A new regulatory pathway for fragile X syndrome?

One out of every 4,500 males suffers cognitive impairment due to alterations in the fragile X gene. The gene has a complicated biology, and it may have just gotten a bit more complex. The fragile X gene product in flies could play a role in regulation by small RNA molecules.

R. Scott Hansen[1] & Charles D. Laird[2]

Research on human fragile X syndrome continues to elicit exciting but humbling discoveries. Once studied primarily because of its major effect on cognition—an IQ score reduction in males by an average of 50 points—and its highly unusual genetics, fragile X syndrome has informed us of many aspects of human biology. The list of phenomena is long but includes triplet-repeat expansions, epigenetic gene inactivation by DNA methylation and translation control[1]. Recent unexpected findings include observations, in some individuals, of hypertranscription rather than silencing of the fragile X gene, *FMR1* (ref. 2). Hints of possible clinical effects of hypertranscription[3] brought a sobering reminder of the complexity of the disease phenotype. As we glimpse new layers of regulation involving the fragile X gene and its products, it now seems unlikely that simple, one-dimensional molecular therapies, such as reactivating mutant *FMR1*, would lead to effective treatment strategies. A deeper understanding of the various molecular networks in human cells will likely be required to intervene effectively in this genetic disorder.

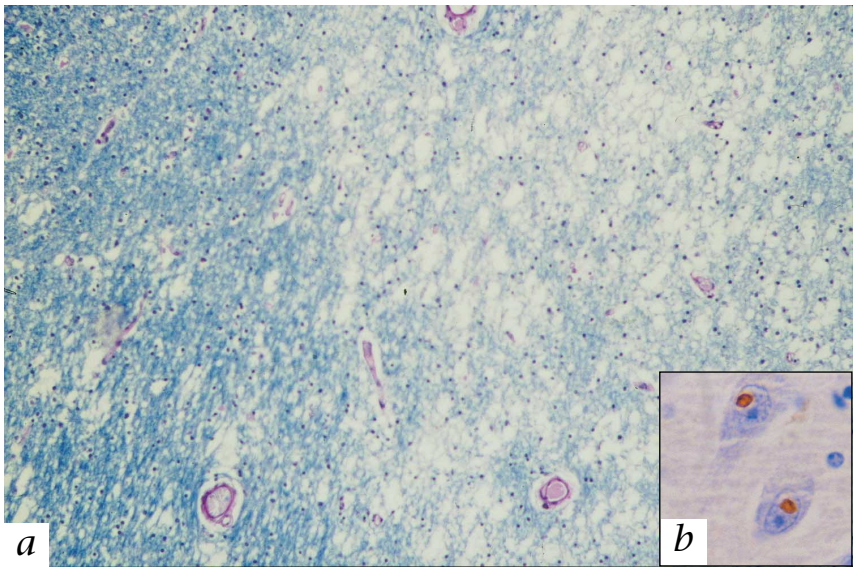

Fig. 1 Some adult males with premutation alleles of the fragile X syndrome have presented with a late-onset, neurological syndrome not evident in patients with classic fragile X syndrome. Elevated (2-4 fold) levels of FMR1 mRNA were present in these individuals[3,15]. Samples of brain tissue at autopsy revealed abnormalities not reported for classic fragile X syndrome. ***a***, Myelin stain of the cerebellum shows areas of "spongy change/vacuolation" in white matter (right) compared with areas that appear normal (left). ***b***, Staining with an anti-ubiquitin antibody reveals neuronal intranuclear inclusions (brown). Credits: *a*, courtesy of C. Greco and P. Hagerman; *b*, reprinted with permission from *Brain*.

What additional molecular network might there be for the fragile X gene and its products? The hottest topic in cell regulation this year involves small RNA molecules. In two papers appearing in *Genes and Development*, Ishizuka, *et al.*[4] and Caudy *et al.*[5] followed different paths to the same conclusion: the only *Drosophila* ortholog of *FMR1*, *dFxr*/*dFmr1*, encodes a protein (dFMR1) that behaves suspiciously as if it were involved in the micro RNA (miRNA), RNA interference (RNAi) pathways or both.

The endogenous miRNAs found in most eukaryotes seem to function in multiple pathways, including RNAi, translation control and chromatin structure. The miRNA and RNAi pathways encompass well-conserved eukaryotic systems with many shared properties[6,7]. miRNAs (~70-nucleotide (nt) hairpin precursors to 21–22-nt RNAs) are often developmentally regulated and can act by inhibiting the translation of certain target mRNAs through interaction with their 3′ proximal sequences.

In RNAi, 21–23-nt small interfering RNAs (siRNAs) are produced from longer double-stranded RNAs, and these lead to catalytic cleavage of complementary target RNAs by the siRNA-directed RNA-induced silencing complex (RISC). This is the activity that has stimulated the increasing use of RNAi as a tool for reverse genetics. Although RNAi probably arose as a defense mechanism to silence RNA viruses and immobilized transposons, these activities appear to have evolved further to include new functions. These include its use in *S. pombe* and probably higher organisms for the establishment of normal heterochromatin domains like those needed for centromere function[8]. Several recent studies suggest further that the miRNA and RNAi pathways are intimately related and quite relevant to human biology. For example, human cells have a catalytically active RNAi pathway associated with an miRNA-containing protein complex[9].

The two new *Drosophila* studies suggest that dFMR1 has a role in at least one of these extraordinary pathways of transcriptional and post-transcriptional regulation. In the Ishizuka *et al.* paper[4], this unexpected conclusion arose from a series of experiments designed to determine which proteins are associated with dFMR1 *in vivo*. Initially, a tandem affinity–tagged version of dFMR1 (dFMR1-TAP) was produced in cultured *Drosophila* S2 cells by transfection, and the proteins associated with it were identified following SDS–PAGE purification and mass-spectroscopy analysis. The various protein associations were verified in reciprocal immunoprecipitations and purifications of tandem affinity–tagged complexes.

Consistent with previous data implicating FMR1 in translation inhibition

and ribosome association[1], two *Drosophila* ribosomal proteins were identified in the complex, L5 and L11, along with 5S RNA that was already known to bind L5. The real surprise was the discovery of Argonaute2 (AGO2) and p68 RNA helicase (Dmp68) in the affinity-purified dFMR–TAP complex. Argonaut proteins are essential components of RISC and are also required in miRNA pathways[6,7]. Ishizuka *et al.*[4] show by RNAi (RNAi of RNAi) that Dmp68, an ortholog of a human double-stranded RNA helicase, is also required for efficient RNAi in S2 cells. Given the shared machinery for RNAi and miRNA, this new observation is consistent with a previous report that a related RNA helicase, GEM3, is present in a human miRNA complex with the Argonaut protein eIF2C2 (ref. 10). However, a general role for dFMR1 in RNAi is not clear from this study, as depletion by RNAi did not have a substantial effect on RNAi of a reporter gene. Perhaps FMR1 adds an additional degree of specificity to RNAi through its unique RNA binding properties.

Caudy *et al.*[5] used an entirely different approach. They were interested in isolating the endogenous RISC complex from cultured *Drosophila* S2 cells to identify the associated proteins. They developed a five-step biochemical purification that followed fractionation using RISC activity and AGO2 protein content. Among the proteins that were consistently co-purified were dFMR1 and an RNA-binding protein, VIG. Little is known about the evolutionarily conserved VIG protein, but it does appear to contain an RGG box, a motif known to bind RNA. An RGG box is also present in FMR1, where it is involved in the recognition of 'G quartet' structures present in its target mRNAs (ref. 1). Like FMR1, PAI-RBP1 (the human VIG) seems to have a role in post-transcriptional regulation in that it affects the stability of the plasminogen-activator inhibitor mRNA through interaction with the 3′ untranslated region (ref. 11).

Caudy *et al.*[5] also used RNAi to explore the possible roles of VIG and dFMR1 in the RNAi pathway, and they found partial inhibition to be associated with reduction of either protein. The authors were able to show that induction of RNAi for a reporter gene resulted in dFMR1-containing RISC complexes that were specific to that mRNA. Expression of a tagged version of dFMR1 allowed testing of a point mutation in the second KH domain known in humans to cause fragile X (ref. 1). The human mutation (I304N) inhibits ribosome association, abolishes translational inhibition activity and prevents homo-oligomerization[1,12]. Biochemical fractionation studies of S2 cells showed that the equivalent *Drosophila* mutation caused a shift of 30% of the protein from the ribosome pellet to a soluble form not associated with AGO2, thus supporting further the biological relevance of the dFMR1–RISC complex. The dFMR1-containing RISC complexes were also shown to contain known miRNAs, thus leaving open the possibility that FMR1 functions in an miRNA pathway. The authors suggest that miRNAs may guide FMR1 to its targets through RNAi complexes and that G-quartet recognition either is required for translational regulation or provides additional specificity.

In humans, males and some females with large, methylated expansions in the *FMR1* triplet CGG repeat, accompanied by delayed replication[13], develop the neurological symptoms of fragile X. Mildly affected males often show marked cell mosaicism for methylation[14]. Other individuals have what is called a 'premutation', a small, unmethylated expansion in the triplet CGG repeat. Premature ovarian failure occurs in some females with premutations even though no cognitive defects are evident. Recently, Hagerman and colleagues reported intention tremor, parkinsonism and generalized brain atrophy in several older males who carried premutation alleles[3,15] (Fig. 1). These phenotypes have not been reported for individuals with large, methylated expansions. It thus seems reasonable that abnormal concentrations of *FMR1* RNA or protein are involved in the phenotypes of some individuals with the premutation allele.

The developing complexity of the fragile X phenotype is certainly consistent with a gene involved in multiple levels of regulation: inhibition of its own expression, both at the transcriptional and translational levels, and altered translation of other mRNAs. These mRNAs include those at the neuronal synaptic cleft[16]. The new findings that *Drosophila* seems to use dFMR1 in the RNAi/miRNA pathways introduces the possibility that loss of these activities contributes to fragile X phenotypes. Potential roles of FMR1 in these pathways include RNAi- or miRNA-directed mRNA degradation, miRNA-directed translational inhibition and miRNA-directed chromatin silencing. Because FMR1 binds its own mRNA, it is also possible that the elevated *FMR1* transcripts in premutation carriers could alter these functions. If involvement in the RNAi/miRNA pathways is confirmed in human cells, it is likely that the fragile X mutation will once again help dissect a fascinating pathway newly revealed to biologists.

1. O'Donnell, W.T. & Warren, S.T. A decade of molecular studies of fragile X syndrome. *Annu. Rev. Neurosci.* **25**, 315–338 (2002).
2. Tassone, F. *et al.* Elevated levels of FMR1 mRNA in carrier males: A new mechanism of involvement in the fragile-X syndrome. *Am. J. Hum. Genet.* **66**, 6–15 (2000).
3. Greco, C.M. *et al.* Neuronal intranuclear inclusions in a new cerebellar tremor/ataxia syndrome among fragile X carriers. *Brain* **125**, 1760–1771 (2002).
4. Ishizuka, A., Siomi, M.C. & Siomi, H. A Drosophila fragile X protein interacts with components of RNAi and ribosomal proteins. *Genes Dev.* **16**, 2497–2508 (2002).
5. Caudy, A.A., Myers, M., Hannon, G.J. & Hammond, S.M. Fragile X-related protein and VIG associate with the RNA interference machinery. *Genes Dev.* **16**, 2491–2496 (2002).
6. McManus, M.T. & Sharp, P.A. Gene silencing in mammals by small interfering RNAs. *Nature Rev. Genet.* **3**, 737–47 (2002).
7. Hutvagner, G. & Zamore, P.D. RNAi: Nature abhors a double-strand. *Curr. Opin. Genet. Dev.* **12**, 225–232 (2002).
8. Allshire, R. RNAi and heterochromatin—a hushed-up affair. *Science* **297**, 1818–1819 (2002).
9. Hutvagner, G. & Zamore, P.D. A microRNA in a multiple-turnover RNAi enzyme complex. *Science* **297**, 2056–2060 (2002).
10. Mourelatos, Z. *et al.* miRNPs: A novel class of ribonucleoproteins containing numerous microRNAs. *Genes Dev.* **16**, 720–728 (2002).
11. Heaton, J.H., Dlakic, W.M., Dlakic, M. & Gelehrter, T.D. Identification and cDNA cloning of a novel RNA-binding protein that interacts with the cyclic nucleotide-responsive sequence in the Type-1 plasminogen activator inhibitor mRNA. *J. Biol. Chem.* **276**, 3341–3347 (2001).
12. Laggerbauer, B., Ostareck, D., Keidel, E.M., Ostareck-Lederer, A. & Fischer, U. Evidence that fragile X mental retardation protein is a negative regulator of translation. *Hum. Mol. Genet.* **10**, 329–338 (2001).
13. Hansen, R.S., Canfield, T.K., Lamb, M.M., Gartler, S.M. & Laird, C.D. Association of fragile X syndrome with delayed replication of the FMR1 gene. *Cell* **73**, 1403–1409 (1993).
14. Stöger, R., Kajimura, T.M., Brown, W.T. & Laird, C.D. Epigenetic variation illustrated by DNA methylation patterns of the fragile-X gene FMR1. *Hum. Mol. Genet.* **6**, 1791–1801 (1997).
15. Hagerman, R.J. *et al.* Intention tremor, parkinsonism, and generalized brain atrophy in male carriers of fragile X. *Neurology* **57**, 127–130 (2001).
16. Weiler, I.J. *et al.* Fragile X mental retardation protein is translated near synapses in response to neurotransmitter activation. *Proc. Natl. Acad. Sci. USA* **94**, 5395–5400 (1997).

[1]*Division of Medical Genetics and*
[2]*Department of Zoology*
University of Washington
Seattle, Washington, USA
Email: cdlaird@u.washington.edu