Promiscuous or discriminating: Has the favored mRNA target of Fragile X Mental Retardation Protein been overlooked?

Aoife C. McMahon\textsuperscript{a,1} and Michael Rosbash\textsuperscript{b}

Fragile X syndrome (FXS), the most common heritable cause of intellectual disability, is caused by the loss of the RNA-binding protein Fragile X Mental Retardation Protein (FMRP). FMRP is thought to bind neuronal mRNA transcripts, traffic them to synapses, and regulate their translation (1). Based on the assumption that insight into disease mechanism can be gained by understanding which targets are regulated, much effort has been put into identifying the transcripts bound by FMRP. The result of combined efforts from multiple laboratories, using multiple approaches, is a large and somewhat overlapping set of FMRP targets (2). To date, the interpretation of these disparate results is that FMRP is a promiscuous binder with a diverse set of largely synaptic function-linked target transcripts. For example, the most heavily cited set of FMRP targets in the mouse (3) comprises some 842 genes and was described by the authors as a “likely underestimate of the true number of targets.” In striking contrast to all previous work, Tabet et al. (4) contend that FMRP has one primary binding target (Fig. 1).

Like many of the previous studies, the authors performed cross-linking immunoprecipitation (CLIP) on FMRP, in their case from cultured mouse cortical neurons. To reduce false positives they compared the immunoprecipitated RNAs to those immunoprecipitated from cells lacking FMRP. Using two different antibodies, the authors find that FMRP associates predominantly with one unique mRNA, diacylglycerol kinase kappa (DgkK), a recently identified master regulator of lipid signaling. As assayed by microarray and quantitative PCR, DgkK mRNA had a CLIP efficiency three- to fourfold greater than the next-most-enriched targets. Notably, these lower-efficiency targets were inconsistent between the experimental methods of the present study and included transcripts previously identified as FMRP targets by other laboratories (Map1B, Dlg4, and APC). The authors suggest that they are due to weak interactions.

What regulatory effect does FMRP have on this newly identified target? Tabet et al. (4) provide evidence that FMRP positively regulates translation of DgkK mRNA. Ribosome loading of DgkK RNA, and DgkK protein expression, was found to be markedly reduced in Fmr1 null mouse neurons. This result challenges the currently accepted model of FMRP translational regulation, where FMRP is believed to repress the translation of target transcripts. Although perplexing, this finding was not addressed any further by the authors, opening an avenue for further investigation.

If DgkK really is the principal target of FMRP, then deficits in DgkK expression should recapitulate phenotypes of an Fmr1 deletion at the cellular and might even be expected to mimic behavioral symptoms of FXS. Deletion of DgkK indeed phenocopied Fmr1\textsuperscript{−/−} mice at the cellular and circuit levels, namely, dendritic spine alterations, decreased long-term potentiation (LTP), and increased long-term depression (LTD). Furthermore, dendritic spine deficits observed in Fmr1\textsuperscript{−/−} neurons were rescued by DgkK overexpression. On a behavioral level, knockdown of DgkK in striatal neurons gave rise to phenotypes reminiscent of FXS-type behaviors (hyperactivity, deficient social interactions, motor stereotypies, etc.).

Dysregulated Lipid Signaling Is Proposed to Be the Cause of FXS-Like Phenotypes

What, then, is the proposed role of DgkK in FXS? DgkK is one of 10 mammalian subtypes of DAG kinases, and as such it regulates the balance between two lipid messengers, diacylglycerol (DAG) and phosphatidic acid (PA). Manipulations of other DAG kinases have resulted in dendritic spine, LTP, and learning phenotypes, and indeed have been suggested as therapeutic targets in neuronal disease, but no data were previously available for DgkK (5).

Concomitant with reduced DgkK expression, Tabet et al. (4) show that in unstimulated Fmr1 null neurons, as well as in postmortem human FXS brain tissue, levels of the DgkK substrate, DAG, are elevated. Conversion of DAG to PA is stimulated in neurons by two lipid messengers, diacylglycerol (DAG) and phosphatidic acid (PA). Manipulations of other DAG kinases have resulted in dendritic spine, LTP, and learning phenotypes, and indeed have been suggested as therapeutic targets in neuronal disease, but no data were previously available for DgkK (5).

Author contributions: A.C.M. and M.R. wrote the paper.

The authors declare no conflict of interest.

See companion article on page E3619.

\textsuperscript{a}European Molecular Biology Laboratory, European Bioinformatics Institute, Wellcome Genome Campus, Hinxton, Cambridge CB10 15D, United Kingdom; and \textsuperscript{b}Howard Hughes Medical Institute, Brandeis University, Waltham, MA 02453

Author contributions: A.C.M. and M.R. wrote the paper.

The authors declare no conflict of interest.

See companion article on page E3619.

\textsuperscript{1}To whom correspondence should be addressed. Email: aoifem@ebi.ac.uk.
activation of group 1 metabotropic glutamate receptors (mGluR1), and Tabet et al. (4) show that this pathway is also defective when FMRP is absent. These new observations suggest that the regulation of DgkK expression by FMRP is the crucial link between synaptic activity and downstream signaling pathways. A large swathe of work already implicates disrupted mGluR5 signaling in FXS pathogenesis (6). The work of Tabet et al. (4) suggests a major revision of the mechanism by which this might occur.

Tabet et al. (4) posit that altered DAG/PA levels are the cause of the broad enhancement of protein translation frequently observed in FXS models (7). Indeed, this effect on translation has generally been viewed as a direct effect of FMRP, either by binding directly to specific mRNAs or to ribosomes. However, Tabet et al. (4) suggest that the major role of FMRP is to regulate DgkK and that downstream effectors of DAG/PA regulate general translation. These candidate effectors include PKCα, a DAG effector that has been shown to be strongly activated in Fmr1 null neurons (8), and mTOR, a PA effector proposed to be abnormally activated in Fmr1 null mice (9). Thus, Tabet et al. (4) propose a new model for how FMRP can be a regulator of protein expression by an indirect rather than a direct mechanism. Importantly, this is still consistent with previous studies showing that altering generalized translation in FMRP null cells can rescue FXS-like phenotypes (10), which in turn suggests that enhanced translation is a critical feature of FXS.

If DgkK Is the Primary Binding Target of FMRP, Why Was It Not Previously Identified?
The obvious facet of this work that is inconsistent with all previous work is the identification of a single primary binding target of FMRP.

Why did no other laboratories ever identify DgkK as a binding target of FMRP? The authors suggest a number of possible reasons.

Normalization. A criticism of CLIP from mixed tissue is that normalization to true input is difficult. FMRP is principally expressed in neurons, but substantial input RNA from brain tissue comes from glial cells where FMRP expression is generally very low (11, 12). A CLIP signal therefore cannot be appropriately normalized to the input, making it impossible to calculate “affinity” as a surrogate of binding strength. Consistent with these ideas, Ouwenga and Dougherty (13) showed that the Darnell et al. (3) FMRP brain target set, which was not normalized because of this neuron/glia issue, is enriched for long and highly expressed genes. A relatively pure neuronal culture allowed Tabet et al. (4) to normalize the CLIP signal to the expression levels of starting mRNA populations. Thus, they calculated a measure of CLIP efficiency, which should identify those transcripts that bind most strongly to FMRP. In addition, the parallel use of Fmr1−/− neurons presumably further reduced the number of false positives (i.e., transcripts that immunoprecipitate with the anti-FMRP antibody in the absence of FMRP).

Nonfragmented CLIP. An obvious technical difference between the experiments of Tabet et al. (4) and previous designs is that the cross-linked and immunoprecipitated RNA was not fragmented. In most CLIP protocols, only the region of RNA covalently bound by the RNA-binding protein escapes fragmentation (to allow identification of the binding footprint). The rationale for avoiding fragmentation was that prior CLIP-sequencing studies failed to identify the G-quadruplex motif, the highest-affinity binding motif...
known for FMRP (3, 14). The authors suggest that maintaining intact mRNAs limits the impact of highly structured regions, possibly FMRP binding motifs, on reverse transcription. However, they also state that DgkK does not actually contain predicted G-quadruplex motifs, so it is unclear why this would have impeded prior identification. How FMRP interacts with DgkK, and how fragmentation could affect its identification are therefore open questions.

**Annotation.** Due to its relatively recent discovery (15), the absence of DgkK probes on microarrays may have precluded its identification in early studies. The authors also suggest that problems with incomplete and poor genome annotation may have led to bioinformatic pipelines missing or filtering out the DgkK gene. Human DGKK was annotated as noncoding until the most recent release of the reference genome, GRCh38, and the mouse reference locus still has an assembly gap and is currently under review.

Additional reasons could be suggested. The Tabet et al. (4) CLIP experiments were performed on cultured neurons at an age when very few functional synapses are present (day 8 in vitro). It will be interesting to see whether these findings can be replicated at all, but especially so at different ages. It could be possible that FMRP has developmental stage-specific target preferences and therefore mechanisms. In fact, Tang et al. (16) have recently shown that Fmr1 deficiency leads to age-dependent alterations in cortical synaptic proteins. In a similar vein, brain region-specific differences in FXS phenotypes could be playing a part.

Many researchers may find these reasons unsatisfying, given the supposed binding strength of FMRP to DgkK, and there are other reasons to receive these surprising results with caution. For example, why did DgkK not appear in proteomic experiments which sought to identify dysregulated proteins (17)? Nonetheless, it remains for other laboratories to attempt to validate or discredit these new findings. Chief among them is the apparent strength of FMRP binding to DgkK as well as the suggestion that many previously identified mRNA targets interact with FMRP weakly and may even be false positives. Similarly, other laboratories now need to address the tantalizing question that Tabet et al. (4) raise: is the translation dysregulation observed in FXS directly mediated by FMRP, or is it an indirect effect mediated primarily through DgkK and disrupted lipid signaling? At a less mechanistic level, it would be satisfying to see rescue of FXS-like behavioral phenotypes by overexpression of DgkK. The authors suggest that DgkK is a promising FXS therapeutic target, a proposal that awaits such comprehensive phenotypic rescue experiments.

---