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RNAi: running interference for the cell

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RNA interference or RNAi is a recently characterized mechanism of eukaryotic gene regulation in which a short sequence of double-stranded RNA (dsRNA) specifically down-regulates expression of the associated gene. Preliminary characterization of this phenomenon has revealed a set of inter-related cellular pathways which appear to represent both a response to foreign RNA and a mechanism of endogenous gene regulation. Introduction of dsRNA into cells by a variety of means, including transfection of synthetic RNA duplexes, triggers the RNAi response resulting in specific suppression of target gene expression. Recent efforts on a genome wide scale have involved application of RNAi as an important new tool in cell biology to elucidate gene function in living cells.

Post-transcriptional gene silencing by dsRNA

Regulation of gene expression in the eukaryotic cell occurs at many levels including synthesis, maturation, and degradation of protein-coding messenger RNAs (mRNAs). Transcription by RNA polymerase II in the nucleus yields precursor-mRNA (pre-mRNA) molecules which are modified in a series of steps, including removal of non-coding intron sequences (splicing) and the addition of a 5′ cap structure and 3′ poly-A tail, before transport to the cytoplasm where translation occurs on the ribosome (Fig. 1A). Mature mRNAs are not all equal in terms of ultimate yield of translated product. Differences in initiation as well as efficiency of translation are key to the regulation of diverse genes.[1] As well, the lifetimes of mRNAs vary from minutes to hours to days—the differing resistance of a particular mRNA to exo- and endonucleolytic degradation is thus an important determinant of the gene expression profile in the eukaryotic cell.[2]

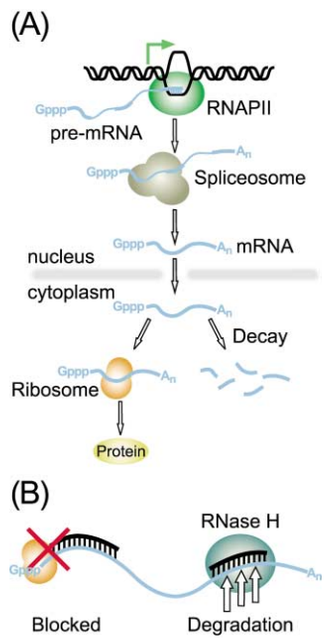

Fig. 1 (A) Synthesis and processing of mRNA in the eukaryotic cell. Nuclear processing of pre-mRNA includes addition of a 5′ cap (Gppp), excision of introns by the spliceosome, and addition of a 3′ poly-A tail (A_n). The mature mRNA is exported to the cytoplasm where it is translated on the ribosome to produce the encoded protein and ultimately degraded. (B) Inhibition of gene expression through hybridization of an anti-sense agent to mRNA. Two possible mechanisms include steric block of translation (left) and RNase H mediated degradation of the RNA portion of an mRNA·anti-sense duplex (right).

The introduction of DNA, RNA, or oligonucleotide analogs into cells has been intensively investigated as a means to modulate gene expression in biological systems.[3] In most cases, the design of such experiments has assumed an antisense mechanism whereby hybridization to a target mRNA would interfere with gene expression by blocking translation or by triggering RNase H mediated degradation of the RNA portion of the duplex (Fig. 1B). In 1998, Fire and colleagues reported the fascinating observation that injection of double-stranded RNA (dsRNA) into the worm *Caenorhabditis elegans* resulted in a specific silencing of the homologous gene's expression

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which was much more potent than that obtained by injection of the antisense or sense RNA alone (Fig. 2A).[4] The silencing was triggered by only a few molecules of dsRNA and was also observed in the progeny of the injected animals. While silencing could be correlated with a reduction in the levels of targeted mRNA, dsRNA corresponding to intron sequences did not trigger silencing, and the levels of pre-mRNA were unaffected in a silencing experiment.[4,5] Together these results suggested a cellular mechanism of suppression occuring after transcription and also after initial RNA processing events such as RNA splicing.

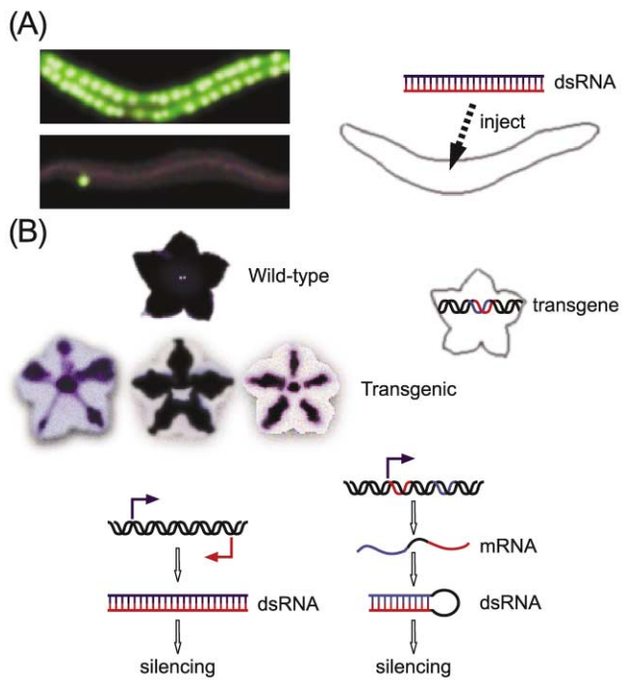

Fig. 2 Post-transcriptional gene silencing (PTGS) in diverse organisms. (A) Injection of double-stranded RNA (dsRNA) corresponding to a luciferase reporter gene potently suppresses luciferase expression in *C. elegans* (adapted from ref. 4); (B) (top) Attempts to intensify petunia colour by creation of transgenic plants with an extra-copy of the pigment pathway enzyme chalcone synthase result in the opposite phenotype (adapted from reference 6). (bottom) Production of dsRNA as a result of both sense and anti-sense transcription (left) or transcription off of inverted repeats (right).

The induction of gene silencing by injection of dsRNA explained, in part, previously mysterious examples of gene repression which had been noted in plants ("cosuppression") and the filamentous fungus *Neurospora crassa* ("quelling").[6,7] In these cases, introduction into the organism of a transgene regulated by a powerful promoter had the unintended effect of suppressing expression of both the transgene and endogenous copy of the gene. For example, when Jorgenson and coworkers attempted to intensify colour in petunias by over-expressing the mRNA for chalcone synthase, a key enzyme in the pigment biosynthetic pathway, the resultant flowers were variegated or in some cases white in colour indicating potent suppression of the synthase gene product (Fig. 2B).[6] In light of the observations of Fire and colleagues, transgene mediated silencing could be explained by the production of dsRNA in the transgenic cell through a combination of sense and anti-sense transcription or *via* read-through transcription of an inverted repeat of the trans-gene (Fig. 2B).

Cosuppression in plants has been described in terms of both post-transcriptional gene silencing (PTGS) involving specific degradation of mRNAs and transcriptional gene silencing (TGS) whereby initial mRNA synthesis is repressed. Interestingly, in plants, some examples of trans-gene mediated silencing have been associated with DNA cytosine methylation[8] which is believed to be an important factor in maintaining the silenced state of heterochromatin in higher eukaryotes. These cases suggested that cosuppression is a complex phenomenon which may occur through related but distinct mechanisms.

The specific, dsRNA mediated silencing observed by Fire and coworkers was named RNA interference or "RNAi". The fact that similar phenomena have been observed in plants suggested the existence of a pathway which might be conserved in diverse eukaryotes and represent a general, perhaps anti-viral, cellular mechanism of response to dsRNA.

The RNAi pathway

An outline of the essential steps in the RNAi response has been established by a combination of genetic and biochemical studies in diverse organisms including worms (*C. elegans*), plants (*Arabadopsis*), flies (*Drosophila*), and humans (Fig. 3). These investigations have also revealed the existence of several distinct but overlapping pathways which represent different forms of genetic regulation involving dsRNA.

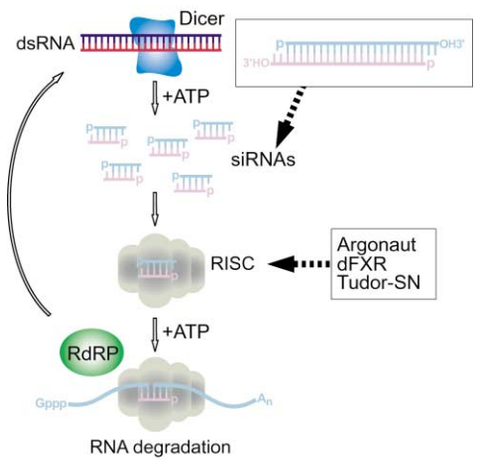

Fig. 3 Gene suppression by RNA interference. dsRNA is processed in an ATP-dependent reaction by the RNase III-type endonuclease Dicer into 21 nucleotide fragments. These siRNAs (inset) contain 19 base-pairs, a 3′ two nucleotide overhang, a 5′ phosphate (p), and 3′ hydroxyl terminus. In the *Drosophila* pathway shown here, the siRNAs are incorporated into the RISC silencing complex which includes members of the Argonaut protein family and the Fragile X protein (dFXR), as well as at least one nuclease activity (Tudor-SN). RISC is activated in an ATP dependent fashion and targeted to a complementary RNA resulting in cleavage of the target sequence near the centre of complementary sequence. In some cases, there may be an amplification of the dsRNA trigger by RNA dependent RNA polymerase activity (RdRP).

Biochemical fractionation of *Drosophila* extracts has resulted in the separation of what may be termed the "initiation" and "effector" steps of the RNAi mechanism. Initiation involves the ATP dependent processing of the triggering dsRNA to short dsRNA fragments, termed "small interfering RNAs" or siRNAs.[9] These RNAs are 21–23 nucleotides in length, with 3′ two nucleotide overhangs and contain 5′ phosphate and 3′ hydroxy termini (Fig. 3). Production of the siRNAs involves the activity of "Dicer" an RNA endonuclease.[10] Dicer is a multi-domain protein containing an N-terminal helicase motif and C-terminal RNase III-like nuclease portion—the crystal structure of the *A. aeolicus* RNase III catalytic domain[11] has been used to suggest that Dicer acts as a dimer to perform the four cleavage reactions required to generate siRNAs. The cytoplasmic localization of Dicer[12] is consistent with an overall localization of the RNAi machinery to the cytosol since targeting of RNAs appears to occur after nuclear processing steps such as splicing.

Dicer is not involved directly in the mRNA degradation or effector stage of RNAi. A complex containing this activity,

RISC (RNA Induced Silencing Complex), has been purified from both *Drosophila* and HeLa cell extracts.[13,14] Incorporation of siRNAs into RISC either involves or is followed by an ATP dependent activation step, as a result of which one strand of the siRNA duplex targets the complex to a complementary mRNA sequence (Fig. 3). This is followed by endonucleolytic cleavage at the centre of the target strand which is believed to expose that RNA to degradation by other (exo) nucleases.[15]

The makeup of RISC has not been fully determined and the composition of the *Drosophila* and mammalian complexes while overlapping are not identical; there may be several distinct RISC particles reflecting the complexity of the RNAi pathway. One common feature of RISC characterized to date is the presence of members of the Argonaute protein family.[16] Argonaute proteins are highly conserved and are involved in the regulation of development in eukaryotes suggesting a role for RNAi in the developmental program. They are characterized by the presence of two domains, PAZ (Piwi/Argonaute/Zwille) and PIWI, the functions of which are not known although it was initially suggested that the PAZ domain might represent a protein–protein interaction module.[17] The recent NMR and X-ray structures of PAZ domains from the *Drosophila* Argonaute-1 and Argonaute-2 proteins reveal a β-barrel structure capped by an α-helix.[18,19] The β-barrel core is an "OB-like" fold that resembles the structure of the RNA binding Sm proteins—as a result of these structural insights, experiments have been performed suggesting that one function of PAZ involves binding of single-stranded RNA possibly mediating recognition of the 3′ overhangs of the siRNAs.

RISC has also been shown to contain a number of other factors. These include the *Drosophila* homolog (dFXR) of the human Fragile X protein FMRP.[20] Fragile X syndrome is one of the most common forms of inherited mental retardation and is caused by the absence or loss of function of FMRP; the discovery of dFXR in RISC suggests that FMRP is involved in repression of gene expression through an RNAi related mechanism.[21] RISC also contains a nuclease, Tudor-SN, with homology to staphlococcal nuclease.[22] This nuclease activity lacks specificity suggesting that it acts at a stage later than the initial endonucleolytic mRNA cleavage step. Although the initiation and effector steps of RNAi are separable biochemically, they must be linked through the incorporation of Dicer generated siRNAs into RISC. A recently characterized Dicer associated protein in *Drosophila*, R2D2, which contains two canonical dsRNA binding domains appears to be involved in the efficient transfer of siRNAs from Dicer into RISC.[23]

As already mentioned, there is a heterogeneity to RISC as currently characterized that may reflect differences between the RNAi pathway in different organisms and/or perhaps hints at a greater complexity of RNAi-related mechanisms.[24] Supporting the latter idea are several observations. Several organisms including *Drosophila* and *Arabidopsis* contain more than one Dicer isoform; more specifically, Dicer family members may be divided into two classes, one of which contains a PAZ domain (but not PIWI) and one which does not. This suggests distinct roles for Dicer isoforms in RNAi-related pathways.

The observation by Fire and coworkers that RNAi is triggered by very small amounts of dsRNA suggests that there may be an amplification step at some point in the pathway. Consistent with this notion, there is evidence in a number of organisms for the participation of RNA dependent RNA polymerases in an amplification of triggering RNAs.[25,26]

miRNAs and cellular gene regulation

Larval development in *C. elegans* is regulated at two separate stages by the "small temporal" RNAs (stRNAs) *lin-4* and *let-7*.[27,28] These 21 nt RNAs act to repress translation by forming imperfect duplexes with sequences in the 3′-untranslated region (3′-UTR) of their respective target mRNAs (Fig. 4A). Homologs of *let-7* are found in a wide range of organisms with bilateral symmetry including flies, frogs, and humans. The identification of short ~21–23 nt RNAs in the RNAi response suggested a possible link between the *lin-4*/*let-7* and RNAi pathways. This was confirmed when it was demonstrated that the processing of the *lin-4* and *let-7* precursor RNAs involved Dicer.[29] Parallel to this work, a large number of small or "micro RNAs" (miRNAs) were cloned from *C. elegans*, flies, and humans.[30–32] Computational methods have been used to estimate that vertebrates possess 200–250 miRNA genes.[33] It is now clear that a significant number of, in some cases evolutionarily conserved, miRNAs are involved in regulation of gene expression in diverse eukaryotes. The identification of embryonic stem (ES) cell specific RNAs in mice suggests that miRNAs play a role in mammalian development.[34] The miRNAs are produced by Dicer from stem-loop precursors; at least some are initially synthesized as polycistronic transcripts which are first cleaved in the nucleus by the Dicer homolog Drosha before export to the cytoplasm for further processing (Fig. 4B).[35,36] It has been shown that a functional siRNA can also inhibit translation by binding to sequences introduced into the 3′-untranslated region of a target gene.[37] This raises that formal possibility that sets of endogenous genes could be regulated by a single dsRNA through a combination of RNAi and the miRNA/translational repression pathway.[38]

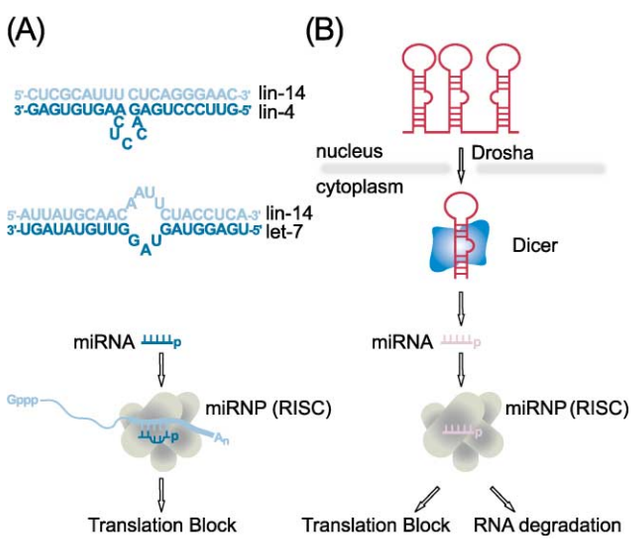

Fig. 4 Gene suppression by miRNAs. (A) Development in *C. elegans* is regulated at two stages by a translational repression mechanism involving imperfect duplex formation between the *lin-4* and *let-7* RNAs and target messages (in this case the *lin-14* mRNA). These small RNAs are part of a larger family of endogenous regulatory RNAs referred to as microRNAs (miRNAs); (B) Polycistronic miRNA precursors are processed by Drosha in the nucleus to hairpin molecules which are in turn cleaved by Dicer to yield mature miRNAs which are incorporated into a RISC-like RNP complex.

Short RNAs and heterochromatin maintenance

Transcriptionally silent DNA in the eukaryotic nucleus is maintained in a highly condensed form referred to as heterochromatin. Large expanses of heterochromatin containing repetitive DNA sequences are associated with the centromere—the region of the chromosome involved in spindle attachment and chromosome segregation during cell division (mitosis). A major mechanism of chromatin silencing involves specific methylation of lysine 9 of histone H3; this also results in recruitment of a protein, Swi6 in fission yeast and HP1 (heterochromatin protein 1), that spreads the block in transcription to adjacent genes.[39]

Martienssen and coworkers recently have made the surprising discovery that deletion of RNAi associated proteins (Argonaute, Dicer, or RNA-dependent RNA polymerase) in

the fission yeast *S. pombe* relieves silencing of genes inserted into the centromeric heterochromatin.[40] Supporting the link between the RNAi machinery and heterochromatin formation, siRNAs derived from transcription of both strands of centromeric repeats have been identified in *S. pombe* by Reinhart and Bartel.[41] It has also been demonstrated that the expression of short RNA hairpins can lead to the RNAi dependent silencing of normally expressed genes through heterochromatin formation.[42] Recently, a complex required for heterochromatin assembly in fission yeast—termed RITS (RNA-Induced Initiation of Tanscriptional Gene Silencing)—has been purified and partially characterized.[43] RITS contains Ago1, the *S. pombe* Argonaute family member, several other protein factors as well as small RNAs homologous to centromeric repeats. This suggests a mechanism whereby an siRNA containing complex recruits silencing factors (possibly the histone methyltransferase Clr4) to specific regions of DNA (Fig. 5).

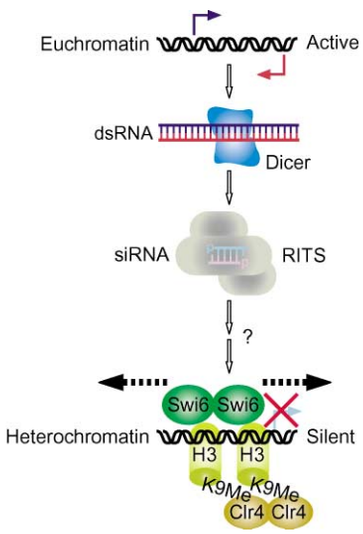

Fig. 5 RNA mediated transcriptional gene silencing (TGS) in the fission yeast *S. pombe*. dsRNA synthesized from transcriptionally active euchromatin is processed by Dicer and incorporated into a silencing complex (RITS) which targets specific DNA sequences and is required for histone methylation by Clr4, recruitment of the silencing factor Swi6, and the establishment of transcriptionally silent heterochromatin at the targeted loci.

It remains to be seen how these observations relate to other RNA-dependent silencing events in eukaryotic cells. For example, it has been shown in plants that expression of a dsRNA can lead to methylation of the homologous gene and consequent silencing of expression. In mammals, female X-inactivation involves association of the *Xist* RNA with one X chromosome an event associated with both histone and DNA methylation.[44] There is no suggestion that *Xist* is part of an RNAi-like pathway; however the mechanism of ultimate transcriptional repression could be related to the RITS-dependent pathway.

RNAi and dsRNA processing

Because dsRNA structures are found in many eukaryotic RNAs, there must be a means of separating the RNAi response and distinct cellular functions of these RNAs; in addition, there may be links between RNAi and other dsRNA processing events in the cell. One example is the adenosine to inosine "editing" of some dsRNAs in eukaryotic cells.[45] Both cellular and viral dsRNAs are substrates for members of the ADAR enzyme family; these proteins act selectively on dsRNA, converting adenosine (A) to inosine (I) through a hydrolytic deamination reaction (Fig. 6A; one form of RNA editing). While non-specific deamination may be part of a general anti-viral response, specific A to I modifications also can effect a codon change in an mRNA since I is read as G by the translational machinery. This latter mechanism has been found to be important in modifying the information content in both cellular (neural transcripts including the serotonin receptor and subunits of glutamate-gated ion channels) and viral (hepatitis δ) RNAs. Bass and coworkers have shown that deletions in each or both of the *C. elegans* ADAR genes is manifested by defects in chemotaxis—wild-type animals respond to a chemoattractant representing a food source by moving towards the attractant while mutant worms are impaired in this response.[46] Recently, these same workers showed that the chemotaxis defect could be rescued by crossing ADAR null worms with RNAi defective strains.[47] This suggests that the wild-type RNA deaminase activity might help control the entry of dsRNA into the RNAi manifold (Fig. 6B). This activity could represent a specific gene regulatory mechanism or could be a means of limiting RNAi triggered by non-specific anti-sense transcription.

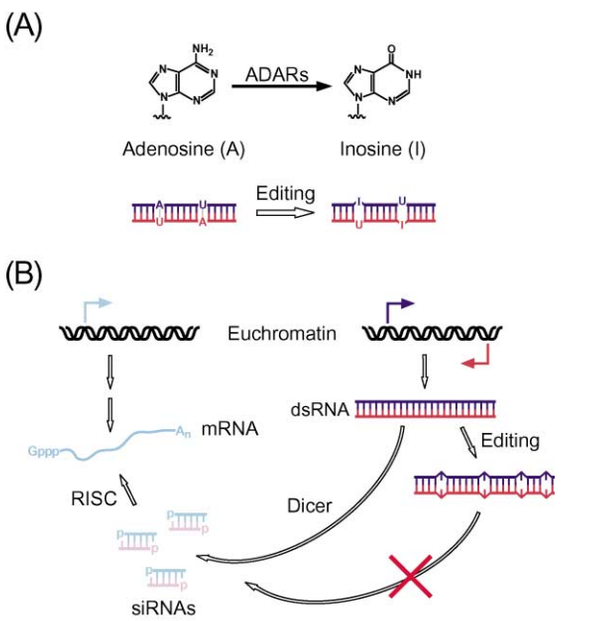

Fig. 6 Intersection of dsRNA processing pathways in the eukaryotic cell. (A) Members of the ADAR enzyme family deaminate adenosine (A) to inosine (I) resulting in the production of I-U mismatches. (B) Model for protection of mRNAs from the RNAi machinery by non-specific deamination. In the absence of ADAR, dsRNA produced from sense and anti-sense transcription or dsRNA in the untranslated region of a single mRNA is a substrate for Dicer yielding siRNAs which then silence expression via the RNAi pathway. Deamination of dsRNA disrupts the dsRNA structure resulting in inefficient processing by Dicer and repression of the RNAi pathway.

RNAi as a tool for cell biology

Attempts to regulate gene expression using anti-sense approaches have been frustrated by a variety of factors including issues of specificity as well as the poor pharmacokinetics of DNA and RNA. The fact that dsRNA elicits a very specific response through a natural pathway and is triggered by very small amounts of RNA suggested the use of RNAi as a tool for cell biology and possible therapeutic. Application of RNAi in the mammalian system was initially complicated by the complexity of the response to dsRNA in mammalian cells. The presence of dsRNA molecules of lengths exceeding 30 nucleotides triggers global suppression of gene expression through several pathways in these cells. In one, the kinase PKR is activated by dsRNA and blocks protein expression by phosphorylation of the translation initiation factor eIF2a.[48]

The discovery that RNAi involves the generation of the short duplex siRNAs suggested that short synthetic RNA duplexes

might initiate the RNAi response in mammalian cells. Since the demonstration by Tuschl and coworkers that this was indeed the case,[49] there has been a huge demand for chemically synthesized RNAs to perform gene "knock-down" experiments. One problem associated with the use of synthetic RNAs for general applications has been the capriciousness of chemical synthesis using monomers bearing 2′-TBDMS protecting groups. This has recently been circumvented by the use of alternate chemistry involving protection of the 2′ position as an orthoester resulting in highly efficient sequence independent synthesis of RNA molecules[50] suitable for use in RNAi experiments. As well, the *in vivo* siRNAs generated from transfected DNA templates effectively suppress gene expression in mammalian cells.[51] These advances in methodology are being rapidly exploited to perform knock-down studies in diverse organisms. As just one example, a systematic examination of genes within *C. elegans* is producing a comprehensive documentation of phenotypes associated with RNAi-based abrogation of individual gene function (see: http://nematoda.bio.nyu.edu/ and http://worm-srv1.mpi-cbg.de/). Combined with array-based analysis of gene expression profiles, RNAi promises to provide dramatic insights into the function of diverse genes in the eukaryotic cell.

Prospects

Initially puzzling observations in plants and worms have been explained and elaborated by the characterization of a set of inter-related gene regulatory mechanisms involving a cellular response to dsRNA. Although the basic outlines of several of these pathways have been sketched out, there is much that remains to be elucidated including the detailed molecular mechanism of RNAi and similar reactions as well as the details of interplay between the related pathways.

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References

1 T. E. Dever, *Cell*, 2002, **108**, 545–556.
2 C. A. Beelman and R. Parker, *Cell*, 1995, **81**, 179–183.
3 S. T. Crooke, *Methods Enzymol.*, 2000, **313**, 3–45.
4 A. Fire, S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver and C. C. Mello, *Nature*, 1998, **391**, 806–811.
5 H. Ngo, C. Tschudi, K. Gull and E. Ullu, *Proc. Natl. Acad. Sci. U. S. A.*, 1998, **95**, 14687–14692.
6 C. Napoli, C. Lemieux and R. Jorgensen, *Plant Cell*, 1990, **2**, 279–289.
7 N. Romano and G. Macino, *Mol. Microbiol.*, 1992, **6**, 3343–3353.
8 I. Ingelbrecht, H. Van Houdt, M. Van Montagu and A. Depicker, *Proc. Natl. Acad. Sci. U. S. A.*, 1994, **91**, 10502–10506.
9 P. D. Zamore, T. Tuschl, P. A. Sharp and D. P. Bartel, *Cell*, 2000, **101**, 25–33.
10 E. Bernstein, A. A. Caudy, S. A. Hammond and G. J. Hannon, *Nature*, 2001, **409**, 363–366.
11 J. Blaszczyk, J. E. Tropea, M. Bubunenko, K. M. Routzahn, D. S. Waugh, D. L. Court and X. Ji, *Structure*, 2001, **9**, 1225–1236.
12 E. Billy, V. Brondani, H. Zhang, U. Muller and W. Filipowicz, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 14428–14433.
13 S. M. Hammond, E. Bernstein, D. Beach and G. J. Hannon, *Nature*, 2000, **404**, 293–296.
14 J. Martinez, A. Patkaniowska, H. Urlaub, R. Luhrmann and T. Tuschl, *Cell*, 2002, **110**, 563–574.
15 S. M. Elbashir, W. Lendeckel and T. Tuschl, *Genes Dev.*, 2001, **15**, 188–200.
16 M. A. Carmell, Z. Xuan, M. Q. Zhang and G. J. Hannon, *Genes Dev.*, 2002, **16**, 2733–2742.
17 L. Cerutti, N. Mian and A. Bateman, *Trends Biochem. Sci.*, 2000, **25**, 481–482.
18 K. S. Yan, S. Yan, A. Farooq, A. Han, L. Zeng and M. M. Zhou, *Nature*, 2003, **426**, 468.
19 J. J. Song, J. Liu, N. H. Tolia, J. Schneiderman, S. K. Smith, R. A. Martienssen, G. J. Hannon and L. Joshua-Tor, *Nat. Struct. Biol.*, 2003, **10**, 1026.
20 A. A. Caudy, M. Myers, G. J. Hannon and S. M. Hammond, *Genes Dev.*, 2002, **16**, 2491–2496.
21 R. W. Carthew, *Curr. Biol.*, 2002, **12**, R852–R854.
22 A. A. Caudy, R. F. Ketting, S. M. Hammond, A. M. Denli, A. M. Bathoorn, B. B. Tops, J. M. Silva, M. M. Myers, G. J. Hannon and R. H. Plasterk, *Nature*, 2003, **425**, 411–414.
23 Q. Liu, T. A. Rand, S. Kalidas, F. Du, H. E. Kim, D. P. Smith and X. Wang, *Science*, 2003, **301**, 1921–1925.
24 M. A. Carmell and G. J. Hannon, *Nat. Struct. Mol. Biol.*, 2004, **11**, 214–218.
25 C. Cogoni and G. Macino, *Nature*, 1999, **399**, 166–169.
26 A. Smardon, J. M. Spoerke, S. C. Stacey, M. E. Klein, N. Mackin and E. M. Maine, *Curr. Biol.*, 2000, **10**, 169–178.
27 R. C. Lee, R. L. Feinbaum and V. Ambros, *Cell*, 1993, **75**, 843–854.
28 B. J. Reinhart, F. J. Slack, M. Basson, A. E. Pasquinelli, J. C. Bettinger, A. E. Rougvie, H. R. Horvitz and G. Ruvkun, *Nature*, 2000, **403**, 901–906.
29 A. Grishok, A. E. Pasquinelli, D. Conte, N. Li, S. Parrish, I. Ha, D. L. Baillie, A. Fire, G. Ruvkun and C. C. Mello, *Cell*, 2001, **106**, 23–34.
30 M. Lagos-Quintana, R. Rauhut, W. Lendeckel and T. Tuschl, *Science*, 2001, **294**, 853–858.
31 N. C. Lau, L. P. Lim, E. G. Weinstein and D. P. Bartel, *Science*, 2001, **294**, 858–862.
32 R. C. Lee and V. Ambros, *Science*, 2001, **294**, 862–864.
33 L. P. Lim, M. E. Glasner, S. Yekta, C. B. Burge and D. P. Bartel, *Science*, 2003, **299**, 1540.
34 H. B. Houbaviy, M. F. Murray and P. A. Sharp, *Dev. Cell*, 2003, **5**, 351–358.
35 Y. Lee, K. Jeon, J. T. Lee, S. Kim and V. N. Kim, *EMBO J.*, 2002, **21**, 4663–4670.
36 Y. Lee, C. Ahn, J. Han, H. Choi, J. Kim, J. Yim, J. Lee, P. Provost, O. Radmark, S. Kim and V. N. Kim, *Nature*, 2003, **425**, 415–419.
37 J. G. Doench, C. P. Petersen and P. A. Sharp, *Genes Dev.*, 2003, **17**, 438–442.
38 Y. Zeng, R. Yi and B. R. Cullen, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 9779–9784.
39 I. M. Hall, G. D. Shankaranarayana, K. Noma, N. Ayoub, A. Cohen and S. I. Grewal, *Science*, 2002, **297**, 2232–2237.
40 T. A. Volpe, C. Kidner, I. M. Hall, G. Teng, S. I. Grewal and R. A. Martienssen, *Science*, 2002, **297**, 1833–1837.
41 B. J. Reinhart and D. P. Bartel, *Science*, 2002, **297**, 1831.
42 V. Schramke and R. Allshire, *Science*, 2003, **301**, 1069–1074.
43 A. Verdel, S. Jia, S. Gerber, T. Sugiyama, S. Gygi, S. I. Grewal and D. Moazed, *Science*, 2004, **303**, 672–676.
44 P. Hajkova and M. A. Surani, *Science*, 2004, **303**, 633–634.
45 B. L. Bass, *Annu. Rev. Biochem.*, 2002, **71**, 817–846.
46 L. A. Tonkin, L. Saccomanno, D. P. Morse, T. Brodigan, M. Krause and B. L. Bass, *EMBO J.*, 2002, **21**, 6025–6035.
47 L. A. Tonkin and B. L. Bass, *Science*, 2003, **302**, 1725.
48 L. Manche, S. R. Green, C. Schmedt and M. B. Mathews, *Mol. Cell. Biol.*, 1992, **12**, 5238–5248.
49 S. M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber and T. Tuschl, *Nature*, 2001, **411**, 494–498.
50 S. A. Scaringe, F. E. Wincott and M. H. Caruthers, *J. Am. Chem. Soc.*, 1998, **120**, 11820–11821.
51 G. Sui, C. Soohoo, B. Affar, F. Gay, Y. Shi, W. C. Forrester and Y. Shi, *Proc. Natl. Acad. Sci. USA*, 2002, **99**, 5515–5520.