Review of: "The Conserved CNOT1 Interaction Motif of Tristetraprolin Regulates ARE-mRNA Decay Independently of the p38 MAPK-MK2 Kinase Pathway"

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Potential competing interests: The author(s) declared that no potential competing interests exist.

The authors are trying to analyze the effects of phosphorylation signals on the destabilization of target mRNAs by the ARE-binding protein TTP biochemically. The details are as follows.

*The authors performed an MS2-tethering pulse-chase decay assay using TTP-CIM and found that CIM itself is sufficient to induce deadenylation and degradation of the target mRNA.
* The authors verified the degree of cooperativity of TTP-CIM with other known functional motifs by using a point mutation of TTP.
* The authors showed that S316 of TTP-CIM is phosphorylated in a PKCα-dependent manner but not by MK2.
* The authors found that phosphorylation of S316 in TTP-CIM negatively regulates mRNA degradation.

These results are significant because they suggest that multiple kinase pathways coordinate to regulate mRNA degradation. However, several points should be considered regarding data analysis methods and their interpretation.

1) I suggest that the numerous phosphorylation sites (S52, S178, S316) that appear in this paper be clearly marked in Figure 1A.
2) Regarding Figure 1C, why does the target mRNA appear to undergo deadenylation even when MS2-GFP is used? Since control mRNA is not degraded, it is possible that the MS2 stem-loop or MS2 protein induces this degradation. It seems inappropriate as an experimental system to verify mRNA degradation.
3) With regards to Figure 1E, I think it is tough to quantify the degree of migration because the bands are not sharp. Referring to other papers using the same technique Chen, Chyi-Ying A et al. "Tob2 phosphorylation regulates global mRNA turnover to reshape transcriptome and impact cell proliferation." RNA (New York, N.Y.) vol. 26,9 (2020): 1143-1159. doi:10.1261/rna.073528.119 , I think it would be more appropriate to graph the distribution of signal intensity.
4) The bands of CTDΔCIM in Figure S2A and WAΔCIM in Figure S2B are saturated. I do not think that this figure can be used for quantification accurately. The quantification should be done accurately by decreasing the number of samples to be transferred and by spacing the lanes apart.

5) In Figure S3D, the bands are saturated, and the figure is inappropriate.

6) Regarding Figure 2, there is no apparent difference in mRNA degradation despite the CTD WA having a lower binding affinity to CNOT1 and CNOT9 than the CTD WT. On the other hand, the rate of mRNA degradation induced by TTP WA is somewhat slower, despite the TTP WA appearing to interact more strongly with CNOT1 than TTP WT. I think there is an insufficient discussion on this result; how do you interpret it?

7) Regarding Figure 2C, why is GAPDH detected in all samples, including the negative control? It seems that the immunoprecipitation experiment itself is not reliable.

8) Given that TTP WA binds to CNOT1 (Fig 2D) and TTP PS;WA does not bind to CNOT1 at all (Fig 3D), I think the mutation (PS) to tetraproline motifs has a significant effect on binding to CNOT1. However, I am concerned that the authors did not confirm the binding of TTP PS to CNOT1.

9) Regarding Figure 4, the 0 hr data shows that endogenous TTP is not expressed, and its expression is promoted by the immune response. Since the band intensity peaks detected by TTP and p-S316-TTP antibodies are not significantly different, this data seems to indicate "induction of TTP expression by the immune response" rather than "induction of phosphorylation by the immune response".

10) With regard to Figure 5A, UPF1 is not appropriate as a loading control.

11) The data is unreliable because many of the bands detected by the p-S178-TTP antibody are non-specific; a quality like Figure 5B would be preferable.

12) Regarding the interpretation of Figure 7B, the authors describe a significant difference even though it is not of statistical significance. What do they mean?
If you want to mention the difference, I think you need to verify it using a different system.

13) On line 275, you mention Supplemental Figure S7B, but isn't that a mistake for S6B? (There is no S7)