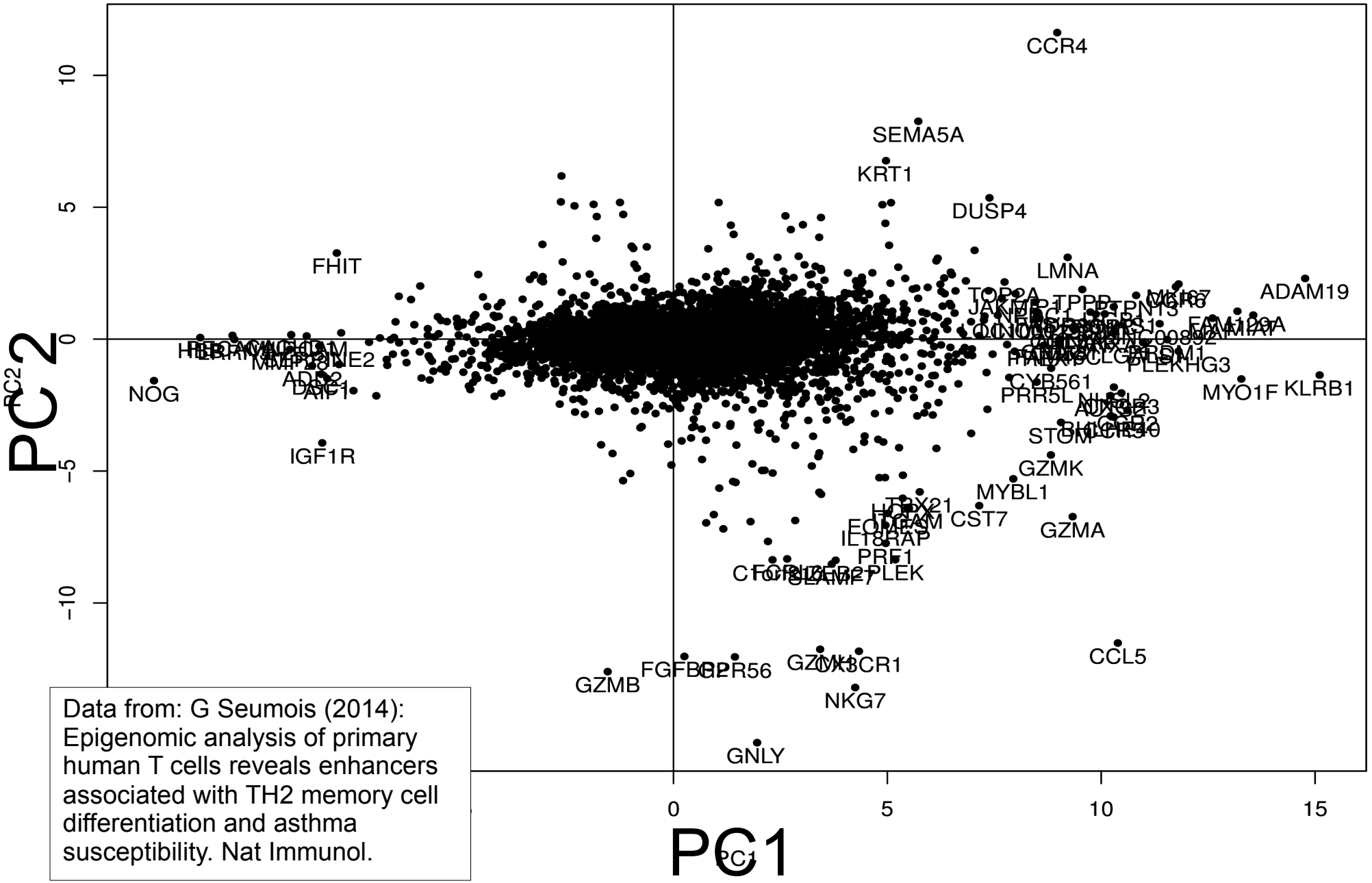
Genes Contributing to PCs



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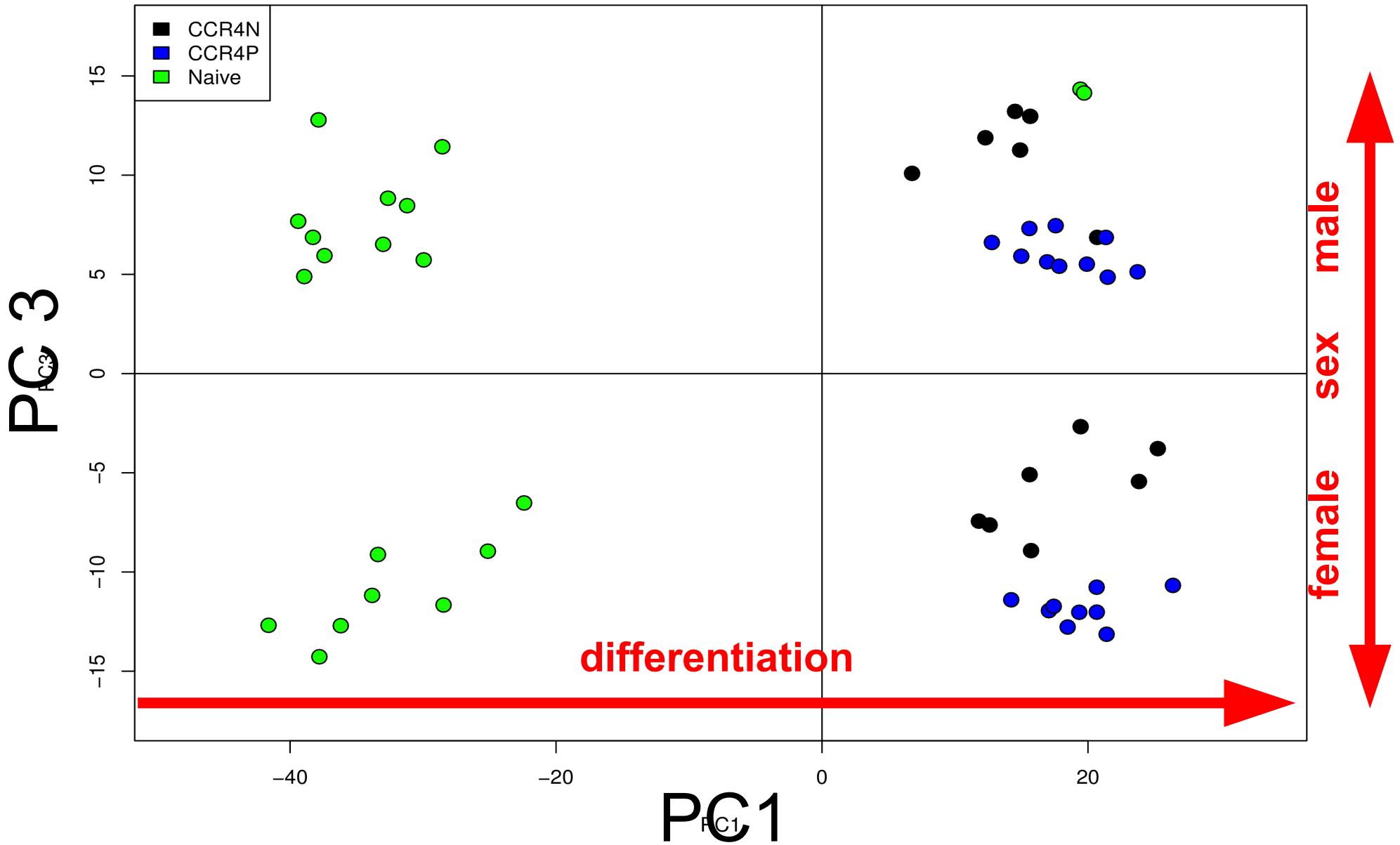
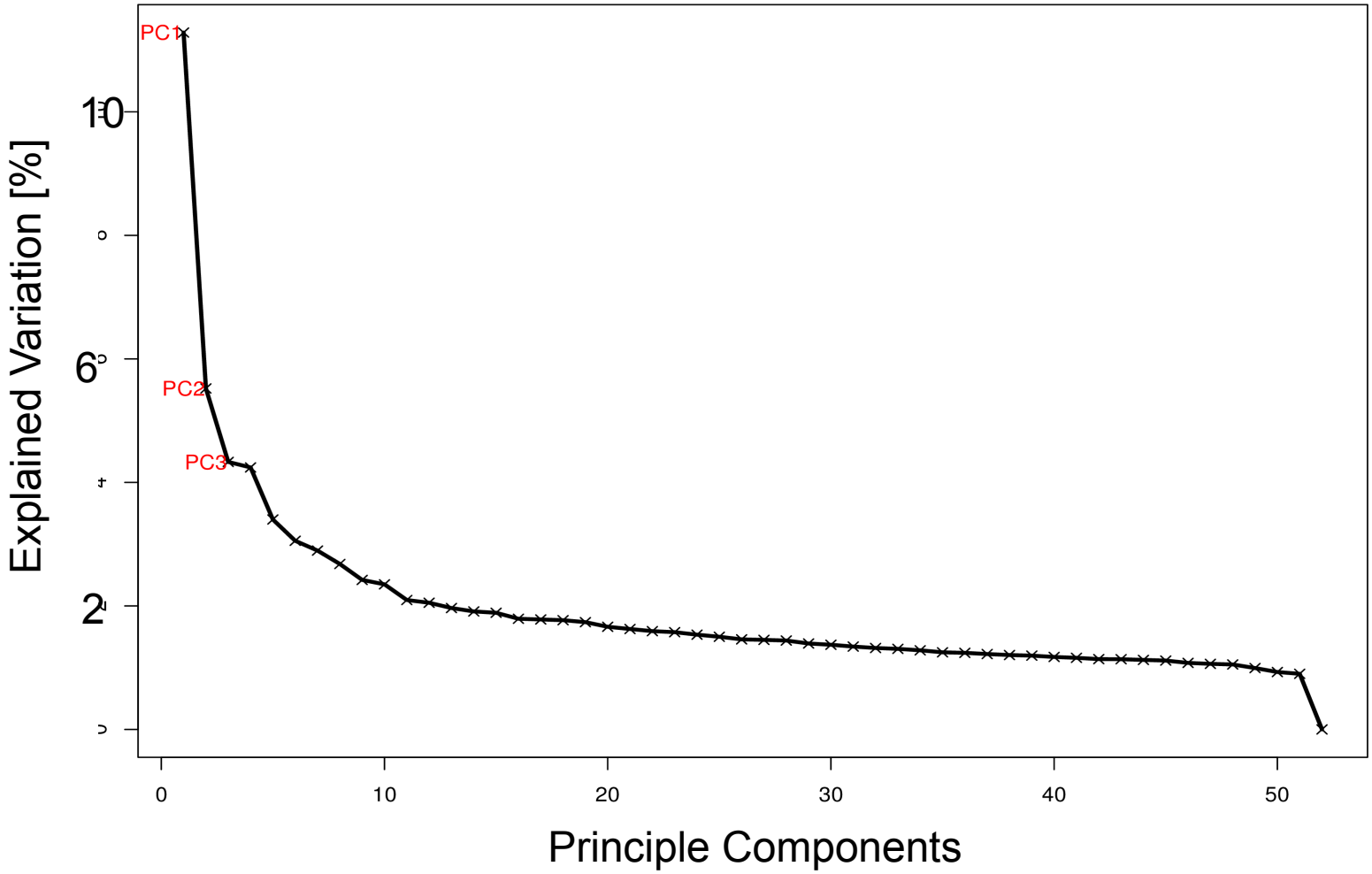
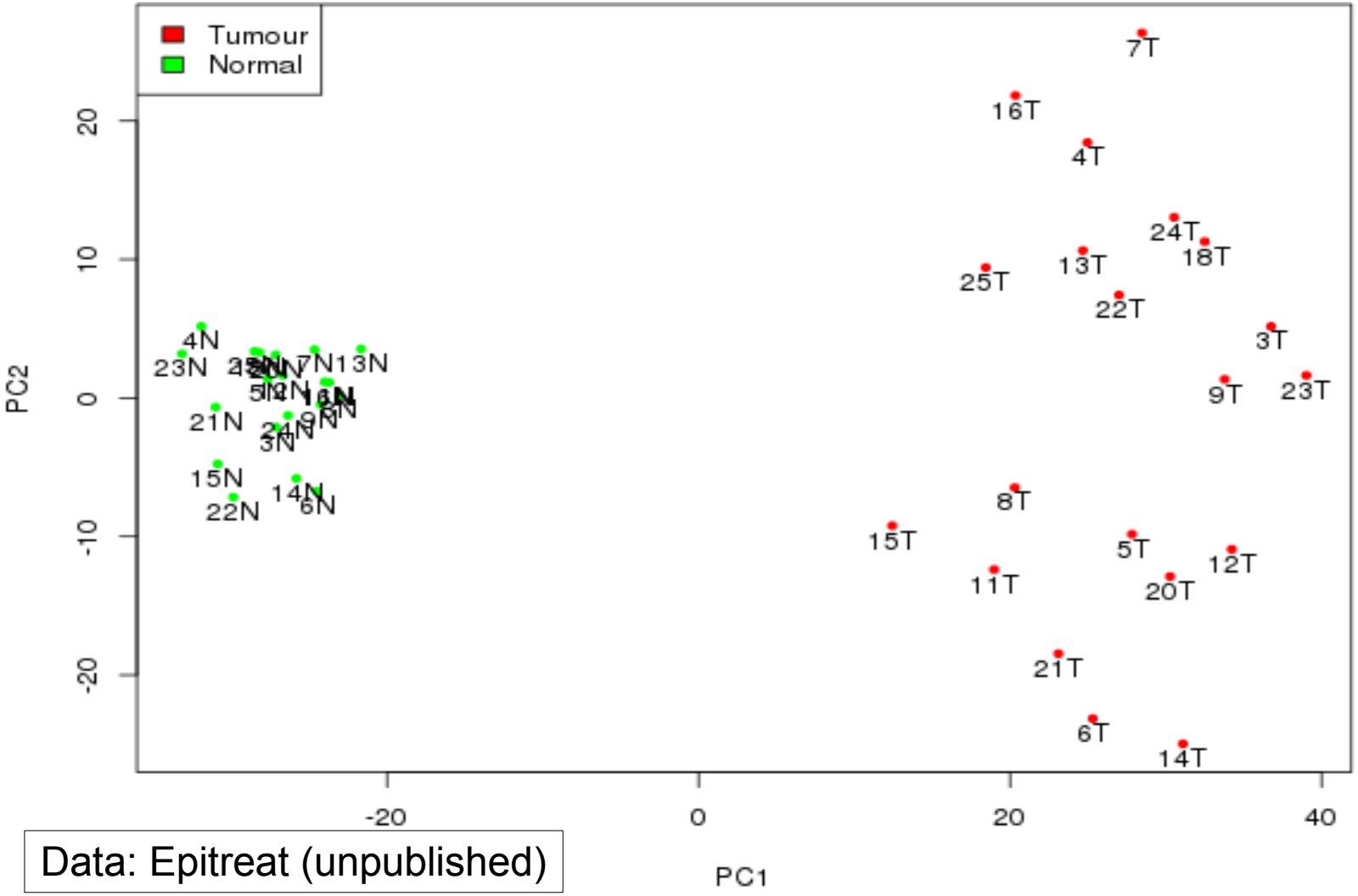
Further Components



Explained Variation of PC



Principle Component Analysis Tumour Example



Data: Epitreat (unpublished)

Summary: PCA / Clustering

Clustering:

- ➕ flexible (distance and clustering functions)
- ➕ Can display nested properties
- ➖ “Binary” decisions
- ➖ “One dimenisonal”

PCA:

- ➕ Separates independent factors
- ➕ PC interpretable as factors
- ➖ Needs sufficient samples
- ➖ Problems with nonlinear dependencies

Differential Gene Expression

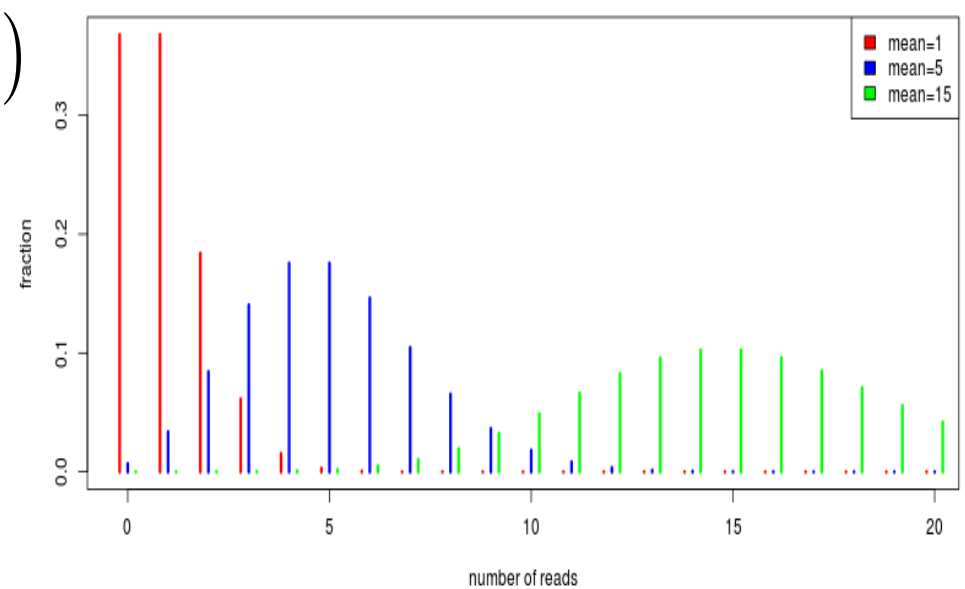
- p-value: probability that H0 is true: gene is expressed in A and B at the same level
  - assuming negative binomial distribution and estimated dispersion
  - If p is “very low” → H0 is “very unlikely”
- Test each gene
  - Multiple testing problem
  - FDR

Poisson Model

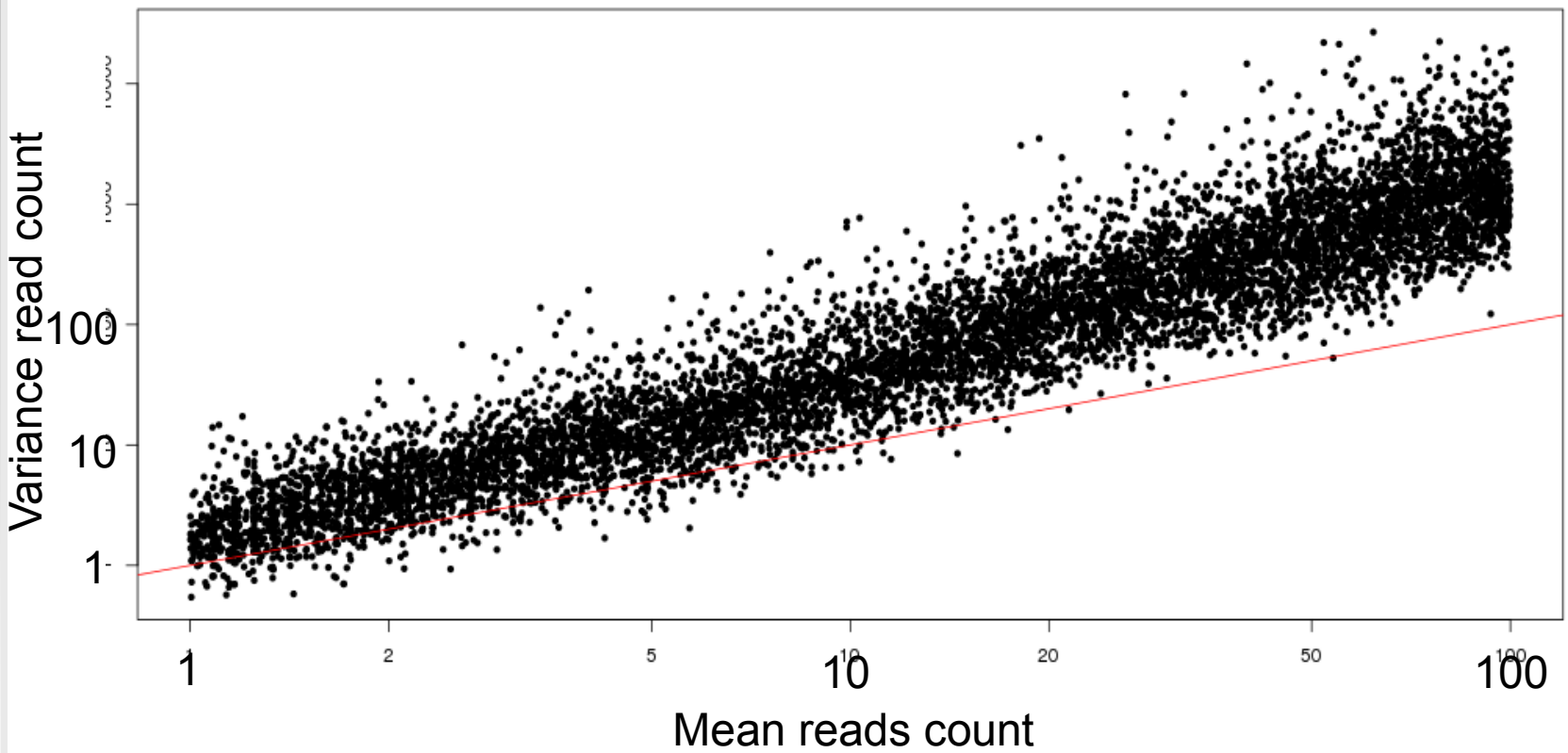
- N_i: total number of reads from sample *i*
- $\pi_{i,g}$: fraction of fragments from gene *g* in *i*
- y_{ig}: number of reads from *g* in *i*
- $\mu_{i,g} = E(Y_{i,g}) = N_i \pi_{i,g}$

$\rightarrow Y_{i,g} \sim Pois(\mu_{i,g})$

$\rightarrow Var(Y_{i,g}) = \mu_{i,g}$



Mean Variance Relation: Overdispersion



Samples are from different cells or organisms

Poisson Mixture Model

$$Y_{i,g} \sim Pois(\mu_{i,g} * \theta)$$

θ : Random variable with

- $E(\theta) = 1$
- $Var(\theta) = \Phi$

$Var(Y_{i,g}) = ?$ (backbord)

$$\rightarrow CV^2(Y_{i,g}) = CV^2_{technical} + CV^2_{biological}$$

Poisson Gamma Mixture

$$Y_{i,g} \sim Pois\left(\mu_{i,g} * \theta\right)$$

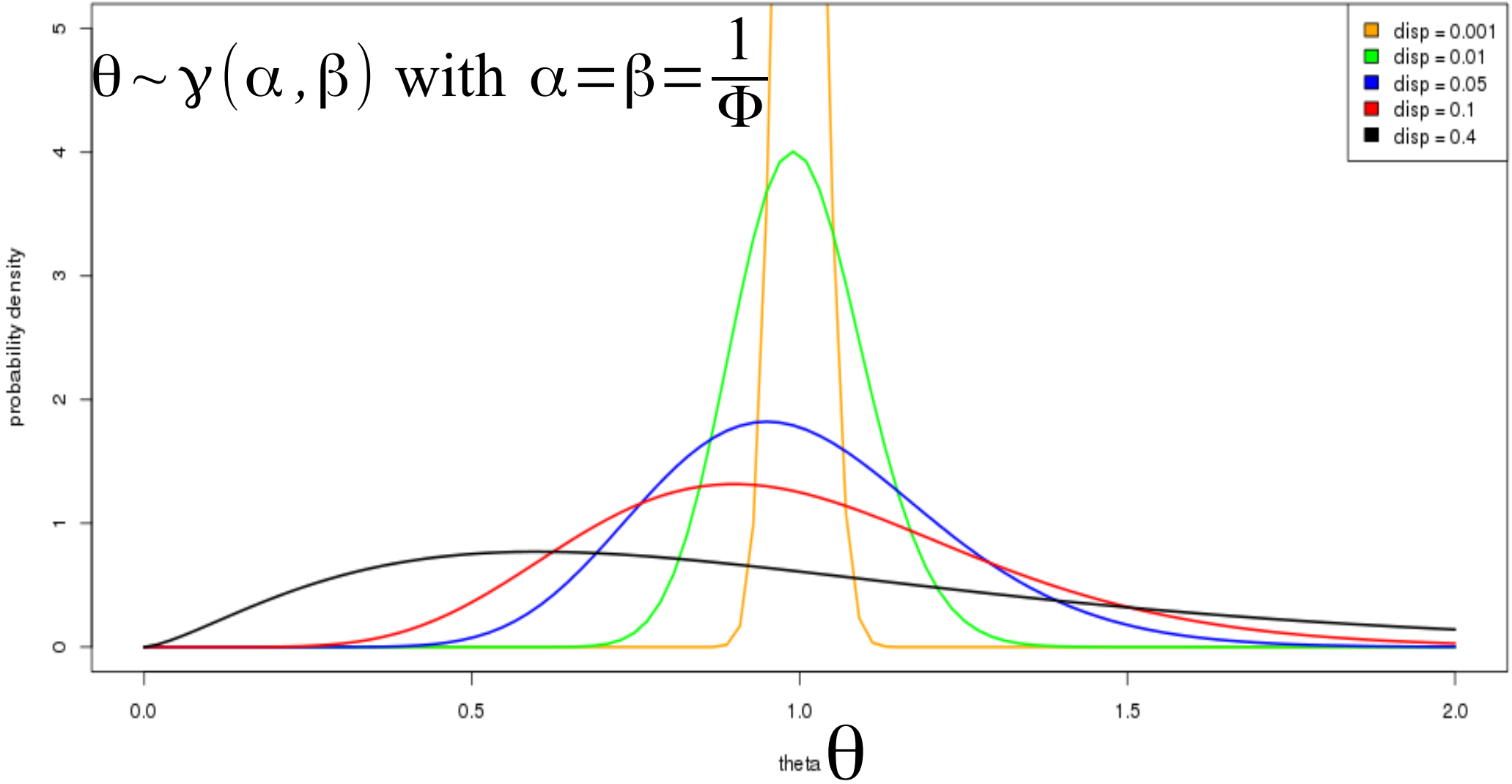
$$\theta \sim \gamma\left(\alpha, \beta\right) \text{ with } \alpha = \beta = \frac{1}{\Phi}$$

$$E\left(\theta\right) = \frac{\alpha}{\beta} = 1$$

$$Var\left(\theta\right) = \frac{\alpha}{\beta^2} = \Phi$$

Poisson Gamma Mixture

Gamma distribution



Poisson Gamma Mixture

$$Y_{i,g} \sim Pois(\mu_{i,g} * \theta)$$

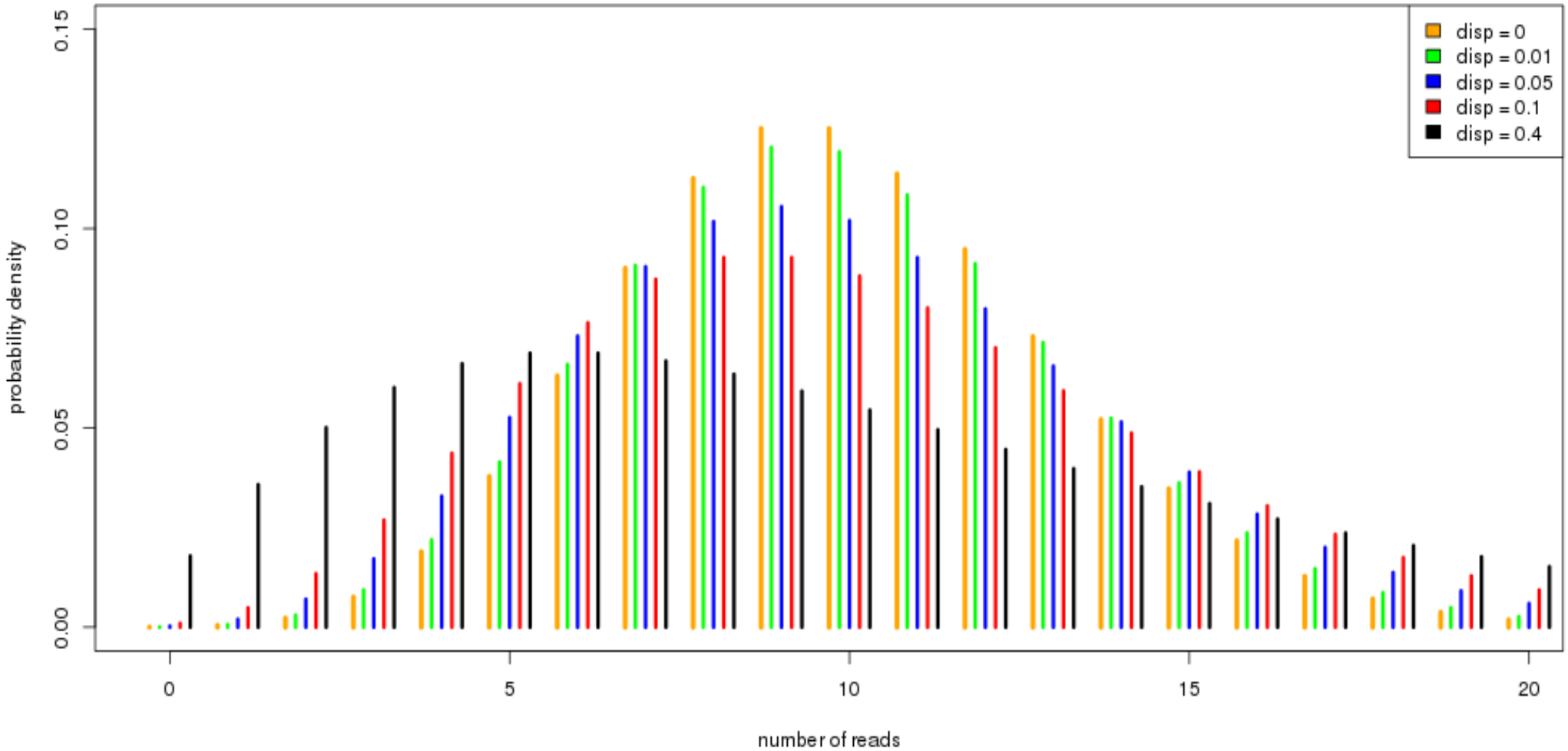
$$\theta \sim \gamma(\alpha, \beta) \text{ with } \alpha = \beta = \frac{1}{\Phi}$$

$$E(\theta) = \frac{\alpha}{\beta} = 1$$

$$Var(\theta) = \frac{\alpha}{\beta^2} = \Phi$$

$$\rightarrow Y_{i,g} \sim NB(k, r) \text{ with } k = \frac{1}{\Phi} \text{ and } r = \frac{1}{\mu * \Phi + 1}$$

Poisson Gamma Mixture



Estimating Dispersion

- edgeR: quantile adjusted conditional maximum likelihood estimate
- Problem: Few samples
  - Share information over genes

    → Common dispersion for all genes

    → Trended dispersion (expression level)

    → dispersion squeezed towards trend

    → dispersion cut by trend

MD Robinson (2008): Small-sample estimation of negative binomial dispersion, with applications to SAGE data. Biostatistics

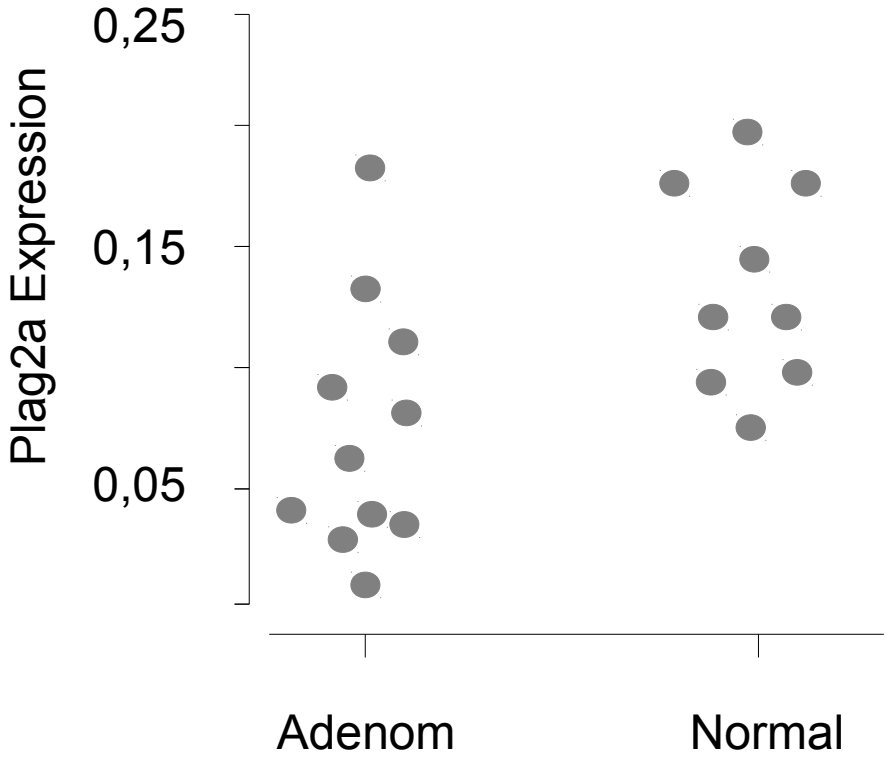
Testing Differential Expression

- H0: Reads for gene g are drawn from the same distribution for groups a and b
- y_T, y_a, y_b: # reads form gene in total, a and b
- N_T, N_a, N_b: total # reads in all, a and b
- $\mu_{0,a}, \mu_{0,b}: E(Y \mid H_{0,} N_x) = \frac{y_T}{N_T} * N_x$

$$pvalue = \sum_{i=0}^{y_T} pr(i \mid \mu_{0,a}, \Phi) * pr(y_T - i \mid \mu_{0,b}, \Phi) * I$$

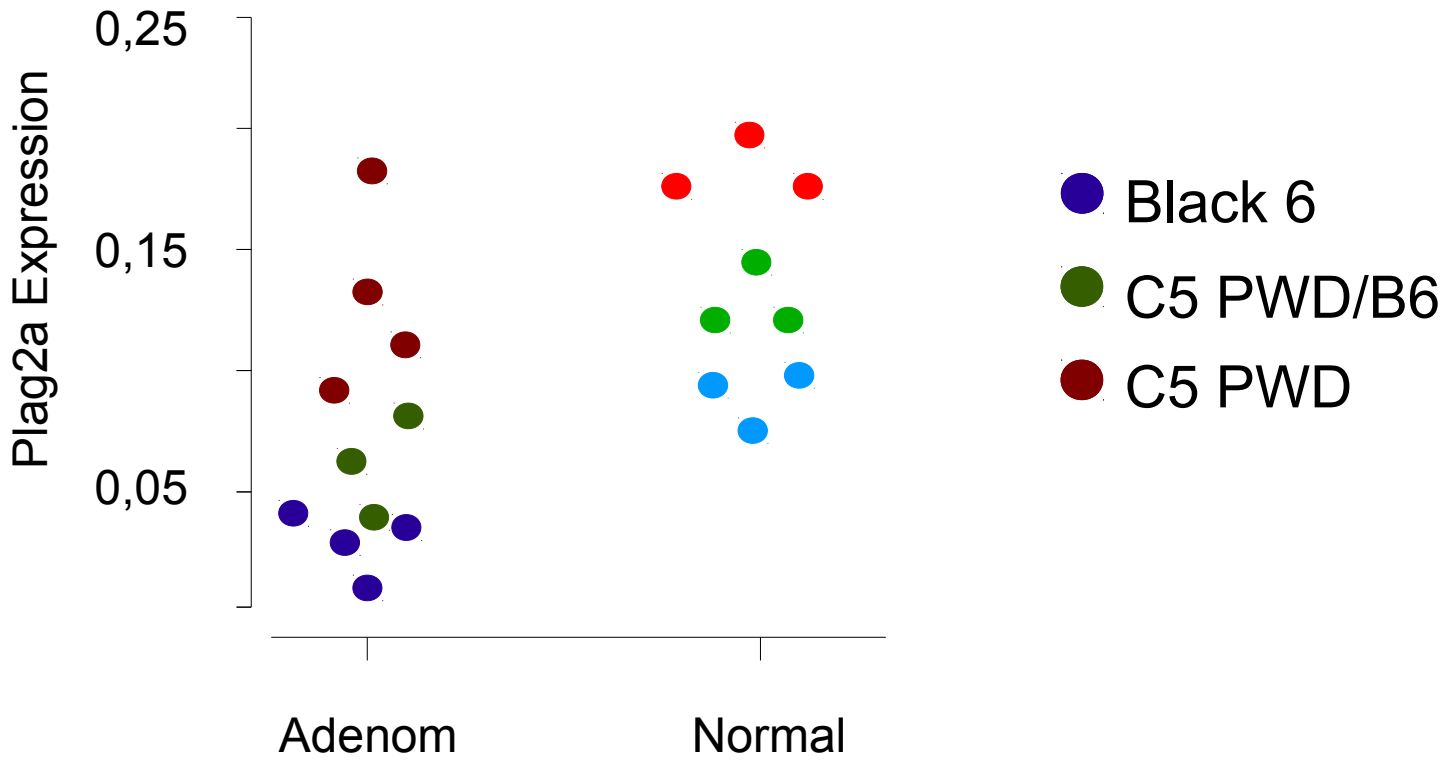
$$I \begin{cases} 1 \text{ if } pr_0(y_a) * pr_0(y_b) \geq pr_0(i) * pr_0(y_T - i) \\ 0 \text{ else} \end{cases}$$

Differentially Expressed?



More Complex Experimental Design

Example: APC-min mice



GLM

$$\log\left(\frac{\mu_{g,i}}{N_i}\right)=\beta_{0,g}+\beta_{1,g}*x_{1,i}+\ldots+\beta_{n,g}*x_{n,i}$$

$$\log(\mu_{g,i})=x_i^T\beta_g+\log(N_i) \longleftrightarrow$$

x_i: Vector of covariats from model matrix

β_g: Vector of regression coefficients

model matrix:

Sample	B6_ad1	B6_ad2	B6_no1	C5F1_ad1	C5F1_no1	C5_ad1	C5_no1
Intercept	1	1	1	1	1	1	1
Adenom	1	1	0	1	0	1	0
Chr5 PWD	0	0	0	1	1	2	2

GLM

$$\log(\mu_{g,i}) = x_i^T \beta_g + \log(N_i)$$

- Find estimates for beta for reduced (null) and full model
- Estimate dispersion under GLM
- Test for DE: likelihood ratio test

$$\frac{L(M_0)}{L(M_1)} \sim \chi^2$$

JD McCarthy et al.(2012): “Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation.” Nucleic Acids Research

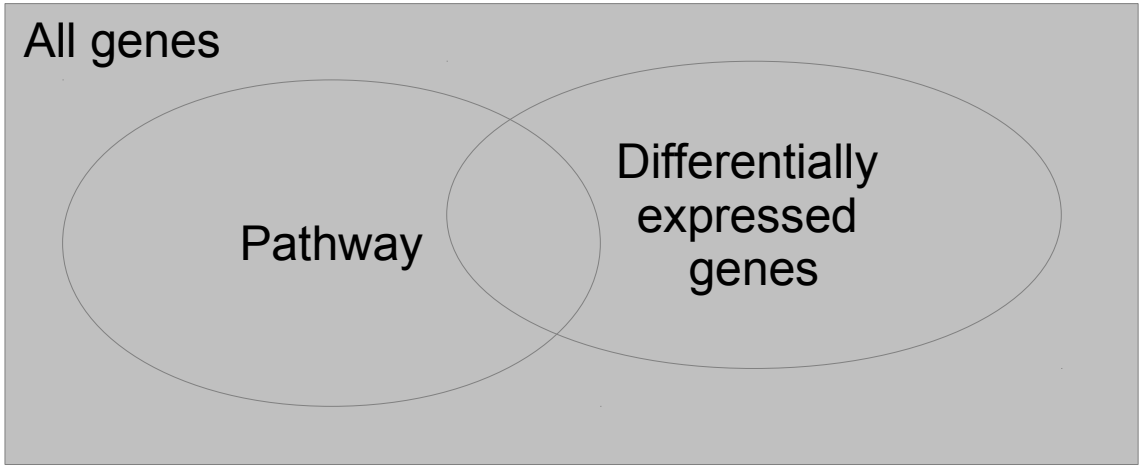
Summary: Tests for Differentially Expressed Genes

- Group A vs group B: exact NB test
- Multi factor test: GLM

→ List of differentially expressed genes

Interpretation of DE Genes

- Overrepresentation analysis
  - Web tools: CPDB, DAVID, ...
  - Integrate GO, Pathway databases, interaction databases, ...
  - Hypergeometric test: is overlap significant?



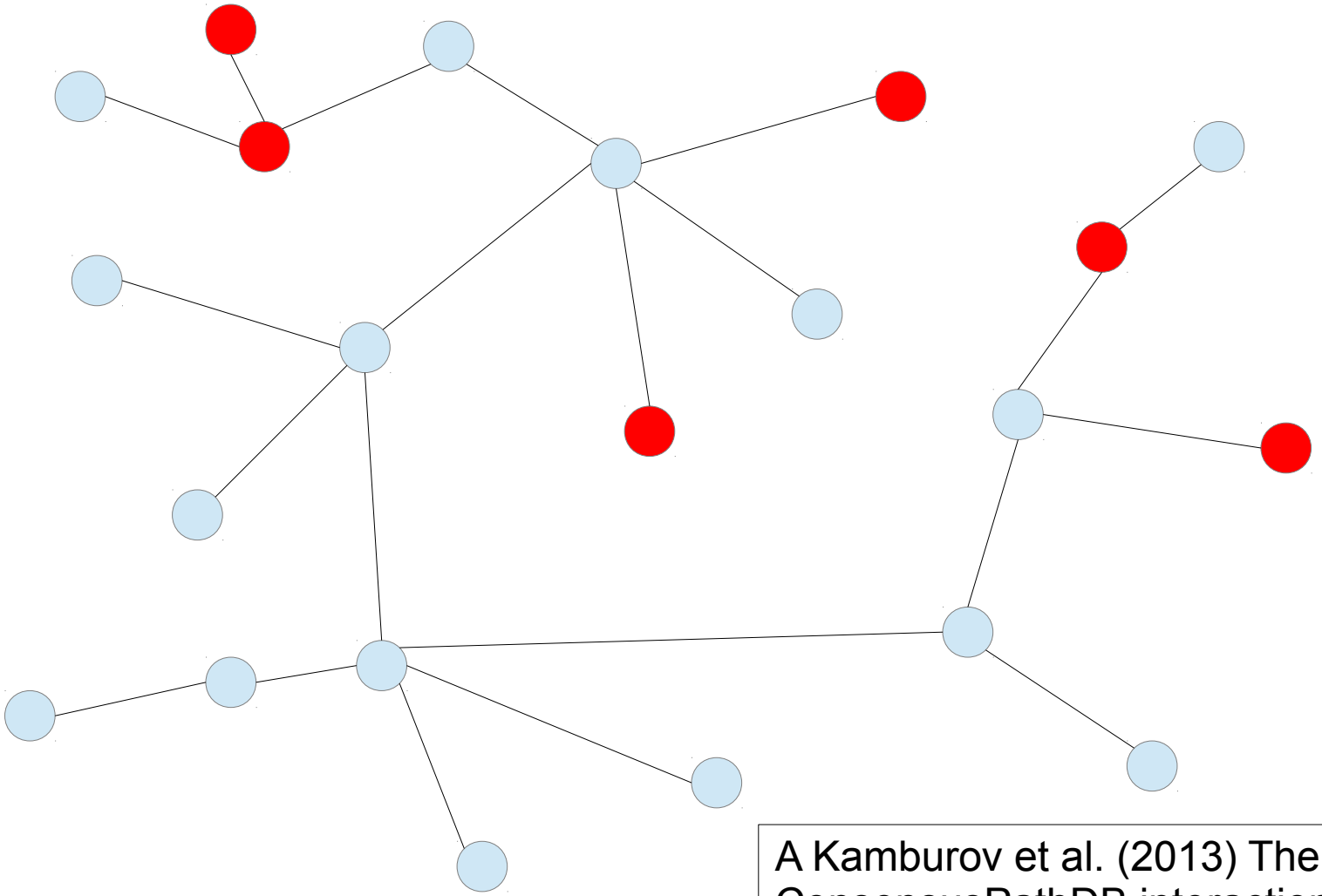
Pathway Analysis

CPDB Over-representation Analysis

pathway name	set size	candidates contained	p-value	q-value	pathway source
Direct p53 effectors	242 (135)	33 (24.4%)	6.52e-06	0.00729	PID
fibrinolysis pathway	20 (14)	8 (57.1%)	3.4e-05	0.019	BioCarta
p53 signaling pathway - Homo sapiens (human)	69 (67)	19 (28.4%)	7.09e-05	0.0244	KEGG
Facilitative Na+-independent glucose transporters	12 (12)	7 (58.3%)	9.15e-05	0.0244	Reactome
Benzo(a)pyrene metabolism	9 (9)	6 (66.7%)	0.000109	0.0244	Wikipathways
Oxidative Stress	29 (26)	10 (38.5%)	0.000251	0.0467	Wikipathways

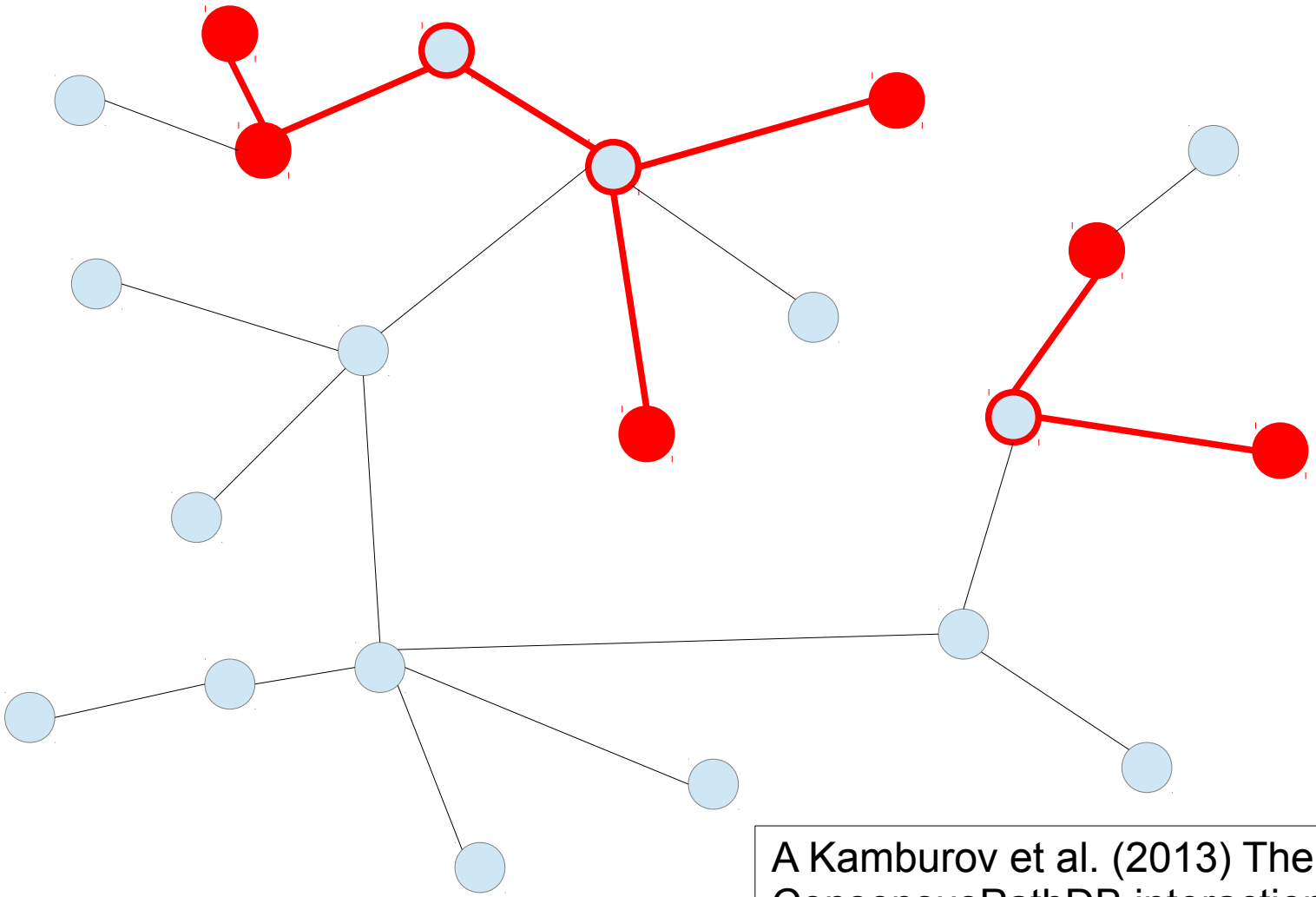
Kamburov, A. et al. (2009) ConsensusPathDB--a database for integrating human interaction networks. Nucleic Acids Res.37:D623-628.

CPDB: Induced networks



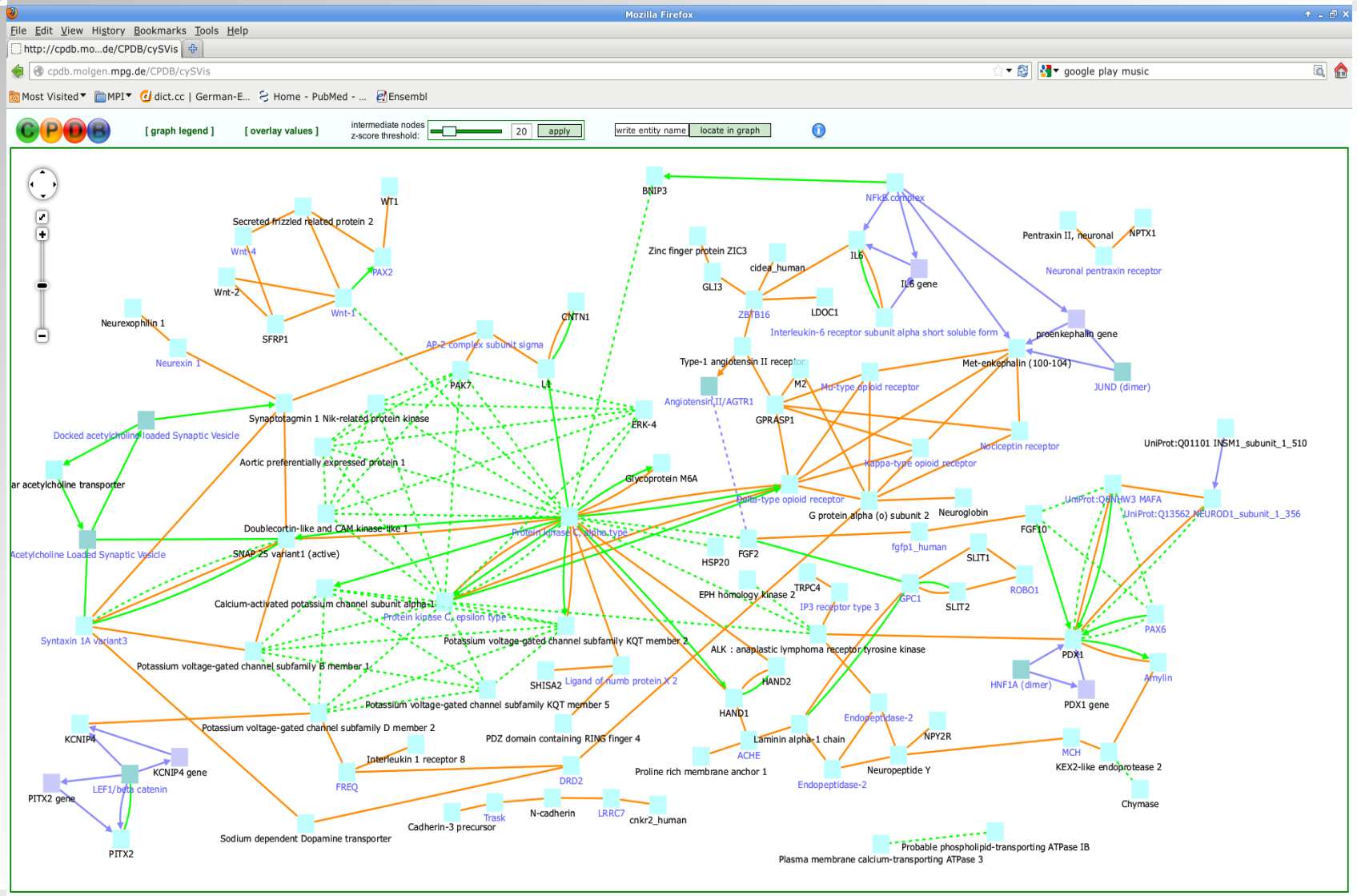
A Kamburov et al. (2013) The ConsensusPathDB interaction database: 2013 update. Nucleic Acids Res.

CPDB: Induced networks

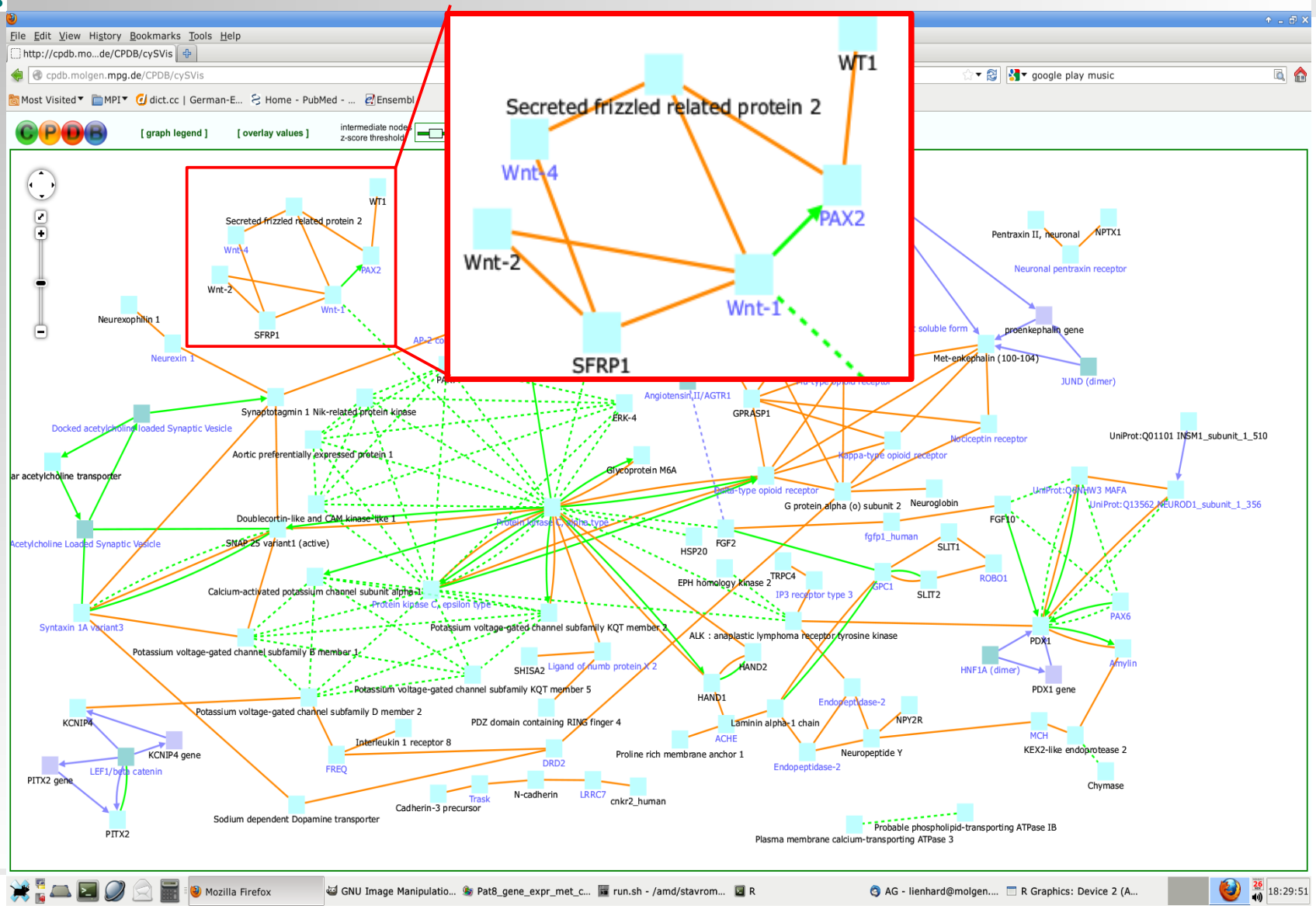


A Kamburov et al. (2013) The ConsensusPathDB interaction database: 2013 update. Nucleic Acids Res.

Induced Network



Induced Network



Summary

- Quality Control: Do data look OK?
- Mapping: Handle reads across exon boundaries
- Quantification: Gene/isoform/exon level
- Exploratory analysis: Relation of Samples?
- Differential Expression: A vs B or GLM?
- Over representation and network analysis: Make sense out of gene lists.