

Reciprocal regulatory relationships between microRNAs and mRNAs

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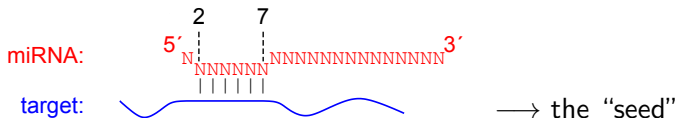
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Computational programs for target prediction: look for seed matches in 3' UTRs, select the ones that were conserved in evolution.

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

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⇒ miRNAs are implicated in every physiological process in animals.

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⇒ 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?

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- ▶ Most computationally predicted targets are not *functionally* targeted (not repressed enough).

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- ▶ Most computationally predicted targets are not *functionally* targeted (not repressed enough).
- ▶ Their phylogenetic conservation means that these binding sites have a function.

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- ▶ That function could be to repress the miRNA by titrating it.

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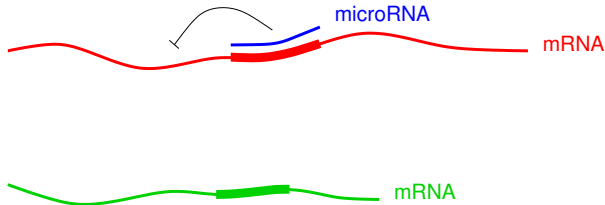
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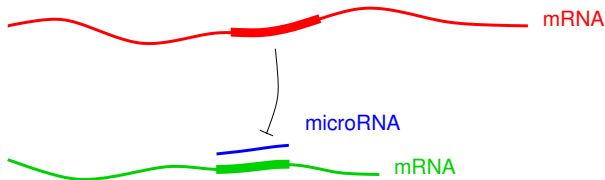
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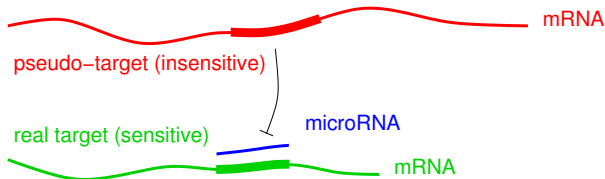
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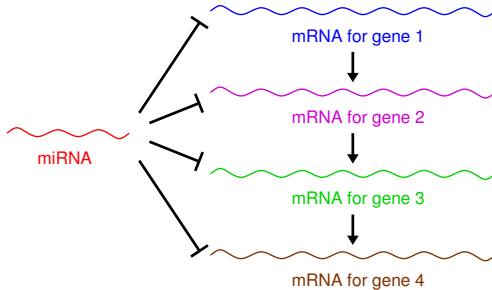
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Targets for a given miRNA often belong to the same biological pathways.



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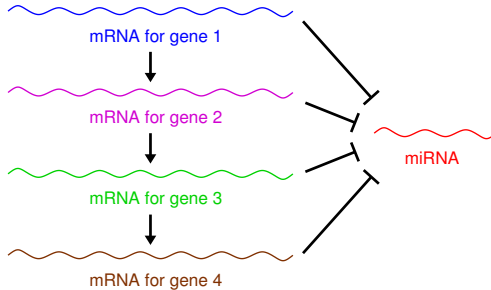
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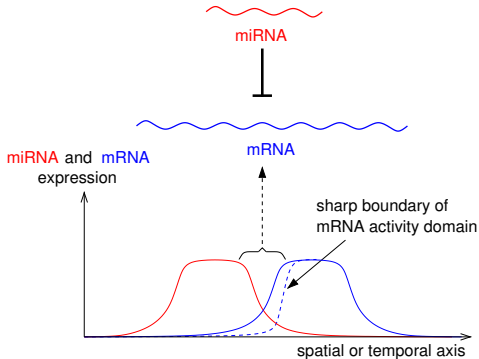
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Expression domains for miRNAs and their predicted targets overlap at their boundaries.



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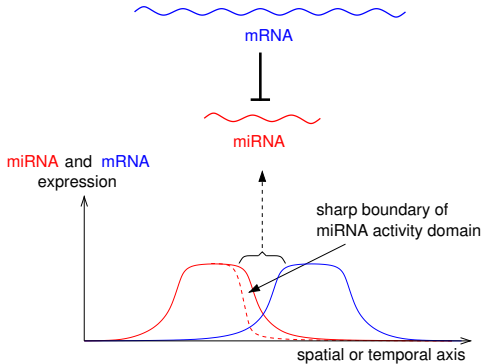
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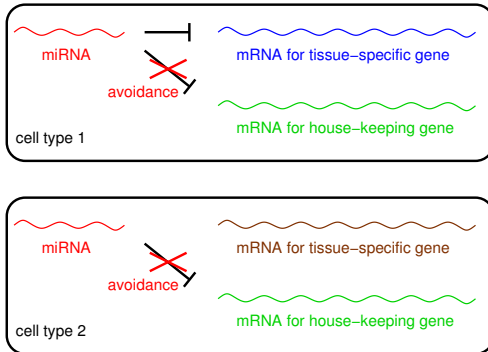
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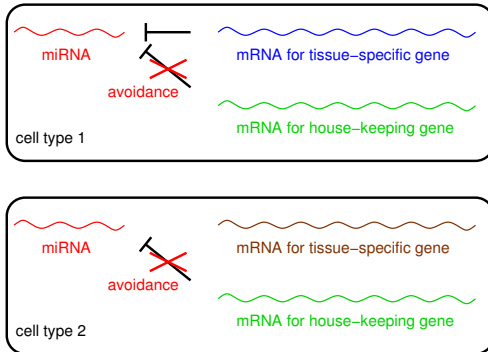
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Discriminative prediction

According to the new hypothesis:

According to the current theory:

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According to the new hypothesis:

- ▶ Pseudo-target expression levels can fluctuate between individuals in a natural population (phenotype is robust).

According to the current theory:

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According to the new hypothesis:

- ▶ Pseudo-target expression levels can fluctuate between individuals in a natural population (phenotype is robust).

According to the current theory:

- ▶ miRNA targets are tightly regulated (inter-individual fluctuation should not exceed miRNA-guided repression).

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Baek *et al.*, 2008: quantification of miR-223-mediated repression in mouse neutrophils.

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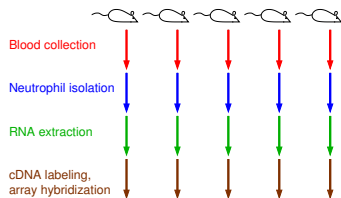
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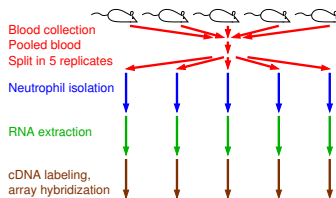
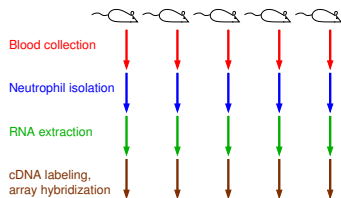
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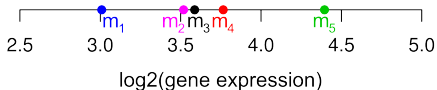
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Gene *Styx*



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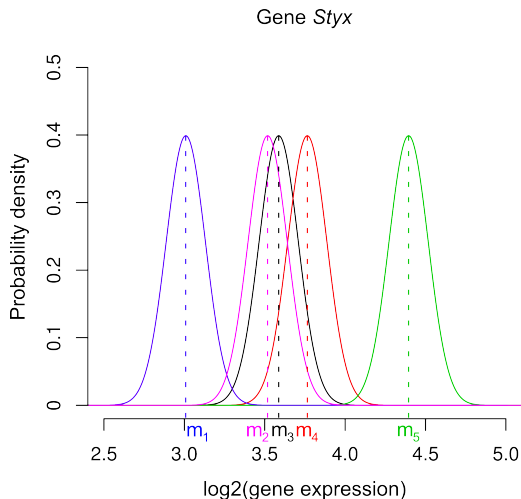
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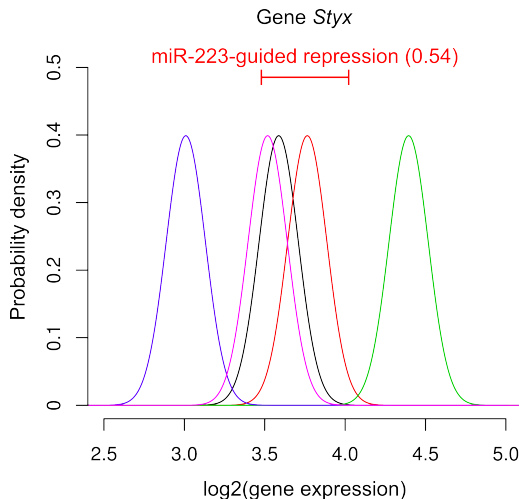
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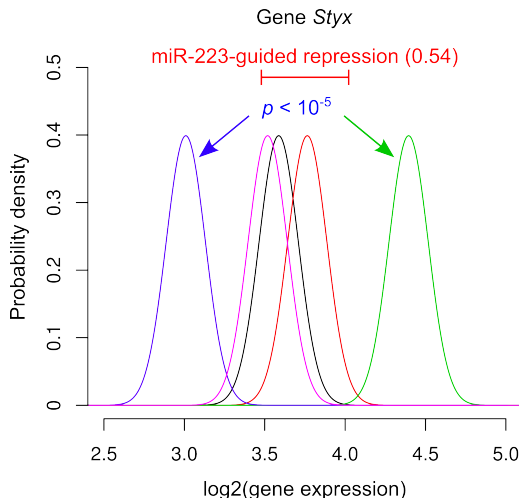
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p : probability that the difference between two individual mice is smaller than miRNA-guided repression

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For 168 predicted targets out of 189:
inter-individual fluctuations across 5 wild-type mice exceeds
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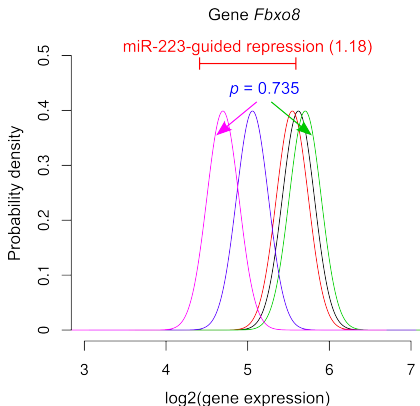
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For mRNAs to titrate miRNAs, they need to be abundant enough.

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► Issues with published examples

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

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

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

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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);

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

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

⇒ 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).

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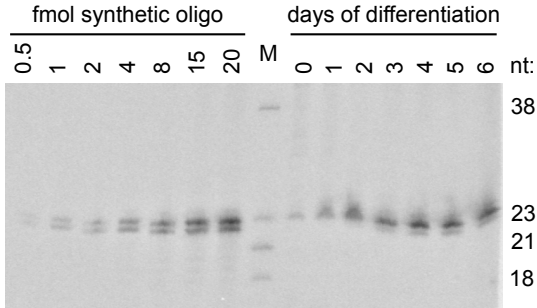
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(quantification of miR-1 and miR-206)

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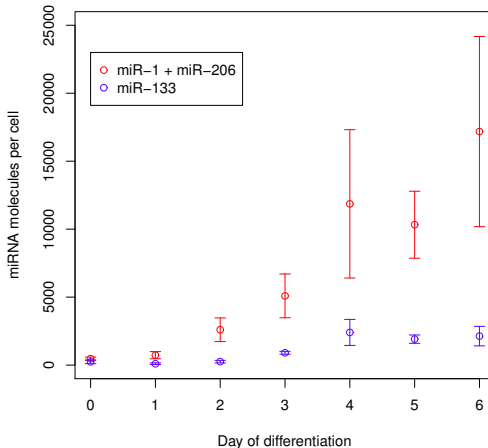
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Very deep sequencing: three time points (day 0, day 3, day 6) in triplicate; each replicate: between 267 and 333 million transcriptome-matching reads.

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27 synthetic spike-ins, for calibration.

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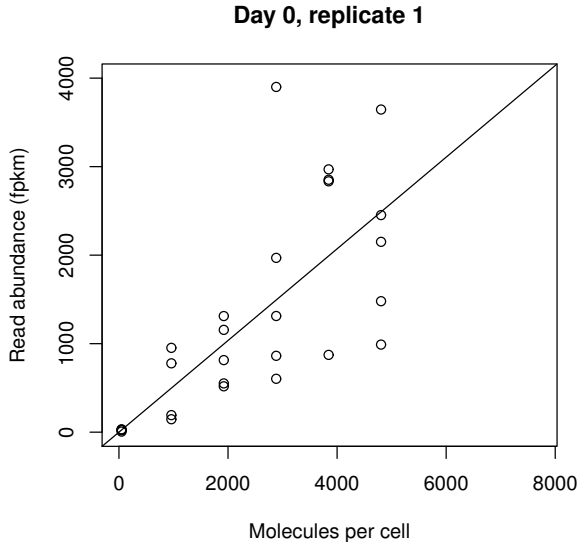
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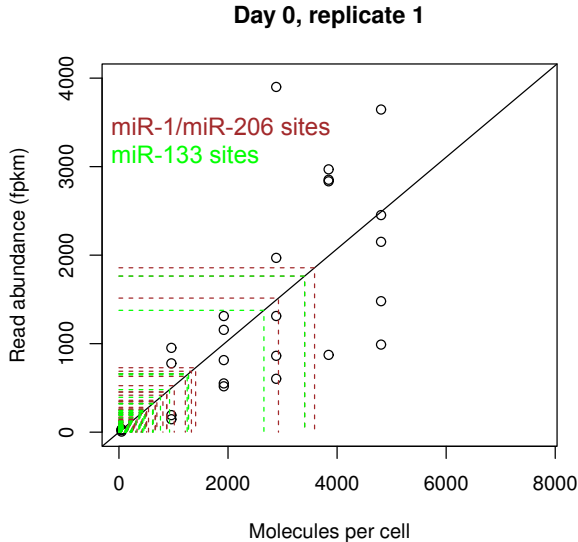
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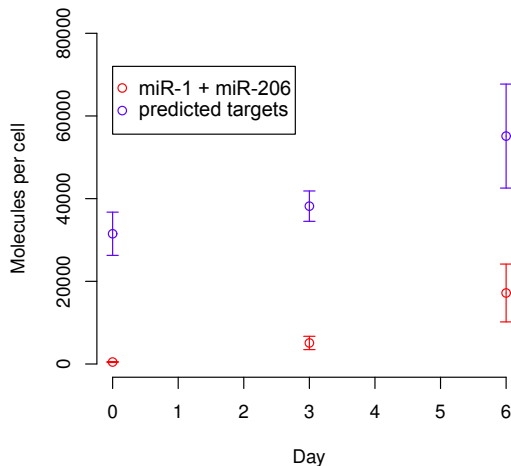
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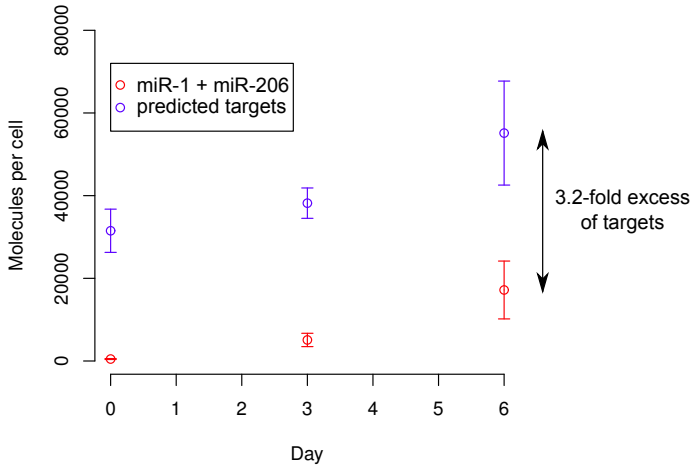
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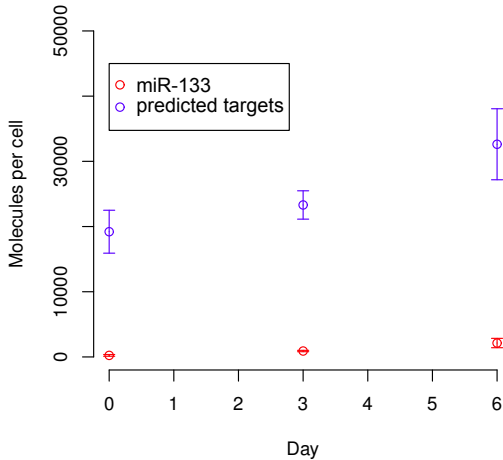
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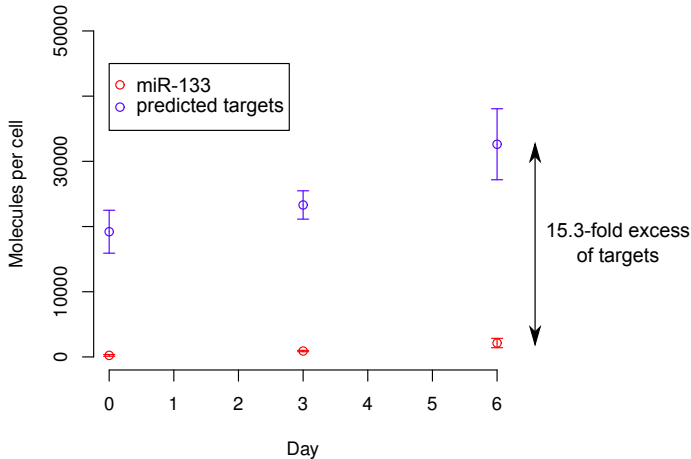
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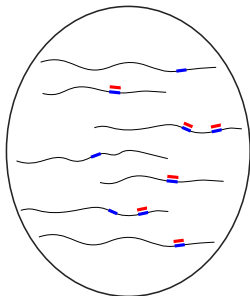
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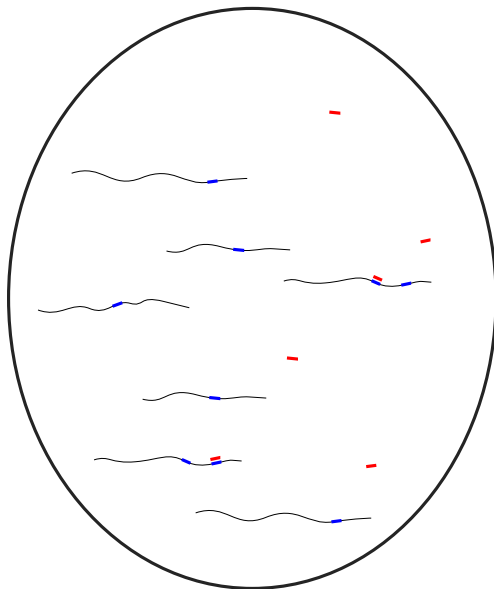
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miRNA family	Predicted targets	
	Total	Above $10 \times K_d$
miR-1 and miR-206	436	31 (7 %)
miR-133	384	22 (6 %)

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

Concentration effects

Titration effect of individual miRNA binding sites:

mRNA	Sample	Increase in free miR-1/miR-206 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 %

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Concentration effects

Titration effect of individual miRNA binding sites:

mRNA	Sample	Increase in free miR-133 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 %

Discriminative prediction

According to the new hypothesis:

According to the current theory:

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Discriminative prediction

According to the new hypothesis:

- ▶ Abundantly expressed pseudo-targets have a stronger miRNA-modulating effect, so their interaction with the miRNA should be better conserved

According to the current theory:

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Discriminative prediction

According to the new hypothesis:

- ▶ Abundantly expressed pseudo-targets have a stronger miRNA-modulating effect, so their interaction with the miRNA should be better conserved $\implies$ mRNA abundance should be positively correlated with miRNA binding site conservation.

According to the current theory:

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Discriminative prediction

According to the new hypothesis:

- ▶ Abundantly expressed pseudo-targets have a stronger miRNA-modulating effect, so their interaction with the miRNA should be better conserved $\implies$ mRNA abundance should be positively correlated with miRNA binding site conservation.

According to the current theory:

- ▶ Each target is a real target, its interaction is more or less conserved depending on the functional importance of the regulation (it is not expected to correlate with gene expression).

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Does mRNA abundance correlate positively with miRNA binding site conservation?

miRNA binding site conservation: measured by TargetScan's " P_{CT} " score. mRNA abundance in published microarray experiments.

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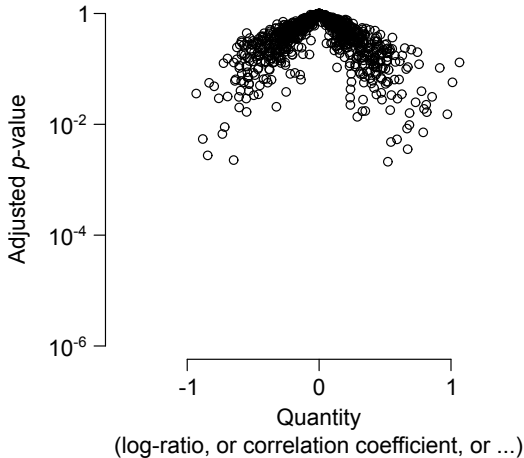
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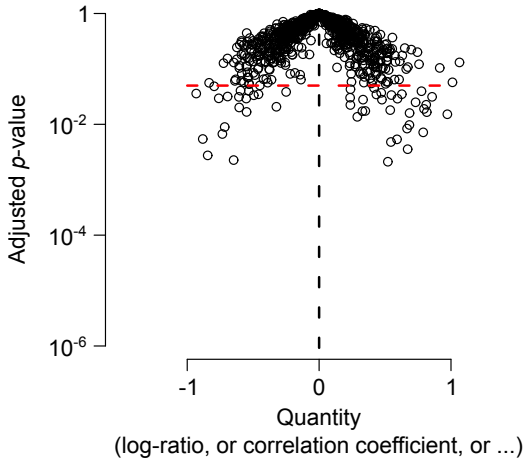
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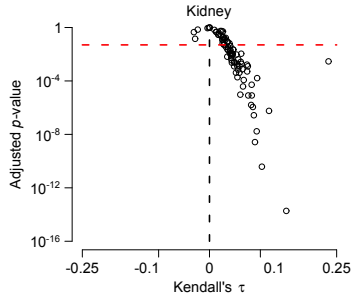
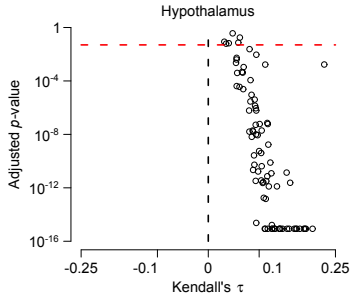
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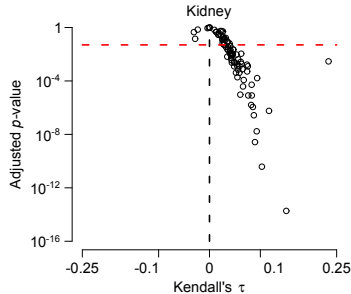
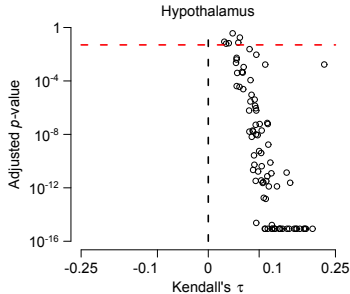
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+34 other mouse tissues, same results:

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+33 fly tissues, same results:

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Every measurable change in gene expression does not translate into a macroscopic, evolutionarily selectable phenotype.

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Conclusion: revisiting miRNA target definition

Every measurable change in gene expression does not translate into a macroscopic, evolutionarily selectable phenotype.

Cells can modulate miRNA activity in a cell type-specific manner (integrating expression information from hundreds of genes on a single regulator).

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Conclusion: revisiting “gene regulation” definition

High-throughput identification of transcription factor or RNA-binding protein targets: thousands of genes are bound, many of them are not under selective pressure to keep these binding sites.

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Microscopic events which are neutral in evolutionary terms.

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Conclusion: revisiting “gene regulation” definition

High-throughput identification of transcription factor or RNA-binding protein targets: thousands of genes are bound, many of them are not under selective pressure to keep these binding sites.

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Microscopic events which are neutral in evolutionary terms.

Are there pseudo-targets for these other regulators?

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High-throughput identification of transcription factor or RNA-binding protein targets: thousands of genes are bound, many of them are not under selective pressure to keep these binding sites.

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Microscopic events which are neutral in evolutionary terms.

Are there pseudo-targets for these other regulators?

A central feature of biological systems: their robustness to external insults. Hard to reconcile with the extreme sensitivity required for fine-tuning (the “butterfly effect” has probably been counter-selected).

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Anna
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Laura
Martinez



Natalia
Pinzón Restrepo



Blaise
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Jessy Presumey and Florence Apparailly (INM, Montpellier, France)

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An alternative hypothesis:

- ▶ Robustness of biological pathways

Testing the hypothesis:

- ▶ Non-coding pseudo-targets?
- ▶ Correlation between dose-sensitivity and targeting conservation
- ▶ Issues with published pseudo-targets
- ▶ RNA-Seq statistics
- ▶ Candidate titrators
- ▶ miRNA tissue-specificity and abundance/conservation correlation

Conclusion:

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

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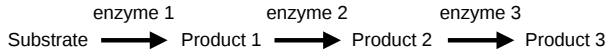
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Biological robustness



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mRNA abundance and seed match conservation

- ▶ Most predicted targets are expected to be pseudo-targets (conservative approximation: we will consider every predicted target).
- ▶ miRNA binding site should correlate with that mRNA's abundance in the cells where miRNA titration is beneficial (conservative approximation: we will consider whole tissues and organs).
- ▶ Poorly abundant mRNAs (without a real titration effect on the miRNA) should not exhibit such correlation (conservative approximation: we will consider every mRNA).

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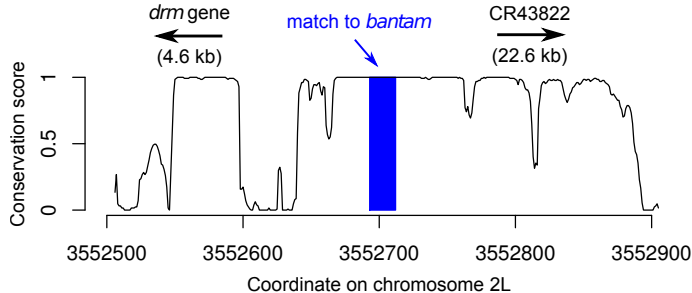
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Non-coding pseudo-targets?



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Reported examples of pseudo-targets

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

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

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

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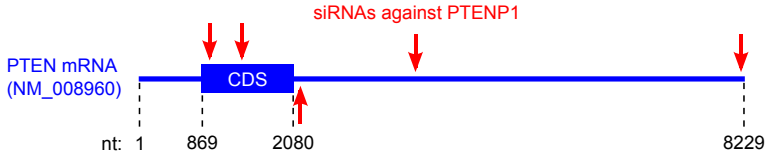
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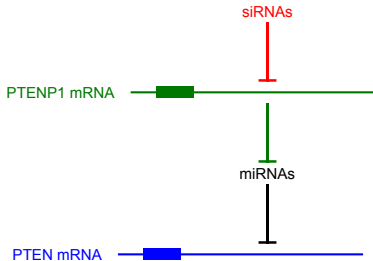
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Proposed by Poliseno *et al* (2010):



More probably:



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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' UUUUUUACUUGGAAAAUUA 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'

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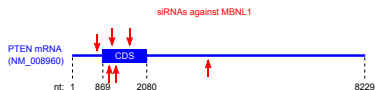
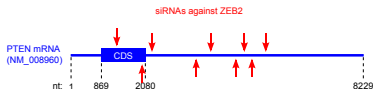
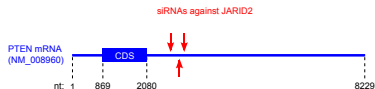
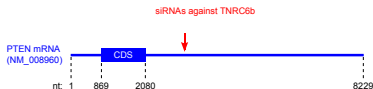
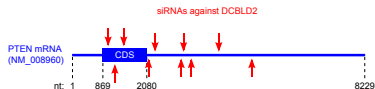
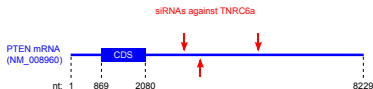
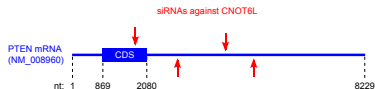
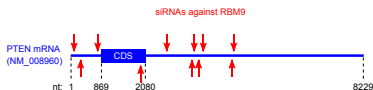
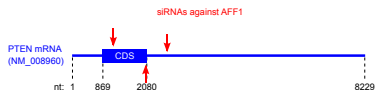
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Deep-sequencing statistics

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

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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).

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- ▶ 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; Lebedeva *et al.*, 2011 and Hafner *et al.*, 2013).

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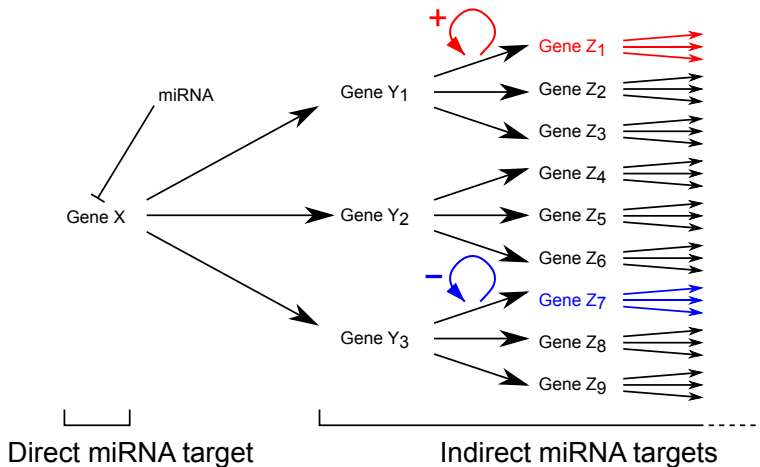
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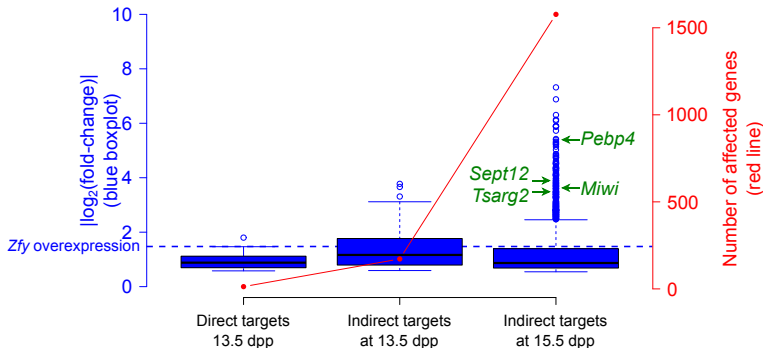
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(in collaboration with H. Royo and J. Turner, MRC, London)

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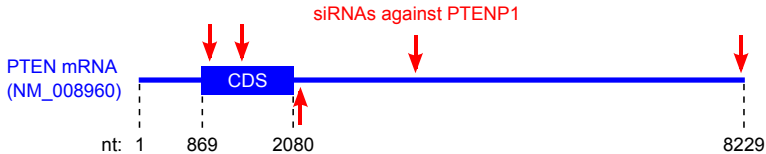
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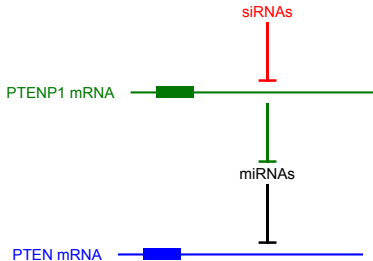
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Proposed by Poliseno *et al* (2010):



More probably:



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siRNA #2 against PTENP1: 5' UAAUAAUCAUUCUGGC 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' UUUUUUACUUGGAAAAAUUA 5'

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

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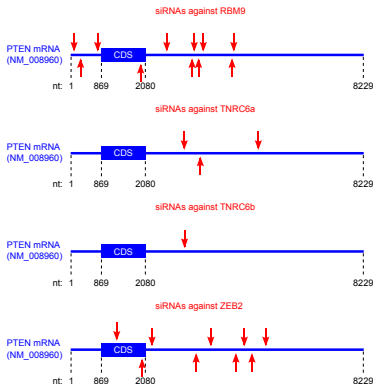
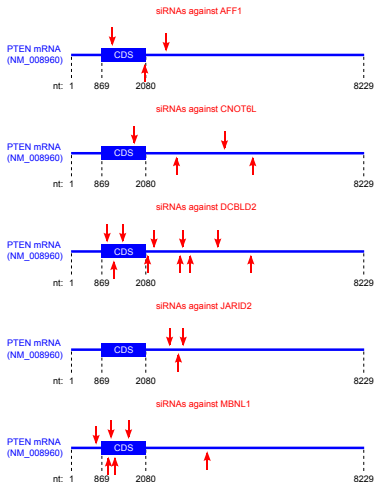
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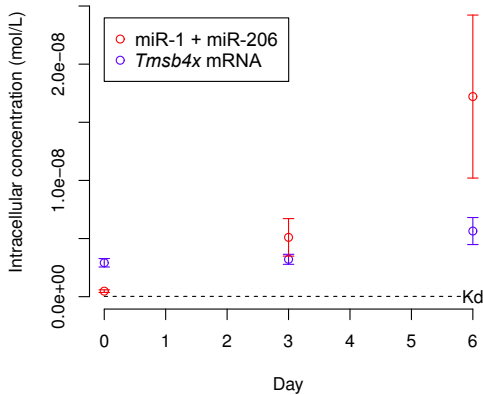
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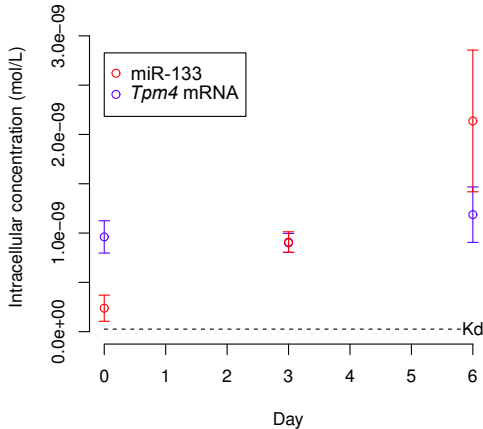
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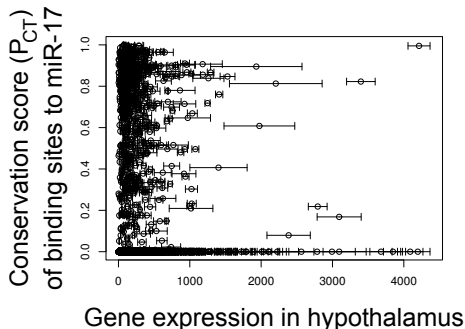
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mRNA abundance and seed match conservation

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



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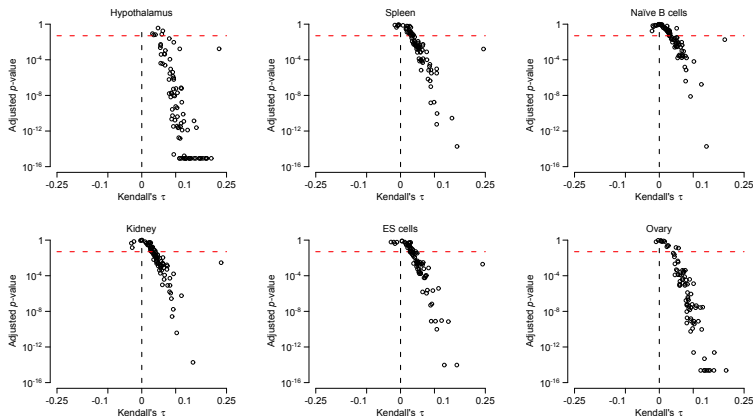
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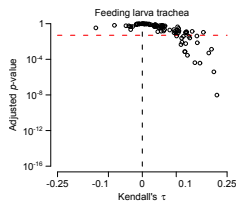
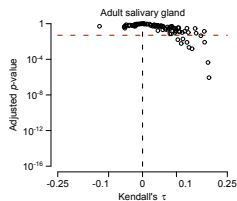
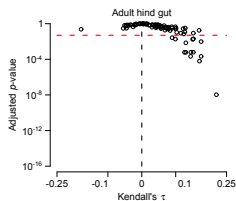
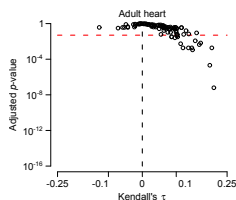
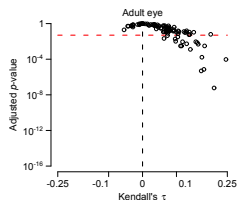
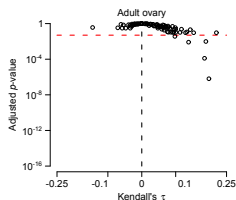
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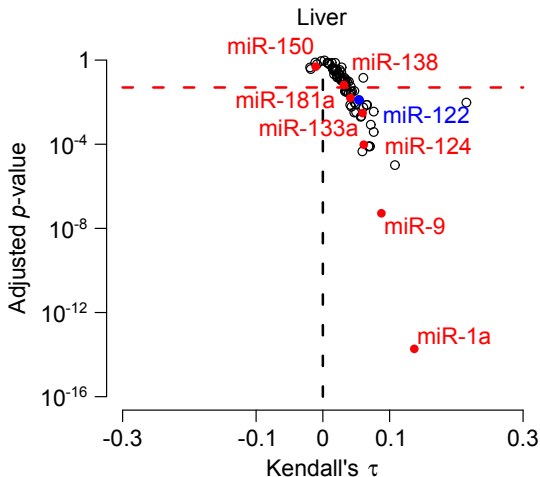
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