Predicted conserved targets of microRNAs are abundant enough to titrate them

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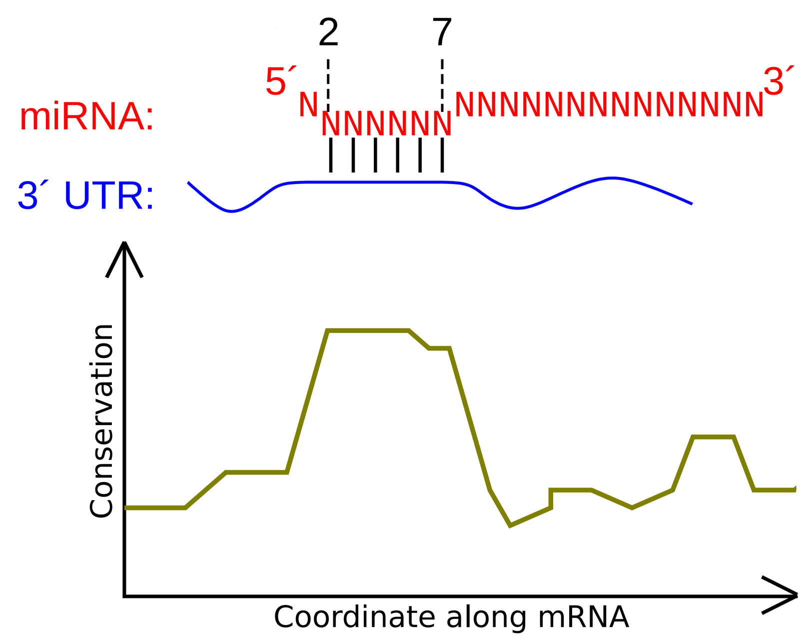
miRNAs as fine-tuners: a paradox

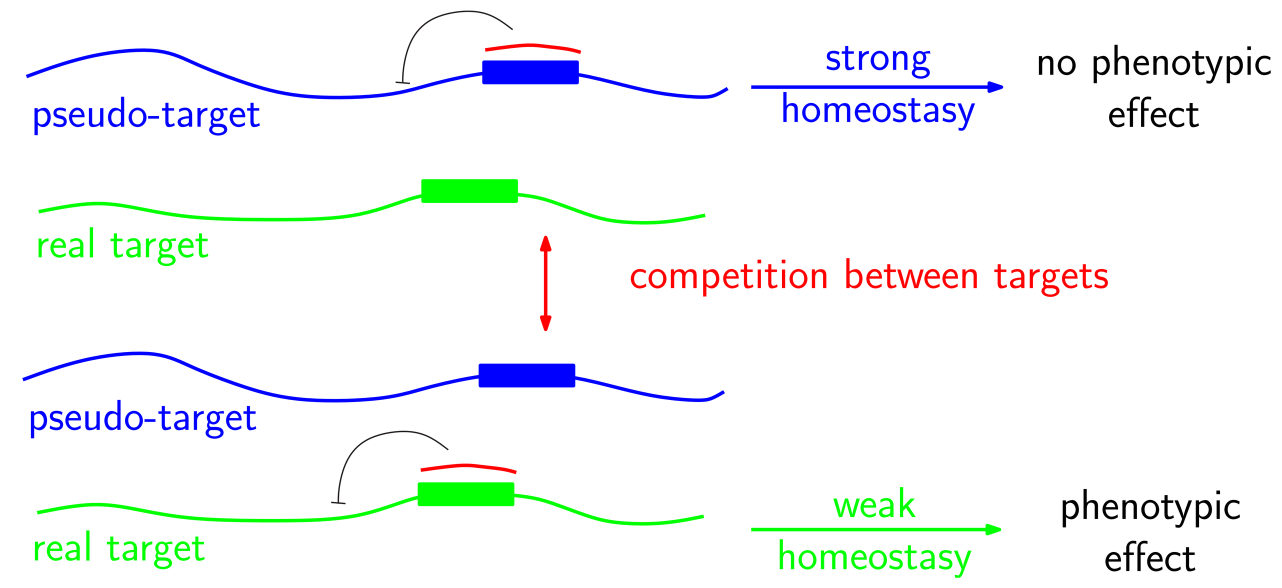
Most microRNA (miRNA) target site prediction programs search for seed matches in 3´UTRs that were conserved during evolution. They typically predict hundreds of targets for a given miRNA, and 60% of the human coding genes are predicted targets for at least one miRNA. **miRNA-mediated repression usually causes a gene expression drop less than 2-fold** (Baek et al., 2008; Selbach et al., 2008). This led to the view that miRNAs act as fine-tuners, precisely setting gene expression levels (Bartel and Chen, 2004).



However, phenotypes seem robust to small variations in gene expression levels. For instance, we have observed that the **expression level of miRNA targets often vary more than 2-fold between individuals** from the same inbred strain (experiments on mouse neutrophils, not shown).
Why are conserved seed-matching sites so numerous in the genome if the effect of miRNA-mediated repression usually doesn't matter so much?

The pseudo-target hypothesis

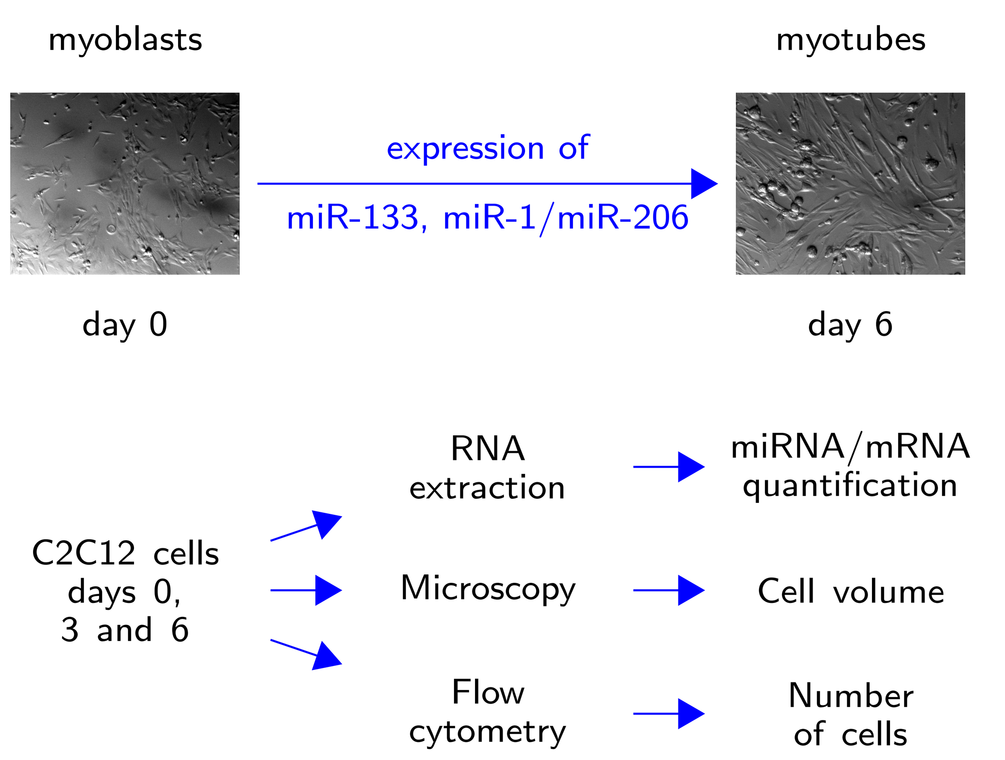
We proposed (Seitz, 2009) the following alternative hypothesis.
The majority of miRNA predicted targets are not functionally repressed, since a small fluctuation in their expression is buffered by homeostatic mechanisms. miRNA action on them does not lead to a phenotypical effect: they are *pseudo-targets*.
These mRNAs bear conserved seed matches: such sites must have a function. This function could be to repress the miRNA by titration, **preventing it from binding its real targets**.



Real targets are those genes that are dose-sensitive: a minor change in their expression affects the phenotype.

Are mRNAs abundant enough to titrate miRNAs?

In order to titrate miRNAs, **target mRNAs should be in large excess**. We tested this by quantifying both RNA populations in differentiating mouse C2C12 cells. This work required RNA extraction, sequencing and annotation, as well as cell number and volume estimation. We were then able to compare miRNA and target concentrations.

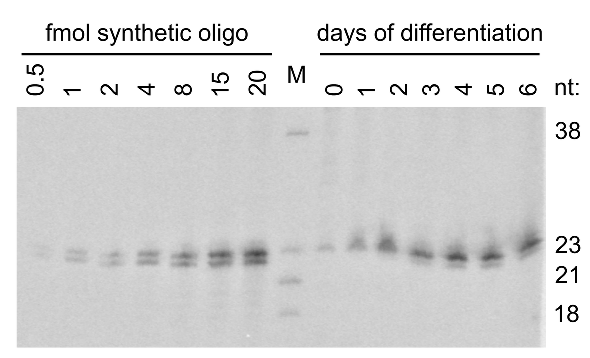


Cell number and volume estimation

An aliquot of cells was stained with thiazole orange and mixed with a known quantity of fluorescent beads. The mix was used for flow cytometry and **the bead count was used to calibrate the cell counts**. This count was used in the RNA quantification phase to **convert the number of molecules in a sample to a number of molecules per cell**.
Cell volume was estimated based on measures taken with a microscope and used to **convert numbers of molecules per cell to concentrations**.

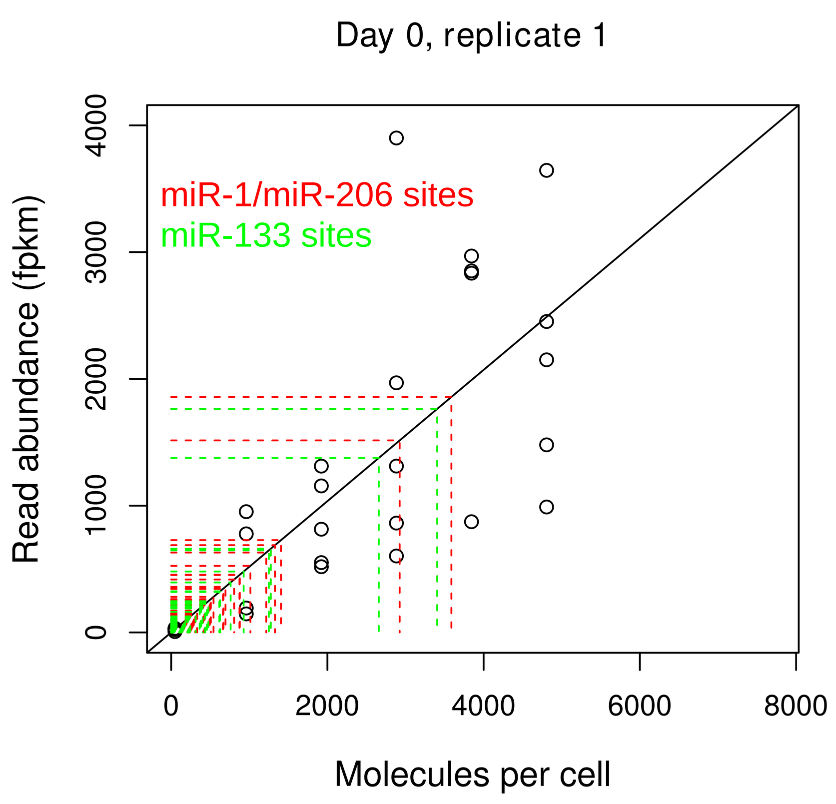
miRNA quantification

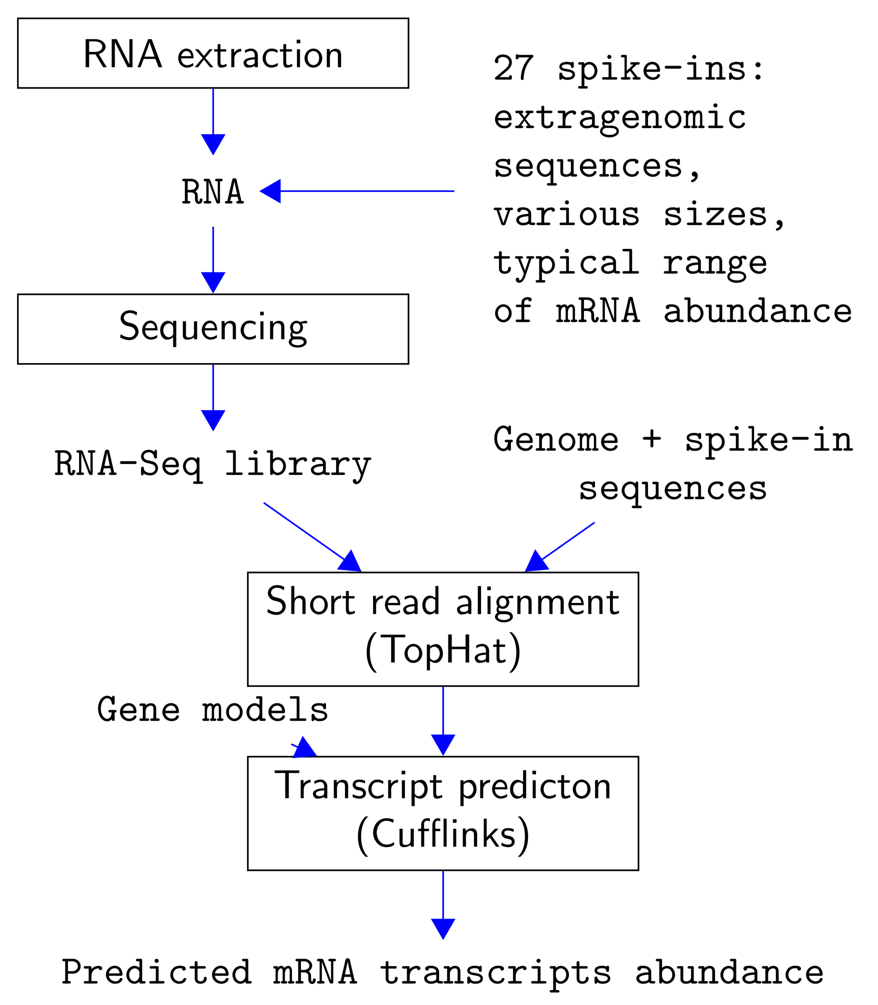
miR-133 and miR-1/miR-206 were quantified by **calibrated Northern blot**. The number of miRNAs molecules per cell could then be estimated, knowing the number of cells from which the deposited RNA was extracted.



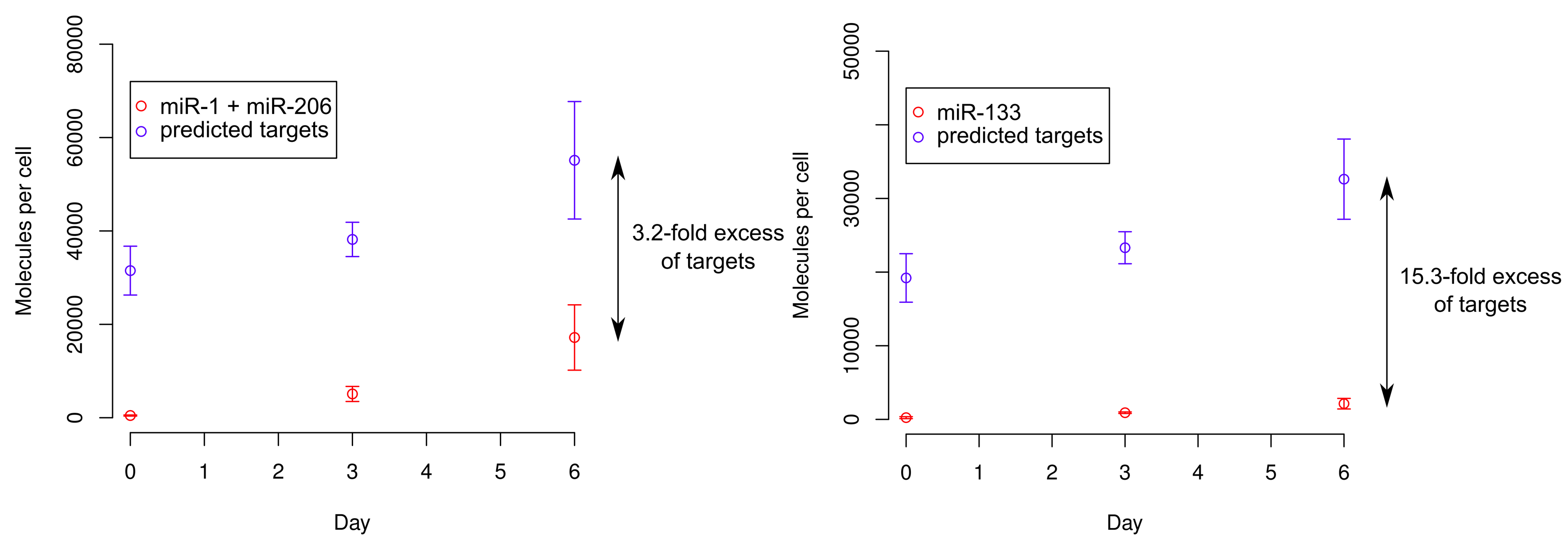
mRNA quantification

mRNA quantification was performed by sequencing cellular RNA extracts to which a controlled proportion of **in vitro transcripts** (*spike-ins*) was **added for calibration**.





Results: titration is possible



⇒ **miRNA predicted targets are in large excess compared to miRNAs.**
In order to estimate the miRNA titrating activity of each mRNA, we calculated the equilibrium between free and bound miRNA in our C2C12 data, as well as in a simulation where a single miRNA binding site was lost in a given mRNA. The increase in the amount of free miRNA after miRNA binding site loss measures the titrating activity of each individual binding site.
⇒ **Several mRNAs are strong titrators.**

mRNA	Sample	free miR-1/mir206 increase if site is lost
Anxa2	Day 0	8 %
Ptma	Day 0	9 %
Tmsb4x	Day 0	10 %
Actb	Day 3	15 %
Tmsb4x	Day 3	9 %
Actb	Day 6	13 %
Tmsb4x	Day 6	11 %

mRNA	Sample	free miR-133 increase if site is lost
Tpm4	Day 0	5 %
Ptma	Day 0	16 %
Eif4a1	Day 0	5 %
Tagln2	Day 0	6 %
Ptma	Day 3	7 %
Eef1a1	Day 3	8 %
Ptma	Day 6	6 %
Eef1a1	Day 6	6 %

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