REPLY TO HOGAN:

Direct evidence of RNA–protein interactions and rewiring

Daniel Wilinski^a, Natascha Buter^b, Andrew D. Klocko^c, Christopher P. Lapointe^d, Eric U. Selker^e, Audrey P. Gasch^f, and Marvin Wickens^{g,1}

We agree with G. J. Hogan (1) that evolution of PUF–RNA interactions is an important problem, but disagree with his characterization of our report (2). First, we cite Hogan et al. (3) and Jiang et al. (4) seven and four times, respectively; credit them with proposing Puf3-regulon rewiring; describe Hogan’s work (3) as “elegant”; and credit it with proposing Puf4/5 duplication and divergence. We also explicitly state that our biochemical data support their earlier analyses.

Second, Hogan’s (1) assertion that “the majority of results presented by the authors have been previously published” is untrue. Using crosslinking and immunoprecipitation, we directly identify mRNAs bound in *Neurospora crassa* by its Puf3 (Nc_Puf3) and Puf4/5 (Nc_Puf4/5) proteins for the first time, and conduct parallel studies in *Saccharomyces cerevisiae* to enable direct comparisons with its Puf3 (Sc_Puf3) and Sc_Puf5 [from our earlier work (5)]. We also determine RNA-binding specificities of Sc_Puf3, Nc_Puf4/5, and *Aspergillus nidulans* Puf4/5 (An_Puf4/5) in three-hybrid assays. Our data (2) provide molecular information not captured by bioinformatics or prior microarray expression analysis (3), and define for the first time mRNAs truly bound by Nc_Puf3 and Nc_Puf4/5. This new information enabled our computational analysis of all PUF target mRNAs, expanding prior analyses anchored primarily on orthologs of Sc_Puf3-bound mRNAs.

Third, in our Discussion (2) we suggest an evolutionary model that best explains our findings. We were careful to present it as a proposal, befitting a Discussion. We had shown that some orthologous mRNAs bound by Sc_Puf3 are bound by Nc_Puf3, in addition to Nc_Puf4/5. Because three modern-day species utilize both Puf3 and Puf4/5 to control these mRNAs (2, 3), we favor a model in which the common ancestor

used both systems. Our three-hybrid data clarify proteins’ specificities, and strongly support a new model in which Nc_Puf4/5 and Sc_Puf5 retained ancestral broad binding specificities, whereas Sc_Puf4 and An_Puf4/5 independently evolved narrower specificities.

We propose that the ancestral PUF regulon may have coregulated cytosolic and mitochondrial ribosome-related functions. Nc_Puf4/5 targets are enriched for ribosomal and translation functions. In one place in our report (2), we indicate Nc_Puf4/5 ribosome (RP) enrichment as “cytosolic,” whereas the enrichment is for the combined group of mitochondrial and cytosolic RPs. We hereby correct this, although it is properly indicated elsewhere in both our text and supplement. Of Nc_Puf4/5-bound mRNAs annotated with “ribosomal” or “translation,” 24% are cytosolic. As shown in figure 5 of our report (2), Sc_Puf3 also binds some cytosolic-RP mRNAs (although they are not orthologous), whereas the Nc_Puf4/5 ortholog Sc_Puf4 binds mRNAs enriched specifically for cytosolic ribosome-biogenesis factors (6). From these associations, we suggest in the Discussion of our report (2) that the ancestral PUF regulon may have coregulated mitochondrial and cytosolic ribosome-related functions. Clearly, this is a hypothesis, advanced within a broader model for multistep regulatory rewiring we summarize. Both Hogan et al. (3) and Jiang et al. (4) acknowledged that multiple evolutionary models were equally parsimonious.

Our work (2) provides biochemical evidence for the previously proposed rewiring event, and sheds new light on evolution of PUF regulons and binding-site preferences. It directly establishes which RNAs bind which PUF proteins in *Neurospora*, data that must be accommodated in models of PUF evolution.

^aDepartment of Molecular, Cellular, and Developmental Biology, University of Michigan, Ann Arbor, MI 48109; ^bPromega Corp, Madison, WI 53711; ^cDepartment of Chemistry and Biochemistry, University of Colorado, Colorado Springs, CO 80918; ^dDepartment of Structural Biology, Stanford University, Stanford, CA 94305; ^eInstitute of Molecular Biology, University of Oregon, Eugene, OR 97403; ^fLaboratory of Genetics, University of Wisconsin–Madison, Madison, WI 53706; and ^gDepartment of Biochemistry, University of Wisconsin–Madison, Madison, WI 54706

Author contributions: D.W., N.B., A.D.K., A.P.G., and M.W. designed research; D.W., N.B., and A.D.K. performed research; D.W., N.B., A.D.K., C.P.L., E.U.S., and A.P.G. contributed new reagents/analytic tools; D.W., N.B., C.P.L., A.P.G., and M.W. analyzed data; and D.W., A.P.G., and M.W. wrote the paper.

The authors declare no conflict of interest.

Published under the [PNAS license](#).

¹To whom correspondence should be addressed. Email: wickens@biochem.wisc.edu.

- 1 Hogan GJ (2017) Clarifying results and prevailing models for the evolution of Puf proteins and their RNA targets. *Proc Natl Acad Sci USA* 114:E10853.
- 2 Wilinski D, et al. (2017) Recurrent rewiring and emergence of RNA regulatory networks. *Proc Natl Acad Sci USA* 114:E2816–E2825.
- 3 Hogan GJ, Brown PO, Herschlag D (2015) Evolutionary conservation and diversification of Puf RNA binding proteins and their mRNA targets. *PLoS Biol* 13:e1002307.
- 4 Jiang H, Guo X, Xu L, Gu Z (2012) Rewiring of posttranscriptional RNA regulons: Puf4p in fungi as an example. *Mol Biol Evol* 29:2169–2176.
- 5 Wilinski D, et al. (2015) RNA regulatory networks diversified through curvature of the PUF protein scaffold. *Nat Commun* 6:8213.
- 6 Gerber AP, Herschlag D, Brown PO (2004) Extensive association of functionally and cytotoxically related mRNAs with Puf family RNA-binding proteins in yeast. *PLoS Biol* 2:E79.