Linear models with multiple factors

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Project next steps - written proposal

- Details here
- Expand upon your proposal lightning talk, incorporating feedback
- Include a plan for how team will work together
  - Aim for a fair balance
  - It is acceptable to modularize your overall workflow, assigning each team member some of the components (e.g. one group member performs planned analysis A, another group member perfroms planned analysis B, etc)
  - It is ***not*** acceptable for one group member to take on *sole responsibility* of the tasks of a certain type (e.g. one group member doing all of the analysis, or one group member defining the research question and hypotheses, etc)

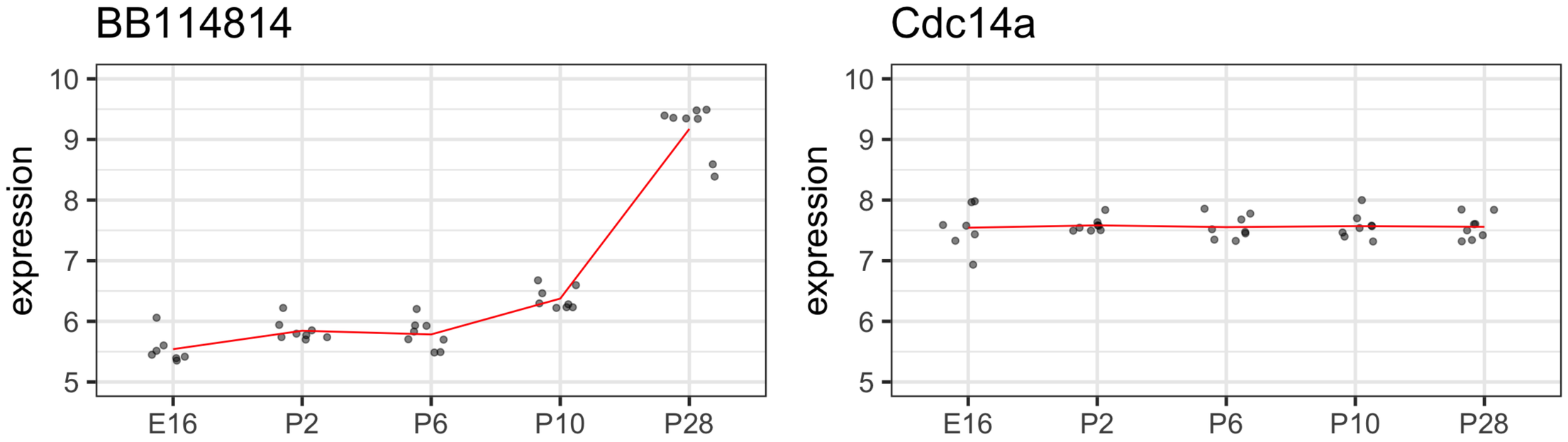
Last class…

1. How to compare means of different groups (2 or more) using a linear regression model
    - indicator variables to model the levels of a qualitative explanatory variable
2. Write a linear model using matrix notation
    - understand which matrix is built by R
3. Distinguish between **single** and **joint** hypotheses
    - t-tests vs F-tests

Comparing more than two groups

- **Biological question**: do gene expression levels differ by developmental stage?
- **Statistical question**: are gene expression generated by a single common distribution across all developmental stages? Or do the distributions differ by timepoint?

▶ Code



Quick review: from t-test to linear regression

2-sample t-test

$$Y \sim F;\ E[Y] = \mu_Y;\ Z \sim G;\ E[Z] = \mu_Z$$

$$H_0 : \mu_Y = \mu_Z$$

↓

How? Why?

↓

Linear regression

$$Y = X\alpha + \epsilon; \qquad H_0 : \alpha_j = 0$$

How: Cell means model using indicator variables

$$Y \sim F;\ E[Y] = \mu_Y;\ Z \sim G;\ E[Z] = \mu_Z$$

$$Y_{ij} = \mu_1 x_{ij1} + \mu_2 x_{ij2} + \varepsilon_{ij};\ i = 1, \ldots, n;\ j = 1, 2$$

$$x_{ij1} = \begin{cases} 1 \text{ if } j = 1 \\ 0 \text{ otherwise} \end{cases}, \quad x_{ij2} = \begin{cases} 1 \text{ if } j = 2 \\ 0 \text{ otherwise} \end{cases}$$

$$
\begin{aligned}
E[Y_{i1}] &= \mu_1 \\
E[Y_{i2}] &= \mu_2
\end{aligned}
$$

$$
\begin{bmatrix} Y_{11} \\ \vdots \\ Y_{n_1 1} \\ Y_{12} \\ \vdots \\ Y_{n_2 2} \end{bmatrix} = \begin{bmatrix} 1 & 0 \\ \vdots & \vdots \\ 1 & 0 \\ 0 & 1 \\ \vdots & \vdots \\ 0 & 1 \end{bmatrix} \begin{bmatrix} \mu_1 \\ \mu_2 \end{bmatrix} + \begin{bmatrix} \varepsilon_{11} \\ \vdots \\ \varepsilon_{n_1 1} \\ \varepsilon_{12} \\ \vdots \\ \varepsilon_{n_2 2} \end{bmatrix}
$$

How: Reference-treatment parameterization using indicator variables

$$Y \sim F;\ E[Y] = \mu_Y;\ Z \sim G;\ E[Z] = \mu_Z$$

$$Y_{ij} = \theta + \tau_2 x_{ij2} + \varepsilon_{ij};\ i = 1, \ldots, n;\ j = 1, 2$$

$$x_{ij2} = \begin{cases} 1 \text{ if } j = 2 \\ 0 \text{ otherwise} \end{cases}$$

How: Using matrix notation

2 group comparison:

$$Y_{ij} = \theta + \tau_2 x_{ij2} + \varepsilon_{ij} \rightarrow \mathbf{Y} = \mathbf{X}\alpha + \epsilon$$

$$\begin{bmatrix} Y_{11} \\ \vdots \\ Y_{n_1 1} \\ Y_{12} \\ \vdots \\ Y_{n_2 2} \end{bmatrix} = \begin{bmatrix} 1 & 0 \\ \vdots & \vdots \\ 1 & 0 \\ 1 & 1 \\ \vdots & \vdots \\ 1 & 1 \end{bmatrix} \begin{bmatrix} \theta \\ \tau_2 \end{bmatrix} + \begin{bmatrix} \varepsilon_{11} \\ \vdots \\ \varepsilon_{n_1 1} \\ \varepsilon_{12} \\ \vdots \\ \varepsilon_{n_2 2} \end{bmatrix}$$

- x_{ij2} is the second column of X (design matrix)
- Tip: examine design matrix in R with `model.matrix()`

$$Y_{11} = 1 * \theta + 0 * \tau_2 + \epsilon_{11} = \theta + \epsilon_{11}$$

$$Y_{12} = 1 * \theta + 1 * \tau_2 + \epsilon_{12} = \theta + \tau_2 + \epsilon_{12}$$

Recall

For comparisons involving more than 2 groups (ANOVA), we add indicator variables (columns of X)

Why: Flexible framework

$\mathbf{Y} = \mathbf{X}\alpha + \epsilon$ gives us a very flexible framework

$$\begin{bmatrix} 1 & 0 & 0 & 0 \\ \vdots & \vdots & \vdots & \\ 1 & 0 & 0 & 0 \\ 1 & 1 & 0 & 0 \\ \vdots & \vdots & \vdots & \\ 1 & 1 & 0 & 0 \\ 1 & 0 & 1 & 0 \\ \vdots & \vdots & \vdots & \\ 1 & 0 & 1 & 0 \\ 1 & 0 & 0 & 1 \\ \vdots & \vdots & \vdots & \\ 1 & 0 & 0 & 1 \end{bmatrix}$$

1 categorical covariate

$$\begin{bmatrix} 1 & 0 & 0 & 0 \\ \vdots & \vdots & \vdots & \\ 1 & 0 & 0 & 0 \\ 1 & 1 & 0 & 0 \\ \vdots & \vdots & \vdots & \\ 1 & 1 & 0 & 0 \\ 1 & 0 & 1 & 0 \\ \vdots & \vdots & \vdots & \\ 1 & 0 & 1 & 0 \\ 1 & 1 & 1 & 1 \\ \vdots & \vdots & \vdots & \\ 1 & 1 & 1 & 1 \end{bmatrix}$$

2 categorical covariates

$$\begin{bmatrix} 1 & 1.22 \\ 1 & 2.02 \\ 1 & 1.42 \\ \vdots & \vdots \\ 1 & 1.89 \\ 1 & 2.01 \\ \vdots & \vdots \\ 1 & 1.56 \\ 1 & 2.17 \\ 1 & 1.51 \end{bmatrix}$$

1 continuous covariate

$$\begin{bmatrix} 1 & 0 & 1.22 & 0 \\ 1 & 0 & 2.02 & 0 \\ 1 & 0 & 1.42 & 0 \\ \vdots & \vdots & \vdots & \vdots \\ 1 & 0 & 1.89 & 0 \\ 1 & 1 & 2.01 & 2.01 \\ \vdots & \vdots & \vdots & \vdots \\ 1 & 1 & 1.56 & 1.56 \\ 1 & 1 & 2.17 & 2.17 \\ 1 & 1 & 1.51 & 1.51 \end{bmatrix}$$

1 continuous 1 categorical

These (and many more) can be accommodated by the design matrix (X)!

Parameterizations

Different ways of writing the $\mathbf{X}\alpha$ = [**design matrix**][**parameter vector**] pair correspond to different **parameterizations** of the model

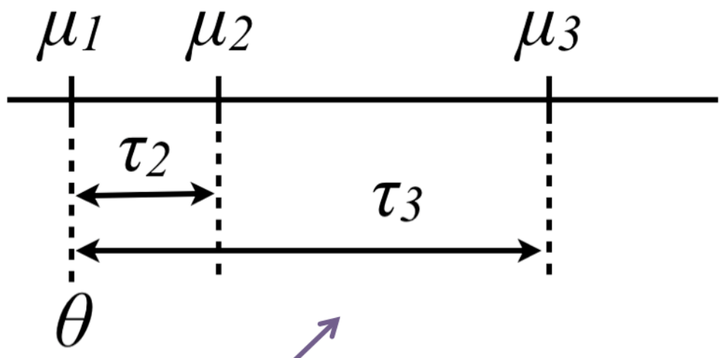
$$\mathbf{Y} = \mathbf{X}\alpha + \varepsilon$$

Understanding these concepts makes it easier:

- to interpret and compare fitted models
- to fit models such that comparisons you care most about are directly addressed in the output

Example: compare means between groups

$$\theta = \mu_1$$
$$\tau_2 = \mu_2 - \mu_1$$
$$\tau_3 = \mu_3 - \mu_1$$



These are **NOT population means**

These are **differences of means**

By default, `lm` estimates group mean differences (with respect to a reference group):

```
filter(twoGenes, gene == "BB114814") %>%
  lm(expression ~ dev_stage, data = .) %>%
  tidy()
```

```
# A tibble: 5 × 5
  term         estimate std.error statistic  p.value
  <chr>           <dbl>     <dbl>     <dbl>    <dbl>
1 (Intercept)     5.54      0.102     54.2  1.31e-34
2 dev_stageP2     0.304     0.140      2.17 3.69e- 2
3 dev_stageP6     0.243     0.140      1.74 9.11e- 2
4 dev_stageP10    0.834     0.140      5.96 9.62e- 7
5 dev_stageP28    3.63      0.140     26.0  5.30e-24
```

We can tell R to use the cell-means parameterization

Write the formula as `Y ~ 0 + x` in the `lm` call to remove the intercept (θ) parameter and fit cell means parameters instead

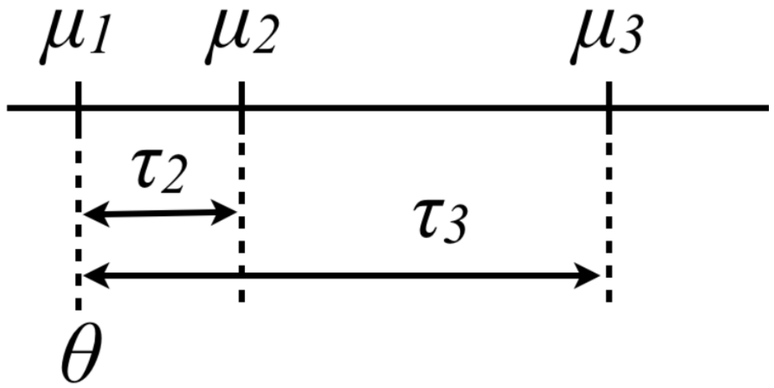
```
filter(twoGenes, gene == "BB114814") %>%
  lm(expression ~ 0 + dev_stage, data = .) %>%
  tidy()
```

```
# A tibble: 5 × 5
  term          estimate std.error statistic  p.value
  <chr>            <dbl>     <dbl>     <dbl>    <dbl>
1 dev_stageE16      5.54    0.102       54.2 1.31e-34
2 dev_stageP2       5.84    0.0956      61.2 2.30e-36
3 dev_stageP6       5.78    0.0956      60.5 3.27e-36
4 dev_stageP10      6.38    0.0956      66.7 1.23e-37
5 dev_stageP28      9.17    0.0956      96.0 5.56e-43
```

What null hypotheses does the *t*-test column now represent?

Converting between parameterizations

$$\mu_1 = \theta \qquad \theta = \mu_1$$
$$\mu_2 = \theta + \tau_2 \qquad \tau_2 = \mu_2 - \mu_1$$
$$\mu_3 = \theta + \tau_3 \qquad \tau_3 = \mu_3 - \mu_1$$



These are **population** means

These are **NOT population** means

These are **ref & TX** effects

```
filter(twoGenes, gene == "BB114814") %>%
  lm(expression ~ 0 + dev_stage, data = .) %>%
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3 dev_stageP6      0.243     0.140      1.74  9.11e- 2
4 dev_stageP10     0.834     0.140      5.96  9.62e- 7
5 dev_stageP28     3.63      0.140     26.0   5.30e-24
```

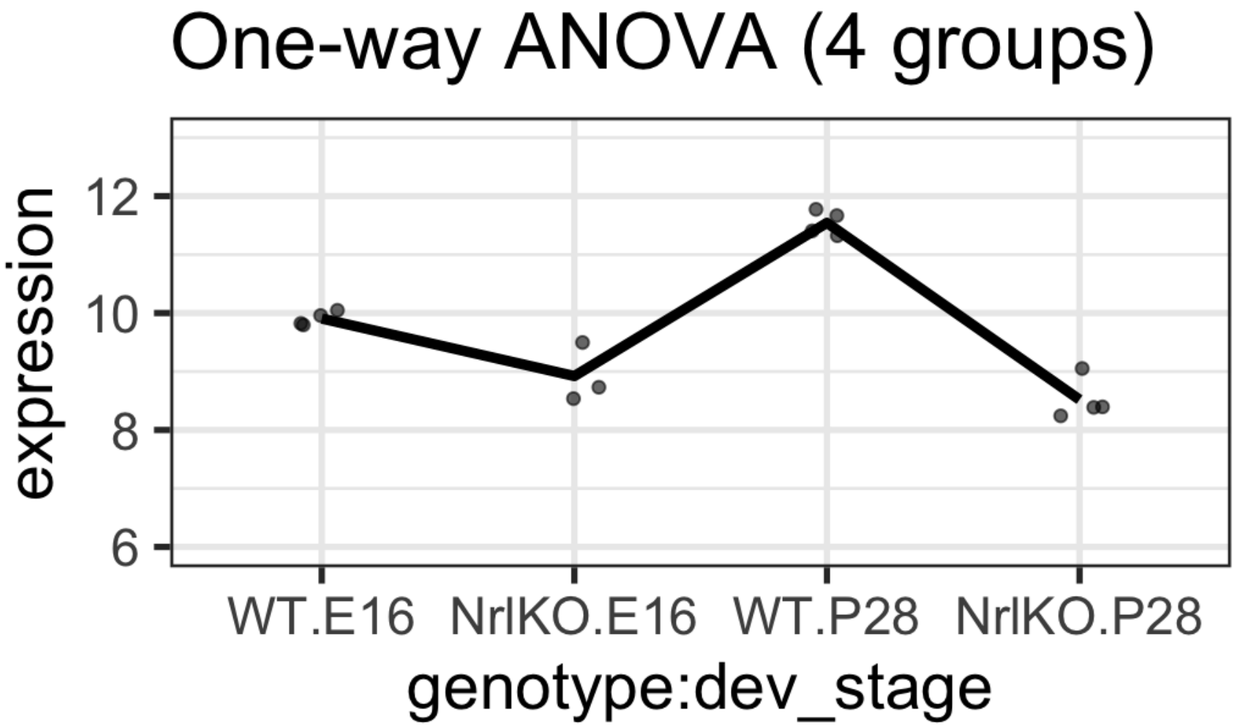
Learning objectives for today

1. Model more than one factor with multiple levels
    - build models with multiple categorical variables and their interaction
2. Distinguish between **simple** and **main** effects
    - `lm` vs `anova` tests
3. Test main effects using **nested** models
    - t-tests vs F-tests

What if you have *2* categorical variables?

For example: `genotype` and `dev_stage` (for simplicity, let's consider only E16 and P28)

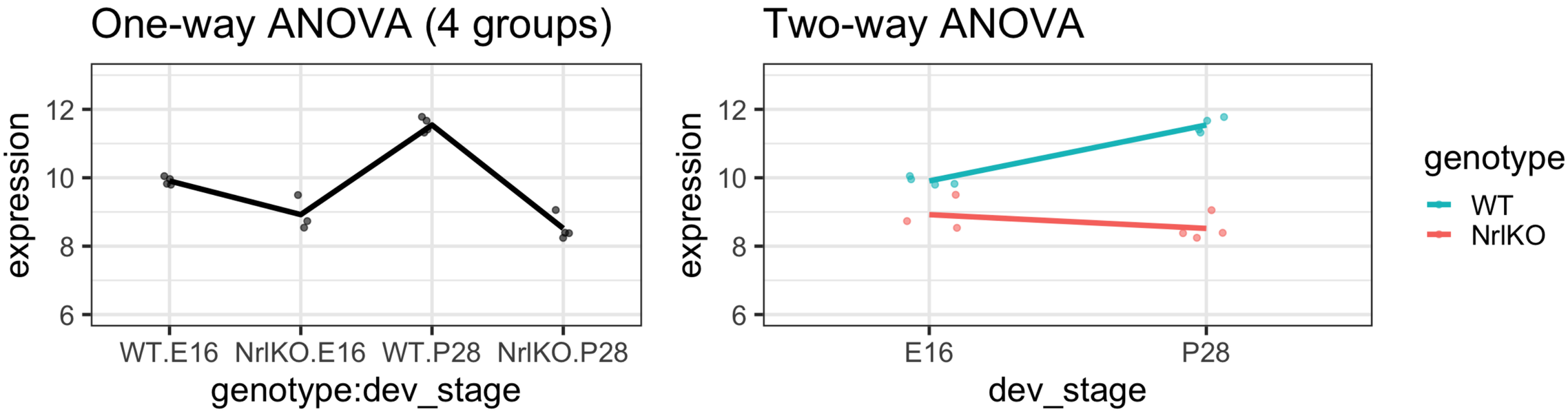
- ANOVA is usually used to study models with one or more categorical variables (factors)
- Can we combine 2 levels in each of 2 factors into 4 groups (treat as one-way ANOVA)?



What if you have *2* categorical variables?

For example: `genotype` and `dev_stage` (for simplicity, let's consider only E16 and P28)

- ANOVA is usually used to study models with one or more categorical variables (factors)
- Can we combine 2 levels in each of 2 factors into 4 groups (treat as one-way ANOVA)?
  - no way to separate effects of each factor, or their interaction



Two-way ANOVA (or a linear model with interaction)

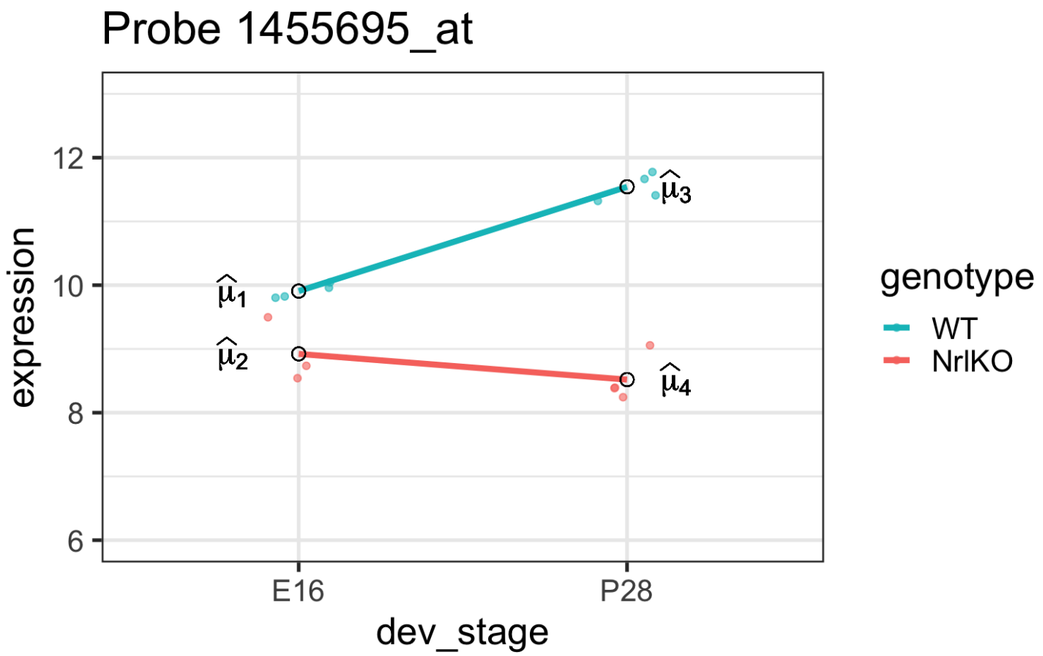
Which group means are we comparing in a model with 2 factors?

$$\mu_1 = E[Y_{(WT,E16)}]$$

$$\mu_2 = E[Y_{(NrlKO,E16)}]$$

$$\mu_3 = E[Y_{(WT,P28)}]$$

$$\mu_4 = E[Y_{(NrlKO,P28)}]$$



Reference-treatment effect parameterization

- By default, `lm` assumes a **reference-treatment effect** parameterization
- Mathematically, we just need *more* indicator variables, see companion notes for more details

```
1 twoFactFit <- lm(expression ~ genotype * dev_stage, oneGene)
2 tidy(twoFactFit)
```

```
# A tibble: 4 × 5
  term                      estimate std.error statistic  p.value
  <chr>                        <dbl>     <dbl>     <dbl>    <dbl>
1 (Intercept)                  9.91      0.157     62.9  2.02e-15
2 genotypeNrlKO               -0.984     0.240     -4.09 1.78e- 3
3 dev_stageP28                 1.64      0.223      7.35 1.44e- 5
4 genotypeNrlKO:dev_stageP28  -2.04      0.328     -6.23 6.47e- 5
```

Cell-means and treatment effects in the two-way model

Why do we need more indicator variables?

```
table(oneGene$dev_stage, oneGene$genotype)
```

```
     WT NrlKO
E16   4     3
P28   4     4
```

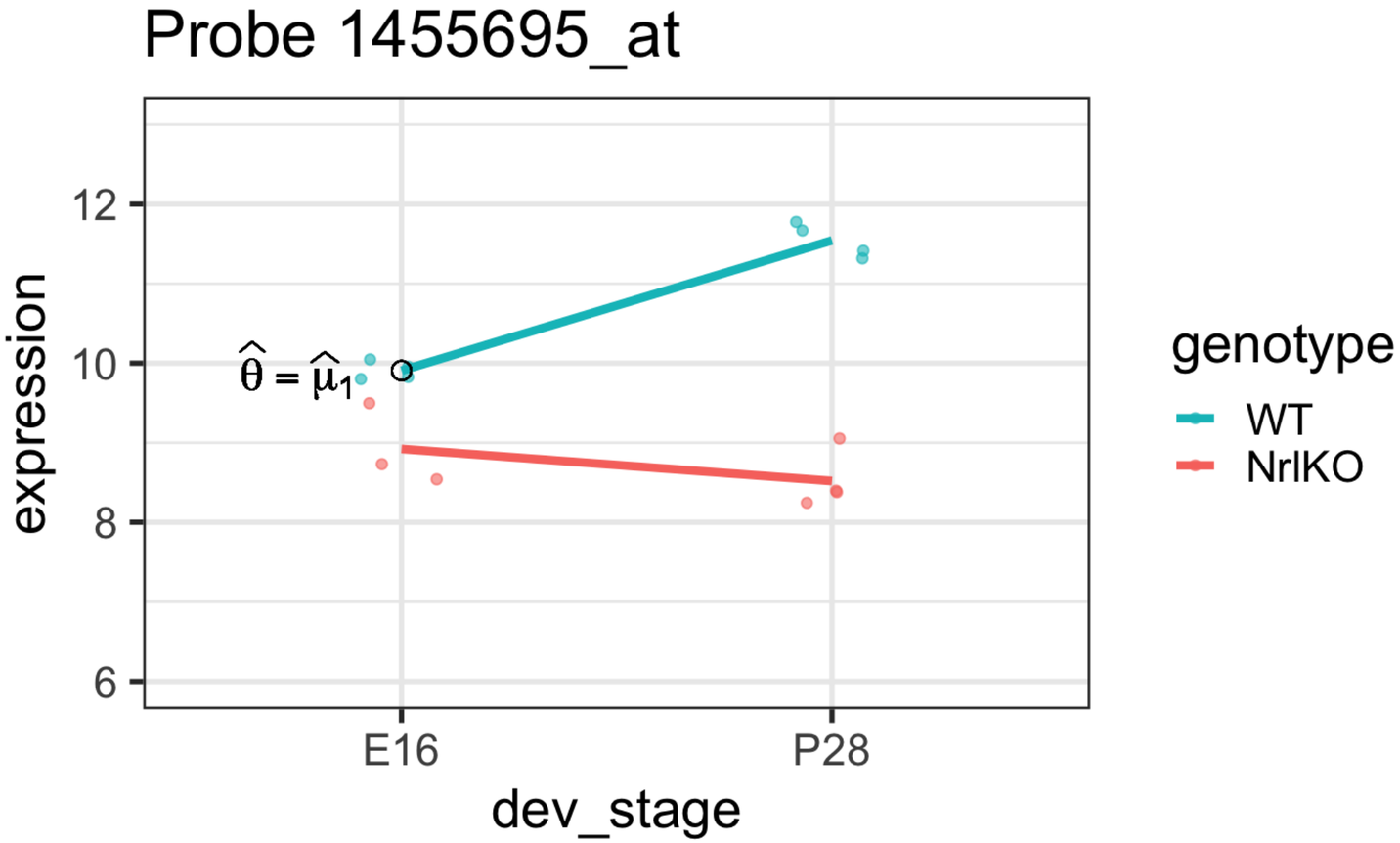
```
(means.2Fact <- oneGene %>%
   group_by(dev_stage, genotype) %>%
   summarize(cellMeans = mean(expression)) %>%
   ungroup() %>%
   mutate(txEffects = cellMeans - cellMeans[1],
          lmEst = tidy(twoFactFit)$estimate))
```

```
# A tibble: 4 × 5
  dev_stage genotype cellMeans txEffects  lmEst
  <fct>     <fct>        <dbl>     <dbl>  <dbl>
1 E16       WT            9.91     0      9.91
2 E16       NrlKO         8.92    -0.984 -0.984
3 P28       WT           11.5      1.64   1.64
4 P28       NrlKO         8.52    -1.39  -2.04
```

What is the reference group here?

Reference group: **WT & E16**

As before, comparisons are relative to a reference but in this case there is a reference level *in each factor*: **WT and E16**



The reference: WT & E16

Mean of reference group: $\theta = E[Y_{WT,E16}]$

`lm` estimate: $\hat{\theta}$ is the sample mean of the group

```
1 tidy(twoFactFit)
```

```
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```
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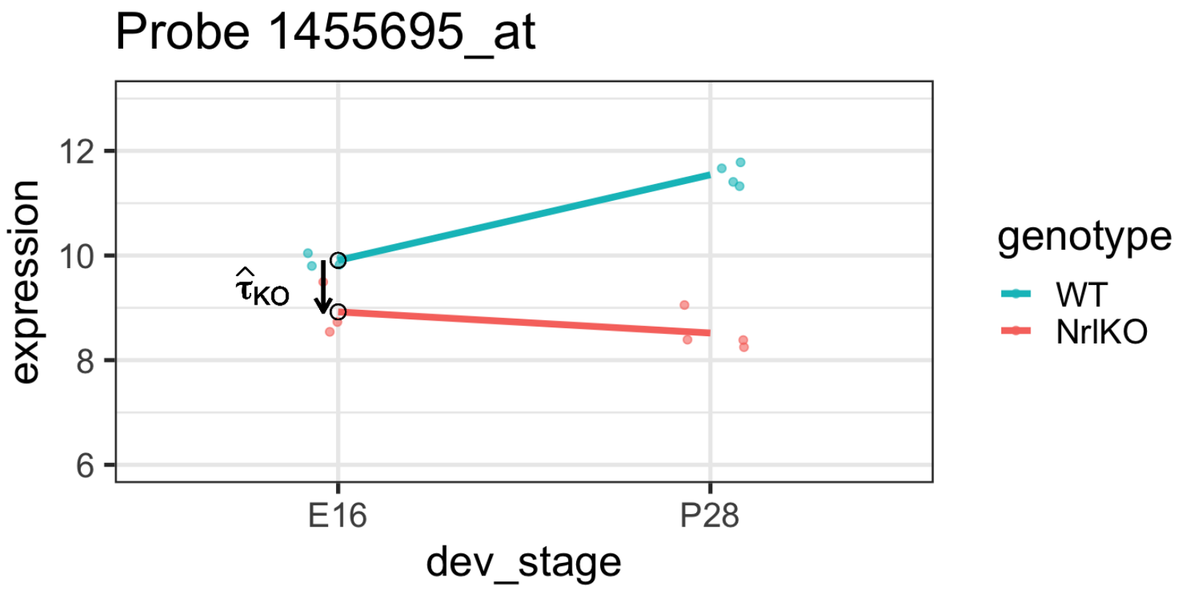
```

In general, one is not interested in: $H_0 : \theta = 0$

Simple genotype effect: WT *vs* NrlKO at E16

And now the “treatment effects”…



Important: Simple/Conditional vs Main/Marginal effects

“Treatment effect” parameters represent **conditional effects**: effects at a given level of the other factor (e.g. effect of genotype at E16). These are also called **simple effects**. They do ***not*** represent marginal effects.

A **marginal effect**, on the other hand, is the overall effect of a factor, averaged over all levels of the other factor (e.g. the overall effect of genotype, averaged over all levels of developmental time). These are also called **main effects**.

Simple genotype effect: WT *vs* NrlKO at E16

Effect of genotype at E16: $\tau_{KO} = E[Y_{NrlKO,E16}] - E[Y_{WT,E16}]$

`lm` estimate: $\hat{\tau}_{KO}$ is the *difference* of sample respective means (check below)

```
1 tidy(twoFactFit)
```

```
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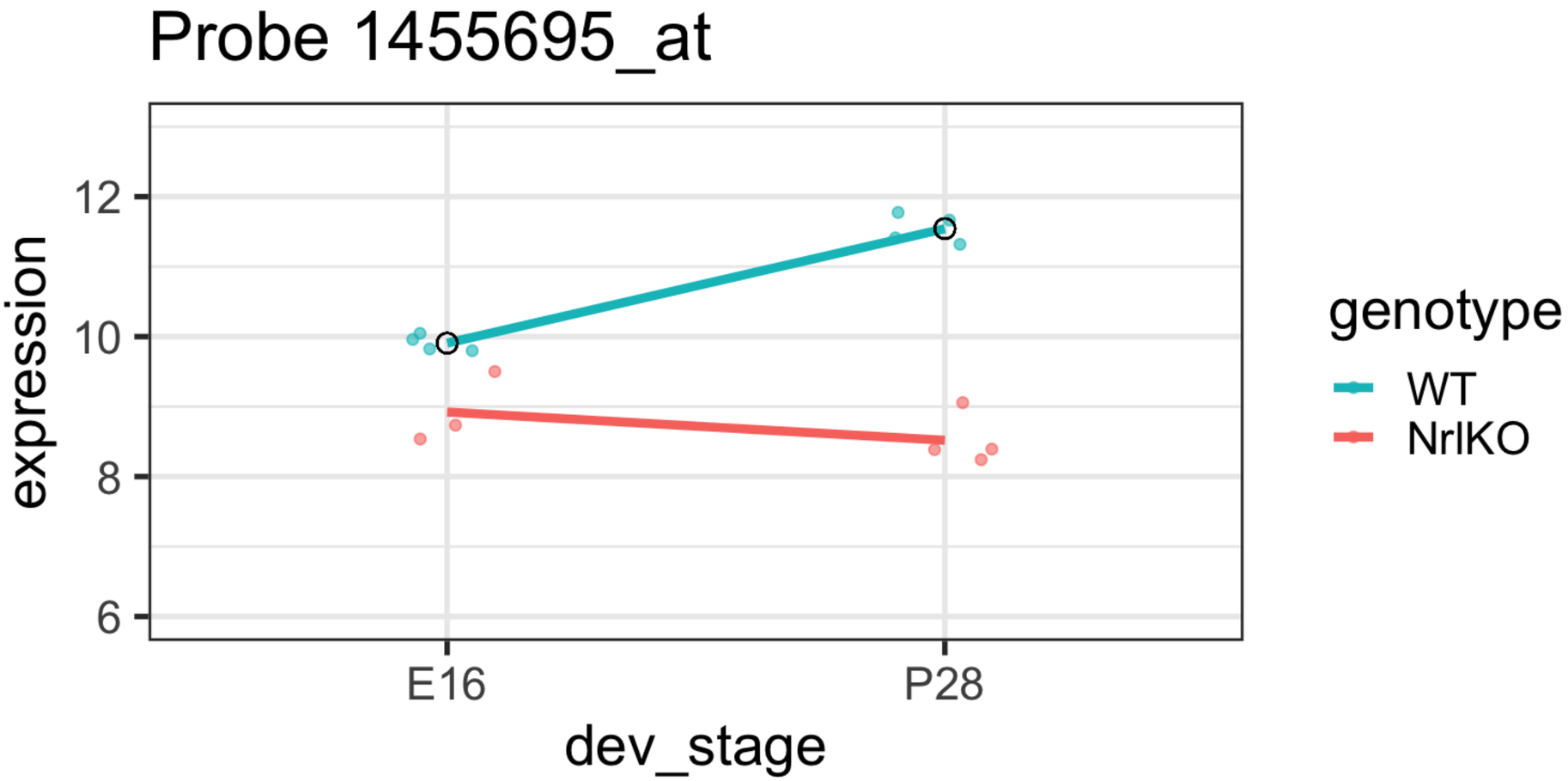
3 P28       WT           11.5       1.64   1.64
4 P28       NrlKO         8.52     -1.39  -2.04
```

But, do you want to test the *conditional* effect at E16: $H_0 : \tau_{KO} = 0$??

Simple developmental effect: E16 *vs* P28 in WT

Similarly, for the other factor: τ_{P28} is the effect of developmental time (P28 vs E16) **in WT**

If $\tau_{P28} = 0$, what would the mean be in the WT group at P28?



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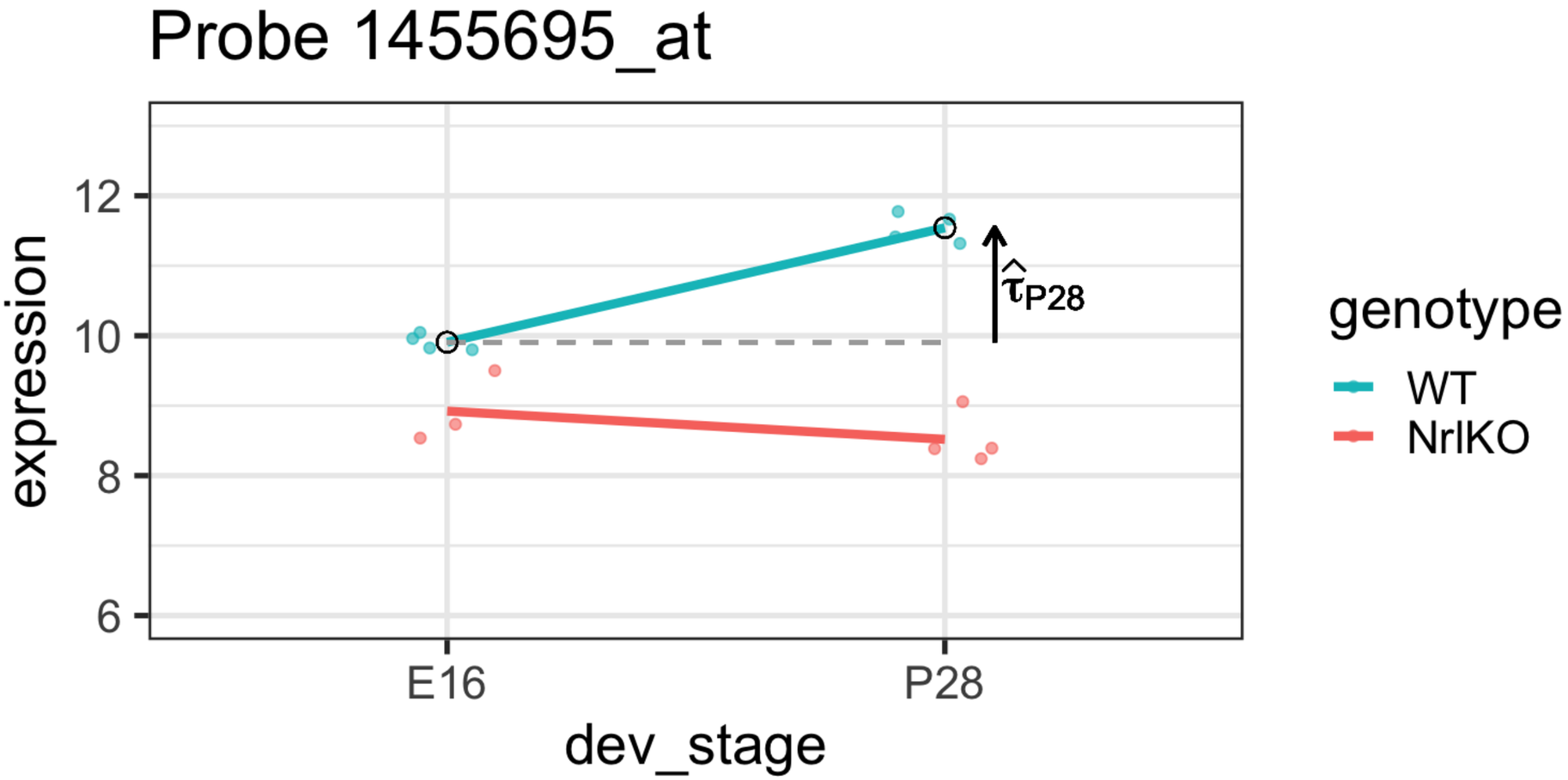

Simple developmental effect: E16 *vs* P28 in WT

Effect of development in WT: $\tau_{P28} = E[Y_{WT,P28}] - E[Y_{WT,E16}]$

`lm` estimate: $\hat{\tau}_{P28}$ is the *difference* of respective sample means (check below)

```
1 tidy(twoFactFit)
```

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```
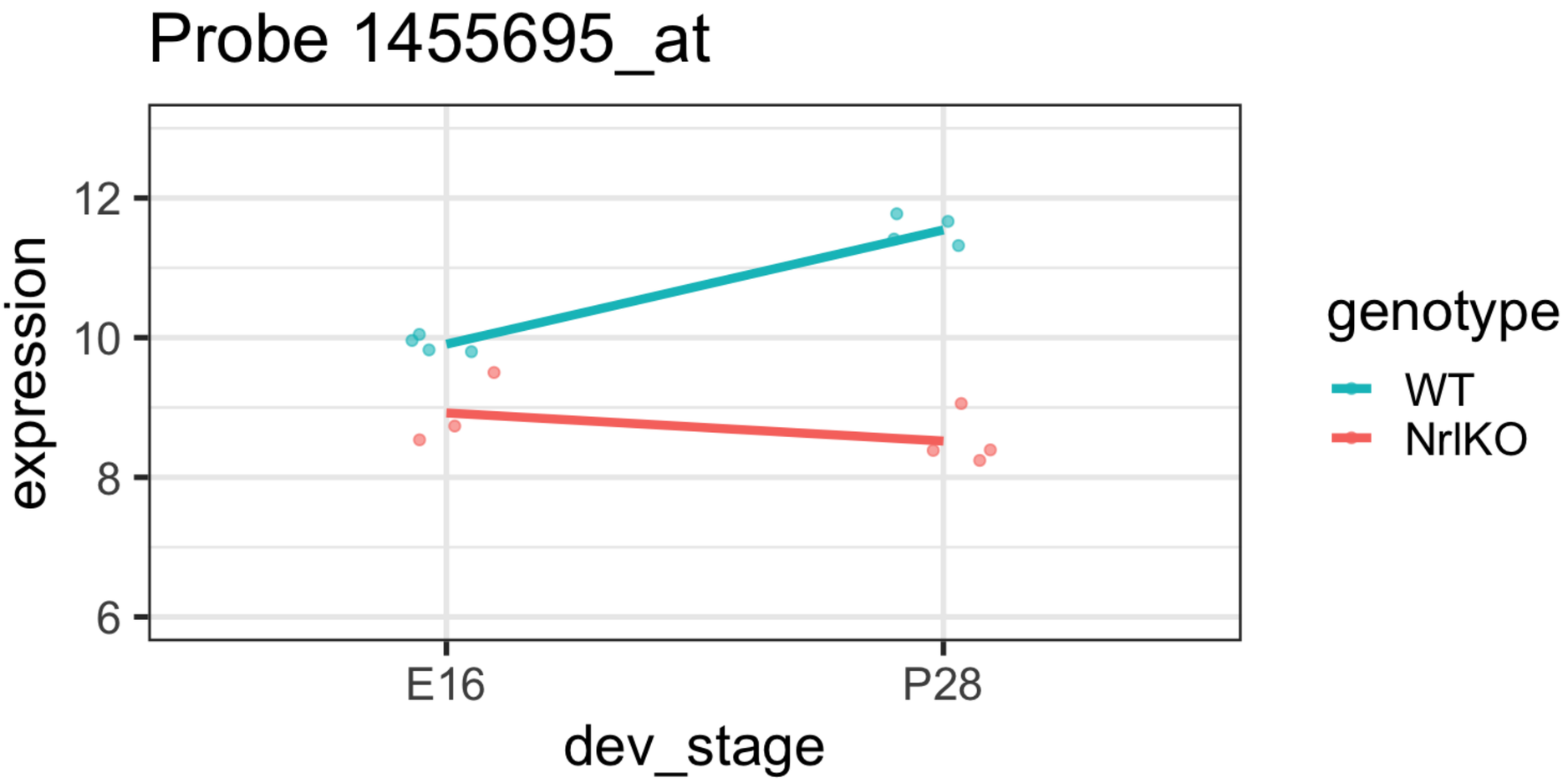

Again, do you want to test the *conditional* effect in WT: $H_0 : \tau_{P28} = 0$??

Interaction effect

Is the effect of genotype the same at different developmental stages?

Equivalently: Is the development effect the same for both genotypes?



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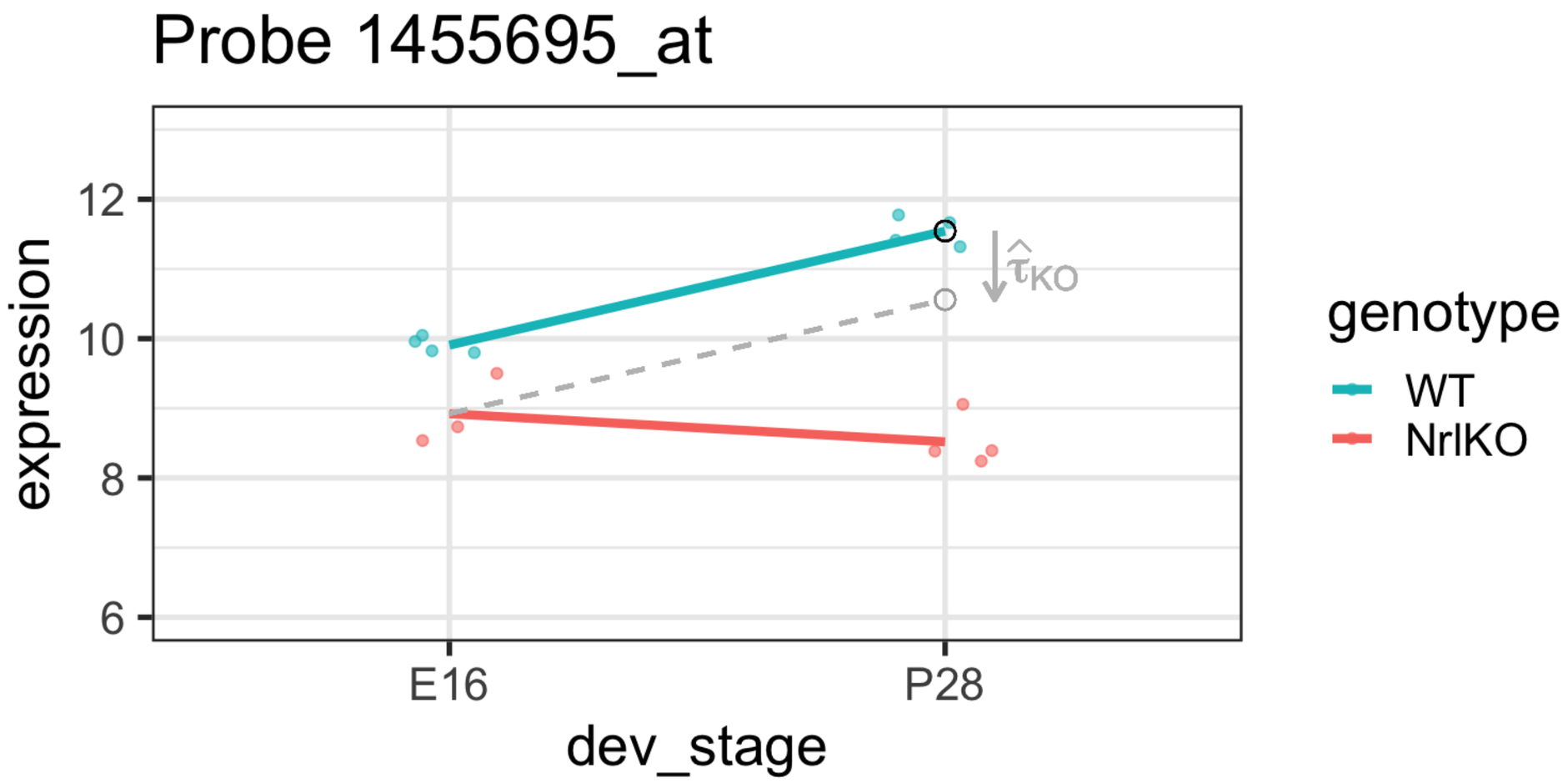

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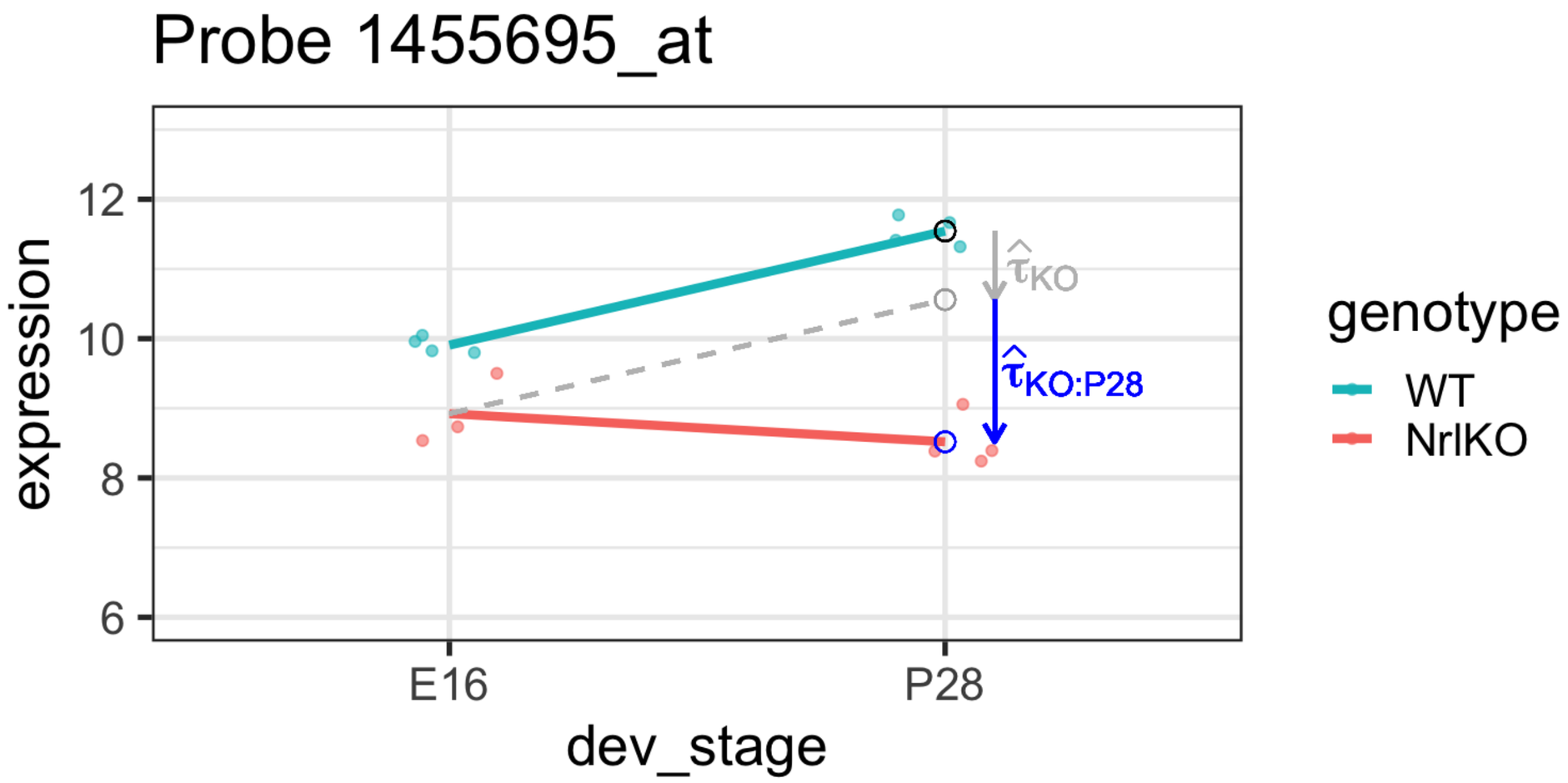
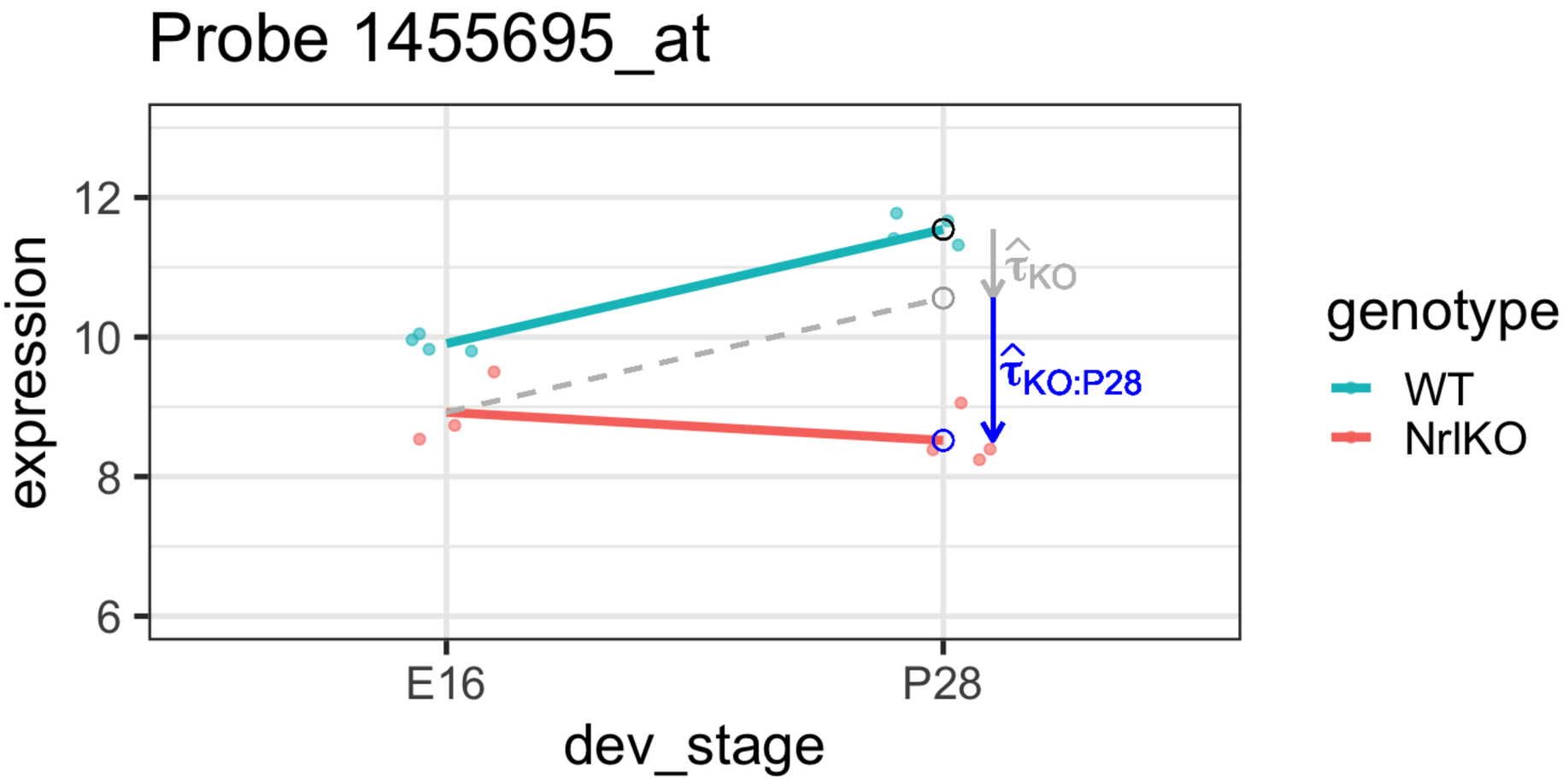


Interaction effect

The genotype effect at E16 is τ_{KO}. However, τ_{KO} does not seem to be the effect at P28.

The difference is the interaction effect! If there's no interaction effect, $\tau_{KO:P28} = 0$



Interaction effect

Difference of differences:

$$\tau_{KO:P28} = (E[Y_{NrlKO,P28}] - E[Y_{WT,P28}]) - (E[Y_{NrlKO,E16}] - E[Y_{WT,E16}])$$

In `lm` output:

```
1 tidy(twoFactFit)
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```

```
1 (means.2Fact$cellMeans[4] - means.2Fact$cellMeans[3]) - (means.2Fact$cellMeans[2] - means.2Fact$cellMeans[1])
```

```
[1] -2.040372
```

Summary of model parameters: with interaction

model parameter	`lm` estimate	stats
θ	`(Intercept)`	$E[Y_{WT,E16}]$
τ_{KO}	`genotypeNrlKO`	$E[Y_{NrlKO,E16}] - E[Y_{WT,E16}]$
τ_{P28}	`dev_stageP28`	$E[Y_{WT,P28}] - E[Y_{WT,E16}]$
$\tau_{KO:P28}$	`genotypeNrlKO:dev_stageP28`	$E[Y_{NrlKO,P28}] - E[Y_{WT,P28}] - \tau_{KO}$

It is important to remember that `lm` reports **simple, not main** effects!

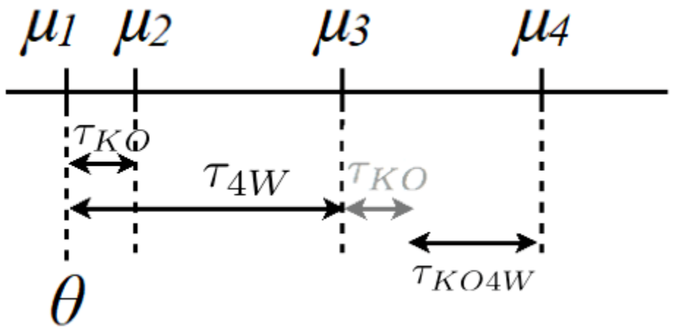
Why? Because of the parameterization used! (see companion notes)

It can also be shown that $\tau_{KO:P28} = E[Y_{NrlKO,P28}] - \tau_{P28} - \tau_{KO} - \theta$ (see previous slide and companion notes)

Let's examine these parameters closer

For our model, `lm` tests 4 hypotheses:

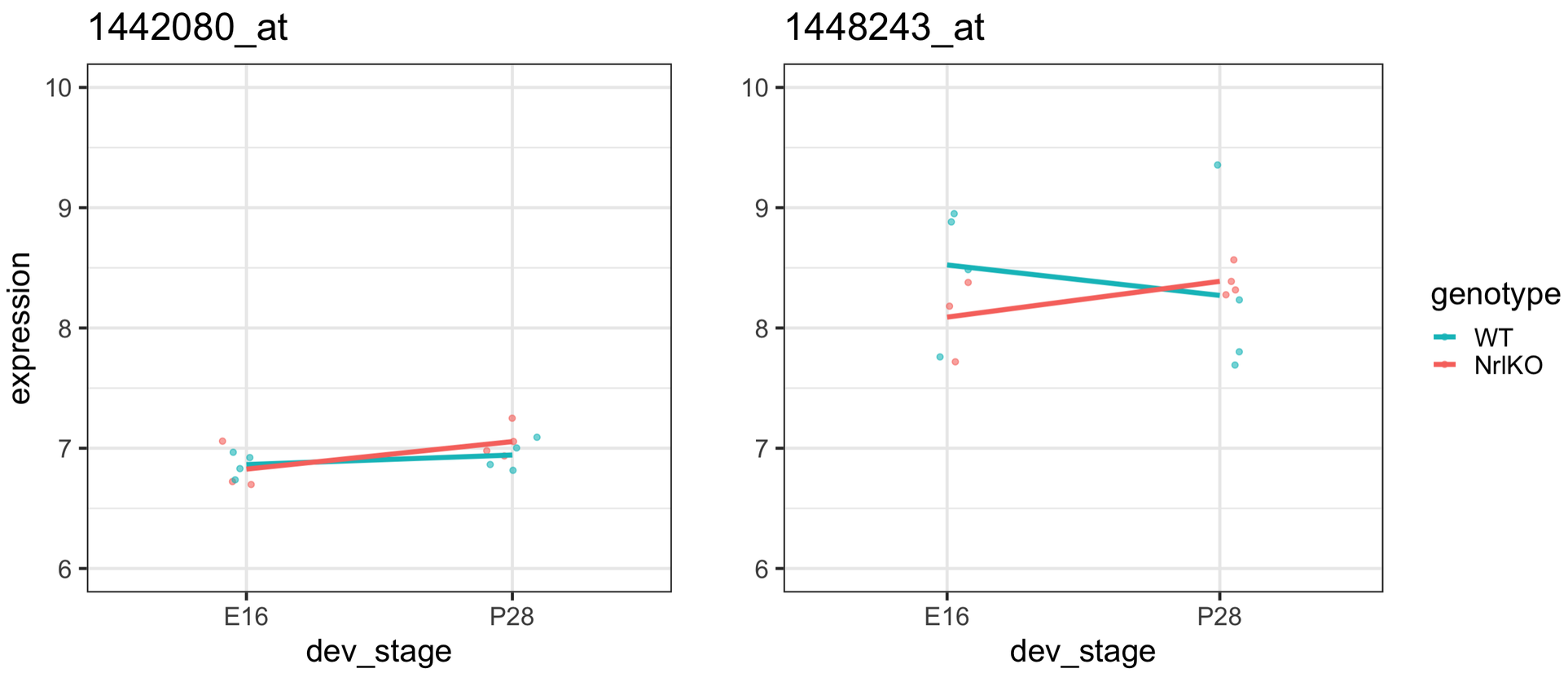
$$H_0 : \theta = 0$$
$$H_0 : \tau_{KO} = 0$$
$$H_0 : \tau_{P28} = 0$$
$$H_0 : \tau_{KO:P28} = 0$$



We may not be interested in these hypotheses, e.g., τ_{KO} and τ_{P28} are *conditional* effects *at* a given level of a factor (*simple effects*)

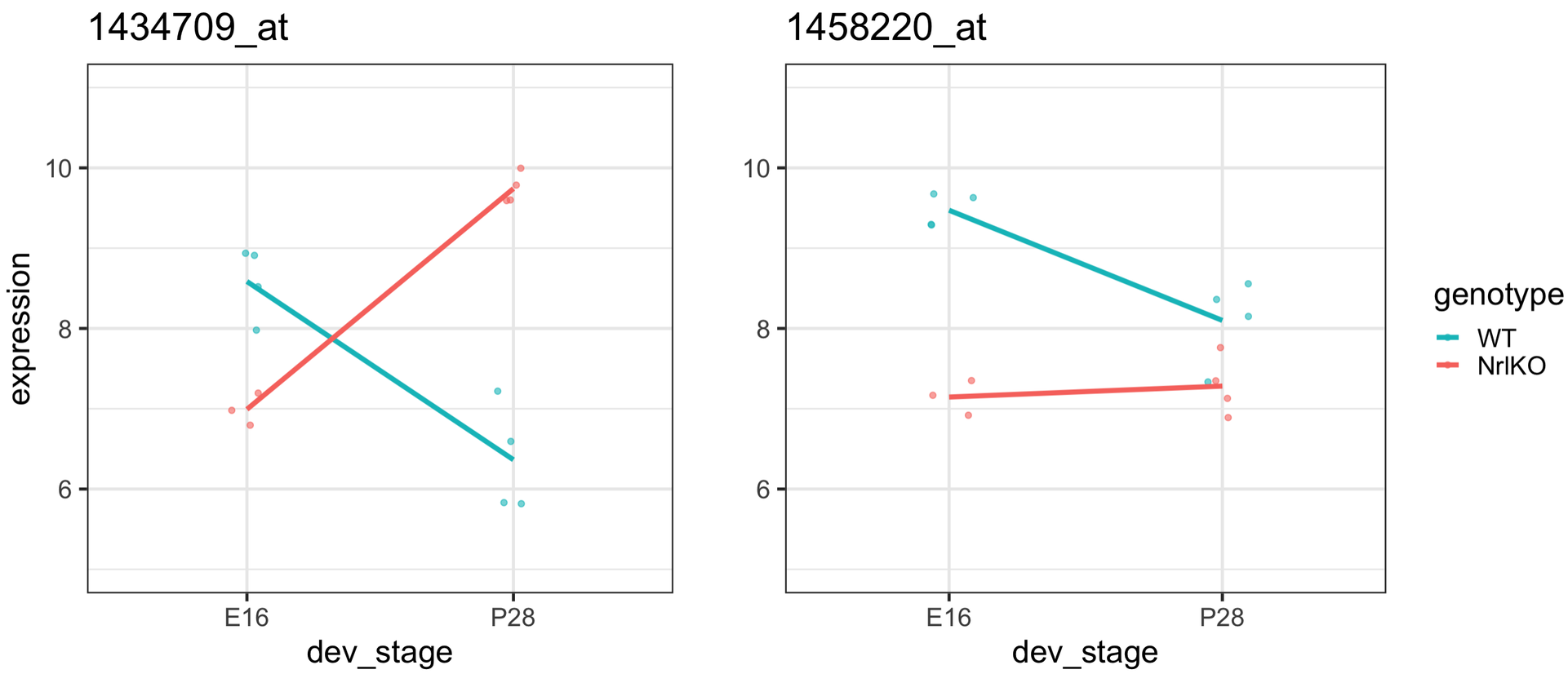
Ex 1: nothing statistically significant, very flat genes

Plots | `lm` output



Ex 2: statistically significant interaction (non-parallel)

Plots | `lm` output



Disagreement in simple effects with interaction

- Note that a significant interaction means the **simple** effects may not agree
- For the gene 1434709_at on the previous slide, compare the effect of genotype at E16 and P28:

Effect	`lm` output	Estimate
Genotype at E16	`genotypeNrlKO`	
Genotype at P28		

- **Main** effects (overall): does genotype have an effect on gene expression?
  - We can't (yet) answer this question! It depends (on the level of `dev_stage`)! (more later)

Ex 3: *BALANCED* & only genotype at E16 is significant

For simplicity here, we'll add a fake observation in the NrlKO & E16 group (close to its mean) so that we have a *balanced* design

> **Note**
>
> In *unbalanced* designs the *main* effects are a *weighted* average of the simple effects, and the weights are not easy to interpret (beyond the scope of this course but worth noting the issue!)

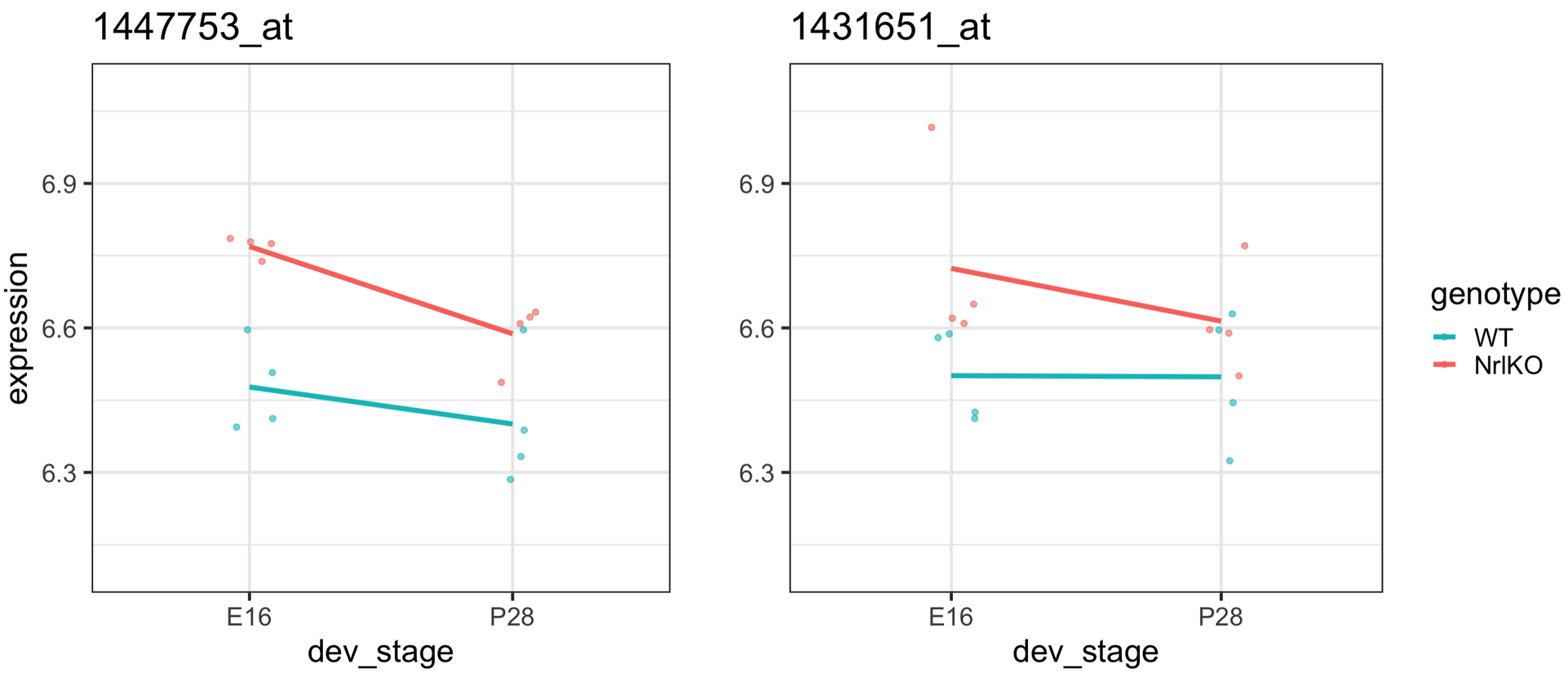
```
# recall our unbalanced design
table(pData(eset)$genotype, pData(eset)$dev_stage)
```

```
       E16 P2 P6 P10 P28
WT       4  4  4   4   4
NrlKO    3  4  4   4   4
```

```
# Duplicate sample GSM92615 (E16 NrlKO) and add noise expression
twoGenes <- filter(twoGenes, sample_id == "GSM92615") %>%
  mutate(expression = expression + rnorm(n(), 0, 0.1)) %>%
  rbind(twoGenes)
```

Ex 3: *BALANCED* & only genotype at E16 is significant

Plots | `lm` output



Ex 3: *BALANCED* & only genotype at E16 is significant

For both of these genes:

- The interaction effect is not significant (almost parallel pattern)
- The effect of developmental stage is not significant for WT (almost flat pattern)
- There is a significant genotype effect at E16
- There may be a genotype effect *regardless* of the developmental stage (**main** effect). However, that hypothesis is **not** tested here!!

- How do we test a **main** effect??

How do we test for a main effect?

- The main effect measures the *overall* association between the response and a factor - it is the (weighted) average of an effect over the levels of the other factor
- `anova()` can be used to test the main effects
- The following is the null hypothesis that there is no main effect of genotype:

$$H_0 : \frac{(E[Y_{KO,E16}] - E[Y_{WT,E16}]) + (E[Y_{KO,P28}] - E[Y_{WT,P28}])}{2} = 0$$

> **Note**
>
> For unbalanced experiments $H_0 : w_1 \text{effect}_{E16} + w_2 \text{effect}_{P28} = 0$, where w_1 and w_2 are sample size weights

Main effects using anova

```
filter(twoGenes, gene == "1447753_at") %>%
  lm(expression ~ genotype * dev_stage, data = .) %>%
  anova() %>%
  tidy()
```

```
# A tibble: 4 × 6
  term                df  sumsq  meansq statistic   p.value
  <chr>            <int>  <dbl>   <dbl>     <dbl>     <dbl>
1 genotype             1 0.230  0.230       28.2   0.000184
2 dev_stage            1 0.0667 0.0667       8.20  0.0142
3 genotype:dev_stage   1 0.0110 0.0110       1.35  0.268
4 Residuals           12 0.0976 0.00813     NA    NA
```

As we suspected, there is a **significant genotype effect** for this probe (1447753_at), i.e., its mean expression changes in NrlKO group (compared to WT), on average over developmental stages.

> ⓘ **Technical note:**
>
> `anova()` uses *type I sums of squares* (sequential; conditional on previous terms), thus order matters in unbalanced designs! See this primer on types of sums of squares for an intuitive explanation.

Main & interaction effects: important notes

- A **significant interaction effect** means that the effect of one factor depends on the levels of another
  - e.g., the effect of genotype depends on developmental stage
- **Main effects**: are the (weighted) average of an effect over the levels of the other factor
- A **non-significant main effect** means that, on average, there's no evidence of a factor's effect
  - e.g., no evidence of a genotype effect, on average over both developmental stages

Danger

If the interaction is significant, it is possible that one or both simple effects are significant but the average effect (i.e., the main effect) is not. This is because the effect of a factor *depends on* the level of the other one. Looking at main effects alone may mask interesting results!

Additive models

- In some applications, we need to/want to test the interaction term
- However, additive models are simpler and smaller
- If there are no statistical or biological grounds to include the interaction term, additive models are preferred
- Additive effects: $E[Y_{NrlKO,P28}] - E[Y_{WT,E16}] = \tau_{KO} + \tau_{P28}$

```
filter(twoGenes, gene == "1447753_at") %>%
  lm(expression ~ genotype + dev_stage, data = .) %>%
  tidy()
```

```
# A tibble: 3 × 5
  term          estimate std.error statistic  p.value
  <chr>            <dbl>     <dbl>     <dbl>    <dbl>
1 (Intercept)      6.50     0.0396    164.   5.90e-23
2 genotypeNrlKO    0.240    0.0457      5.24 1.59e- 4
3 dev_stageP28    -0.129    0.0457     -2.83 1.43e- 2
```

Additive models and balanced designs

- In an additive model, the `lm()` parameters for balanced designs are **average effects**, over the levels of the other factor - same as in `anova()`!
  - Note the agreement between `lm` and `anova`; this is gone in unbalanced designs since weights are computed differently!
- The intercept parameter is now $\bar{Y} - \bar{x}_{ij,KO}\hat{\tau}_{KO} - \bar{x}_{ij,P28}\hat{\tau}_{P28}$

> **Note**
>
> *Type III sum of squares* (partial; conditional on all other terms in the model) are required for agreement in unbalanced designs (use `car::Anova()` to obtain) - beyond our scope

Parameters in additive models represent main effects

```
(fit <- filter(twoGenes, gene == "1447753_at") %>%
  lm(expression ~ genotype + dev_stage, data = .)) %>%
  tidy()
```

```
# A tibble: 3 × 5
  term          estimate std.error statistic  p.value
  <chr>            <dbl>     <dbl>     <dbl>    <dbl>
1 (Intercept)      6.50     0.0396    164.   5.90e-23
2 genotypeNrlKO    0.240    0.0457      5.24 1.59e- 4
3 dev_stageP28    -0.129    0.0457     -2.83 1.43e- 2
```

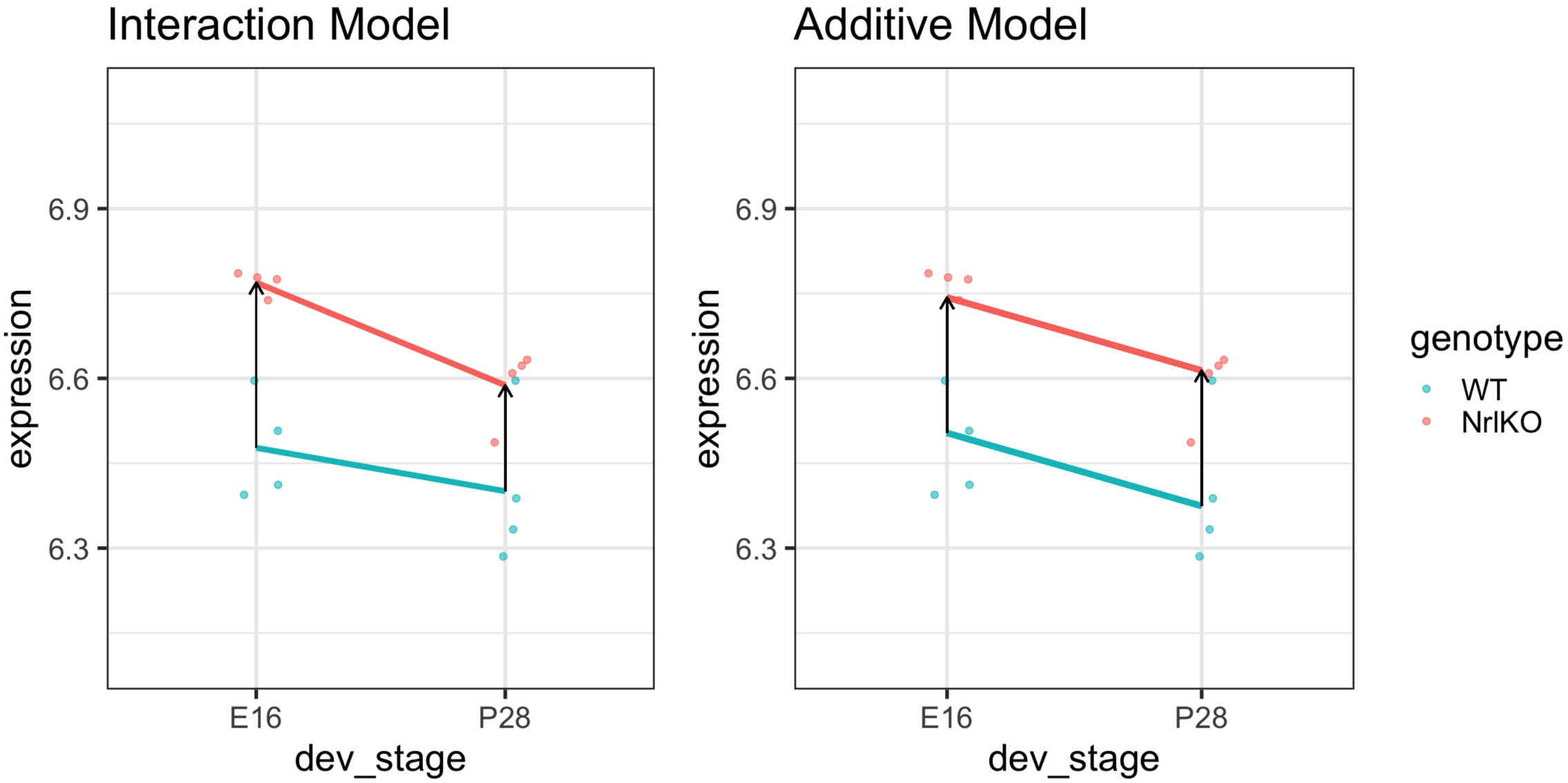
```
tidy(fit)$statistic[2]^2
```

```
[1] 27.49729
```

```
fit %>% anova() %>% tidy()
```

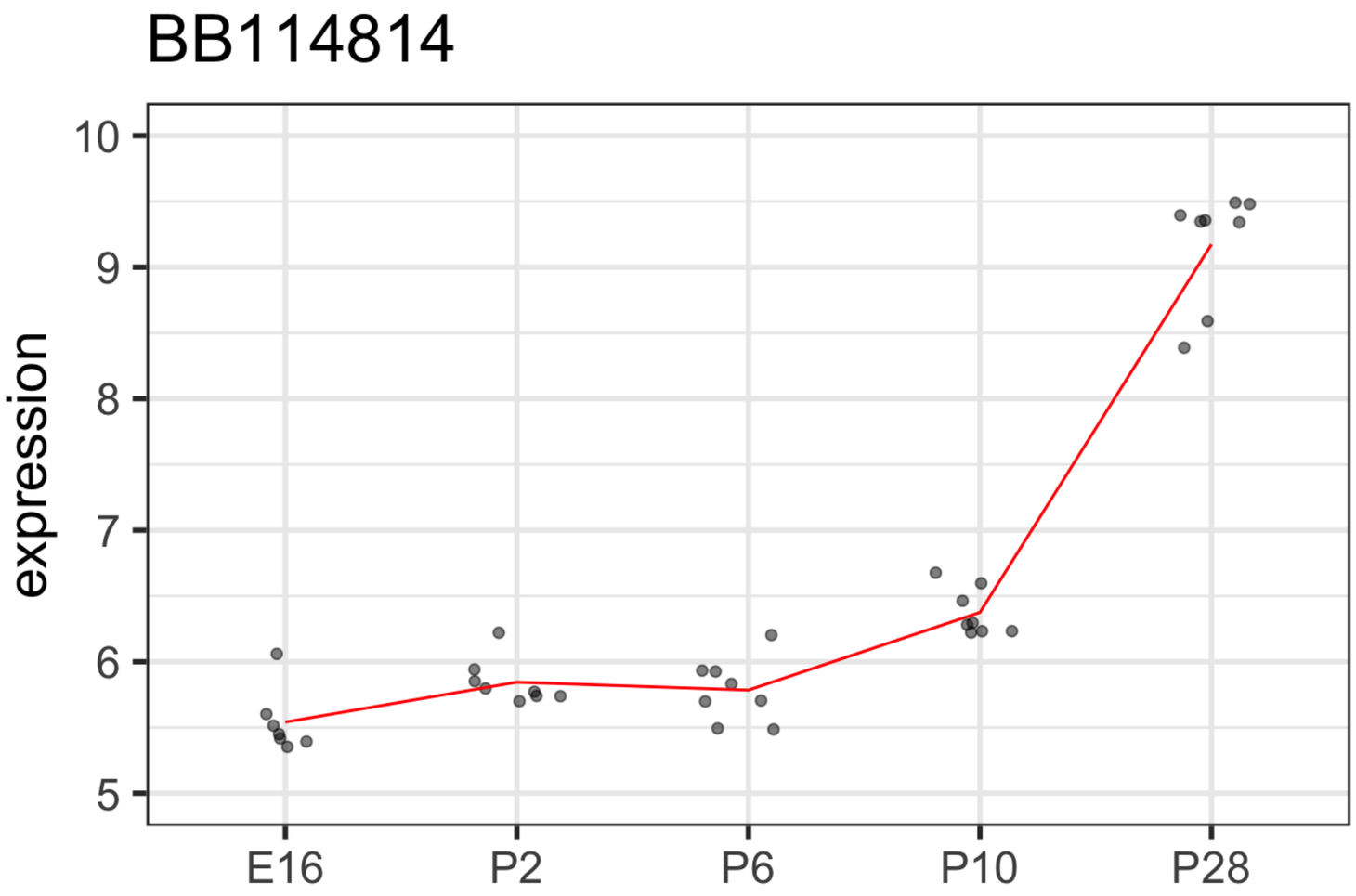
```
# A tibble: 3 × 6
  term         df  sumsq  meansq statistic   p.value
  <chr>     <int>  <dbl>   <dbl>     <dbl>     <dbl>
1 genotype      1 0.230  0.230       27.5   0.000159
2 dev_stage     1 0.0667 0.0667       7.99  0.0143
3 Residuals    13 0.109  0.00835     NA    NA
```

Additive vs interaction models



Interactions with multi-level factors (more than 2 groups)

Back to our old friend the BB114814 gene



Interactions with multi-level factors (more than 2 groups)

We can generalize the regression model to factors with more levels (e.g., E16, P2, P10 and P28): we just add more indicator variables (and parameters)!

With interaction

▶ Code

```
# A tibble: 10 × 5
   term                        estimate std.error statistic  p.value
   <chr>                          <dbl>     <dbl>     <dbl>    <dbl>
 1 (Intercept)                   5.43        0.124    43.8    4.76e-28
 2 genotypeNrlKO                 0.252       0.189     1.33   1.95e- 1
 3 dev_stageP2                   0.399       0.175     2.27   3.05e- 2
 4 dev_stageP6                   0.195       0.175     1.11   2.75e- 1
 5 dev_stageP10                  0.920       0.175     5.24   1.29e- 5
 6 dev_stageP28                  3.96        0.175    22.6    5.97e-20
 7 genotypeNrlKO:dev_stageP2    -0.226       0.258    -0.877  3.88e- 1
 8 genotypeNrlKO:dev_stageP6     0.0599      0.258     0.232  8.18e- 1
 9 genotypeNrlKO:dev_stageP10   -0.208       0.258    -0.804  4.28e- 1
10 genotypeNrlKO:dev_stageP28   -0.694       0.258    -2.69   1.18e- 2
```

Note

All the `dev_stage` parameters are still **simple** effects, but we now have more: one for each level compared to the reference

Factors with multiple levels (cont.)

Without interaction: additive

```
(addFit <- lm(expression ~ genotype + dev_stage, data = bb1gene)) %>%
  tidy()
```

```
# A tibble: 6 × 5
  term           estimate std.error statistic  p.value
  <chr>             <dbl>     <dbl>     <dbl>    <dbl>
1 (Intercept)      5.53      0.110     50.2   9.62e-33
2 genotypeNrlKO    0.0317    0.0878     0.361 7.21e- 1
3 dev_stageP2      0.302     0.142      2.13  4.11e- 2
4 dev_stageP6      0.241     0.142      1.70  9.87e- 2
5 dev_stageP10     0.832     0.142      5.86  1.44e- 6
6 dev_stageP28     3.63      0.142     25.6   2.43e-23
```

Parameters are now **main** effects (on average over the levels of the other factor), but we have more!

> **Question**
>
> Does developmental stage have a significant effect on this gene's expression?

We haven't tested that!!

Recall: *F*-test and overall significance

- the *t*-test in linear regression allows us to test single hypotheses; these are given in the summary of `lm`

$$H_0 : \tau_i = 0$$

$$H_A : \tau_j \neq 0$$

- but we often like to test multiple hypotheses *simultaneously*:

$$H_0 : \tau_{P2} = \tau_{P6} = \tau_{P10} = \tau_{P28} = 0 \text{ [AND statement]}$$

$$H_A : \tau_j \neq 0 \text{ for at least one j [OR statement]}$$

the *F*-test allows us to test such compound tests

Overall effects: compound tests

Interaction model with two factors: genotype and (5-level) developmental time

`lm` output tests the following null hypotheses (OR):

> $H_0 : \tau_{KO} = 0$ (1 df)
> $H_0 : \tau_{P2} = \tau_{P6} = \tau_{P10} = \tau_{P28} = 0$ (**in WT!**, 4 df)
> $H_0 : \tau_{KO:P2} = \tau_{KO:P6} = \tau_{KO:P10} = \tau_{KO:P28} = 0$ (4 df)

`anova` output: tests overall effects of a factor (AND) controlling for the previous ones

```
1 anova(itxFit) %>% tidy()
```

```
# A tibble: 4 × 6
  term                df   sumsq  meansq statistic   p.value
  <chr>            <int>   <dbl>   <dbl>     <dbl>     <dbl>
1 genotype             1  0.0693  0.0693      1.13  2.97e- 1
2 dev_stage            4 71.0    17.8       288.    6.72e-23
3 genotype:dev_stage   4  0.689   0.172       2.80  4.43e- 2
4 Residuals           29  1.78    0.0616     NA    NA
```

Overall effects: compound tests (cont.)

Additive model with genotype and development time (5-level); no interaction

`lm` output tests the following null hypotheses (OR)

> $H_0 : \tau_{KO} = 0$ (1 df)
> $H_0 : \tau_{P2} = \tau_{P6} = \tau_{P10} = \tau_{P28} = 0$ (**on average!**, 4 df)

`anova` output tests overall effects of a factor (AND) controlling for the previous ones

```
1 anova(addFit) %>% tidy()
```

```
# A tibble: 3 × 6
  term          df   sumsq  meansq statistic   p.value
  <chr>      <int>   <dbl>   <dbl>     <dbl>     <dbl>
1 genotype       1  0.0693  0.0693     0.925  3.43e- 1
2 dev_stage      4 71.0    17.8      237.     8.45e-24
3 Residuals     33  2.47    0.0750    NA      NA
```

> **Note**
>
> The t-test in `lm` and the F-test (1 df) in `anova` for genotype are not equivalent here due to unbalancedness (order matters)

These examples are just special cases of *nested models*

For example: does development have a significant effect on gene expression?

Compare the models with and without `dev_stage`!

Model 1: `expression ~ genotype`

Model 2: `expression ~ genotype + dev_stage`

Mathematically:

Model 1: $Y_{ijk} = \theta + \tau_{KO} x_{KO,ijk} + \varepsilon$

Model 2: $Y_{ijk} = \theta + \tau_{KO} x_{KO,ijk} + \tau_{P2} x_{P2,ijk} + \tau_{P6} x_{P6,ijk} + \tau_{P10} x_{P10,ijk} + \tau_{P28} x_{P28,ijk} + \varepsilon$

$$H_0 : \tau_{P2} = \tau_{P6} = \tau_{P10} = \tau_{P28} = 0$$

The $x_{**,ijk}$ are indicator variables (see companion notes)

More general: F-test to compare nested models

$H_0 : \alpha_{k+1} =. . . = \alpha_{k+p}$

$$F = \frac{(SS_{reduced} - SS_{full})/(p)}{SS_{full}/(n-p-k-1)} \sim \mathbf{F}_{p,\, n-p-k-1}$$

This F-statistic compares the following two models:

- Reduced (k + 1 parameters):

$$y_i = \alpha_0 + \alpha_1 x_{i1} + . . . + \alpha_k x_{ik} + \epsilon_i$$

- Full (p + k + 1 parameters):

$$y_i = \alpha_0 + \alpha_1 x_{i1} + . . . + \alpha_k x_{ik} + . . . + \alpha_p x_{ip} + \epsilon_i$$

A *significant* F-statistic here means that the full model explains significantly more variation in the outcome variable than the reduced model

Nested models in R

```r
addReduced <- lm(expression ~ genotype, data = bb1gene)
addFull <- lm(expression ~ genotype + dev_stage, data = bb1gene)
anova(addReduced,addFull)
```

```
Analysis of Variance Table

Model 1: expression ~ genotype
Model 2: expression ~ genotype + dev_stage
  Res.Df    RSS Df Sum of Sq      F    Pr(>F)
1     37 73.497
2     33  2.474  4    71.023 236.84 < 2.2e-16 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```r
anova(addFull) %>% tidy()
```

```
# A tibble: 3 × 6
  term         df   sumsq  meansq statistic   p.value
  <chr>     <int>   <dbl>   <dbl>     <dbl>     <dbl>
1 genotype      1  0.0693  0.0693     0.925  3.43e- 1
2 dev_stage     4 71.0    17.8      237.     8.45e-24
3 Residuals    33  2.47    0.0750    NA      NA
```

Another special case: overall goodness of fit!

Compare the full *vs* the intercept-only models (compound test)!

$$H_0 : \tau_{KO} = \tau_{P2} = \tau_{P6} = \tau_{P10} = \tau_{P28} = 0 \ \ (5 \text{ df})$$

```
addReduced <- lm(expression ~ 1, data = bb1gene)
anova(addReduced,addFull)
```

```
Analysis of Variance Table

Model 1: expression ~ 1
Model 2: expression ~ genotype + dev_stage
  Res.Df    RSS Df Sum of Sq      F    Pr(>F)
1     38 73.566
2     33  2.474  5    71.092 189.66 < 2.2e-16 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Goodness of fit also given in output of `lm`

```
1 summary(addFull)
```

```
Call:
lm(formula = expression ~ genotype + dev_stage, data = bb1gene)

Residuals:
     Min       1Q   Median       3Q      Max
-0.80137 -0.12454 -0.03212  0.17038  0.50036

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)   5.52734    0.11012  50.192  < 2e-16 ***
genotypeNrlKO 0.03167    0.08785   0.361   0.7207
dev_stageP2   0.30152    0.14185   2.126   0.0411 *
dev_stageP6   0.24102    0.14185   1.699   0.0987 .
dev_stageP10  0.83185    0.14185   5.864 1.44e-06 ***
```

Summary so far

- ***t*-tests** can be used to test the equality of **2** population means
- **ANOVA** can be used to test the equality of **more than 2** population means simultaneously (main effects)
- **Linear regression** provides a general framework for modelling the relationship between a response and different type of explanatory variables
  - *t*-tests are used to test the significance of **simple effects** (*individual* coefficients)
  - *F*-tests are used to test the significance of **main effects** (*simultaneously* multiple coefficients)
  - *F*-tests are used to compare nested models (**overall** effects or **goodness of fit**)
- Next up: continuous explanatory variables! Multiple genes!