nature cell biology

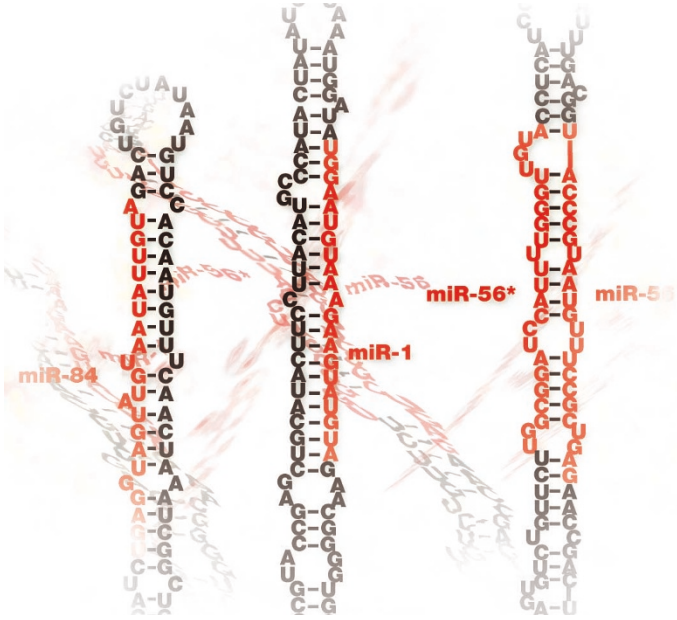
Whither RNAi?

The generally rapid progress of molecular biology methodologies is punctuated on occasion by discoveries that do nothing less than revolutionise the field. Historical examples include DNA and protein sequencing, restriction enzymes, PCR and the two-hybrid assay. The most recent addition to the methods hall of fame is RNA interference (RNAi). The discovery of a whole universe of small RNAs with regulatory functions of fundamental importance was entirely unexpected, and it is as remarkable for its conceptual and biological importance as it is for the tremendously powerful techniques based on this mechanism that allow selective ablation of gene expression. The explosive growth of the RNAi literature, from just 19 papers in 1999 to well over 180 in the first four months of this year, attests to the meteoritic impact of this nascent field on all areas of molecular biology.

One of the key experimental approaches to elucidate the function of a gene *in vivo* is the selective ablation of its expression or activity by one of several loss-of-function (LOF) approaches. Of the traditional arsenal of LOF techniques, gene disruption remains the most definitive approach; however, it has the disadvantage of being a far from trivial strategy in higher model organisms. Dominant-negative mutants, either designed or isolated through screens, also represent a useful tool. The disadvantage is that it is usually impossible to understand fully how the mutant actually functions in the cell. Other approaches that target the protein of interest include small-molecule inhibitors and antibody microinjection. Although often powerful, these techniques are rarely immune from the concerns of specificity.

Antisense technologies heralded a new era for LOF experiments. The technique remains extremely useful, especially in experimental systems such as Zebrafish and *Xenopus*. A general problem has been the need to screen a significant number of oligonucleotides to find a selective and effective agent, and concerns about specificity remain. The arrival of morpholinos re-invigorated the field with their higher specificity and efficacy. Nevertheless, nothing beats siRNAs (small-interfering RNAs) for the specific degradation of target mRNAs. Efficiency, efficacy and cost compare so favourably with other techniques that RNAi has quickly become the method of choice for LOF. This is especially true after a major hurdle was recently overcome with the use of short siRNAs that seem to bypass the interferon response; previously this had excluded the use of RNAi in mammalian cells. The recent shift in investment from antisense-based technologies to RNAi seems to mirror the enthusiasm of researchers for this nascent field.

Although the apparent ease of RNAi is fantastic news, it has also led to the publication of many papers that display RNAi data without sufficient controls. The problem has become so acute that the organizers of the Horizon symposium on RNA in early May set aside a whole session to debate the issue of standards in the field. The outcome of this debate was keenly observed by editors from several *Nature* journals and reflects closely internal discussions at this journal on the controls required for publication of definitive LOF data utilizing siRNAs.



We now wish to set out some of the best controls that are, in our view, currently available for the technique. We appreciate the technical challenges of some of the controls listed and realize that the field is still rapidly evolving, with new modes of siRNA action still emerging, so we are not setting the criteria for publication in stone. However, we will be assessing manuscripts closely for the overall level of proof provided by an RNAi, and indeed any other LOF, experiment. We will make every effort to avoid publishing LOF data that is clearly ambiguous and below par. At the same time, we will carefully assess what level of controls can be expected of any given study: this obviously varies between model systems, as well as approach (for example, specific ablation versus high-throughput analysis).

The following suggestions reflect the broad general opinion of many of the stakeholders present at the Horizon meeting on RNA, as well as the editors of this journal:

1. Mismatch or scrambled siRNAs: These are often of somewhat limited value. A scrambled sequence is too unrelated to the 'active' probe to function as a truly informative control. Occasionally, a one- or two-base-pair change in the middle of the antisense can be a useful negative control if the siRNA effect is clearly ablated. However, such a change may have unanticipated effects by converting an siRNA into a miRNA (micro RNA) that inhibits translation through a pathway closely related to siRNA.
2. Basic controls: siRNAs exert their effects through a growing number of surprisingly diverse mechanisms in addition to selective degradation of the targeted mRNA, such as specific effects at the chromatin level. Currently, siRNAs, unlike long double-stranded RNA (dsRNA), are not thought to trigger general translational attenuation through the interferon response. However, this also

remains a hotly debated possibility for some short siRNAs, or at least mixed populations retaining some longer dsRNA species. In addition, the closely related miRNAs do function through target-specific translational attenuation. Thus, it is important to show reduction of expression at the mRNA and protein level, as well as a functional readout where available. If both message and protein are ablated, the response is 'classical' RNAi. In contrast, if only the protein is reduced, the chances are that a miRNA-related translational mechanism is at work. It should be noted that although this set of controls should be regarded as best practice, the functional control listed below renders analysis of the mRNA non-essential.

Additional useful controls are available for unintentional activation of global translational repression through the interferon response (commercial assay kits or expression of unrelated proteins). Although centromeric or other emergent chromatin effects are harder to generate controls for, global gene expression may function as a control for any non-specific effects on gene expression (see, for example, the June issue of *Nature Biotechnology*; DOI: 10.1038/nbt831).

3. Quantitative controls: Titration of the siRNA is strongly encouraged. RNAi is often extremely effective already at minimal concentrations, and titration to the lowest possible levels reduces the chance of side effects as well as providing a graded readout of the effect. This is especially important because the RNAi machinery (the RISC complex in particular) is saturable, at least in some settings. Again, the rescue control outlined below is especially important when high levels of siRNA must be used.

   Protein levels should be assessed with quantitative techniques, such as quantitative western blotting, to allow for an accurate estimate of the level of reduction.
4. Functional controls: the ultimate control for any RNAi experiment remains rescue by expression of the target-gene in a form refractory to siRNA (ideally within the physiological range). This can often be achieved by utilizing one or more silent third-codon point mutations within the targeted region, although controls for fortuitous miRNA effects are desirable. Translational effects can be avoided by utilizing siRNAs targeted against 3′-untranslated regions (UTRs), which are non-essential for rescue expression from a plasmid.

   The use of recently developed vector-based RNAi systems will alleviate some of the technical hurdles of rescue expression (often with the added benefit of providing an inducible system; see also p513 of this issue).
5. Multiplicity controls: a good way to enhance confidence in RNAi data is to demonstrate a similar effect with two or more siRNAs targeted to different sites in the message under study. Alternatively, the RNAi approach is usefully supplemented by alternative methods, such as those described above.

The rescue control has to be regarded as the control of choice, given the multiple as yet ill-defined modes of action of this powerful mechanism. However, this may not always be possible and we will not insist on it if convincing alternative controls are enclosed, especially of category 5. In our eyes, the criteria outlined in points 2 and 3 will have to be satisfied to a significant extent to provide a convincing case for a LOF experiment. Obviously in the case of large-scale screens, controls have to be more minimal. However, in cases such as this we would suggest validation of a critical number of key targets, identified at least at the level of additional siRNAs against the same target.

Finally, we should like to emphasize that a very similar set of controls applies to antisense techniques, and this list will hopefully also inspire researchers favouring techniques complementary to RNAi to assess the level of evidence of their data carefully before submission.

Further information on siRNA, miRNA and other exciting RNA-related developments can be obtained from the Horizon symposium website at http://www.nature.com/horizon/rna/index.html.

Spring clean

You will find yourself reading a different-look NCB this month. This redesign has been borne of a critical evaluation of the various *Nature* Research journals, leading to a distillation of the most successful design elements into the new look you see before you. Aside from integrating strengths that have evolved independently across the *Nature* research journals, one of the explicit aims of this exercise is to facilitate cross-reading between the titles. In addition to the more integrative designs, the *Nature* journals now also feature a more consistent use of journal sections (see also our March editorial).

In a few months, this will be followed by an overhaul of the journal's website design. Again, the aim is improve clarity, accessibility and functionality, as well as to establish more consistency with the design of the other *Nature* journals. The reader's perspective on these redesigns is of critical importance to help precipitate further improvements, so comments, suggestions and criticisms are welcome. □