

# MicroRNA titration by pseudo-targets: an alternative explanation to seed match conservation

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Introduction

An alternative hypothesis

Testing the hypothesis

miRNA quantification

mRNA quantification

Relative abundance of miRNAs and mRNAs

Conclusion

Supplementary data

# microRNA target identification

MicroRNA  
titration by  
pseudo-targets

## Introduction

An alternative  
hypothesis

Testing the  
hypothesis

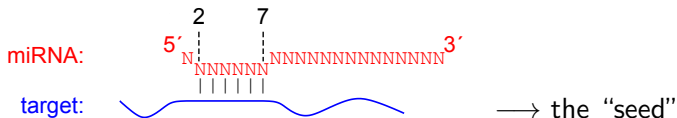
miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# microRNA target identification

MicroRNA  
titration by  
pseudo-targets



Introduction

An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# microRNA target identification

Computational programs for target prediction: look for seed matches in 3' UTRs, select the ones that were conserved in evolution.

Introduction

An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

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Such short matches are very frequent (60 % of human coding genes seem to be targeted: Friedman *et al.*, 2009).

Introduction

An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

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⇒ miRNAs are implicated in every physiological process in animals.

Introduction

An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

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Such short matches are very frequent (60 % of human coding genes seem to be targeted: Friedman *et al.*, 2009).

⇒ miRNAs are implicated in every physiological process in animals.

miRNA-mediated repression is very modest (usually  $< 2$ -fold): lower than well tolerated fluctuations in gene expression (e.g., haplosufficiency). Why have these sites been conserved if they are not functional?

Introduction

An alternative  
hypothesis

Testing the  
hypothesis

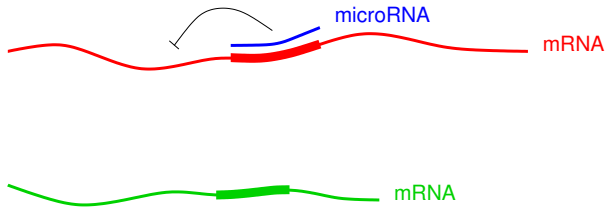
miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# An alternative hypothesis

MicroRNA  
titration by  
pseudo-targets



Introduction

An alternative  
hypothesis

Testing the  
hypothesis

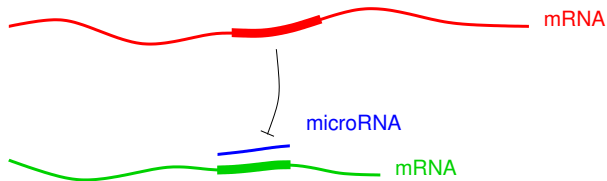
miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# An alternative hypothesis

MicroRNA  
titration by  
pseudo-targets



Introduction

An alternative  
hypothesis

Testing the  
hypothesis

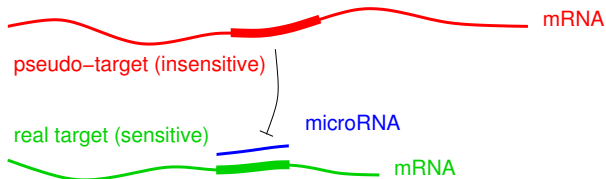
miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# An alternative hypothesis

MicroRNA  
titration by  
pseudo-targets



Introduction

An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# Absolute RNA quantification

For mRNAs to titrate miRNAs, they need to be abundant enough.

► Issues with published examples

Introduction

An alternative hypothesis

Testing the hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary data

# Absolute RNA quantification

For mRNAs to titrate miRNAs, they need to be abundant enough.

► Issues with published examples

Murine cell line “C2C12”: differentiate in myotubes;  
expresses muscle-specific miR-1, miR-206 and miR-133.

Introduction

An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

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miR-1 and miR-206 (at least) control C2C12 differentiation (Goljanek-Whysall *et al.*, 2012).

Introduction

An alternative hypothesis

Testing the hypothesis

miRNA quantification

mRNA quantification

Relative abundance of miRNAs and mRNAs

Conclusion

Supplementary data

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⇒ absolute quantification of miR-1, miR-206, miR-133 and their predicted targets in differentiating C2C12.

Introduction

An alternative hypothesis

Testing the hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary data

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Introduction

An alternative hypothesis

Testing the hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary data

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- miRNAs: Northern blot (calibrated with RNA oligos);

Introduction

An alternative hypothesis

Testing the hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary data

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⇒ absolute quantification of miR-1, miR-206, miR-133 and their predicted targets in differentiating C2C12.

- miRNAs: Northern blot (calibrated with RNA oligos);
- mRNAs: RNA-Seq (calibrated with *in vitro* transcripts).

Introduction

An alternative hypothesis

Testing the hypothesis

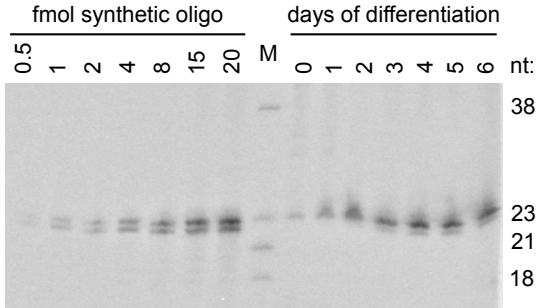
miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary data

# miRNA quantification

MicroRNA  
titration by  
pseudo-targets



(quantification of miR-1 and miR-206)

Introduction

An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# miRNA quantification

MicroRNA  
titration by  
pseudo-targets

Introduction

An alternative  
hypothesis

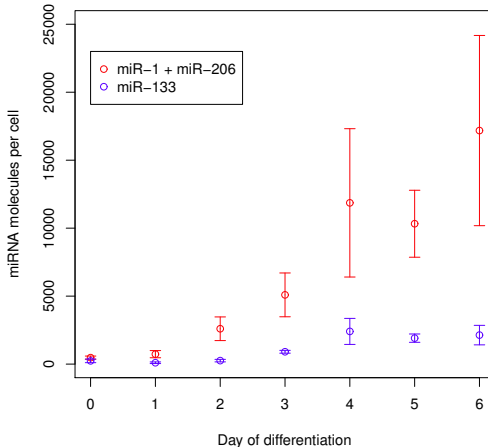
Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

**miRNA abundance during C2C12 differentiation**



# mRNA quantification

MicroRNA  
titration by  
pseudo-targets

Introduction

An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification

**mRNA quantification**

Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# mRNA quantification

Very deep sequencing: three time points (day 0, day 3, day 6) in triplicate; each replicate: between 267 and 333 million transcriptome-matching reads.

► Statistics



Introduction

An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
**mRNA quantification**  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

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► Statistics



27 synthetic spike-ins, for calibration.

Introduction

An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# mRNA quantification

MicroRNA  
titration by  
pseudo-targets

Introduction

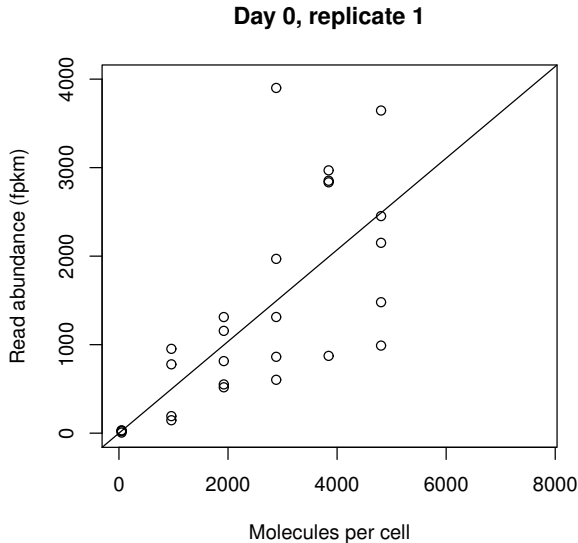
An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
**mRNA quantification**  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data



# mRNA quantification

MicroRNA  
titration by  
pseudo-targets

Introduction

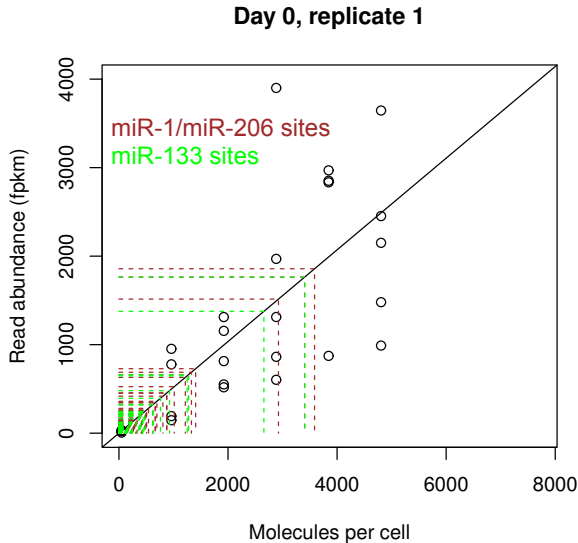
An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

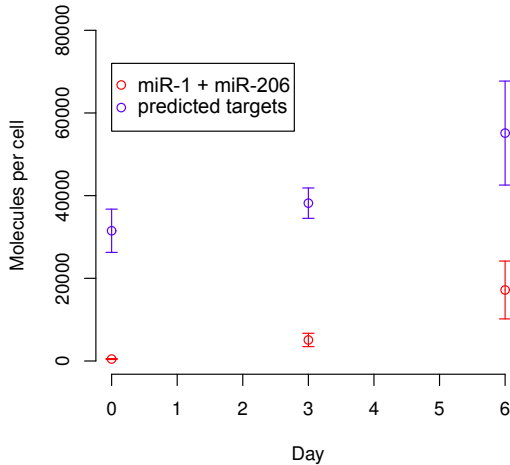
Conclusion

Supplementary  
data



# mRNA quantification

MicroRNA  
titration by  
pseudo-targets



Introduction

An alternative  
hypothesis

Testing the  
hypothesis

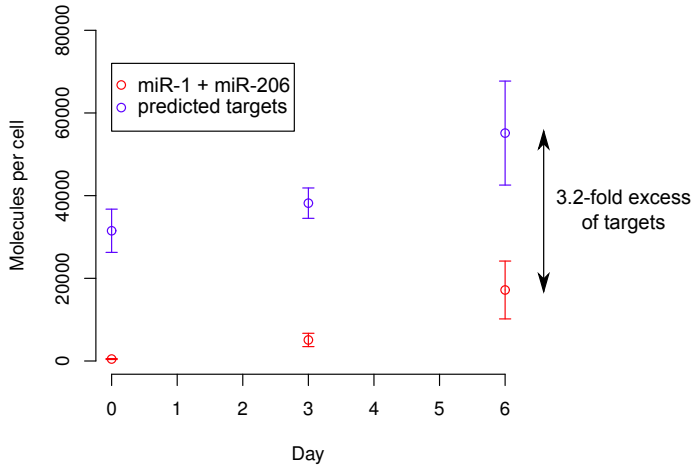
miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# mRNA quantification

MicroRNA  
titration by  
pseudo-targets



Introduction

An alternative  
hypothesis

Testing the  
hypothesis

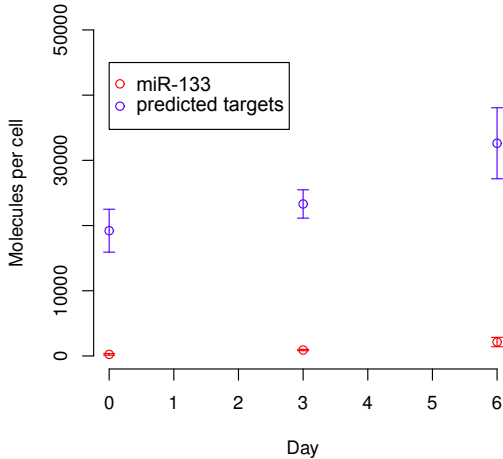
miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# mRNA quantification

MicroRNA  
titration by  
pseudo-targets



Introduction

An alternative  
hypothesis

Testing the  
hypothesis

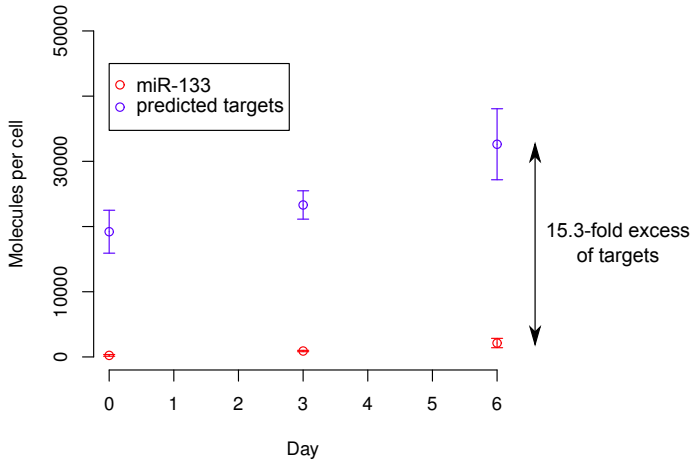
miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# mRNA quantification

MicroRNA  
titration by  
pseudo-targets



Introduction

An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# Concentration effects

MicroRNA  
titration by  
pseudo-targets

Introduction

An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification

mRNA quantification

Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# Concentration effects

MicroRNA  
titration by  
pseudo-targets

Introduction

An alternative  
hypothesis

Testing the  
hypothesis

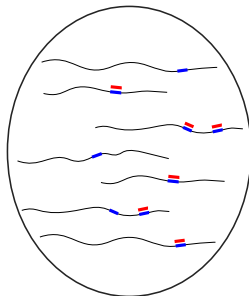
miRNA quantification

mRNA quantification

Relative abundance of  
miRNAs and mRNAs

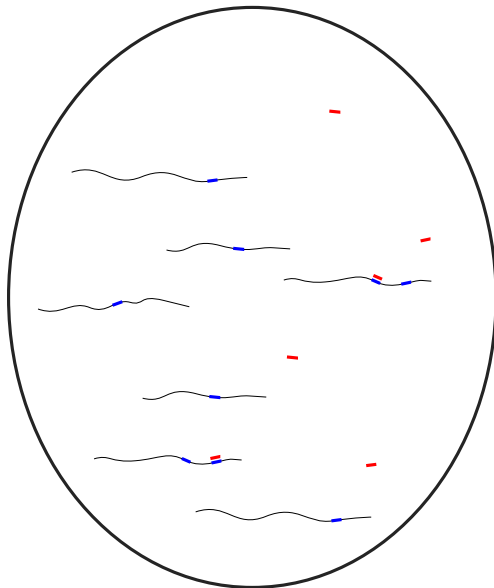
Conclusion

Supplementary  
data



# Concentration effects

MicroRNA  
titration by  
pseudo-targets



Introduction

An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

## Concentration effects

| miRNA family      | Predicted targets |                       |
|-------------------|-------------------|-----------------------|
|                   | Total             | Above $10 \times K_d$ |
| miR-1 and miR-206 | 436               | 31 (7 %)              |
| miR-133           | 384               | 22 (6 %)              |

( $K_d$  value taken from Wee *et al.*, 2012: 26 pM)

Introduction

An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

## Concentration effects

Titration effect of individual miRNA binding sites:

| mRNA          | Sample | Increase in free miR-1/miR-206<br>if site is lost |
|---------------|--------|---------------------------------------------------|
| <i>Anxa2</i>  | Day 0  | 8 %                                               |
| <i>Ptma</i>   | Day 0  | 9 %                                               |
| <i>Tmsb4x</i> | Day 0  | 10 %                                              |
| <i>Actb</i>   | Day 3  | 15 %                                              |
| <i>Tmsb4x</i> | Day 3  | 9 %                                               |
| <i>Actb</i>   | Day 6  | 13 %                                              |
| <i>Tmsb4x</i> | Day 6  | 11 %                                              |

Introduction

An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# Concentration effects

Titration effect of individual miRNA binding sites:

| mRNA          | Sample | Increase in free miR-133<br>if site is lost |
|---------------|--------|---------------------------------------------|
| <i>Tpm4</i>   | Day 0  | 5 %                                         |
| <i>Ptma</i>   | Day 0  | 16 %                                        |
| <i>Eif4a1</i> | Day 0  | 5 %                                         |
| <i>Tagln2</i> | Day 0  | 6 %                                         |
| <i>Ptma</i>   | Day 3  | 7 %                                         |
| <i>Eef1a1</i> | Day 3  | 8 %                                         |
| <i>Ptma</i>   | Day 6  | 6 %                                         |
| <i>Eef1a1</i> | Day 6  | 6 %                                         |

# Conclusion

MicroRNA  
titration by  
pseudo-targets

Introduction

An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification

mRNA quantification

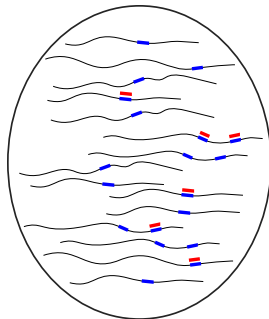
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# Conclusion

miRNA titration by mRNAs *does* occur.



Introduction

An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# Conclusion

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Introduction

An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# Conclusion

miRNA titration by mRNAs *does* occur.

Every cell where miR-1, miR-206, miR-133 titration is beneficial may express a different set of miRNA titrators.

Introduction

An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# Conclusion

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Every cell where miR-1, miR-206, miR-133 titration is beneficial may express a different set of miRNA titrators.

⇒ It cannot be assumed that every conserved binding site mediates functional target repression.

Introduction

An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

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Every cell where miR-1, miR-206, miR-133 titration is beneficial may express a different set of miRNA titrators.

⇒ It cannot be assumed that every conserved binding site mediates functional target repression.

The number of miRNA targets has probably been overestimated.

Introduction

An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# Acknowledgements

Anna  
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Laura  
Martinez



Natalia  
Pinzón Restrepo



Blaise  
Li



Introduction

An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

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MicroRNA  
titration by  
pseudo-targets

Anna  
Sergeeva



Laura  
Martinez



Natalia  
Pinzón Restrepo



Blaise  
Li



Introduction

An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data



# Supplementary data

## An alternative hypothesis:

- ▶ Robustness of biological pathways
- ▶ Non-discriminative predictions (coordinated targeting)
- ▶ Non-discriminative predictions (expression domains)
- ▶ Non-discriminative predictions (house-keeping genes)

## Testing the hypothesis:

- ▶ RNA-seq technical reproducibility
- ▶ RNA-seq biological reproducibility
- ▶ Issues with published examples
- ▶ Experimental procedure
- ▶ Amount of RNA per cell
- ▶ Differentiation controls
- ▶ Cell volume
- ▶ Sequencing depth
- ▶ Titration expression profiles
- ▶ Abundance vs. conservation

## Conclusion:

- ▶ Pseudo-targets for other regulators?
- ▶ Propagation of gene expression perturbation

Introduction

An alternative hypothesis

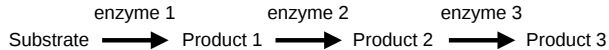
Testing the hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary data

# Biological robustness



MicroRNA  
titration by  
pseudo-targets

Introduction

An alternative  
hypothesis

Testing the  
hypothesis

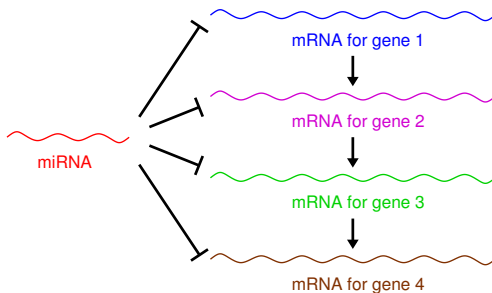
miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# Non-discriminative predictions

Targets for a given miRNA often belong to the same biological pathways.



Introduction

An alternative  
hypothesis

Testing the  
hypothesis

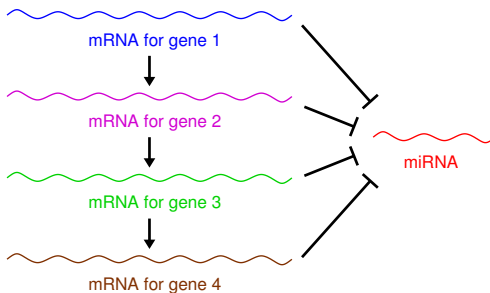
miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

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Introduction

An alternative  
hypothesis

Testing the  
hypothesis

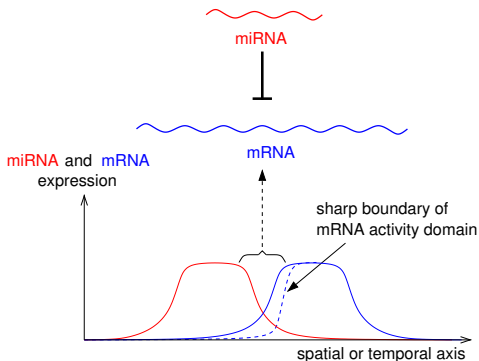
miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# Non-discriminative predictions

Expression domains for miRNAs and their predicted targets overlap at their boundaries.



Introduction

An alternative  
hypothesis

Testing the  
hypothesis

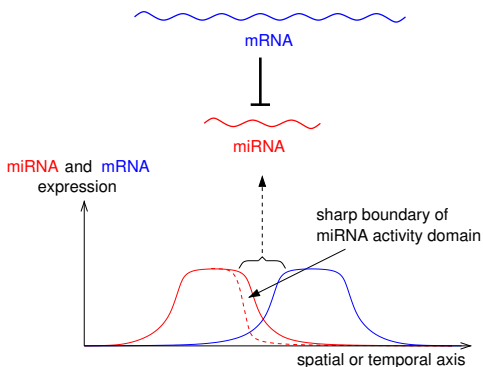
miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

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Introduction

An alternative  
hypothesis

Testing the  
hypothesis

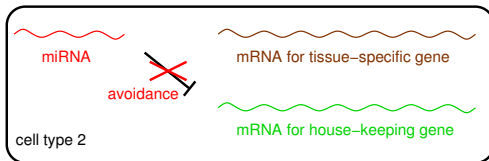
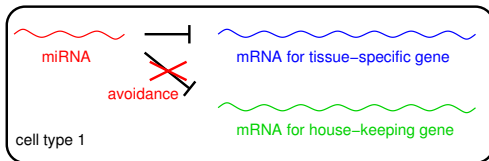
miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# Non-discriminative predictions

House-keeping genes are rarely predicted to be targeted.



Introduction

An alternative  
hypothesis

Testing the  
hypothesis

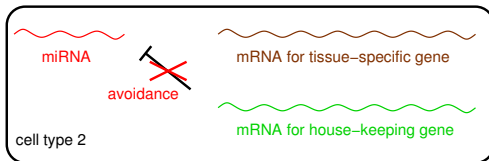
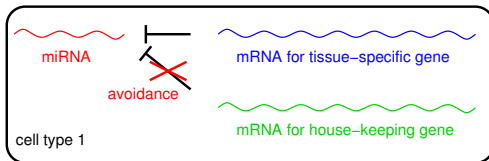
miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

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Introduction

An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# Technical reproducibility of RNA-seq

MicroRNA  
titration by  
pseudo-targets

Introduction

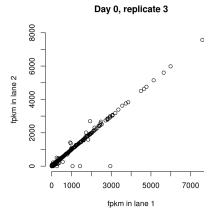
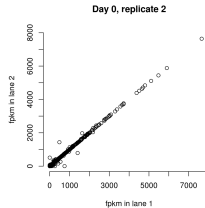
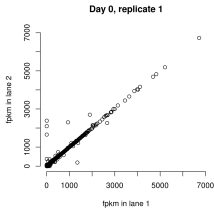
An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data



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MicroRNA  
titration by  
pseudo-targets

Introduction

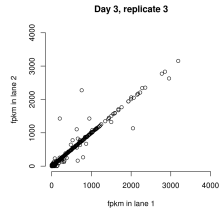
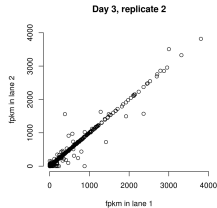
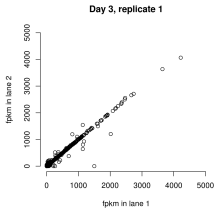
An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data



# Technical reproducibility of RNA-seq

MicroRNA  
titration by  
pseudo-targets

Introduction

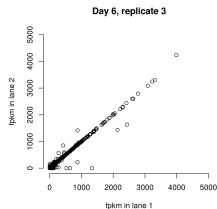
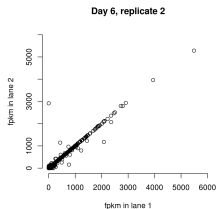
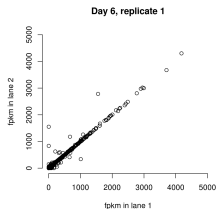
An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

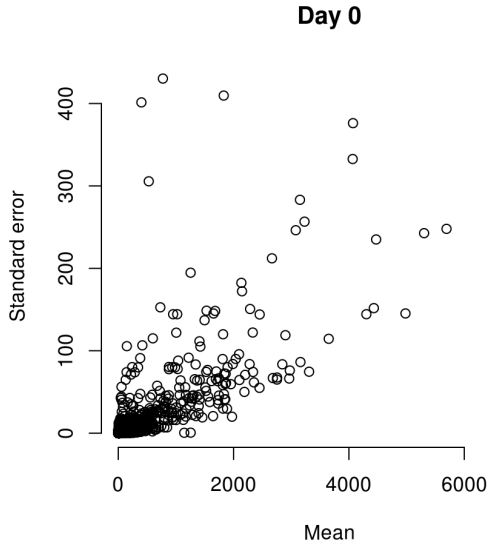
Conclusion

Supplementary  
data



# Biological reproducibility of RNA-seq

MicroRNA  
titration by  
pseudo-targets



Introduction

An alternative  
hypothesis

Testing the  
hypothesis

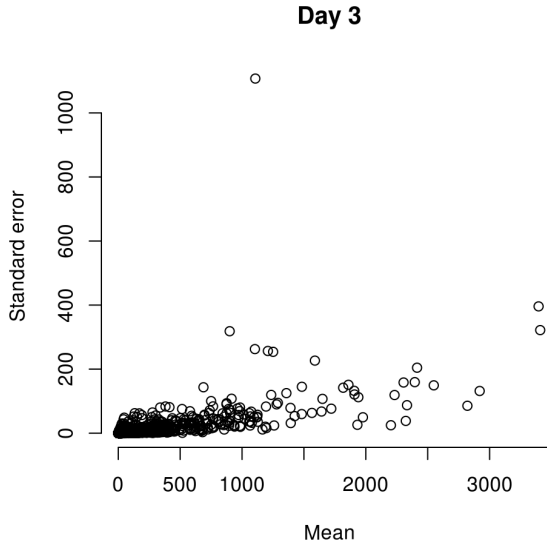
miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# Biological reproducibility of RNA-seq

MicroRNA  
titration by  
pseudo-targets



Introduction

An alternative  
hypothesis

Testing the  
hypothesis

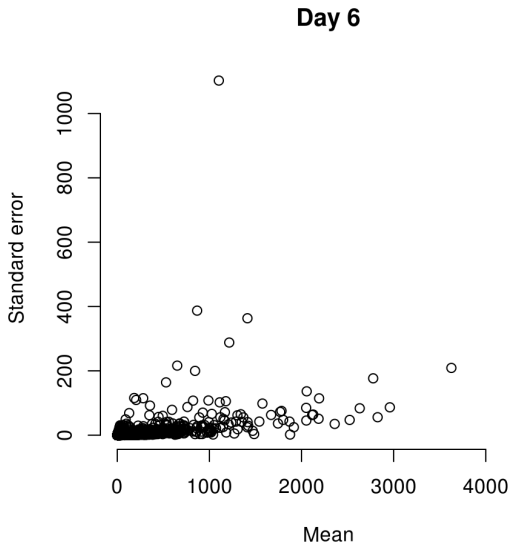
miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# Biological reproducibility of RNA-seq

MicroRNA  
titration by  
pseudo-targets



Introduction

An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# Reported examples of pseudo-targets

A proposed pseudo-target: PTENP1, that de-repressed PTEN (Poliseno *et al.*, 2010).

Introduction

An alternative hypothesis

Testing the hypothesis

miRNA quantification

mRNA quantification

Relative abundance of miRNAs and mRNAs

Conclusion

Supplementary data

# Reported examples of pseudo-targets

A proposed pseudo-target: PTENP1, that de-repressed PTEN (Poliseno *et al.*, 2010).

But PTENP1 mRNA is  $\approx 100$  times less abundant than the PTEN mRNA (Ebert and Sharp, 2010).

Introduction

An alternative hypothesis

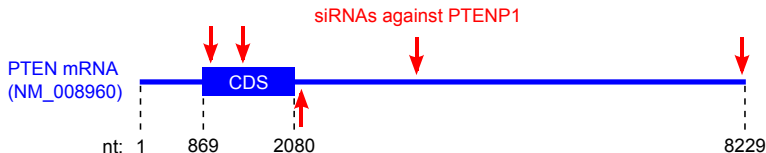
Testing the hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary data

# Reported examples of pseudo-targets



Introduction

An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# Reported examples of pseudo-targets

MicroRNA  
titration by  
pseudo-targets

Introduction

An alternative  
hypothesis

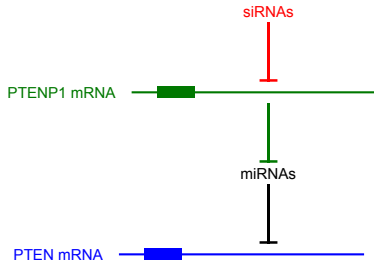
Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

Proposed by Poliseno *et al* (2010):



More probably:



# Reported examples of pseudo-targets

MicroRNA  
titration by  
pseudo-targets

siRNA #2 against PTENP1: 5' UAAUAAUCAUCAUUCUGGC 3'  
||||| ||| |||  
PTEN mRNA (nt 948-961): 3' GUUAUUA-----UAA-ACCU 5'

siRNA #2 against PTENP1: 5' UAAUAAUC-AUCAUUCUGGC 3'  
||||| |:| |:|  
PTEN mRNA (nt 8189-8208): 3' UUUUUUACUUGGAAAAUUU 5'

siRNA #2 against PTENP1: 5' UAAUAAUCAUCAUUCUGGC 3'  
||||| ||| :| |  
PTEN mRNA (nt 1383-1401): 3' AUUAUUUAUGUAUCGCGG 5'

siRNA #3 against PTENP1: 5' UCCUAUA--UGAUCUCUGAUG 3'  
||||| || |||||:  
PTEN mRNA (nt 2192-2212): 3' AGGAUAUUGACGUUAGACUGU 5'

siRNA #2 against PTENP1: 5' UAAUAAUCAUCAUUCUGGC 3'  
||||| |||  
PTEN mRNA (nt 3769-3782): 3' AUUAUUACC-----GACCU 5'

Introduction

An alternative  
hypothesis

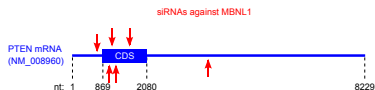
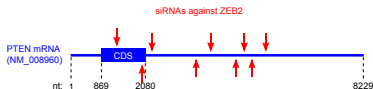
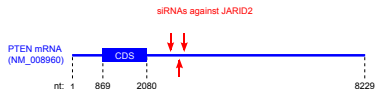
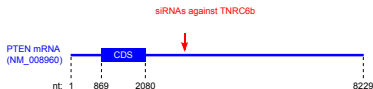
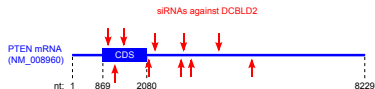
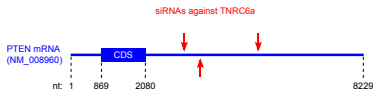
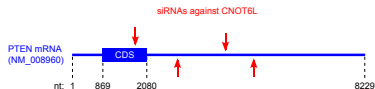
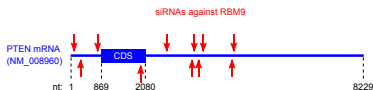
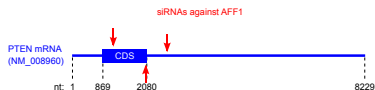
Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# Reported examples of pseudo-targets



Introduction

An alternative  
hypothesis

Testing the  
hypothesis

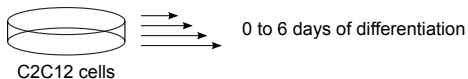
miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# Absolute RNA quantification

MicroRNA  
titration by  
pseudo-targets



Introduction

An alternative  
hypothesis

Testing the  
hypothesis

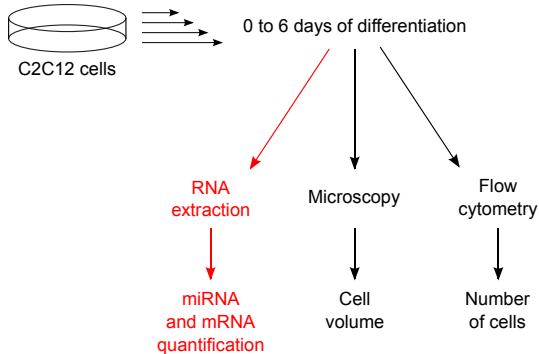
miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# Absolute RNA quantification

MicroRNA  
titration by  
pseudo-targets



Introduction

An alternative  
hypothesis

Testing the  
hypothesis

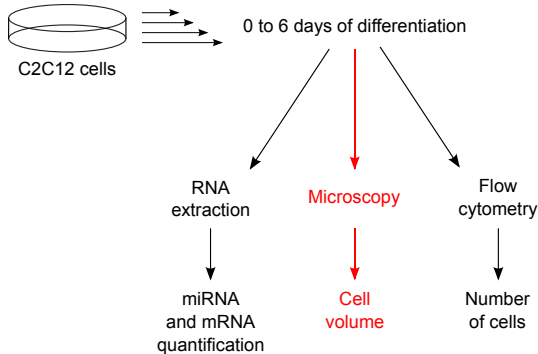
miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# Absolute RNA quantification

MicroRNA  
titration by  
pseudo-targets



Introduction

An alternative  
hypothesis

Testing the  
hypothesis

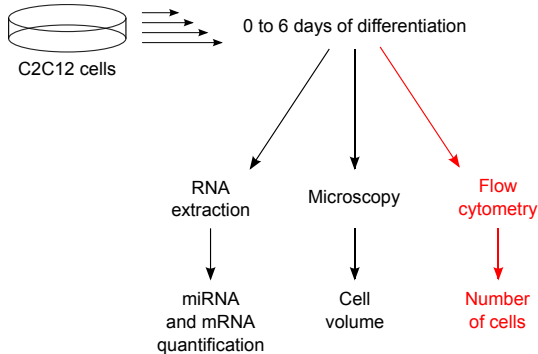
miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# Absolute RNA quantification

MicroRNA  
titration by  
pseudo-targets



Introduction

An alternative  
hypothesis

Testing the  
hypothesis

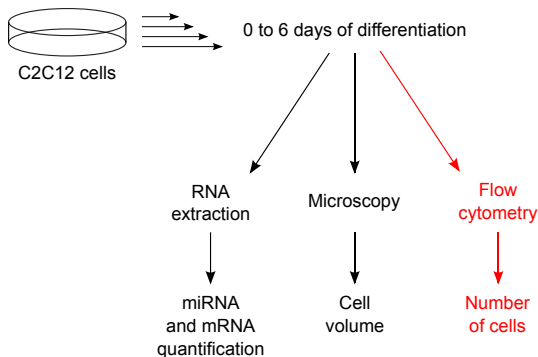
miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# Absolute RNA quantification

MicroRNA  
titration by  
pseudo-targets



(three biological replicates)

► Amount of RNA per cell

► Differentiation controls

◀ Return

Introduction

An alternative  
hypothesis

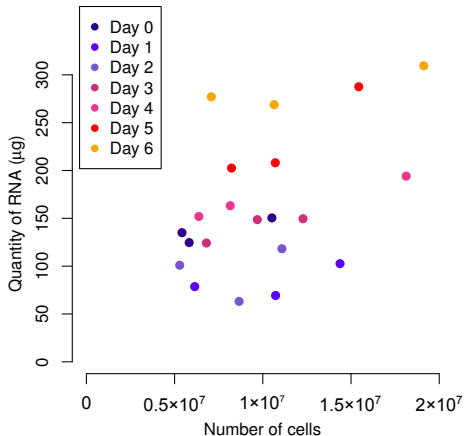
Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# Absolute RNA quantification



Introduction

An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# Differentiation controls

MicroRNA  
titration by  
pseudo-targets

Introduction

An alternative  
hypothesis

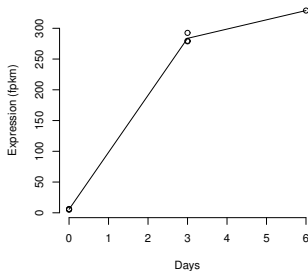
Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

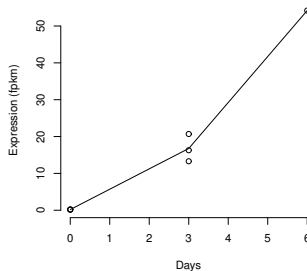
Conclusion

Supplementary  
data

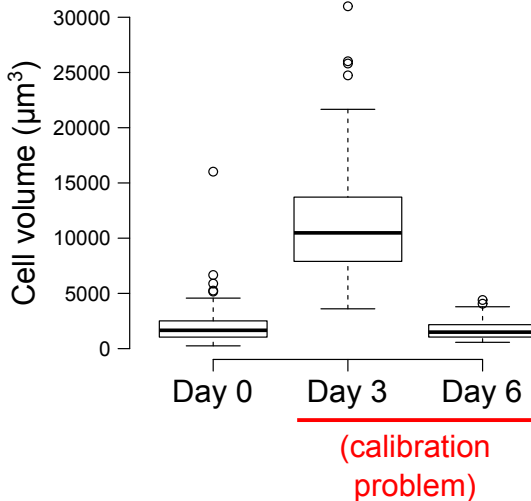
**Myogenin**



**Myosin heavy chain polypeptide 1**



## Cell volume



Introduction

An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

## Deep-sequencing statistics

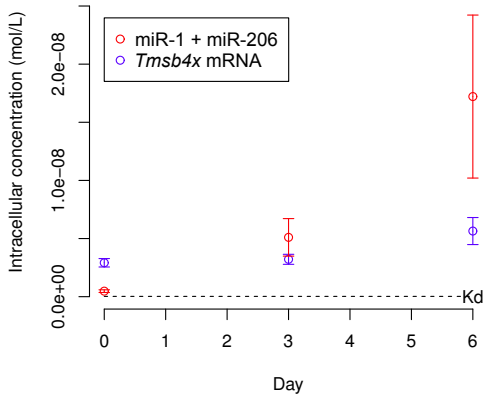
Number of reads per kb (median transcript, excluding spike-ins):

|       |             |        |
|-------|-------------|--------|
| Day 0 | Replicate 1 | 159.26 |
|       | Replicate 2 | 140.53 |
|       | Replicate 3 | 150.03 |
| Day 3 | Replicate 1 | 213.09 |
|       | Replicate 2 | 167.77 |
|       | Replicate 3 | 198.48 |
| Day 6 | Replicate 1 | 223.57 |
|       | Replicate 2 | 243.86 |
|       | Replicate 3 | 220.23 |

[Introduction](#)[An alternative hypothesis](#)[Testing the hypothesis](#)[miRNA quantification](#)  
[mRNA quantification](#)  
[Relative abundance of miRNAs and mRNAs](#)[Conclusion](#)[Supplementary data](#)

# Concentration effects

MicroRNA  
titration by  
pseudo-targets



Introduction

An alternative  
hypothesis

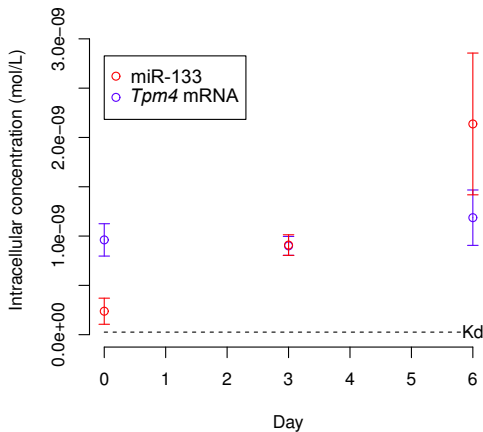
Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# Concentration effects



Introduction

An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

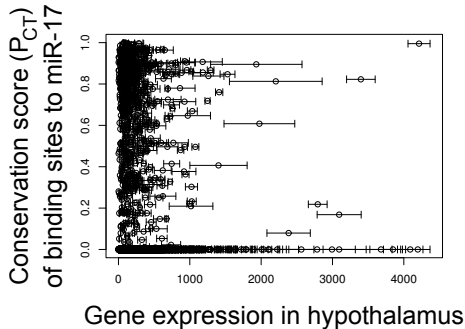
Conclusion

Supplementary  
data

# mRNA abundance and seed match conservation

MicroRNA  
titration by  
pseudo-targets

Kendall's  $\tau = 0.0873$   
( $p$ -value =  $1.18 \cdot 10^{-8}$ )



Introduction

An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# mRNA abundance and seed match conservation

MicroRNA  
titration by  
pseudo-targets

## Introduction

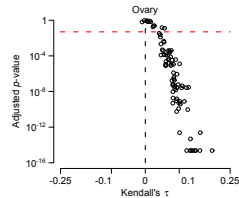
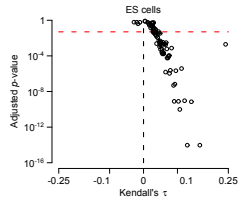
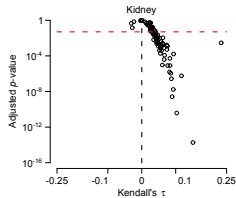
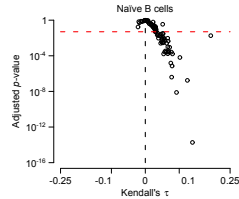
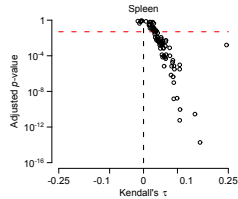
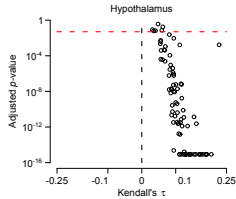
### An alternative hypothesis

### Testing the hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

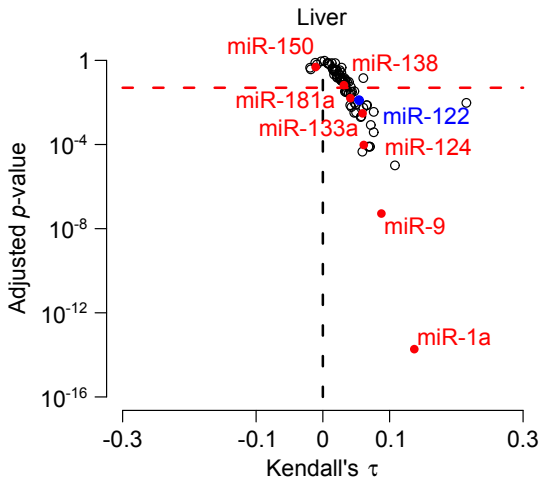
### Conclusion

### Supplementary data



# mRNA abundance and seed match conservation

MicroRNA  
titration by  
pseudo-targets



Introduction

An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

## Pseudo-targets for other regulators?

- ▶ Transcription factors: experimentally identified binding sites are poorly conserved among vertebrates (Odom *et al.*, 2007 and Schmidt *et al.*, 2010).

Introduction

An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

## Pseudo-targets for other regulators?

- ▶ Transcription factors: experimentally identified binding sites are poorly conserved among vertebrates (Odom *et al.*, 2007 and Schmidt *et al.*, 2010).
- ▶ RNA-binding proteins are poorly specific (thousands of experimentally validated targets for each analyzed protein: Hafner *et al.*, 2010 and Lebedeva *et al.*, 2011).

Introduction

An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

## Pseudo-targets for other regulators?

- ▶ Transcription factors: experimentally identified binding sites are poorly conserved among vertebrates (Odom *et al.*, 2007 and Schmidt *et al.*, 2010).
- ▶ RNA-binding proteins are poorly specific (thousands of experimentally validated targets for each analyzed protein: Hafner *et al.*, 2010 and Lebedeva *et al.*, 2011).

Real molecular events, which are neutral in evolutionary terms?

Introduction

An alternative hypothesis

Testing the hypothesis

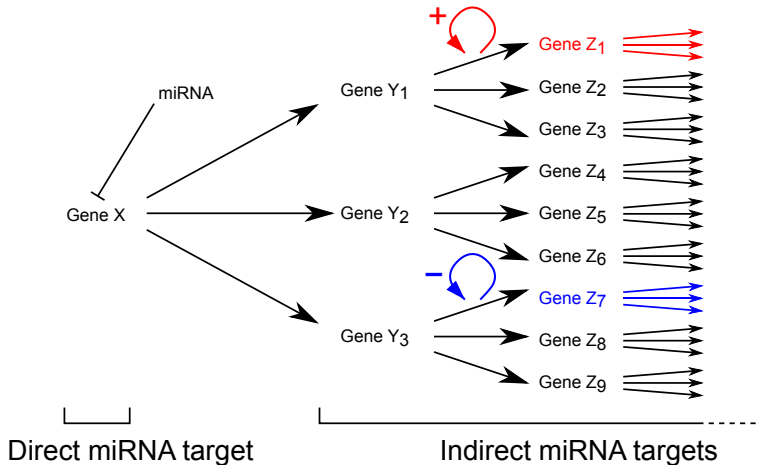
miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary data

# Propagation of gene expression perturbation

MicroRNA  
titration by  
pseudo-targets



Introduction

An alternative  
hypothesis

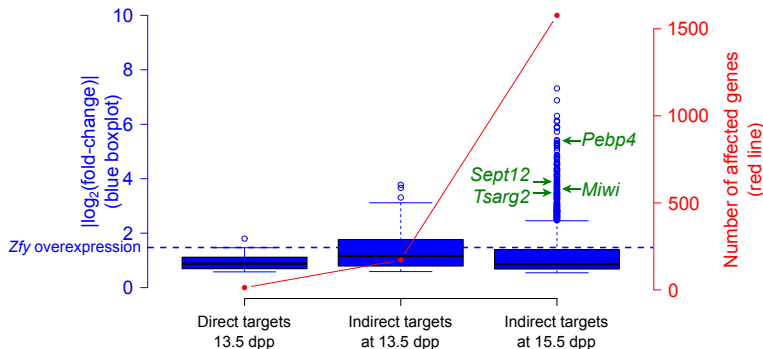
Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data

# Propagation of gene expression perturbation



(in collaboration with H. Royo and J. Turner, MRC, London)

Introduction

An alternative  
hypothesis

Testing the  
hypothesis

miRNA quantification  
mRNA quantification  
Relative abundance of  
miRNAs and mRNAs

Conclusion

Supplementary  
data