Two group comparisons

Keegan Korthauer

January 24, 2023

STAT 540

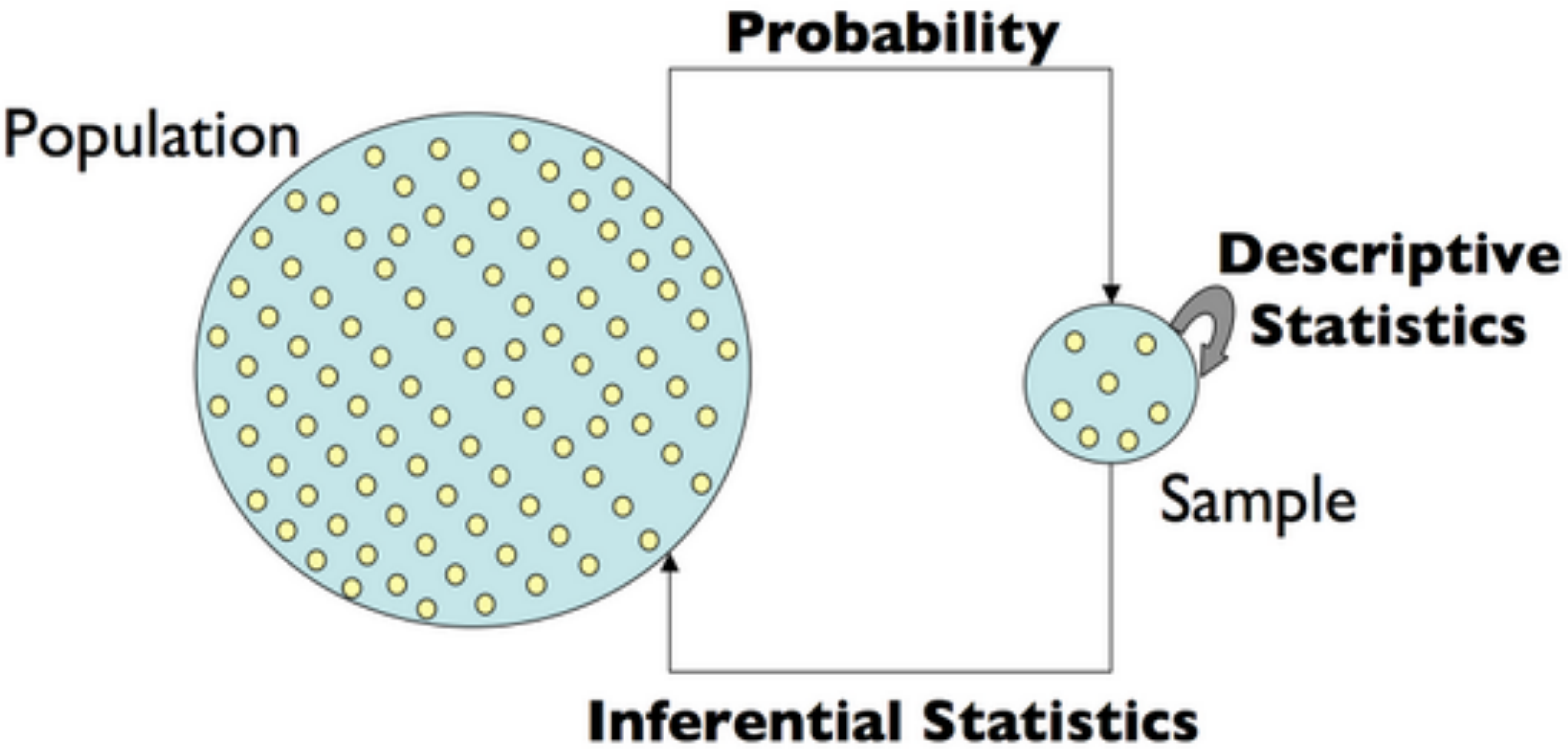
Reminders

- Intro Assignment due **today** at 11:59pm
- Project groups posted to Canvas last week
- Project Proposal Lightning Talks in class Tuesday Jan 31 (Slides due Monday Jan 30 11:59pm)

Today’s learning objectives

- Understand **how** and **when** to carry out a t-test for comparing two population means
- Identify when alternative approaches (e.g. nonparametric) are more appropriate
- Avoid common pitfalls in interpretation of hypothesis tests and p-values

Central dogma of statistics

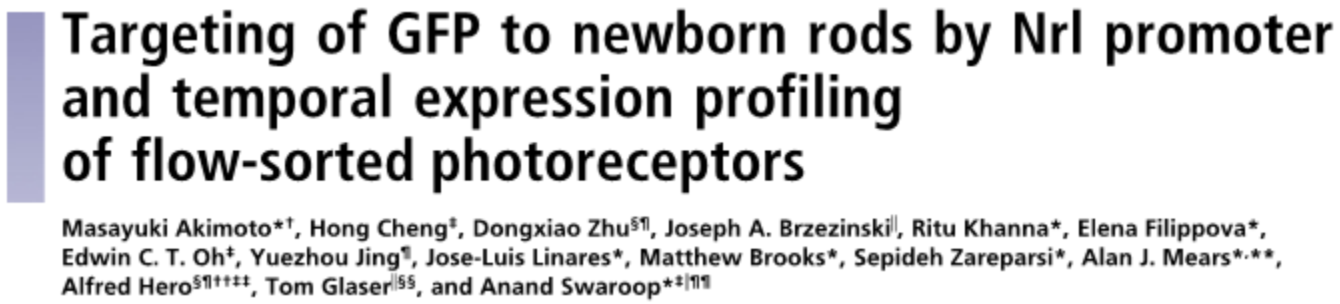


We want to understand a **population** (e.g. all individuals with a certain disease) but we can only study a **random sample** from it

Image source: Josh Akey’s Lecture notes

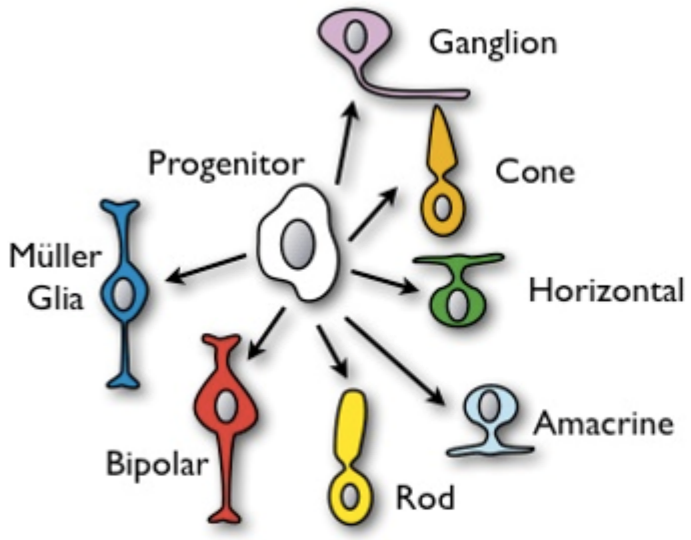
Hypothesis Testing in Genomics

- Retina presents a model system for investigating **regulatory networks** underlying neuronal differentiation
- **Nrl** transcription factor is known to be important for Rod development



Targeting of GFP to newborn rods by Nrl promoter and temporal expression profiling of flow-sorted photoreceptors

Masayuki Akimoto[*,†], Hong Cheng[‡], Dongxiao Zhu[§,¶], Joseph A. Brzezinski[||], Ritu Khanna[*], Elena Filippova[*], Edwin C. T. Oh[‡], Yuezhou Jing[¶], Jose-Luis Linares[*], Matthew Brooks[*], Sepideh Zareparsi[*], Alan J. Mears[*,**], Alfred Hero[§,¶,††,‡‡], Tom Glaser[||,§§], and Anand Swaroop[*,‡,||,¶¶]



Akimoto et al. (2006)

What happens if you delete *Nrl*?

Why a Hypothesis Test?

From the Akimoto et al. (2006) paper:

> “we hypothesized that Nrl is the ideal transcription factor to gain insights into gene expression changes …”

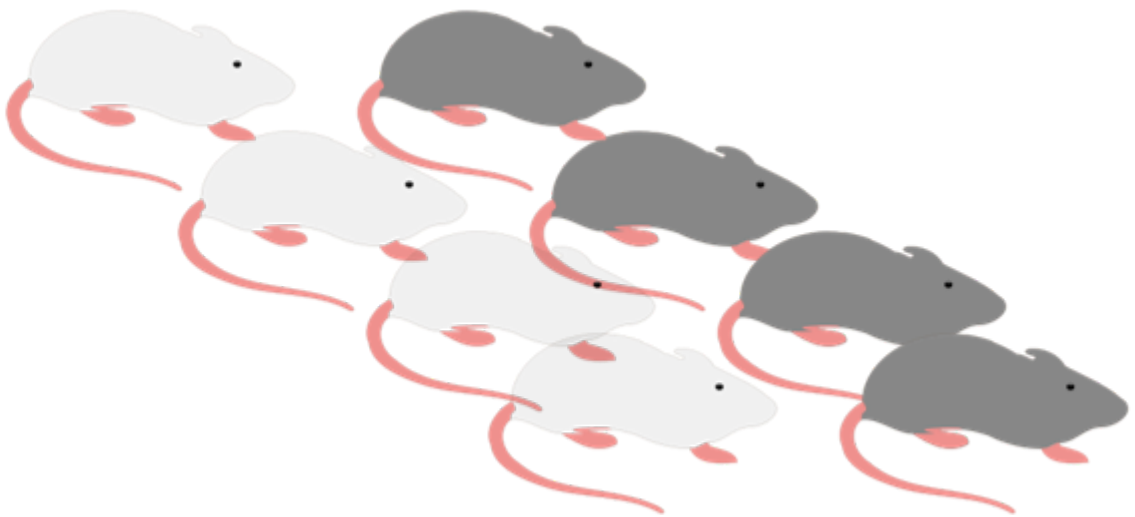
ⓘ **Biological question**

Is the expression level of gene *A* affected by knockout of the *Nrl* gene?

We can use **statistical inference** to answer this biological question!

Statistical inference

- Let’s observe and study a **random sample** to make conclusions about a population: measure gene expression on a random sample of mice
- **Experimental design**:
  - 5 developmental stages (E16, P2, P6, P10, 4Weeks)
  - 2 genotypes: Wild type (WT), Nrl Knockout (NrlKO)
  - 3-4 replicates for each combination



Reading in / exploring the data

- Data obtained from the Gene Expression Omnibus (GEO) repository (accession GSE4051)
- Load directly into R session using `GEOquery` package - see code below (which also refactors some of the metadata for convenience)
- Practice with this in Seminars 4 and 5 (Review lecture 3 for general principles)

```r
# load libraries
library(GEOquery)
library(gridExtra)
library(tidyverse)
theme_set(theme_bw(base_size = 20))

# download and read in dataset
eset <- getGEO("GSE4051", getGPL = FALSE)[[1]]

# recode time points
pData(eset) <- pData(eset) %>%
  mutate(sample_id = geo_accession) %>%
  mutate(dev_stage =  case_when(
    grepl("E16", title) ~ "E16",
    grepl("P2", title) ~ "P2",
    grepl("P6", title) ~ "P6",
    grepl("P10", title) ~ "P10",
    grepl("4 weeks", title) ~ "4_weeks"
  )) %>%
```

```
ExpressionSet (storageMode: lockedEnvironment)
assayData: 45101 features, 39 samples
  element names: exprs
protocolData: none
phenoData
  sampleNames: GSM92610 GSM92611 ... GSM92648 (39 total)
  varLabels: title geo_accession ... genotype (39 total)
  varMetadata: labelDescription
featureData: none
experimentData: use 'experimentData(object)'
  pubMedIds: 16505381
Annotation: GPL1261
```

Two example genes: *Irs4* and *Nrl*

ⓘ Biological questions

1. Is the expression level of gene *Irs4* truly different in NrlKO compared to WT?
2. Is the expression level of gene *Nrl* truly different in NrlKO compared to WT?

We can’t answer these questions in general; we can *only* study these genes in collected data (**gene expression values from a random sample of mice**)

Extract the two genes of interest

```r
# function to convert to tidy format
toLongerMeta <- function(expset) {
    stopifnot(class(expset) == "ExpressionSet")

    expressionMatrix <- exprs(expset) %>%
    as.data.frame() %>%
    rownames_to_column("gene") %>%
    pivot_longer(cols = !gene,
                 values_to = "Expression",
                 names_to = "sample_id") %>%
    left_join(pData(expset) %>% select(sample_id, dev_s
          by = "sample_id")
  return(expressionMatrix)
}

# convert to tidy format and extract two genes
twoGenes <- toLongerMeta(eset) %>%
  filter(gene %in% c("1422248_at", "1450946_at")) %>%
  mutate(gene = ifelse(gene == "1422248_at", "Irs4", "N
```

```
# A tibble: 78 × 5
   gene  sample_id Expression dev_stage genotype
   <chr> <chr>          <dbl> <fct>     <fct>
 1 Irs4  GSM92610        7.71 4_weeks   NrlKO
 2 Irs4  GSM92611        7.77 4_weeks   NrlKO
 3 Irs4  GSM92612        7.73 4_weeks   NrlKO
 4 Irs4  GSM92613        7.57 4_weeks   NrlKO
 5 Irs4  GSM92614        7.95 E16       NrlKO
 6 Irs4  GSM92615        7.52 E16       NrlKO
 7 Irs4  GSM92616        8.08 E16       NrlKO
 8 Irs4  GSM92617        7.71 P10       NrlKO
 9 Irs4  GSM92618        7.87 P10       NrlKO
10 Irs4  GSM92619        7.75 P10       NrlKO
# … with 68 more rows
```

What do you notice?

Visualizing *Irs4* and *Nrl* genes in our sample

Code Output

```r
irsLim <- filter(twoGenes, gene == "Irs4") %>%
  ggplot(aes(y = Expression, x = genotype, colour = genotype)) +
  geom_jitter(size = 2, alpha = 0.8, width = 0.2) +
  labs(title = "Irs4 gene") +
  theme(legend.position = "none")

nrlLim <- filter(twoGenes, gene == "Nrl") %>%
  ggplot(aes(y = Expression, x = genotype, colour = genotype)) +
  geom_jitter(size = 2, alpha = 0.8, width = 0.2) +
  labs(title = "Nrl gene") +
  theme(legend.position = "none")

grid.arrange(irsLim + ylim(5, 13), nrlLim + ylim(5, 13), ncol = 2)
```

Formulating our hypotheses

- **Experimental design:** (ignoring developmental time for now)
  - 2 conditions: WT *vs* NrlKO
  - observe the expression of many genes in a random sample of ~20 mice from each condition
- **Biological hypothesis:** for *some* genes, the expression levels are different between conditions
- **Statistical hypotheses:** (for each gene $g = 1, \ldots, G$)
  - H_0 (null hypothesis): the expression level of gene g is the ***same*** in both conditions
  - H_A (alternative hypothesis): the expression level of gene g is ***different*** between conditions

How might we test H_0?

Notation[1]

Random variables and estimates (we can observe):

- Y_i : expression of gene g in the WT sample i
- Z_i: expression of gene g in NrlKO sample i
- $Y_1, Y_2, \ldots, Y_{n_Y}$: a **random sample** of size n_Y WT mice
- $Z_1, Z_2, \ldots, Z_{n_Z}$: a **random sample** of size n_Z NrlKO mice
- $\bar{Y} = \frac{\sum_{i=1}^{n_Y} Y_i}{n_Y}$: sample mean of gene g expression from WT mice
- $\bar{Z} = \frac{\sum_{i=1}^{n_Z} Z_i}{n_Z}$: sample mean of gene g expression from NrlKO mice

[1] ignoring subscript for gene g for now

Notation[1]

Population parameters (unknown/unobservable):

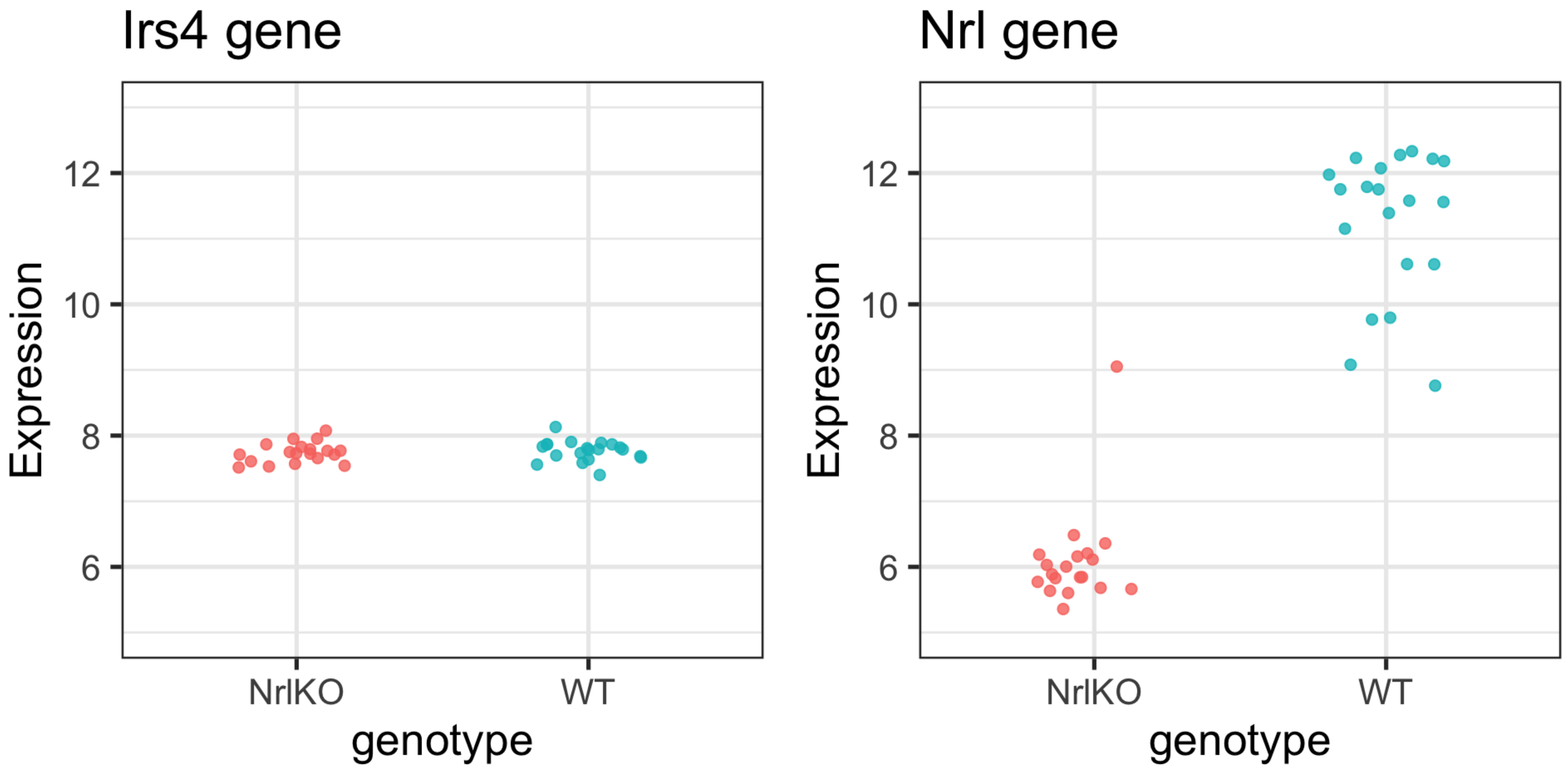
$\mu_Y = E[Y]$: the (population) expected expression of gene g in WT mice

$\mu_Z = E[Z]$: the (population) expected expression of gene g in NrlKO mice

[1] ignoring subscript for gene g for now

Is there enough evidence to reject H_0?

$$H_0 : \mu_Y = \mu_Z$$



Statistical Inference: random samples are used to learn about the population

What we observe: sample averages: $\bar{Y}$ vs $\bar{Z}$

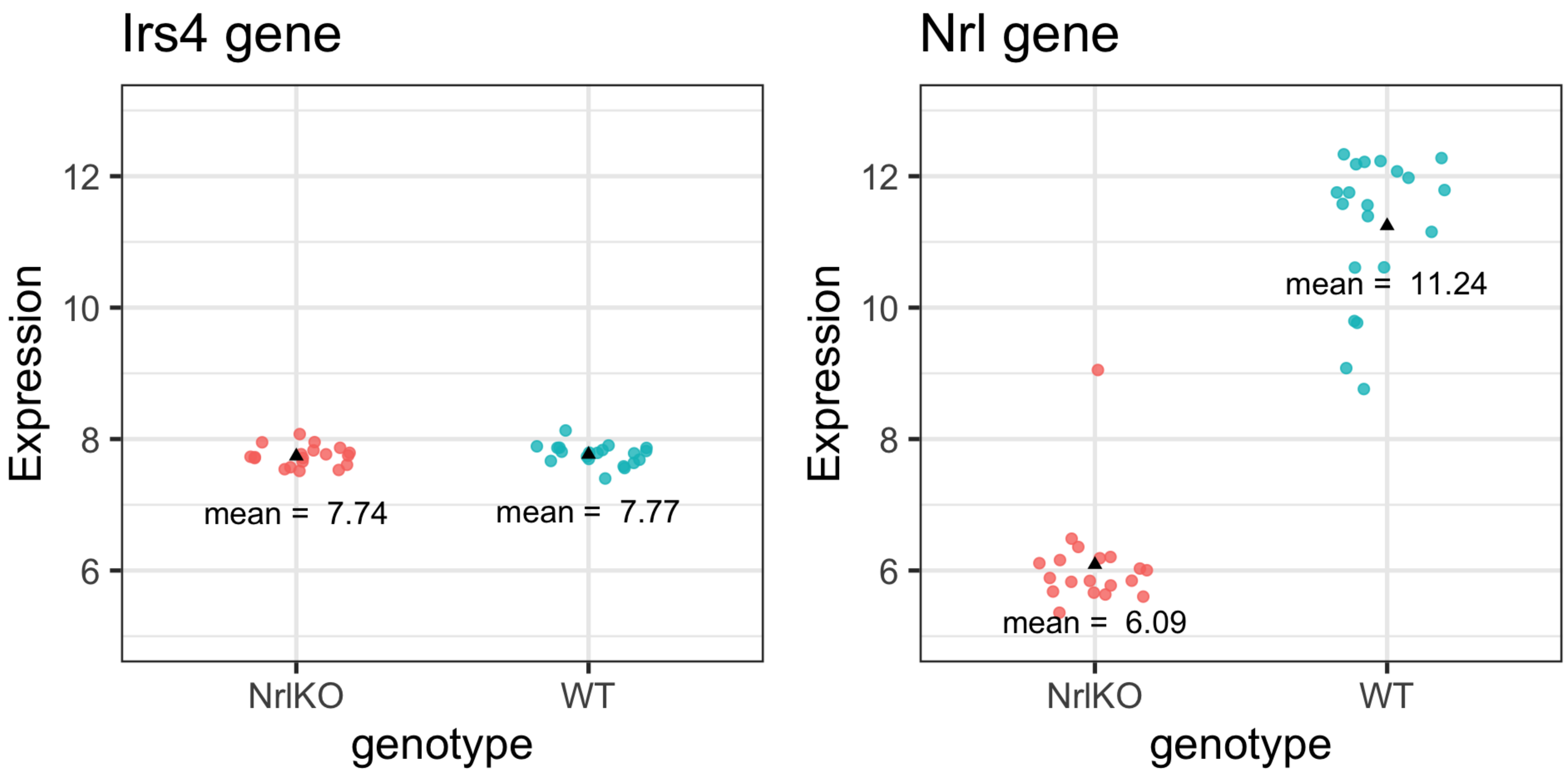
```r
# calculate mean of each gene and genotype
meanExp <- twoGenes %>%
  group_by(gene, genotype) %>%
  summarize(meanExpr = mean(Expression)) %>%
  pivot_wider(names_from = genotype, values_from = mean
  mutate(diffExp = NrlKO - WT)
meanExp
```

```
# A tibble: 2 × 4
# Groups:   gene [2]
  gene  NrlKO    WT diffExp
  <chr> <dbl> <dbl>   <dbl>
1 Irs4   7.74  7.77 -0.0261
2 Nrl    6.09 11.2  -5.15
```

This code uses tidy data wrangling functions to calculate:

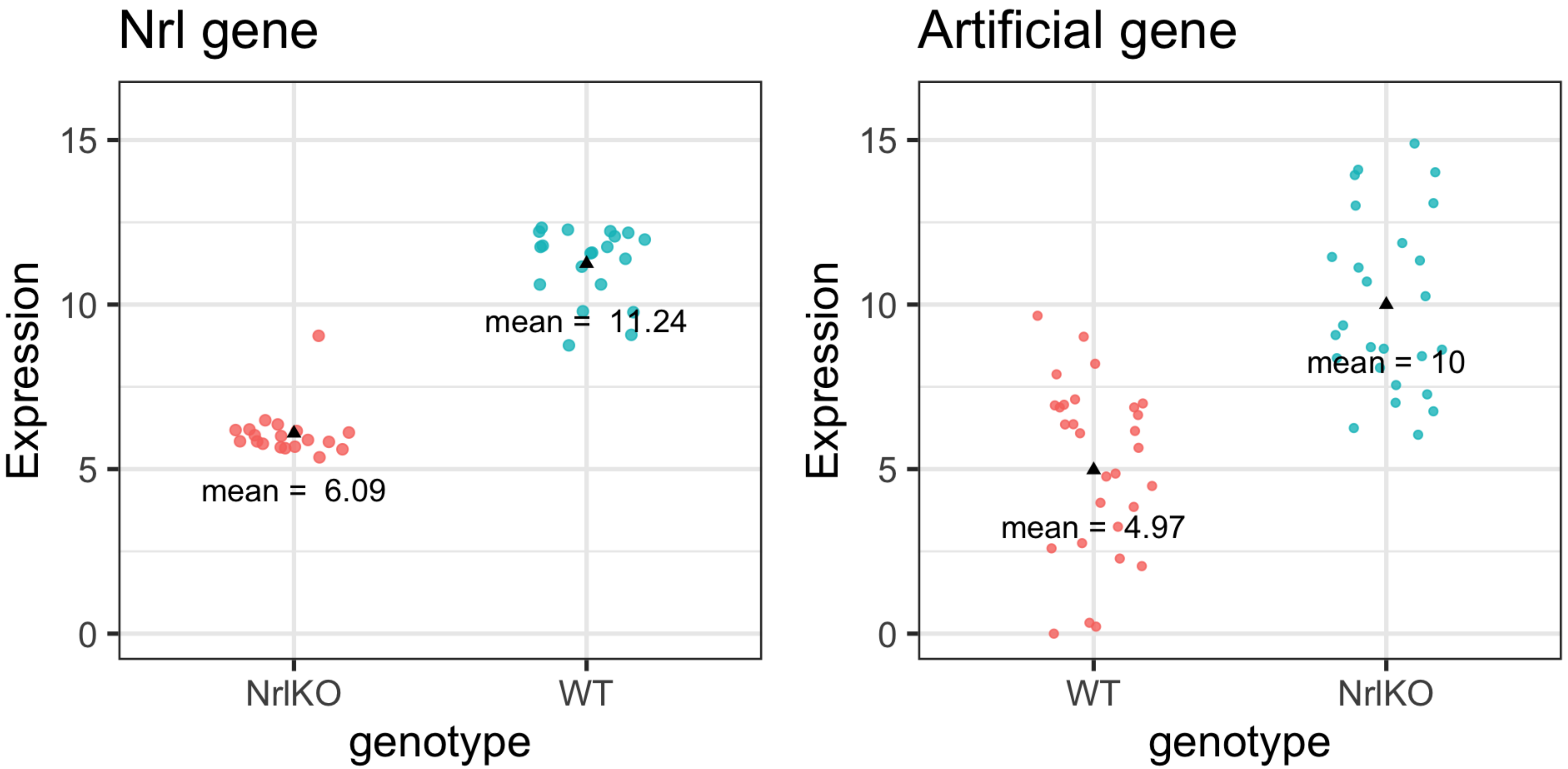
- the mean expression of each gene per genotype group
- the difference in mean expression of each gene in Nrl KO vs WT groups

Is the difference between $\bar{Y}$ and $\bar{Z}$ enough to reject H_0?



- The sample means, $\bar{Y}$ vs $\bar{Z}$, by themselves are not enough to make conclusions about the population
- What is a “large” difference? “Large” relative to what?

Consider this artificial version of *Nrl*

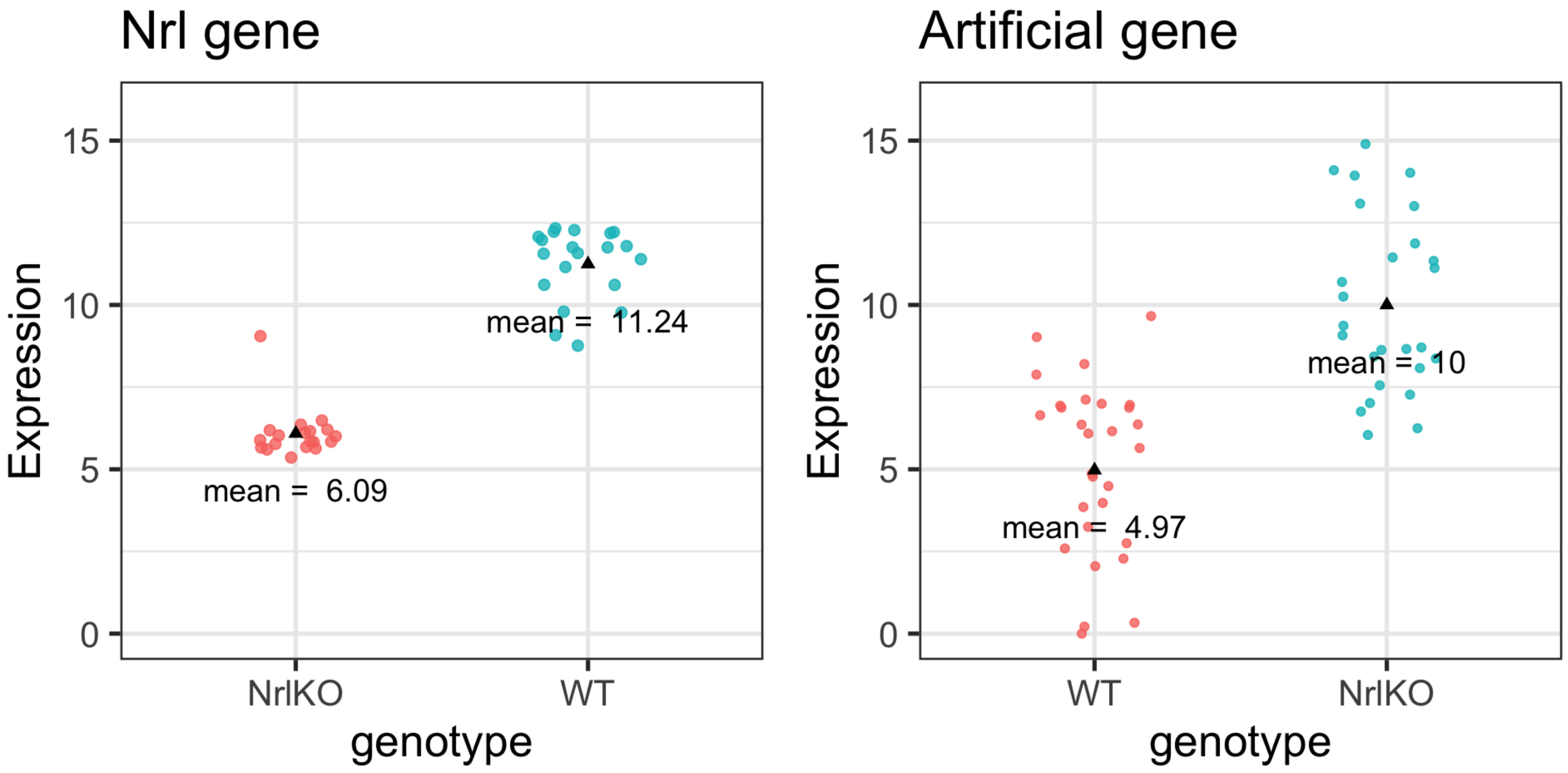


What can we use to interpret the size of the mean difference? $\frac{\bar{Y}-\bar{Z}}{??}$

“Large” difference relative to what?

“Large” relative to the **observed variation:**

$$\frac{\bar{Y} - \bar{Z}}{\sqrt{Var(\bar{Y} - \bar{Z})}}$$



Quantifying observed variation

- Recall that if $Var(Y_i) = \sigma_Y^2$, then $Var(\bar{Y}) = \frac{\sigma_Y^2}{n_Y}$
- Assume that the random variables within each group are *independent and identically distributed* (iid), and that the groups are independent. More specifically, that
  1. $Y_1, Y_2, \ldots, Y_{n_Y}$ are iid,
  2. $Z_1, Z_2, \ldots, Z_{n_Z}$ are iid, and
  3. Y, Z are independent. Then, it follows that

$$Var(\bar{Z} - \bar{Y}) = \frac{\sigma_Z^2}{n_Z} + \frac{\sigma_Y^2}{n_Y}$$

- If we also assume equal population variances: $\sigma_Z^2 = \sigma_Y^2 = \sigma^2$, then

$$Var(\bar{Z} - \bar{Y}) = \frac{\sigma_Z^2}{n_Z} + \frac{\sigma_Y^2}{n_Y} = \sigma^2 \left[\frac{1}{n_Z} + \frac{1}{n_Y} \right]$$

Reflect

🚧 **Stop!**

But how can we calculate population variance σ if it is **unknown**?

…using the sample variances (combined, somehow)!

Combining sample variances

Plug these sample variances into your chosen formula for the variance of the difference of sample means:

- Assuming **equal** variance of Y’s and Z’s

$$\widehat{Var}(\bar{Z}_n - \bar{Y}_n) = \hat{\sigma}^2_{\text{pooled}} \left[\frac{1}{n_Y} + \frac{1}{n_Z} \right]$$

$$\hat{\sigma}^2_{\text{pooled}} = S^2_Y \frac{n_Y - 1}{n_Y + n_Z - 2} + S^2_Z \frac{n_z - 1}{n_Y + n_Z - 2}$$

- Assuming **unequal** variance of Y’s and Z’s (Welch’s t-test)

$$\widehat{Var}(\bar{Z}_n - \bar{Y}_n) = \hat{\sigma}^2_{\bar{Z}_n - \bar{Y}_n} = \frac{S^2_Y}{n_Y} + \frac{S^2_Z}{n_Z}$$

Recall: the ‘hat’ (^) is used to distinguish an ‘estimate’ from a ‘parameter’

Test Statistic

‘Manual’ calculation of $T = \frac{\bar{Z}_n - \bar{Y}_n}{\sqrt{\hat{Var}(\bar{Z}_n - \bar{Y}_n)}}$ (for illustration):

Pooled variances | t-statistics

```r
## compute sample variance of each gene/genotype
theVars <- twoGenes %>%
  group_by(gene, genotype) %>%
  summarize(groupVar = var(Expression))

## compute sample size in each group
nY <- with(twoGenes, sum(genotype == "WT" & gene == "Nrl"))
nZ <- with(twoGenes, sum(genotype == "NrlKO" & gene == "Nrl"))

## assuming unequal true variance
s2DiffWelch <- theVars %>%
    mutate(s2Welch = groupVar / ifelse(genotype == "WT", nY, nZ)) %>%
    group_by(gene) %>%
    summarize(s2Welch = sum(s2Welch))
meanExp$s2DiffWelch <- s2DiffWelch$s2Welch

## assuming equal true variance
s2Pooled <- theVars %>%
```

Can we now say whether the observed differences are ‘big’?

The difference is about half a standard deviation for *Irs4* and ~17 standard deviations for *Nrl*

What to do with this statistic?

- The test statistic T is a **random variable** because it's based on our **random sample**
- We need a measure of its **uncertainty** to determine how extreme our observed T is:
  - If we were to repeat the experiment many times, what's the probability of observing a value of T **as extreme** as the one we observed?
- We need a probability distribution!
- However, this is unknown to us so we need to **make more assumptions**

Null distribution assumptions

- If we know how our statistic behaves when the *null hypothesis is true*, then we can evaluate how extreme our observed data is
  - The **null distribution** is the probability distribution of T under H_0

- Let's assume that Y_i and Z_i follow (unknown) probability distributions called F and G:

$$(Y_i \sim F, \text{ and } Z_i \sim G)$$

- Depending on the assumptions we make about F and G, theory tells us specific **null distributions** for our test statistic

Willing to assume that F and G are normal distributions?

2-sample *t*-test (equal variances):

$$T \sim t_{n_Y + n_Z - 2}$$

Welch's 2-sample *t*-test (unequal variances):

$$T \sim t_{<something\ ugly>}$$

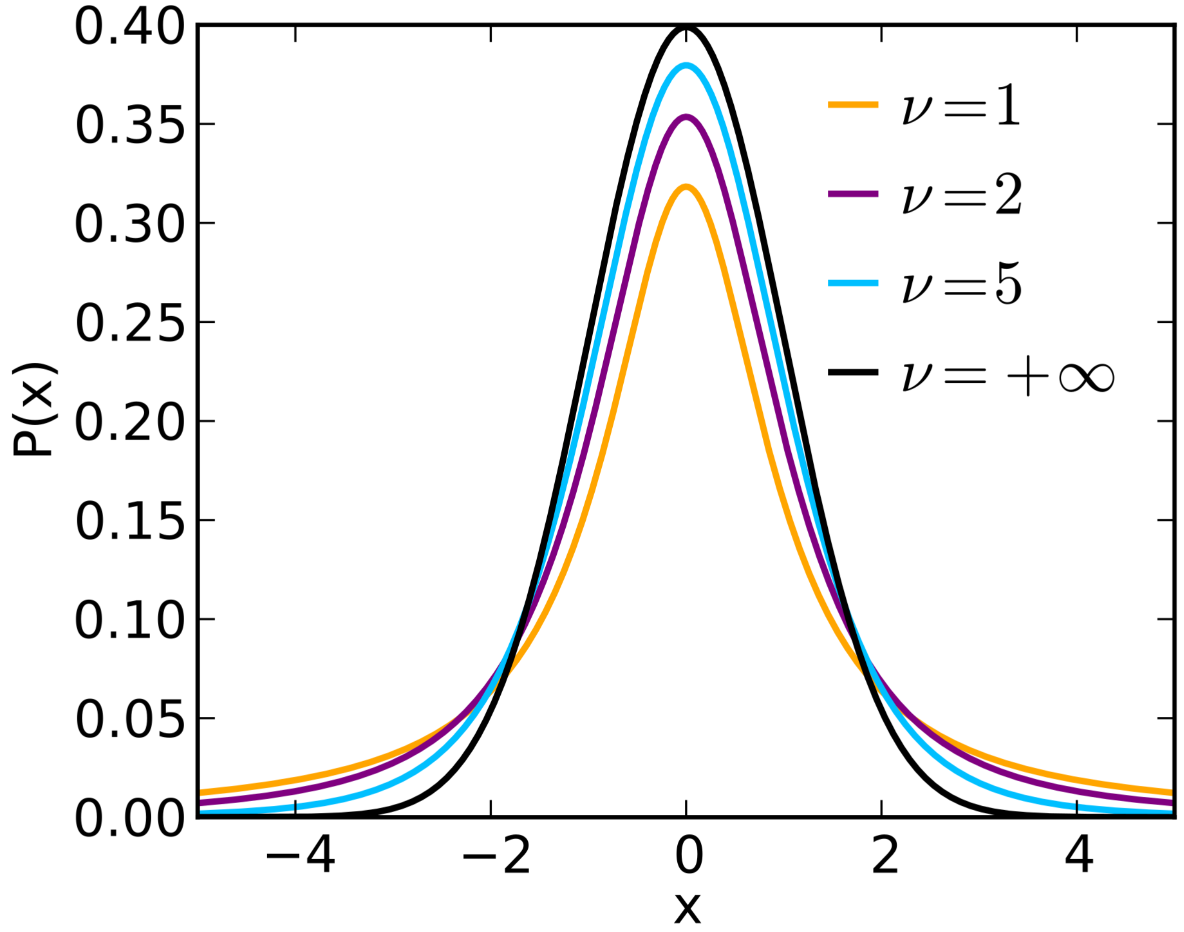
Unwilling to assume that F and G are normal distributions?

But you feel that n_Y and n_Z are large enough?

Then the t-distributions above (or even a normal distribution) are decent approximations

Student's *t*-distribution

Summary: $T = \frac{\bar{Z}_n - \bar{Y}_n}{\sqrt{\hat{Var}(\bar{Z}_n - \bar{Y}_n)}}$ is a **random variable**, and under certain assumptions, we can prove that T follows a *t*-distribution



Recall that the *t*-distribution has one parameter: df = degrees of freedom

Hypothesis testing: Step 1

1. Formulate your hypothesis as a statistical hypothesis

In our example:

$$H_0 : \mu_Y = \mu_Z \text{ vs } H_A : \mu_Y \neq \mu_Z$$

Hypothesis testing: Step 2

2a. Define a test statistic

In our example: 2-sample *t*-test

2b. Compute the observed value for the test statistic

For our two example genes:

```r
twoGenes %>%
  group_by(gene) %>%
  summarize(t = t.test(Expression ~ genotype,
                            var.equal=TRUE)$statistic)
```

```
# A tibble: 2 × 2
  gene        t
  <chr>   <dbl>
1 Irs4   -0.529
2 Nrl   -16.8
```

Tip

This code uses a shortcut to computing the t-statistic using the `t.test` function

Hypothesis testing: Step 3

3. Compute the p-value

Definition

p-value: Probability of observing a test statistic at least as extreme as that observed, under the *null sampling distribution*

For our two example genes:

```
twoGenes %>%
  group_by(gene) %>%
  summarize(pvalue = t.test(Expression ~ genotype,
                            var.equal=TRUE)$p.value)
```

```
# A tibble: 2 × 2
  gene    pvalue
  <chr>    <dbl>
1 Irs4  6.00e- 1
2 Nrl   6.73e-19
```

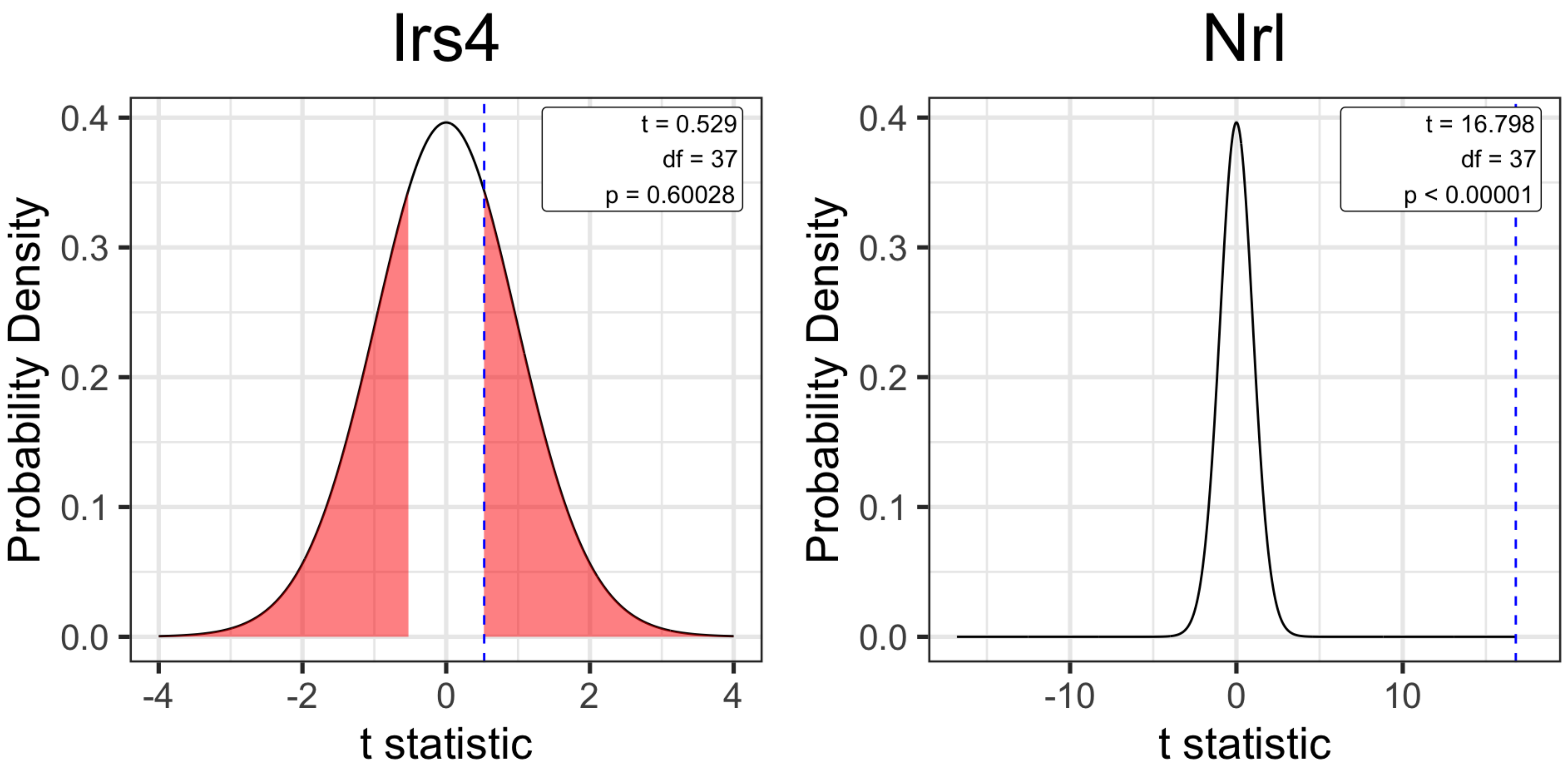
Tip

The `t.test` function also computes the p-value for us

In other words, assuming that H_0 is true:

For *Irs4*, the probability of seeing a test statistic as extreme as that observed $(t = -0.53)$ is pretty high $(p = 0.6)$.

But for *Nrl*, the probability of seeing a test statistic as extreme as that observed $(t = -16.8)$ is extremely low $(p = 6.76 \times 10^{-19})$



Hypothesis Testing: Step 4

4. Make a decision about significance of results

- The decision should be based on a pre-specified significance level (α)
- α is often set at 0.05. However, this value is arbitrary and may depend on the study.

Irs4

Using $\alpha = 0.05$, since the p-value for the Irs4 test is greater than 0.05, we conclude that there is **not enough evidence** in the data to claim that Irs4 has differential expression in WT compared to NrlKO models.

We do not reject H_0!

Nrl

Using $\alpha = 0.05$, since the p-value for the Nrl test is much less than 0.05, we conclude that there is **significant** evidence in the data to claim that *Nrl* has differential expression in WT compared to NrlKO models.

We reject H_0!

`t.test` function in R

Assuming equal variances

```r
twoGenes %>% filter(gene == "Nrl") %>%
  t.test(Expression ~ genotype,
         var.equal=TRUE, data = .)
```

```

    Two Sample t-test

data:  Expression by genotype
t = -16.798, df = 37, p-value < 2.2e-16
alternative hypothesis: true difference in means between
group NrlKO and group WT is not equal to 0
95 percent confidence interval:
 -5.776672 -4.533071
sample estimates:
mean in group NrlKO    mean in group WT 
           6.089579           11.244451 
```

Not assuming equal variances

```r
twoGenes %>% filter(gene == "Nrl") %>%
  t.test(Expression ~ genotype,
         var.equal=FALSE, data = .)
```

```

    Welch Two Sample t-test

data:  Expression by genotype
t = -16.951, df = 34.01, p-value < 2.2e-16
alternative hypothesis: true difference in means between
group NrlKO and group WT is not equal to 0
95 percent confidence interval:
 -5.772864 -4.536879
sample estimates:
mean in group NrlKO    mean in group WT 
           6.089579           11.244451 
```

Tip

Check out `?t.test` for more options, including how to specify one-sided tests

Interpreting p-values

Which of the following are true? (select all that apply)

a. If the effect size is very small, but the sample size is large enough, it is possible to have a statistically significant p-value

b. A study may show a relatively large magnitude of association (effect size), but a statistically insignificant p-value if the sample size is small

c. A very small p-value indicates there is a very small chance the finding is a false positive

Common p-value pitfalls

Caution

Valid inference using p-values depends on accurate assumptions about null sampling distribution

Caution

A p-value is **NOT**:

- The probability that the null hypothesis is true
- The probability that the finding is a “fluke”
- A measure of the size or importance of observed effects

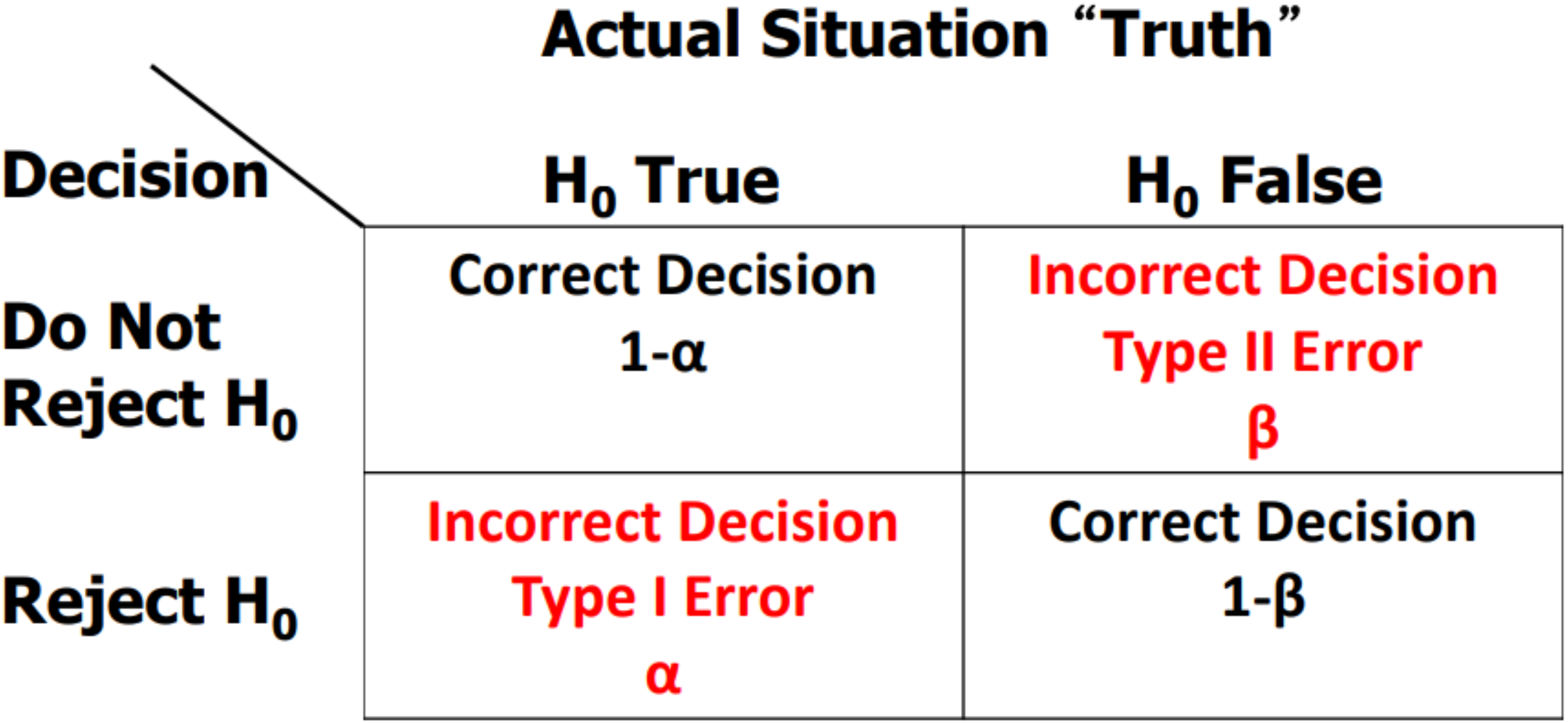
Preview: “Genome-wide” testing of differential expression

- In genomics, we often perform thousands of statistical tests (e.g., a *t*-test per gene)
- The distribution of p-values across all tests provides good diagnostics/insights
- Is it mostly uniform (flat)? If not, is the departure from uniform expected based on biological knowledge?
- We will revisit these topics in greater detail in later lectures

Different kinds of *t*-tests:

- One sample *or* **two samples**
- One-sided *or* **two sided**
- Paired *or* **unpaired**
- Equal variance *or* unequal variance

Types of Errors in Hypothesis Testing



Actual Situation “Truth”

Decision	H_0 True	H_0 False
Do Not Reject H_0	Correct Decision 1-α	Incorrect Decision Type II Error β
Reject H_0	Incorrect Decision Type I Error α	Correct Decision 1-β

$$\alpha = P(\text{Type I Error}),\ \beta = P(\text{Type II Error}),\ \text{Power} = 1 - \beta$$

H_0: "*Innocent until proven guilty*"

- The default state is H_0 → we only reject if we have enough evidence
- If H_0: Innocent and H_A: Guilty, then
  - Type I Error (α): Wrongfully convict innocent (*False Positive*)
  - Type II Error (β): Fail to convict criminal (*False Negative*)

Willing to assume that F and G are normal distributions?

2-sample *t*-test (equal variances):

$$T \sim t_{n_Y+n_Z-2}$$

Welch’s 2-sample *t*-test (unequal variances):

$$T \sim t_{<something\ ugly>}$$

Unwilling to assume that F and G are normal distributions?

But you feel that n_Y and n_Z are large enough?

Then the t-distributions above (or even a normal distribution) are decent approximations

> **Stop!**
>
> What if we aren’t comfortable assuming the underlying data generating process is normal **AND** we aren’t sure our sample is large enough to invoke the CLT?

What are alternatives to the *t*-test?

- First, one could use the t test statistic but use a **permutation approach** to compute its p-value; we'll revisit this topic later
- Alternatively, there are *non-parametric* tests that are available:
  - **Wilcoxon rank sum test**, aka Mann Whitney, uses ranks to test differences in population means
  - **Kolmogorov-Smirnov test** uses the empirical CDF to test differences in population cumulative distributions

Wilcoxon rank sum test

Rank all data, **ignoring the grouping** variable

Test statistic = sum of the ranks for one group (optionally, subtract the minimum possible which is $\frac{n_Y(n_Y+1)}{2}$)

(Alternative but equivalent formulation based on the number of y_i, z_i pairs for which $y_i \geq z_i$)

The null distribution of such statistics can be worked out or approximated

`wilcox.test` function in R

Irs4 *Nrl*

```r
wilcox.test(Expression ~ genotype,
            data = twoGenes %>% filter(gene == "Irs4"))
```

```
	Wilcoxon rank sum exact test

data:  Expression by genotype
W = 160, p-value = 0.4115
alternative hypothesis: true location shift is not equal to 0
```

Kolmogorov-Smirnov test (two sample)

Null hypothesis: F = G, i.e. the distributions are the same

Estimate each CDF with the empirical CDF (ECDF)

$$\hat{F}(x) = \frac{1}{n} \sum_{i=1}^{n} I[x_i \leq x]$$

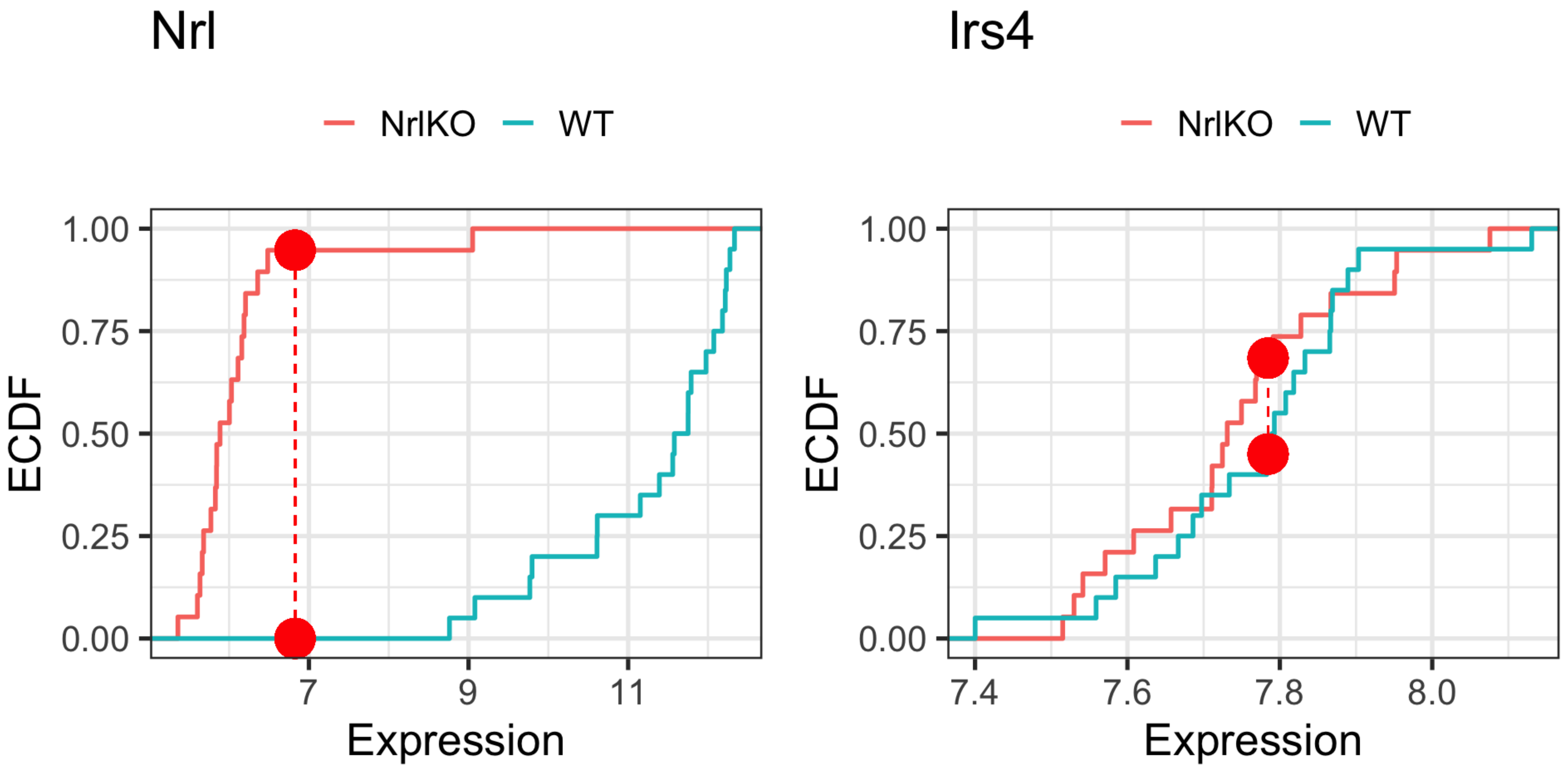
Test statistic is the maximum of the absolute difference between the ECDFs[1]

$$max|\hat{F}(x) - \hat{G}(x)|$$

Null distribution does not depend on F, G (!)

[1] I'm suppressing detail here

Kolmogorov-Smirnov test (two sample)



`ks.test` function in R

Irs4 *Nrl*

```r
Irs4gene <- twoGenes %>% filter(gene == "Irs4")
ks.test(Irs4gene$Expression[Irs4gene$genotype == "WT"],
        Irs4gene$Expression[Irs4gene$genotype == "NrlKO"])
```

```

    Exact two-sample Kolmogorov-Smirnov test

data:  Irs4gene$Expression[Irs4gene$genotype == "WT"] and Irs4gene$Expression[Irs4gene$genotype == "NrlKO"]
D = 0.28421, p-value = 0.3278
alternative hypothesis: two-sided
```

Discussion

1. What test(s) might be appropriate if your sample size is just barely large enough to invoke CLT, but you also have suspected outliers?
2. If more than one test is appropriate (e.g. *t*-test, Wilcoxon, and KS), which should we report?
3. What is generally more important for results interpretation: the effect size or the p-value?
4. What should you do if methods that are equally appropriate and defensible give very different answers?