Competition between target sites of regulators shapes post-transcriptional gene regulation

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Abstract | Post-transcriptional gene regulation (PTGR) of mRNA turnover, localization and translation is mediated by microRNAs (miRNAs) and RNA-binding proteins (RBPs). These regulators exert their effects by binding to specific sequences within their target mRNAs. Increasing evidence suggests that competition for binding is a fundamental principle of PTGR. Not only can miRNAs be sequestered and neutralized by the targets with which they interact through a process termed 'sponging', but competition between binding sites on different RNAs may also lead to regulatory crosstalk between transcripts. Here, we quantitatively model competition effects under physiological conditions and review the role of endogenous sponges for PTGR in light of the key features that emerge.

The diversity of cell types in multicellular organisms can only be understood by dissecting gene regulation — although each cell contains approximately the same DNA sequences, gene expression is tissue-specific and regulated at many levels. The concepts underlying gene regulation were initially formulated for *trans*-acting factors binding to specific *cis*-elements in either DNA (transcriptional regulation) or RNA[1] (post-transcriptional gene regulation (PTGR)[2]). However, only recently has PTGR received growing attention, perhaps as a result of recent improvements in biochemical assays to quantify RNA–protein interactions transcriptome-wide, the increasing precision and sensitivity of profiling cellular RNA by sequencing technology, and the discovery of important regulatory mechanisms acting at the transcript level.

After transcription and splicing, mature mRNAs must be properly localized, translated or degraded. In human cells, these tasks are regulated by thousands of microRNAs (miRNAs)[3–6] and by at least 800 RNA-binding proteins (RBPs)[7–12], the numbers of which are roughly comparable to the at least ~1,400 transcription factors[13]. The interactions of RBPs and miRNAs with mRNAs have been dubbed the 'post-transcriptional regulatory code' (REF. 14) (FIG. 1). Analogous to enhancers within DNA, which are bound by transcription factors to modulate gene expression through the regulation of transcription (BOX 1), untranslated regions (UTRs) of mRNAs contain binding sites for the post-transcriptional regulators (that is, miRNAs and RBPs). By interacting with binding sites, post-transcriptional regulators confer information about the state of a cell on an mRNA. Specifically, their local concentrations determine binding site occupancies, which in turn dictate the regulatory impact on mRNA stability and localization, as well as on protein production (FIG. 1).

Following the description of transcripts that can sequester miRNAs and thereby inhibit miRNA function[15–17], it was suggested that competition between binding sites on different RNAs is a fundamental principle of PTGR (reviewed in REF. 18). In general, each and every binding site for a regulator necessarily reduces, to some extent, the amount of regulator available to other sites. To determine whether this can amount to a regulatory impact requires quantitative modelling of binding site competition. As binding sites in transcripts can exist in high copy numbers and vary across cell states and types, this is especially relevant for PTGR (BOX 1). Advances in our understanding of how cellular concentrations of RBPs and miRNAs translate into regulatory control are important to the entire field of gene regulation. Predicting, quantitatively, how a regulator binds to transcripts in a cell is not trivial because simple models for binding — for example, the binding equation (FIG. 1) — describe the idealized case of isolated binding sites and do not apply if competition between sites is strong[19,20].

Here, we begin by reviewing the dynamics of the transcriptome and its regulators. We describe how the combinatorial control of mRNAs is determined by the occupancy of RNA binding sites, and how

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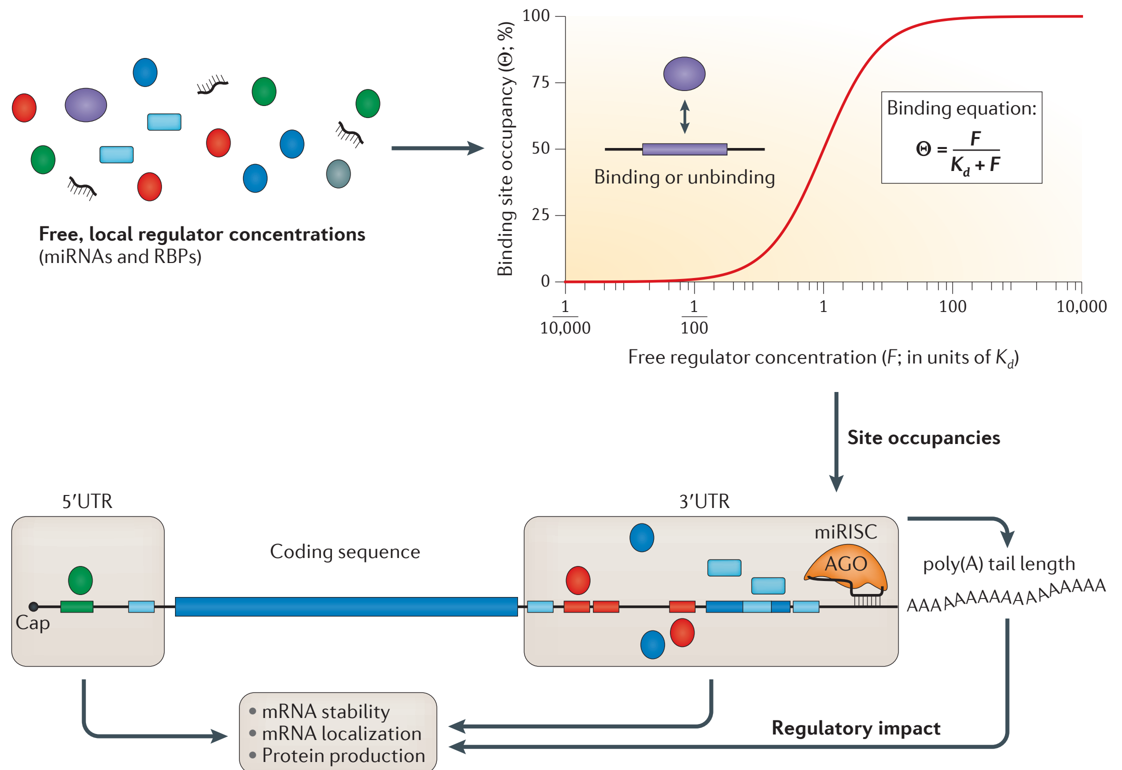

Figure 1 | **RBPs and miRNAs regulate protein output and mRNA fate.** mRNA untranslated regions (UTRs) flank the coding sequence and are bound by post-transcriptional regulators (RNA-binding proteins (RBPs) and microRNAs (miRNAs)), which collectively control mRNA stability, mRNA localization and protein production. miRNAs are short ~22-nucleotide non-coding RNAs that repress gene expression. Processed, mature miRNAs are loaded into Argonaute (AGO) proteins to form the miRNA-induced silencing complex (miRISC). Base-pairing between the loaded miRNA (guide RNA) and 3′UTRs of mRNAs guides miRISC to its target sites. Additional factors are recruited to induce translational repression and mRNA destabilization through deadenylation and decapping[3,5,55,59,60,125]. RBPs contain specialized RNA-binding domains[39], which form sequence- and structure-specific interactions with RNA (not shown). The local concentrations of post-transcriptional regulators determine binding site occupancies and thus their regulatory impact. Occupancy of a binding site (Θ) as a function of available regulator and strength of binding is described by the binding equation. Θ (value between 0% and 100%) is the site occupancy, *F* is the free concentration of the ligand (for example, the RBP), and K_d is the dissociation constant, which quantifies the strength of interaction between binding site and ligand. For a single site, the ratio between regulator concentration (*F*) and dissociation constant (K_d) is the relevant parameter. For example, a binding site with K_d = 1 nM (~1,000 molecules per cell) is bound with a probability of 50% if 1 nM of free regulator is available. Below this threshold, the binding site quickly becomes unbound; above this threshold it is saturated. Cooperativity between binding factors can change the slope of the function around the threshold but is not considered here. The concentration (*F*) in units of K_d is plotted on a logarithmic scale, and occupancy is plotted on a linear scale to emphasize the relevance of absolute occupancy changes.

concentrations of RNA binding sites can be of a comparable order of magnitude to or exceed the concentrations of regulators. We then combine the binding equations for many different binding sites[20] with the constraint that the total number of regulator molecules is not changed by binding or unbinding. This yields a simple steady-state model of site occupancies that can be numerically solved for any number and combination of binding sites. We use this model to investigate the type of competition effects — such as 'sponging', buffering or regulatory crosstalk — that can occur. We estimate concentrations for regulators and target sites at which competition is expected to induce gene regulatory effects under physiological conditions and compare these estimates to published experimental results on artificial RNA sponges and, qualitatively, to RBP perturbation experiments to test the validity of our approach. We conclude by discussing the phenomenon of regulatory crosstalk and implications of our findings for the competing endogenous RNA (ceRNA) hypothesis[21] and the role of ceRNAs in PTGR. We mostly discuss competition for miRNA binding, which occurs at a relatively narrow range of binding energies, but the general effects also hold for RBPs.

Dynamics of mRNA targets and regulators

Spatiotemporal dynamics of the transcriptome. Why is it important to consider the concentrations of both the regulator and its binding sites? Transcription generates a single copy to thousands of copies of mRNA molecules

Competition
Competition occurs if different ligands can form a complex with the same molecule (for example, an RNA-binding protein). In biochemistry this is often used to characterize an interaction. However, in biochemistry the term 'competition' describes a situation with multiple available species of ligands, regardless of whether these are bound or unbound, which essentially means that 'alternatives are present'. In this Analysis, we use the term for situations in which the binding factor is limited and distributed among many possible ligands (binding sites); that is, we refer to the unbound ligands as 'competing'. This is closer to the intuitive meaning used to describe, for example, markets or sports, where competition is introduced by either limited money or limited trophies, which cannot be awarded to everybody.

Binding equation
At equilibrium, the binding equation gives the site occupancy (Θ) as a function of free ligand concentration (*F*) and the dissociation constant (K_d). More generally, it describes a system with components that can only be in one of two states, which differ in energy (in this context, bound or unbound). The equation therefore arises in many contexts and is known, for example, as Langmuir isotherm or Fermi function. As we consider non-cooperating, independent binding sites, the binding equation is equivalent to the more general Hill equation, with the Hill coefficient equal to 1.

Occupancy
The probability with which a particular binding site is bound by a regulator, for example, an RNA-binding protein.

Competing endogenous RNA (ceRNA) hypothesis
In its current form, a hypothesis stating that competition for microRNA binding can introduce crosstalk between RNA transcripts, including mRNAs and pseudogenes.

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Box 1 | **Binding models for transcriptional regulation**

Sequence-specific interactions between proteins and nucleic acids have first been studied for transcription factors (TFs) and DNA, starting with the bacterial *lac* operon[1]. Lactose metabolism in *Escherichia coli* is regulated by a repressor protein, which binds to several sites on a specific locus of DNA. If cell divisions are discounted, then the concentration of these binding sites is constant, whereas the concentration of TF is regulated. Consequently, an effective model only needs to account for the TF concentration. This scenario applies widely to transcriptional regulation, as the concentration of binding sites (which is determined by the genome) is constant. Furthermore, when it is lower than that of the available TF, the amount of TF bound to an individual site is negligible compared to the total TF. Under this assumption, binding does not alter the free TF concentration, and each binding site independently interacts with the complete pool of TF molecules (that is, without competition). This simple picture is known to break down for complex eukaryotic enhancers with cooperativity between TFs[19]. However, competition between different enhancers is rarely considered, although 'super-enhancers' may, in principle, sequester TFs available for other enhancers[121]. The inhibition of TF activity via titration by additional binding sites was recently measured[122].

per cell, depending on cell type and state. Accordingly, the concentrations of binding sites for RNA regulators, which are contained within the transcripts, vary in the same way (BOX 1). Pronounced changes in the transcriptome of a cell can also occur without transcription. An extreme example is the fertilization of an oocyte, the reprogramming of which into a totipotent zygote is independent of transcription in all studied animals[22–24]. In *Caenorhabditis elegans*, thousands of mRNAs are turned over within ~30 minutes[25]. Many others are organized into P-granules, which are dense foci of aggregated RNA and proteins[26]. Such granules can concentrate both RNA and RBPs in a small volume, possibly altering the equilibrium between binding and unbinding of post-transcriptional regulators. Later in development, when zygotic transcription begins, maternal transcripts are selectively degraded; in the zebrafish embryo this process depends on a specific miRNA[27]. Moreover, differentiation into another cell type can alter the expression of thousands of genes[19]. These examples highlight that the copy number of RNA binding sites and their spatial distribution are highly dynamic. It is now possible to profile transcript abundances in single cells[28,29] using RNA sequencing (RNA-seq)[30], such that the concentration of any RNA sequence may be approximated to good precision. However, the P-granules mentioned above should be seen as a warning that the biologically relevant concentrations of transcripts may differ from whole-cell averages. The question remains: how many sites are actually bound in the transcriptome of a given cell at a given point in time by a particular regulator?

Widespread binding of RBPs and miRNAs. Recent experiments have mapped RBP–transcriptome interactions using CLIP–seq, which consists of crosslinking of protein and RNA, followed by immunoprecipitation and sequencing of bound RNA fragments[31–34]. RBPs were typically found to interact reproducibly and specifically with thousands of target genes and tens of thousands of binding sites[35–38], which is consistent with the specific recognition of only 4–8 nucleotides by most RBPs that bind to single-stranded RNA[39,40]. The same holds true for miRNAs. After loading into Argonaute (AGO) proteins, miRNAs predominantly base-pair to their targets through a region of 6–8 nucleotides at their 5′ ends[3,41] (FIG. 1). AGO–CLIP[38,42–45] or CLASH[46] experiments[47,48] confirm the widespread binding of miRNAs. Importantly, these experiments yield an average binding profile because millions of cells are mixed together. Furthermore, the current protocols are not quantitative enough to directly infer the frequency with which an individual binding site is used. Consequently, the extent to which the uncovered interactions are functional is a matter of ongoing research, but there is evidence that the number of binding sites can be of a comparable order of magnitude to that of the corresponding regulators (RBPs or miRNAs) or can even exceed them, as has been suggested experimentally for miR-122 in hepatocytes[49]. This observation complicates the quantitative modelling of regulatory interactions of RBPs and miRNAs with the transcriptome because the simple assumption that regulatory molecules are in excess over binding sites, which can be posited for simple models of transcription factor binding to DNA[19] (BOX 1), does not apply. Hence, to be of use, quantitative models must enable the computation of binding site occupancies also when binding sites are in excess of the regulator.

Combinatorial control of RNA

Catalytic and indirect PTGR. How does the occupancy of a binding site relate to the regulatory impact of binding? A number of RBPs can enzymatically alter bound mRNA; an example is the small interfering RNA (siRNA)-directed cleavage by AGO[50,51]. However, this function is usually not used for regulation by miRNAs in animals, with exceptions among non-bilateria[52]. More recently, reversible mRNA methylation has been recognized as a regulatory mechanism (reviewed in REF. 53). Such catalytic modes of PTGR require a description that includes kinetics, and steady-state models do not apply in these cases. However, several important RNA-binding factors do not chemically alter RNA but act indirectly by changing the accessibility of other binding sites (through steric blocking or RNA structure[54]); through protein–protein interactions with other (potentially enzymatic) factors[55]; or by restricting the location of a bound mRNA[56]. These interactions are highly relevant to gene expression, and their regulatory impact should depend directly on binding site occupancy. Moreover, the half-life of most mRNAs (hours[57]) is much longer than the diffusion time of a regulator (~10 seconds[58]) or the equilibration of binding and unbinding events (minutes[41]). Thus, a steady-state model can provide a good approximation. To simplify a quantitative model, the indirect effects of binding on mRNA levels — for example, de-stabilization by miRNA binding — can be disregarded, as they are already accounted for in the steady-state levels. However, it should be noted that this additional simplification can only be expected to yield a useful approximation if the changes of mRNA levels induced by the indirect action of post-transcriptional regulators considered in the model are weak.

Binding energies
The energies of molecules in a complex, which are contributed by the physical interactions (for example, hydrogen bonding) between them. It is often expressed in kcal mol^{-1}; it determines the dissociation constant (K_d), which describes the concentration at which binding and unbinding are in equilibrium.

CLIP–seq
(Crosslinking and immunoprecipitation followed by sequencing). A biochemical technique to extract RNA-binding protein (RBP)-bound fragments of RNA with high specificity and sensitivity, which are then subjected to high-throughput sequencing to map RBP interactions transcriptome-wide at nucleotide resolution.

Argonaute
(AGO). A functional protein component of the microRNA (miRNA) effector complex, RNA-induced silencing complex (RISC). When AGO is loaded with a miRNA, it is guided to a miRNA target site on a target mRNA. It reduces stability and protein production of a target mRNA.

CLASH
(Crosslinking, ligation and sequencing of hybrids). A method that uses high-throughput sequencing to profile and computationally analyse RNA–RNA interactions (for example microRNA–target binding).

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Excess
Species A is in excess over species B if its concentration is greater. In the context of binding or simple complex formation with a 1:1 stoichiometry, this may be an indication that most molecules of B are bound. However, the fraction of B that is bound by A is described by the binding equation and strongly depends on the dissociation constant (K_d). For example, weak binding may require many times more A than B to be present before substantial amounts of complex are formed.

Small interfering RNA (siRNA)-directed cleavage by AGO
A process by which target mRNAs are cleaved by the endonucleolytic ('slicing') activity of Argonaute (AGO) proteins, which is triggered when complementarity extends beyond position 11 of the guide RNA. Base-pairing with positions 10 and 11 distinguishes siRNA function (slicing) from microRNA function (no slicing).

Dissociation constant
(K_d). In the absence of competition effects, the concentration of the unbound, free regulator (for example, an RNA-binding protein) at which a binding site is bound or unbound with equal probability. It is derived from the binding energy (E) of the regulator bound to the site: $K_d = \exp(E/k_B T) \times [\text{mol}/L]$, where k_B is the Boltzmann constant and T is the temperature in Kelvin. As binding energies are negative, strong binding corresponds to a small K_d. K_d can also be defined as the ratio of the off-rates and on-rates: $K_d = k_{off}/k_{on}$.

Threshold concentration
(Also known as equivalence point of titration). The total ligand concentration at which occupancy is 50%. Without competition effects, this is equal to the dissociation constant (K_d). With many competing binding sites, the free ligand concentration can be much lower than the total concentration, which leads to increased threshold concentrations.

The strength of post-transcriptional regulatory effects. Although miRNAs can regulate hundreds of target genes, the effect of perturbing a miRNA on individual target mRNA levels or protein output only rarely exceeds two-fold[3,5,59,60]. This seems to also be the case for many RBPs[61–65], although their influence on protein production can be much larger than that of miRNAs[66,67]. Generally, although PTGR constitutes an essential layer of control, as exemplified by the numerous diseases caused by its disruption[9–11,14,65], the measured impact of individual post-transcriptional regulators on the expression of target mRNAs is typically small, only rarely exceeding a two-fold upregulation or downregulation. However, 3′UTRs are subject to multiple interactions by RBPs and miRNAs, which together can impart substantial changes in gene expression[54,68–71].

Regulation requires absolute changes in binding site occupancy. The response of an mRNA to changes in regulator concentration depends intrinsically on the interactions that mediate binding. This is described by the binding equation (FIG. 1), which expresses the occupancy (Θ) of a site (that is, the probability to be bound) as a function of the free regulator concentration (F) and the dissociation constant (K_d). A single binding site responds in a nonlinear, sigmoidal manner to the regulator concentration (FIG. 1). At low or high concentrations, the site is essentially unbound or saturated, respectively. In both cases, changes of regulator concentrations have negligible consequences. For example, if the occupancy of a site increases by 1,000-fold from 0.0001% to 0.1%, then the consequences are certainly below those that can be attributed to stochastic noise. By contrast, a two-fold change from 40% to 80% occupancy may well alter the fate of an mRNA. Therefore, we argue that regulation requires occupancies to be variable within a relevant window, for example, between 5% and 95%, for changes to measurably alter gene expression.

The threshold concentration demarcates the relevant concentration range within which a binding site is responsive to changes. *In vitro*, this is the K_d, which can be measured, for example, in gel-shift assays or by surface plasmon resonance[72]. Recently, K_d values have been experimentally measured for miRNAs and miRNA-induced silencing complex (miRISC)[41]. In addition, high-throughput technologies are being developed to simultaneously measure relative K_d values of an RBP for tens of thousands of RNA sequences[72,73] (BOX 2), which may soon allow on-rates and off-rates to be measured as well[74]. The binding equation describes an idealized situation in which a few binding sites are studied in isolation. Therefore, F should be considered the free concentration, which is the concentration that is effectively available locally to a single binding site (FIG. 2a). How F relates to the total concentration, in the presence of a large number of binding sites (FIG. 2b), is key to understanding how competition influences gene regulation. In essence, we need to take into account the extent to which each binding site reduces the amount of available regulator.

A simple model for regulator binding

We consider only non-cooperative binding and a well-mixed environment in equilibrium (FIG. 3a); hence, all binding sites of the same strength (K_d) 'see' the same 'local' concentration of free regulator and therefore have the same occupancy, which means that they can be grouped accordingly (FIG. 3b). However, all sites are connected by competition for the same, limited regulator (FIG. 3c). This model is equivalent to standard formulations in biophysical chemistry for mixtures of many different ligands[20]. For the case of only two competing types of binding sites, the equations can be solved explicitly and are widely applied in biochemical competition assays, for example, to measure the affinity of a mutated sequence relative to the wild type. Using the constraint that regulator molecules must be either bound or unbound, the model can be solved numerically[75] for any combination of binding site abundances and affinities. It thereby allows one to study the impact of increased binding site concentrations on binding site occupancies (FIG. 3d). Our source code to solve this model is freely available on doRiNA — RNA competition[76].

Modelling miRNA–transcriptome interactions. As an example, we model binding sites for the human miRNA miR-20a in the transcriptome of monocytes (FIG. 4). We use mRNA expression data[77] to estimate relative mRNA abundances.

To estimate the abundance of miRNA binding sites, we counted computationally predicted, conserved miRNA targets for miR-20a (updated PicTar[78,79] predictions downloaded from the doRiNA database[76]). Each binding site was weighted with the estimated relative abundance of the harbouring mRNA. Furthermore, we estimated the K_d for each binding site by projecting the predicted base-pairing between target and miRNA onto the respective guide RNA segments[41], such that the binding energy is mostly contributed by base-pairing of the 'seed match' with the 'seed' (miRNA positions 2–7) (BOX 2). Assuming that the transcriptome comprises 250,000 mRNA molecules per cell, in line with recent reports (50,000–500,000 mRNAs per cell[28,29] depending on cell type or stage), and that, furthermore, only half of the binding sites are accessible, we estimate that ~22,700 binding sites per cell compete for miR-20a. Given the large uncertainties, we also solved models with one-fourth and four times this number of binding sites (FIG. 4a,b). Finally, to exclude a bias due to the details of conserved binding site selection, we counted all 6-, 7- and 8-nucleotide matches in 3′UTRs and weighted them with the mRNA expression. For 250,000 mRNAs per cell, this amounts to ~151,000 binding sites, which is likely to represent an upper bound.

Competition effects on miRNA function. Although these models of a monocyte comprise drastic simplifications, they capture the key elements for competition: occupancies are described quantitatively, and a large number of sites compete for the same, limited regulator. The prime consequence of a large pool of binding sites is the discrepancy between the total amount of regulator (RBP

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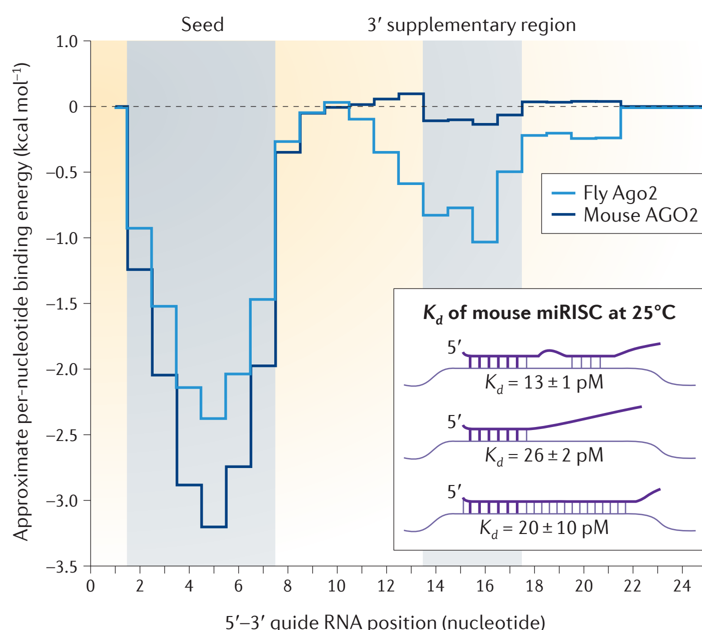
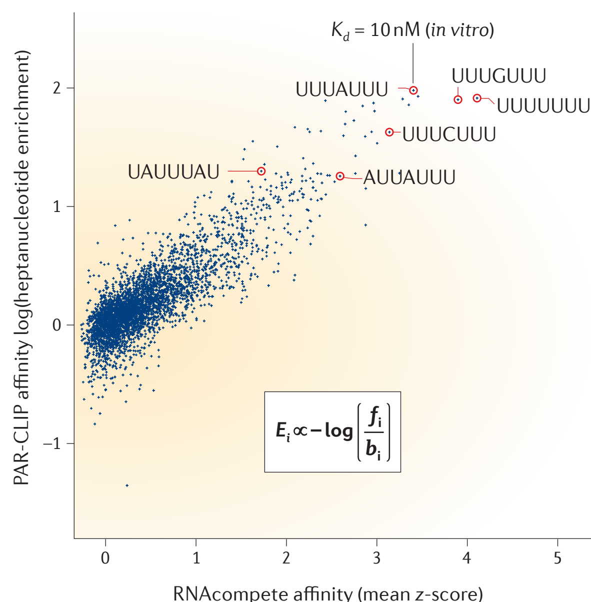
Surface plasmon resonance
A precise technique to measure dissociation constants, for example, for binding of RNA-binding proteins to RNA sequences.

or miRNA) and the amount that is free and available for binding to an individual site (FIG. 4a). In our monocyte example, a large fraction of total miR-20a is sequestered by the pool of binding sites over a wide range of miRNA concentrations. Regulation can only occur when free miR-20a concentrations approach the dissociation constants of binding sites (FIG. 1). The corresponding total concentrations (that is, threshold concentrations) are orders of magnitude larger than those *in vitro*, where they would directly correspond to K_d (BOX 2; FIG. 4b). This discrepancy is an important consequence of competition between the binding sites in a cell.

Competition versus excess. Importantly, although in classical biochemistry 'competition' simply denotes the presence of alternative ligands or binding sites, regardless of

Box 2 | Energy models for the binding of RBPs and miRNAs





The binding of regulators such as RNA-binding proteins (RBPs) and microRNAs (miRNAs) is a result of physical interactions (for example, hydrogen bonding) that contribute binding energy. For sequence-specific binding, the binding energy depends strongly on the part of nucleic acid sequence that is in direct contact with the regulator. This is often further simplified by assuming that each nucleotide contributes to the total binding energy independently. The binding energy directly determines the dissociation constant (K_d), and vice versa. An energy model for the binding of a post-transcriptional regulator therefore allows one to quantitatively derive the relationship between regulator concentrations and binding site occupancies.

Purified fly RNA-induced silencing complex (RISC) has recently been extensively analysed to elucidate the impact of mismatches between guide RNA and target for all positions along the guide RNA[41]. We used these data to construct an approximate per-nucleotide binding energy model (see the figure, part **a**). Briefly, we reasoned that the log-ratios of perfect and mismatched K_M (related to K_d but includes catalytic turnover; $K_M=(k_{off}+k_{cat})/k_{on}$) should be proportional to the change in binding energy introduced by the mutation. Averaging all the available data for each guide RNA position, we obtained pseudo-energies contributed by each position of the guide RNA if they were paired to the target. We scaled these pseudo-energies such that their sum corresponds to the best reported binding (3.7 pM at 25 °C). For mammalian (mouse) Argonaute (AGO), three distinct modes of binding (see the figure, part **a** inset) were measured[37]. We used these data to scale the fly Ago energies in each of the three described guide RNA segments to arrive at an approximate energy model for mammalian AGO. Strong binding requires base-pairing with the 'seed' (miRNA nucleotides positions 2–7), which nucleates the base-pairing between guide and target before additional 3′ pairs may form[123]. Pairing that extends the seed match up to position 9 of the miRNA and supplementary base-pairing of the 3′ part of the miRNA (around positions 13–16) can further stabilize binding[41]. As additional 3′ pairing leads only to a slightly reduced K_d (that is, slightly stronger binding), the seed region contributes the majority of binding energy. By contrast, complementarity beyond position 16 and at positions 10–11 was observed to weaken binding. We estimated the K_d of arbitrary miRNA binding sites using this approximate energy model. Of note, the details of how the per-nucleotide binding energies are derived from the measurements in REF. 41, and the precise values of the binding energies do not affect our conclusions. For example, the temperature can be reduced to 25 °C (stronger binding) or increased to 40 °C (weaker and more-uniform binding) with very minor effects on the scenarios for competition by additional binding sites (that is, 'sponge' effects) that we investigate. Our source code for this miRNA energy model is freely available on doRiNA — RNA competition[76]. Sequence-specific binding energies of RBPs also manifest as enrichments in CLIP–seq experiments[43], artificial RNA-binding arrays[73] or novel sequencing-based methods[72]. The log-ratio of how frequently a given sequence i was bound (f_i) versus its background frequency (b_i) is directly related to the binding energy (E_i)[124]. Given the K_d values of a few binding sites, the sequence enrichments can be mapped to dissociation constants (see the figure, part **b**). The example is taken from REF. 75 and shows the enrichment of heptanucleotides bound by the human RBP ELAV-like protein 1 (ELAVL1) as seen in a photoactivatable-ribonucleoside-enhanced CLIP (PAR-CLIP) experiment[61], compared to an *in vitro* RNA-binding assay for affinity (RNAcompete[73]).

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On-rates and off-rates
The rates at which a complex of two molecules is formed (on-rate, measured in $M^{-1} s^{-1}$) and decays (off-rate, measured in s^{-1}). They are determined by the series of structural and energetic changes that both molecules undergo upon binding or unbinding. These intrinsic rates depend on temperature and the solvent, but not on the concentrations of the molecules that form the complex. Kinetic (time-dependent) models of molecular interactions require knowledge of these rates.

Seed match
The core region of a microRNA (miRNA) binding site. It is complementary to nucleotides 2–7 (the seed) of the miRNA (guide RNA) and forms a duplex with the miRNA upon binding, which contributes most of the binding energy.

whether these are bound or not, we use the term here in analogy to other fields such as economics or sports: where there are more participants to a market or tournament than the number of investments or prizes available, competition occurs. In this sense, the less bound a binding site is (as a result of the 'success' of the many other sites), the more it is challenged by competition. Therefore, this competition occurs when the presence of many sites lowers the free regulator concentration below the K_d value of a site, while the total concentration would be higher than this threshold. This definition takes into account the concentration of the regulator, as well as the concentration of the binding sites and their K_d values. It is therefore more specific than the expression 'an excess of binding sites', which is sometimes used in the discussion of competition[49]. There can never be more sites bound than the number of regulator molecules present. Thus, it is true that an excess of binding sites implies strong competition effects. However, the inverse, which may suggest that crosstalk effects are strongest at equimolar concentrations of binding sites and regulator, is generally not true because it ignores the affinity of binding sites.

Nonlinear competition effects. Using the definition described above, competition in our miR-20a model is reduced when miR-20a levels increase: as strong binding sites become highly occupied, they cease to bind to additional miRNAs. The number of competing binding sites effectively decreases, and changes in total miRNA concentration lead to larger relative changes in free concentration. This phenomenon has been described as ultrasensitivity[80] because the slope of the free regulator in FIG. 4a can become very steep. As a consequence, the occupancy curves in FIG. 4b also have altered slopes. This effect can be more or less pronounced depending on the distribution of K_d values in the transcriptome. The binding of RBPs with a broader spectrum of binding energies (BOX 2), such as ELAV-like protein 1 (ELAVL1; also known as HuR), seems to be more strongly affected by this phenomenon[75].

At very high miRNA concentrations, at which all sites are effectively saturated, the distinction between total and free concentrations becomes irrelevant, and the curves converge. The inverse scenario, in which a miRNA is completely sequestered by highly overexpressed transcripts (and effectively unbound binding sites), was previously described as an 'escape' (REF. 81) from miRNA regulation and has a qualitatively very similar plot.

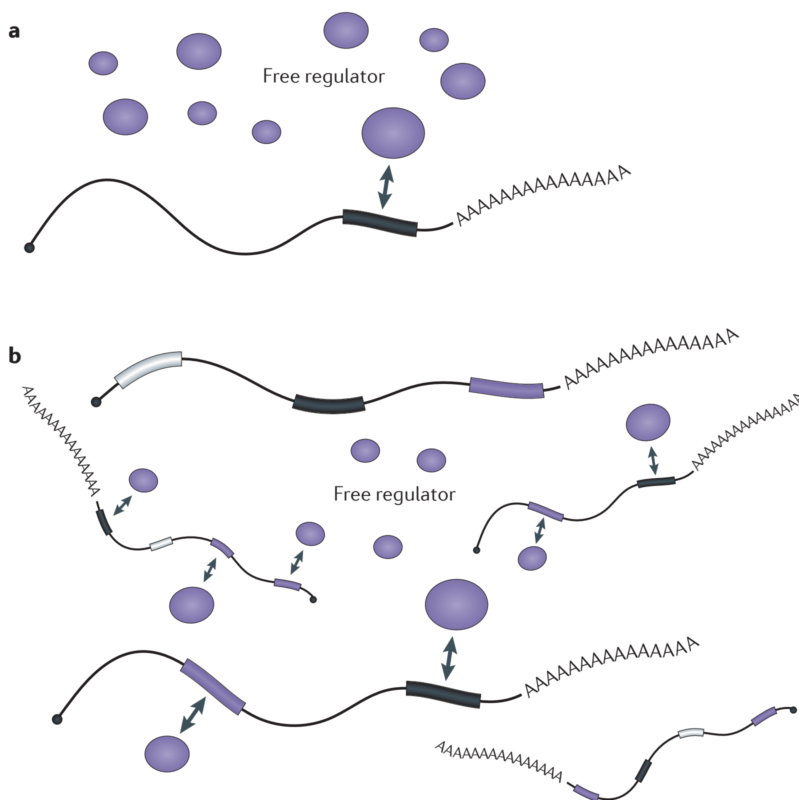

Figure 2 | **RNA competition.** **a** | A regulator (circle) — which can be an RNA-binding protein (RBP) or a microRNA (miRNA) — interacts with a single binding site (black rectangle) on an mRNA. **b** | Binding sites of different strength (black, purple and grey rectangles) on multiple mRNAs compete for regulators.

Reconciling model and experimental data

How do the findings presented above relate to the experimental characterization of post-transcriptional regulators?

Why regulation correlates with the strength of binding. In the cases where binding to RNA has been measured quantitatively, K_d values (BOX 2) range from tens of picomolar to hundreds of nanomolar[40,41,67,72,73], corresponding to a few dozen to hundred-thousands of molecules per cell (a K_d of 1 nM corresponds to ~1,000 molecules per cell[58]). Furthermore, K_d values of strong binding sites can differ from weak, but functional, binding sites by 1–2 orders of magnitude. When considering individual binding sites, this constitutes a riddle because most RBPs or miRNAs that exert a regulatory effect are expressed at levels that well exceed the K_d values of strong binding sites. At a concentration at which the weakest sites confer regulation, the strongest sites would already be highly saturated. In this scenario, siRNA knockdowns (which may reduce regulator concentrations by 1–2 orders of magnitude) would barely be able to reduce the occupancies of strong sites. This is a contradiction to experimental evidence that finds the strongest sites (the top targets in CLIP experiments, best motif matches and the strongest miRNA seed matches) to frequently exhibit the strongest responses when perturbing the regulator[59–62]. By contrast, the presence of other binding sites in the transcriptome tends to not only increase the effective threshold concentrations (FIG. 4b) of different binding sites but also bring them closer together (owing to the steep slope in FIG. 4a). In this way, competition helps to explain why strong sites react strongly in real experiments. The increase in effective threshold concentrations grows with the number of considered binding sites. For miRNAs, a similar qualitative connection between the abundance of targets

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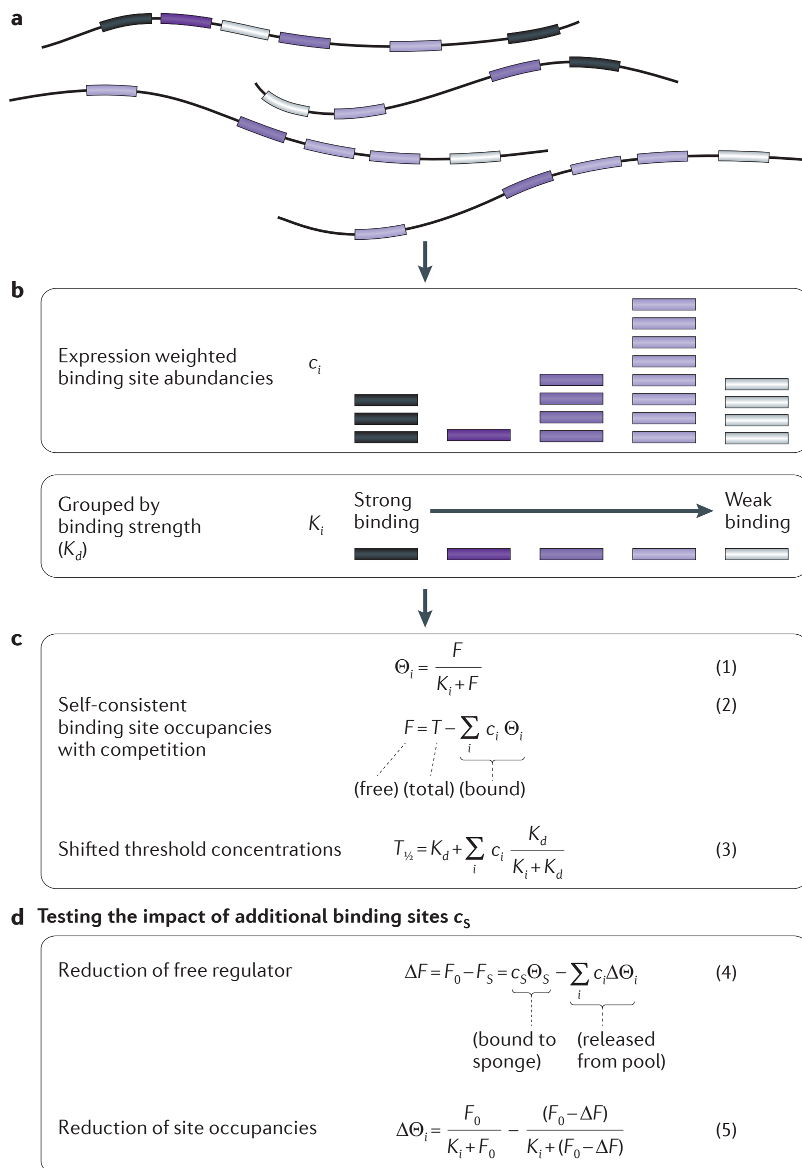

Figure 3 | **Steady-state model of the transcriptome to study RNA competition effects.** **a** | By combining the binding equations for many different binding sites with the constraint that the total number of regulator molecules is not changed by binding or unbinding, we developed a simple steady-state model of binding site occupancies that can be numerically solved for any number and combination of binding sites. To simplify this model, we have not considered the indirect effects of binding on mRNA levels, for example, destabilization by microRNA (miRNA) binding. Black, purple and grey rectangles represent different binding sites of RNA-binding proteins (RBPs) or miRNAs. **b** | Expression data allow one to estimate concentrations (c_i) of all binding sites of a given strength (K_i; represented by different colours). For non-cooperative binding sites, the mRNA on which they are found does not matter, and they are grouped by affinity. **c** | Equation 1 is the binding equation. At equilibrium, all sites of a given K_i have the same occupancy (Θ_i), which is determined by the amount of free regulator (F) and the binding equation (FIG. 1). Equation 2 expresses that all regulator (T) is either free or bound (that is, the sum of all binding sites, each weighted with its occupancy and concentration[20]). This constraint introduces competition effects when the aggregate amount of bound regulator (the sum over $c_i\Theta_i$) leads to a substantial difference between total and free concentrations. This manifests in increased threshold concentrations $T_{½}$, described by Equation 3. **d** | Expression of a 'sponge' RNA is modelled by increasing the concentration of strong binding sites (c_S). This reduces the free regulator concentration from F_0 to F_S and thereby reduces binding site occupancies (Θ_i). The source code to solve this model is freely available on doRiNA — RNA competition[76].

and the miRNA concentration range in which regulation can occur has been proposed and is supported by experimental data[77,82–84].

Given that competition between all binding sites 'sets the stage' on which PTGR functions, can competition also provide a mechanism for specific regulatory control? Potentially, changes in the levels of one transcript could affect the regulation of another because upregulation of that transcript increases the abundance of the contained binding sites. If a repressor, such as a miRNA, is sufficiently sequestered by such an increase in binding site concentration, then its targets could become derepressed.

Target mimics. This mechanism was first described in plants, in which the miRNA miR-399 can be functionally inhibited through sequestration by the non-coding RNA *IPS1*[15]. Importantly, and in contrast to animal miRNAs, plant miRNAs usually (but perhaps not always[85]) induce cleavage of their targets. By contrast, *IPS1* is not cleaved by miR-399, which enhances its potency as a miRNA inhibitor and clearly distinguishes it from typical plant miRNA targets. It was therefore termed a 'target mimic' (REF. 15). Most animal miRNAs usually do not induce target cleavage, and a clear distinction between target and mimic is not possible. However, the transcriptomes of animal cells harbour large numbers of potential miRNA seed matches. Therefore, competition for binding is equally relevant in animals.

RNA decoys. The idea that not all of the many potential miRNA binding sites are functional led Hervé Seitz to revisit the definition of a miRNA target[86]. Seitz argued that, in addition to merely being bound, a target ought to be sensitive to the regulatory impact conferred by a miRNA. According to Seitz, 'fine-tuning' of mRNA levels can only be evolutionarily selected for if the regulatory impact of the miRNA outweighs the variability in expression observed between cells or individuals. Ideally, a target gene would be responsible for a phenotypic change upon perturbation of the targeting miRNA. In this view, all other binding sites represent 'decoys'. This distinction depends on the cellular context and the researcher's interpretation: a gene could be considered a target in one cell type and a decoy in another. The term decoy suggests non-functional and would therefore imply the absence of natural selection on any particular decoy site to retain its binding capability. However, some miRNA binding sites could be evolutionarily selected to purely act as decoys and thereby acquire an alternative function: to sequester miRNAs from the 'real' targets. The extent to which evolution has indeed separated the aspect of decoy from other functions is unclear; below, we estimate lower bounds on the amount of decoy binding sites that have a measurable effect.

RNA sponges. In 2007, the functional sequestration of animal miRNAs was tested experimentally. Insertion of artificial RNAs — adding a total of ~10,000 binding sites for a miRNA — into cells resulted in sufficient sequestration of the miRNA to observe derepression

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Equimolar
Pertaining to the situation where the same number of different molecules is present in the same volume. The concentrations of these molecules are the same.

Target mimic
A plant microRNA binding site that is resistant to cleavage.

miRNA sponge
A highly expressed RNA transcript that carries an unusually large number (~5 or more) of binding sites for the same microRNA (miRNA). The term was originally introduced for artificial constructs designed to inhibit miRNA function.

of its targets[16]. Consistent with the limited strength of miRNA repression, the impact on the tested endogenous target *E2F1* was overall mild (1.5–2.5-fold upregulation). To highlight the large number of strong binding sites for a single miRNA that were engineered into the artificial transcripts, in contrast to physiological mRNAs, the term 'miRNA sponge' was coined[16]. The levels of miRNAs were only mildly perturbed in these experiments (1.3–3-fold), arguing that the dominant effect was indeed via sequestration. Conceptually, miRNA sponges can be described

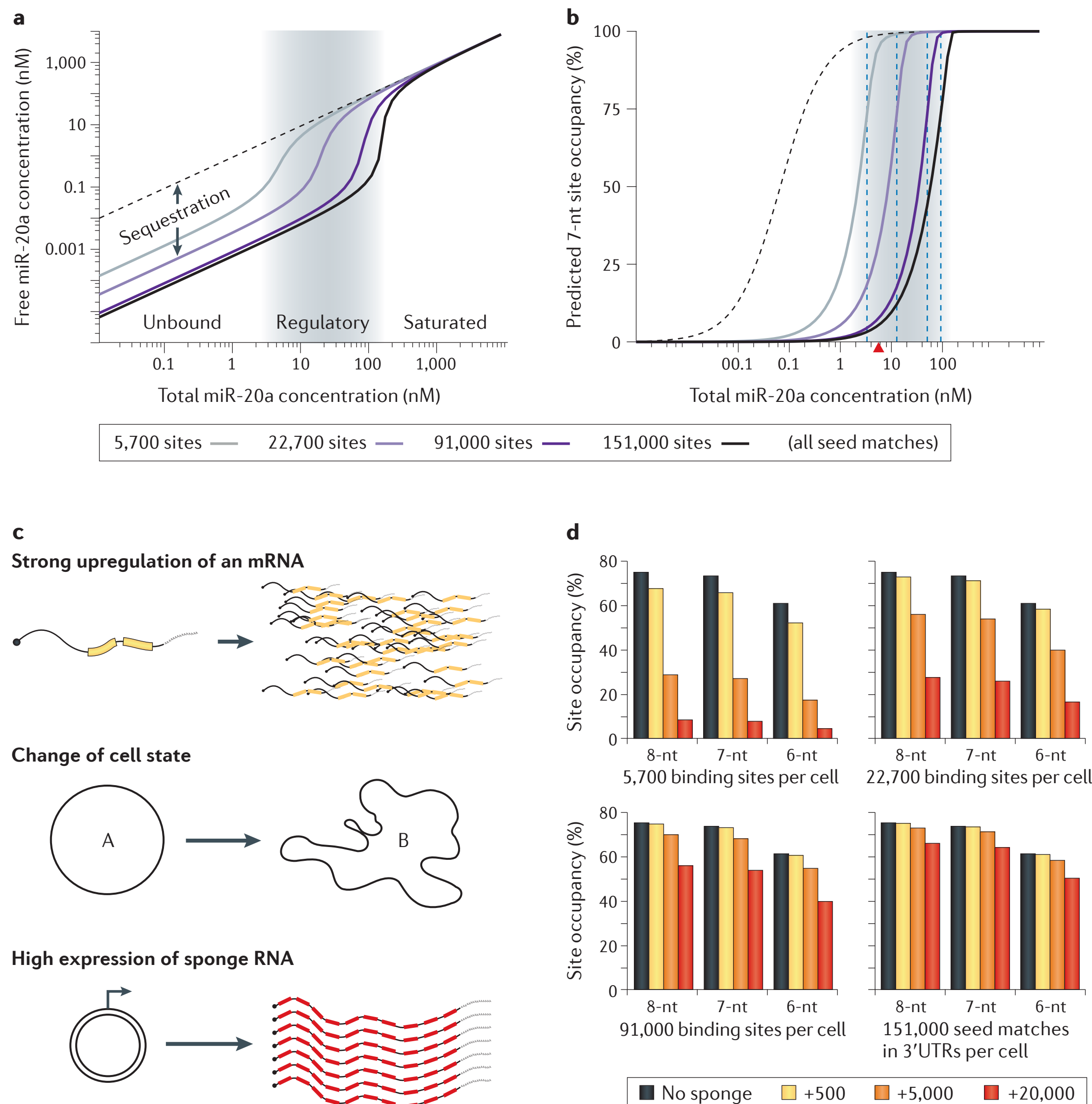

Figure 4 | **Quantitative modelling of competition effects for miR-20a binding.** **a** | Free miR-20a concentration — computed for different models of a monocyte (solid lines) — is plotted as a function of total miR-20a concentration on a logarithmic scale. Sequestration of microRNA (miRNA) is substantial (dashed line indicates where free miR-20a concentration is the same as total miR-20a concentration). Regulation requires free miRNA concentration around the dissociation constant (K_d) of binding sites (shaded region). Saturation requires unphysiological levels of miRNA (>100,000 copies per cell). **b** | Predicted occupancies of 7-nucleotide (nt; position 2–8) seed matches are shown. The presence of many sites increases the 50% occupancy ($\Theta = 50\%$) thresholds (solid lines) relative to the *in vitro* measured K_d (dashed line). Blue dashed lines indicate total concentrations at which $\Theta \approx 75\%$ for an 8-nt site. Red triangle indicates the approximate miR-20a concentration (~5,600 copies per cell), assuming that ~150,000 Argonaute proteins are expressed per cell[126] and that miR-20a abundance is proportional to its read count[77]. **c** | Scenarios for increasing binding site concentrations are shown. In the top panel, a single mRNA with two miRNA binding sites is strongly upregulated (+500 sites). In the middle panel, the collective transcriptome changes by cellular differentiation (+5,000 sites). In the bottom panel, an artificial 'sponge' RNA (+20,000 sites) roughly doubles the total binding site concentration. **d** | Scenarios from part **c** are evaluated computationally. Predicted occupancies of miRNA binding sites at the miR-20a concentration indicated in part **b** are plotted for 8-nt, 7-nt and 6-nt seed matches (the approximate K_d values of which are 61pM, 67pM and 118pM, respectively, at 37 °C).

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as artificially produced miRNA decoys that are optimized to add a very large number of binding sites for maximum sequestration of a miRNA.

Naturally occurring sponges for miRNAs or RBPs. Bacterial PTGR includes several examples of sequestration as a means of regulation[87,88]. In eukaryotes, non-coding RNAs were reported to regulate the activity of RBPs such as Dicer by sequestration[89]. However, until recently, there were no reports about endogenous, potent miRNA sponges with high expression levels, multiple strong binding sites, high stability and resistance to AGO endonuclease activity, although biological scenarios have been identified in which such an antagonist for a miRNA would be useful[90]. Given the stability of miRISCs (which have half-lives of days or more)[91], how can cells remove the repressive effect of a miRNA, for example, during differentiation or in response to signals?

Sequestration through rapid transcription of an endogenous sponge seems to be a plausible mechanism, especially given that miRNA seed matches could evolve easily[92]. A striking candidate for a naturally occurring miRNA sponge is the circular RNA (circRNA) CDR1 antisense RNA (*CDR1-AS*; also known as *ciRS-7*)[93], which in humans contains ~74 binding sites for miR-7 and is very highly expressed in the brain. Cell line experiments and overexpression of this circRNA in zebrafish demonstrate that it can function as a miR-7 sponge[45,94], although the *in vivo* function in its natural context in the mammalian brain awaits further elucidation.

Recently, a viral transcript was described that binds to and degrades miR-27 (REF. 17). The degradation mechanism is unclear but is specific to a particular binding site. Some strong binding sites have been observed to destabilize bound miRNAs[91]. Transcripts that induce miRNA degradation inhibit a miRNA more strongly than those that inhibit a miRNA by pure competition, but this discussion is beyond the scope of this Analysis. In summary, experimental evidence clearly shows that miRNAs can be functionally sequestered or sponged by binding alone. However, in cases where no catalytic turnover of either the miRNA or its targets is involved, the experiments indicate that very high expression of sponge sites is required to substantially relieve miRNA repression.

Regulatory crosstalk

The following questions remain. To what extent can changes in the levels of natural transcripts, such as mRNAs, affect miRNA binding on other transcripts? Can competition effects amount to a relevant relief of repression and thereby mediate a form of mRNA crosstalk?

In 2010, one study reported that the expression level of *PTENP1*, a pseudogene of the tumour suppressor phosphatase and tensin homologue (*PTEN*), affected PTEN protein levels and that this transcript contained shared binding sites for five different miRNAs[95]. Function of a pseudogene, which potentially titrates a regulator that also targets the coding gene, was described previously[96] but later disproved[97]. As pointed out elsewhere[98], the levels of *PTENP1* RNA are much lower than those of *PTEN* RNA, which has led to a debate on the interpretation of the results as competition for miRNAs. Nonetheless, these results motivated the work that led to the ceRNA hypothesis[21], which posits that transcripts functionally interact via competition for common miRNAs. Given that even complete relief from repression by a miRNA usually has only mild effects on an individual mRNA, this theory highlights the importance of sharing binding sites for different miRNAs to yield substantial crosstalk[21,99].

By this logic, pairs of mRNAs that can be repressed by multiple (~5 or more) shared miRNAs would represent the best ceRNA candidates. Such mRNA pairs can be searched computationally[100,101]. As miRNAs and their target mRNAs are often expressed in a tissue- or cell type-specific manner, a number of shared miRNA binding sites between functionally related genes should be expected[92,102], making it difficult to construct a null model and to identify ceRNA-like signatures based on computation alone. Furthermore, such a computational search enriches for long 3′UTRs, a hallmark of many transcription factors and other regulators with many potential downstream targets (indirectly shown in REF. 103).

Indeed, a transposon-based mutagenesis screen identified 320 genes that drive tumorigenesis of melanoma[104], and a subset of these genes were predicted to share 7 or more miRNAs with *PTEN* and were considered candidate ceRNAs. From the seven of eight tested genes that were found to reduce *PTEN* expression upon knockdown, three encode transcription factors linked to proliferation (*AFF1*, *JARID2* (jumonji, AT-rich interactive domain 2) and zinc-finger E-box-binding homeobox 2 (*ZEB2*)); two encode splicing regulators linked to differentiation and development (muscleblind-like splicing regulator 1 (*MBNL1*) and RBFOX2 (also known as RBM9)); and the remaining two are trinucleotide repeat-containing 6A (*TNRC6A*) and *TNRC6B*, human homologues of *GW182*, which are required for global repression by miRNAs[105,106]. Of note, transposon insertions that disrupt only the 3′UTR were not described in this study. This leaves the possibility that tumorigenicity and changes in *PTEN* expression could be driven by loss of these proteins.

A different example is the long non-coding RNA *LINC-MD1* (REFS 107,108). This transcript is transiently expressed at high levels (~1,000 copies per cell; I. Legnini, personal communication) in a cell line system for myoblast differentiation and harbours one of three precursors for miR-133. Knockdown of *LINC-MD1* strongly impairs muscle differentiation in cell culture[107]. Unlike the ceRNA candidates discussed above, *LINC-MD1* could compete for only two miRNAs with the two assayed target genes — mastermind-like 1 (*MAML1*; via miR-133) and myocyte enhancer factor 2C (*MEF2C*; via miR-135). Of note, the proposed miR-133 binding site in *LINC-MD1* is part of the pre-miR-133 hairpin and is likely to have low accessibility. Thus, although the molecular mechanism by which *LINC-MD1* functions can be linked to miRNAs, it is not

miRNA decoys
MicroRNA binding sites that are not selected for conferring repression on its target transcript; they are non-functional or act by sequestration.

Pseudogene
A mutated copy of a protein-coding gene that has lost a functional open reading frame. Some pseudogenes seem to be conserved, but their function is unclear.

Long non-coding RNA
An RNA transcript of at least 200 nucleotides in length that cannot be translated. Many long non-coding RNAs are spliced and capped RNA polymerase II transcripts with poly(A) tails; the function of most long non-coding RNAs is unknown.

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entirely clear that, at endogenous expression levels of *LINC-MD1*, crosstalk via miRNA competition suffices to explain its functionality.

Quantifying requirements for substantial crosstalk. Given that crosstalk through competition for miRNAs may not be the only explanation for the experimental results currently described as ceRNA effects, is it at least a probable explanation? Below, we estimate the number of additional binding sites that is required to substantially reduce occupancies by sequestration. This determines the limits for the efficacy of any transcript, mRNA or artificial sponge to regulate other transcripts solely by competition for miRNAs, as proposed by the ceRNA hypothesis.

A single binding site is 75% occupied when the available miRNA concentration is 3 times the K_d and 25% occupied at one-third of the K_d, which is roughly one order of magnitude lower. For the strongest miRNA binding observed[41], K_d is 13 pM, a concentration corresponding to ~12 molecules in the cytoplasm. Thus, if only a single binding site were present in the cell, then ~40 miRNA molecules would lead to 75% occupancy. To relieve repression and switch this site to 25% occupancy, at least 35 of the 40 miRNA molecules would have to be sequestered. However, to achieve this by sequestration, many more binding sites have to be added: competing sites, even in an artificial sponge, are only strong binding sites and cannot sequester regulators (RBPs or miRNAs) at 100% efficiency. As new binding sites sequester free miRNA, all occupancies decrease, including those of sponge sites, which diminishes their impact. Similarly, as free miRNA levels decrease, the (typically large number of) other binding sites present in the cell react with a net release of miRNAs, further attenuating the reduction of free miRNA by the sponge (FIG. 3d). As outlined above, the relative amounts and affinities of the targeted, endogenous binding sites and the sponge sites need to be taken into account.

Scenarios for crosstalk. We used our monocyte models to investigate three scenarios for the effects of an increase in miR-20a binding sites on occupancies (FIG. 4c,d). Importantly, when only conserved miRNA targets are considered, the model contains relatively few but strong binding sites, which is a favourable condition for crosstalk. In the simplest case, we consider the upregulation of an mRNA. Most expressed mRNAs have no more than 10–100 copies per cell. Nonetheless, to estimate an upper bound for mRNA crosstalk, we assume that an mRNA with two strong binding sites may be highly induced to up to 250 additional copies per cell, adding in a total of ~500 binding sites. A potentially larger effect could be expected if a cell undergoes differentiation or other large-scale changes, and multiple transcripts with strong binding sites are collectively upregulated. We consider a scenario with ~5,000 extra binding sites. The most extreme scenario considered is the expression of an artificial sponge construct[16], which adds ~20,000 binding sites. This corresponds to roughly doubling the target abundance in our model with 22,700 binding sites.

Given the binding site and target abundance estimated above, at which miRNA concentration would the system be susceptible to crosstalk effects? miR-20a actively represses targets in monocytes[77], suggesting that targets have reasonably high occupancies. Derepression (that is, reduction of site occupancy) by crosstalk cannot exceed the initial site occupancy before sponge expression (FIG. 3d, equation 5), suggesting that strong crosstalk requires high occupancies. Conversely, high occupancies are indicative of saturation, and occupancies are most sensitive to changes in free miRNA concentration around K_d, which corresponds to 50% occupancy (FIG. 1). Thus, for each model of the monocyte, we chose the miR-20a concentration such that strong binding sites are initially at 75% occupancy, a favourable assumption for ceRNA crosstalk. We note that uncertainties in the binding energies, which lead to different estimates of K_d, shift these estimates for miR-20a concentrations but leave the general behaviour of the system largely unchanged; by this logic, the estimate of ~6,000 miR-20a molecules per cell would predict notable binding to strong sites in the model with ~22,700 sites, a reassuring consistency between our estimates and the observation that miR-20a is active in monocytes. Even under the above assumptions, which were always selected to maximize crosstalk, the strong upregulation of a single mRNA could not notably affect gene regulation by competition (FIG. 4d). Substantial reductions in site occupancy require an increase in binding site abundance that is at least 10 times higher than that in the mRNA scenario and become comparable to a complete relief from miRNA repression only for the sponge scenario described earlier.

ceRNA crosstalk requires unphysiological amounts of additional binding sites. Our estimates are consistent with recently published work[49], in which significant derepression of endogenous miRNA targets in liver cells is only observed when the target abundance is effectively doubled by the expression of additional binding sites. In our model, when simplified to assume that all binding sites have equal strength, a reduction from 75% to 25% occupancy requires exactly twice the total number of binding sites in the cell, independent of the estimates of K_d or precise copy numbers. It is a general feature of RNA competition that it emerges from a global, collective effect of all binding sites. Thus, small changes in binding site concentrations become negligible when realistic numbers of binding sites for miRNAs or RBPs are considered. A recent theoretical ceRNA analysis finds that substantial crosstalk requires a small number of competing target sites[109]. The authors propose that ceRNA function may require a channel of 'stoichiometric decay', in which a bound miRNA needs to be destabilized, at least partially, by a ceRNA or functionally depleted by other mechanisms, such as trapping in P-bodies. Thus, it demarcates an important departure from the original ceRNA hypothesis that was based on competition alone.

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Taking together these results[109], recent experiments[49] and our own estimates, we conclude that a typical mRNA, expressed at ~10–100 copies per cell and with 1 or 2 binding sites for a miRNA, is not capable of significantly relieving miRNA repression on other mRNAs by sequestration alone. Crosstalk by competition for miRNAs between mRNAs requires unphysiologically high expression levels that are comparable to those of artificial sponges[16]. As the expected changes in site occupancy induced by even large fluctuations of mRNA levels on a realistic background of other sites in the transcriptome amount to no more than a few percent, additional interactions with groups of miRNAs cannot fundamentally change this result. We conclude that the current ceRNA concept is likely to require extension, modification and diversification to offer an explanation for the experimentally observed regulatory effects of non-coding RNAs such as *LINC-MD1*.

Testing functional crosstalk. Does this mean that RNA crosstalk by competition is not possible under physiological conditions? The large number of binding sites (74) for miR-7 and the exceptional stability of *CDR1-AS*[45,94] indicate that potent endogenous sponges may indeed exist. However, although ample biochemical data argue that *CDR1-AS* is efficiently bound by miR-7, the function of this circRNA as a competitor of miR-7 has so far not been tested by loss-of-function experiments.

Of note, neither overexpression of a putative ceRNA nor siRNA knockdown is a direct test for the hypothesis of interaction by competition for miRNAs. In the case of overexpression, almost any RNA may start to function as a potent sponge owing to unphysiologically high copy numbers. In siRNA knockdown, in addition to overwhelming the miRNA machinery with siRNAs[110], direct effects cannot be easily distinguished from indirect effects that are not mediated through competition for miRNAs. This is especially true for long and highly conserved 3′UTRs, which probably partake in manifold complex interactions. In such cases, target-site protectors[111], or mutagenesis using the clustered regularly interspaced short palindromic repeat–CRISPR-associated protein (CRISPR–Cas) system[112,113], could be used to disrupt, precisely, the miRNA binding sites that are presumed to render a ceRNA functional in the endogenous context. Knockdown and miRNA inhibition experiments may be repeated in this background, while overexpression of the ceRNA can be avoided. A meaningful rescue experiment would express a minimal construct bearing only the presumed competing binding sites to endogenous levels (or a construct with twice the number of the binding sites to half of that level). Furthermore, occupancies on endogenous RNA can now be monitored by crosslinking experiments[7,114], which allow changes upon perturbation of miRNAs or disruption of binding sites to be monitered. Finally, even without detectable mutual influences in expression level, another functional consequence of multiple transcripts being targeted by joint repressors could be the processing or coupling of noise in gene expression[109,115–117].

Conclusions

The amount and diversity of RNA sequences, together with the typically low sequence specificity of post-transcriptional regulators, suggest that, in animals, most RNA binding sites are in excess over their regulators. The resulting competition effects can substantially raise the threshold concentrations of binding sites, preventing saturation. Therefore, competition between sites might represent the natural context for the function and evolution of PTGR. Competition on a large scale (that is, between all binding sites in the cell) renders PTGR more robust and keeps mRNAs responsive to changes in regulator concentrations (buffering regime) (FIG. 5). However, this robustness also diminishes the potential for crosstalk between individual transcripts by competition.

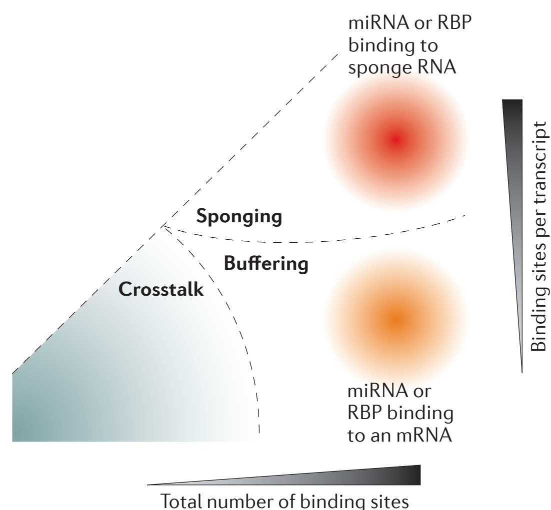

Figure 5 | **An overview of competition effects.** Crosstalk requires that the number of competing binding sites is small enough for an individual target (for example, an mRNA species) to influence binding site occupancies (FIG. 3d). For typical mRNA copy numbers, crosstalk in a well-mixed environment therefore requires that the total number of binding sites must be small as well. Furthermore, the binding sites need to be notably occupied and sensitive to changes in free concentration (F close to the dissociation constant (K_d)), suggesting very specific interactions. However, for most post-transcriptional regulators, sequence specificity is low. Consequently, the total number of potential binding sites is large (at least thousands of sites), which vastly exceeds the contribution of an individual mRNA species. In this scenario, the effects of competition consist of increased threshold concentrations (FIG. 3c,4b) and a more linear response to concentration changes because saturation effects are strongly suppressed. We refer to this as 'buffering'. Only when the number of binding sites contributed by a single transcript species becomes comparable to the total number of binding sites in the transcriptome can a substantial reduction of site occupancies be expected. In contrast to the crosstalk scenario, this requires a number of RNA copies and binding sites per transcript that are not observed for physiological mRNAs. Such transcripts are therefore termed 'sponges'.

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The ceRNA hypothesis seems to offer an explanation for the functionality of non-coding RNAs and pseudogenes. However, marked regulatory effects due to a global competition for miRNAs can, as a rule of thumb, only be expected when changes in binding site concentration approach the target abundance. For miRNAs, this commonly amounts to tens of thousands of binding sites per cell and thus requires high expression of transcripts with many binding sites (sponge regime) (FIG. 5). This result is consistent with experimental data for miR-122 in hepatocytes[49]. We conclude that the current ceRNA model is neither a compulsory nor a likely explanation for the experimental results. In particular, endogenous changes in mRNA levels are unlikely to substantially alter the total concentrations of miRNA binding sites [49,118].

By contrast, binding site occupancies, and therefore PTGR, are determined by local concentrations. The cell is a very complex environment, and the cytoplasm is only crudely approximated as a single well-mixed compartment. Neurons, for example, can have substantial differences in RNA composition depending on subcellular localization[119]. Local concentrations of binding sites and miRNAs or RBPs may deviate from the average by orders of magnitude. Similarly, the reduced volume available in RNA granules can influence local concentrations. These effects could enhance the relevance of local crosstalk by competition.

Newly developed technology allows sequencing of mRNAs *in situ*[120], which offers the exciting possibility to quantify the localization of transcripts for thousands of genes. Turning the argument around, strong crosstalk effects may also be seen as an indication that localization renders the number of effectively interacting binding sites very small (crosstalk regime) (FIG. 5), or that multiple binding sites could cooperate and react more sensitively to small changes in concentrations. Sometimes small changes in mRNA or miRNA levels can be amplified to yield a clear phenotype. For example, the 3′UTR of the transcription factor c-Myb harbours three binding sites for miR-150, which cooperatively confer strong and dose-dependent repression; reduction of c-Myb expression by only ~30% is sufficient to alter cell-fate decisions[69]. Thus, extensions of the simple quantitative model applied in this Analysis may become important in the future.

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Competing interests statement
The authors declare no competing interests.

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