Response to Reviewers' comments

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The revised manuscript by Bi & Shen details the protocol for the FbioCLIP-seq method. The clarification that this should be seen as a method pairing to the Mol Cell paper clarifies many issues, and I appreciate the language corrections addressing other concerns.

Major Concerns:

One major remaining concern is the level of detail in figures -

Figure 5D - what are the p-values (and fraction observation) of these motifs?

Response #1

We have added the p-values and percentage of targets for the motifs in Figure 5D.

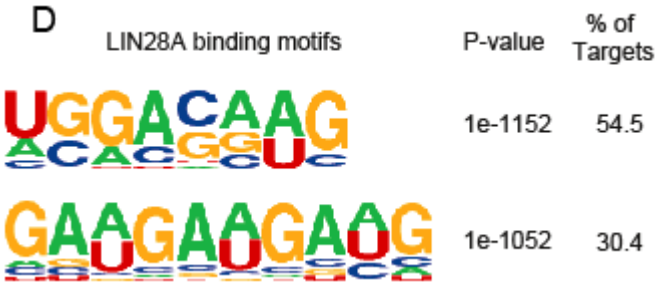
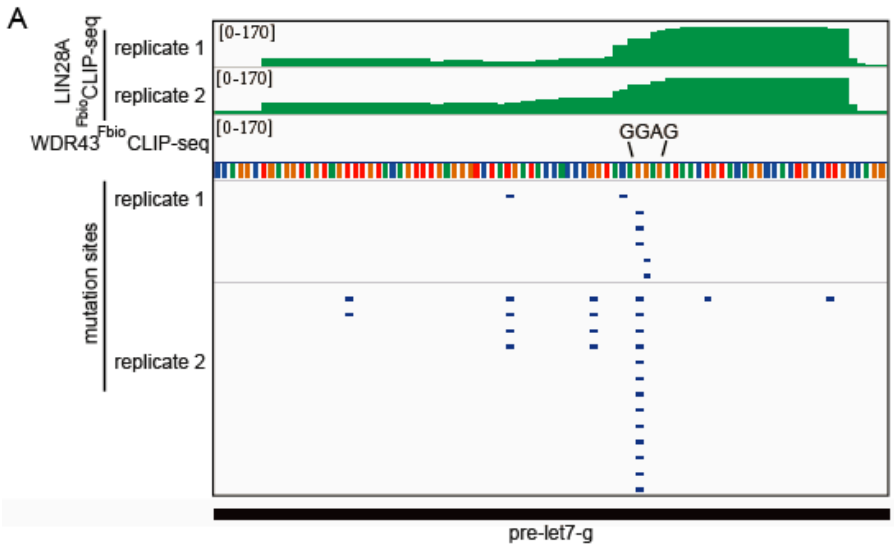


Figure 5A&E - having a non-LIN28 control here would help (I appreciate the addition of LIN28 to figure 6 in response to the prior review) - can the WDR43 data be included here as a negative control? I'm not clear that this figure shows anything without having some non-LIN28A control (whether total RNA-seq, wild-type with no tagged protein, or a different protein).

Response #2

We have added the WDR43 result as a control in the Figures.



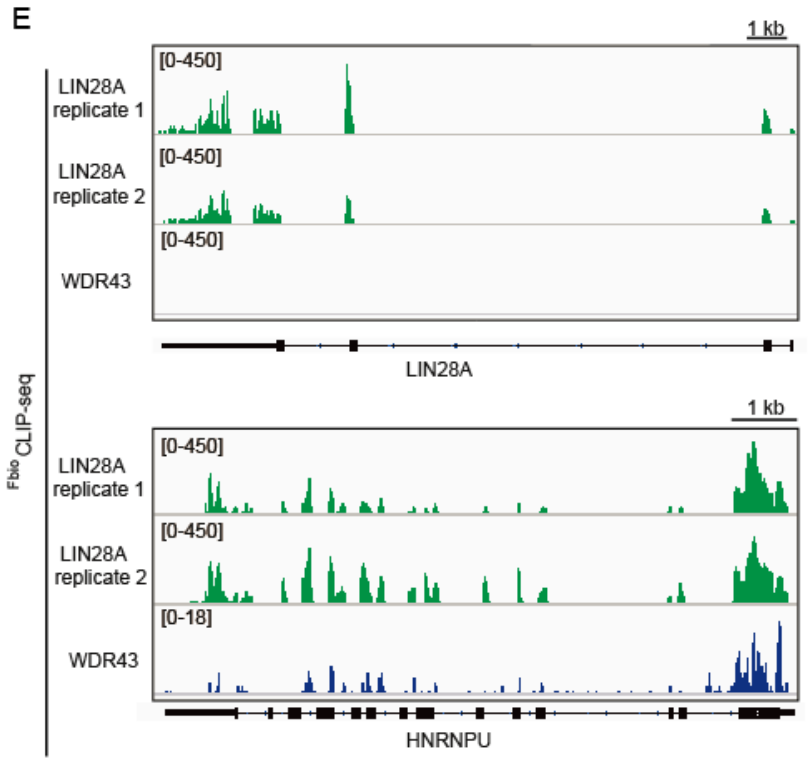


Figure 5A-B - can the 4 positions highlighted in B be added in A? As displayed I'm not clear which bases in B reflect which positions in A.

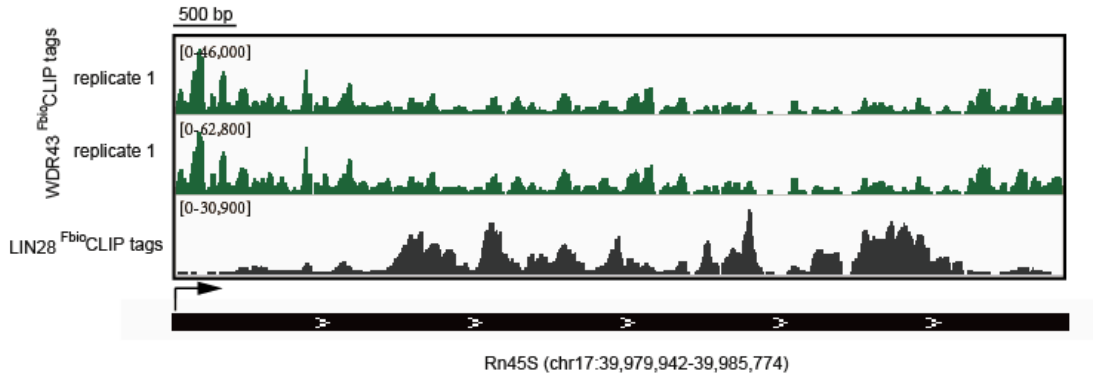
Response #3

The GGAG sequences are labeled in the revised Figure 5A. Please refer to Response #2.

Figure 6 - the positions (x-axis) being shown on the 45S rRNA should be indicated

Response #4

The positions (x-axis) have been indicated by the coordinates of the Rn45S RNA.



Another issue that should be addressed in the text regards the RNA ligations. One of the original insights in iCLIP was that reverse transcription often terminated at the site of crosslinking (and thus these fragments would be lost if the 5' adapter ligation was performed prior to reverse transcription, as was done in original HITS-CLIP methods). As the FbioCLIP method appears to use the HITS-CLIP strategy of 5' RNA linker ligation prior

to reverse transcription, the authors should mention whether they believe this will limit recovery in cases where reverse transcription termination is high (and whether they recommend particular analyses as a result of this design choice), or if their reverse transcription conditions are designed to alleviate this issue.

Response #5:

We appreciate the reviewers comment about the potential limitation of 5' adaptor ligation strategy by HITS-CLIP. We agreed with the comment.

However, we need to point out that despite transcription often terminates at the site of crosslinking, a portion of the reverse transcriptase can overcome the crosslinking "barrier". For these events that successfully cross the "barrier", the reverse transcriptase will stop at the end of RNA molecules instead of the crosslinking site. In such a situation, the iCLIP-seq analysis will treat it as a potential crosslinking site, which actually is a false positive event. For those events fails to overcome the "barrier" of crosslinked peptides, they simply can not be detected by HITS-CLIP or ^{Fbio}CLIP-seq, which is potentially a false negative. In other words, the iCLIP strategy may lead to 'false positive', while HITS-CLIP strategy may lead to 'false negative'. Logically, this limitation of HITS-CLIP strategy can be partially solved by using more input sample to get more RT events that can overcome the "barrier". Still, we agreed the concern of the reviewer and added the statement in the discussion: "Ligation of 5' adaptor to the RNA directly may limit the recovery of the signals because a significant portion of reverse transcription will be terminated by residual crosslinked peptides" (Line 440-442).

Minor Concerns:

I appreciate the response 1.5 with respect to PCR cycles and unique RNA molecules. These numbers (particularly the number of unique reads observed for LIN28A FbioCLIP) would be useful to include in the text to give context for what users of the method should expect if they follow or attempt to repeat the LIN28A experiment as described.

Response #6:

We have added the information in the figure legend.

Reviewer #2:

Manuscript Summary:

Revised manuscript.

Technical concerns were addressed.

Major Concerns:

Missing from Introduction: The strategy behind UV crossing linking, generating mutations, and criteria for a hit identification is missing and needs to be added.

Response #7:

We have added this statement in the text: "The key feature of the methodology is the induction of covalent crosslinks between RNA-binding protein and its directly bound RNA molecules (within ~ 1 Å) by UV irradiation[7]. The RBP footprints can be determined by CLIP tag clustering and peak

calling, which usually have a resolution of 30-60 nt. Alternatively, the reverse transcription step of CLIP may lead to indels (insertions or deletions) or substitutions to the crosslinking sites, which allows a single-nucleotide resolution of the protein binding sites on the RNAs. Pipelines like novoalign and CIMS have been developed for the analysis of the high-throughput sequencing results of CLIP-seq." (Line 43-49). Since the bioinformatics analysis is not the main focus of this protocol, we referred to the available pipelines and the literature for the readers.

Improve names of buffers, at present they are hard to follow
Line 157 'high salt wash buffer"…… 5x PBS or do you mean 1x? is this stringent wash buffer I?
Line 194/5 terminology 'stringent wash buffer I' …stringent wash buffer II'
Make terminology consistent. Ie label each wash buffer I II III or A B C

Response #8:

We have renamed the buffers as suggested by the reviewer to make it easier for reading. And 5x PBS indicates a 5 fold concentration of PBS (~ 750 mM salt).

Need to clean up typos, lack of prepositions.
in-efficient is one word
typo Figure 1 Highthrouput ----------high-throughput

Response #9:

We have revised the typos as suggested.