Supplementary Material

Pseudouridine and N-6 methyladenosine modifications weaken PUF protein/RNA interactions

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**A**

Figure S1

- Bound RNA
- Unbound RNA

**B**

- [Competitor RNA, nM]

- Bound RNA
- Unbound RNA

**C**

- C\textsubscript{2}C U\textsubscript{1}G\textsubscript{U}\textsubscript{A} AA U\textsubscript{A}

- Fraction RNA Bound
- [hPUM2], nM

- $K_D = 0.14 \ (0.11, 0.18) \text{ nM}$

**D**

- C\textsubscript{2}C U\textsubscript{1}G\textsubscript{U}\textsubscript{A} AA U\textsubscript{A}

- Fraction RNA Bound
- [Unlabeled RNA], nM

**E**

- UCU UGUAAUAUA UA

- Fraction RNA Bound
- [hPUM2], nM

- $K_D = 0.33 \ (0.15, 0.65) \text{ nM}$

**F**

- UCU UGUAAUAUA UA

- Fraction RNA Bound
- [Unlabeled RNA], nM

- $K_D = 0.33 \ (0.15, 0.65) \text{ nM}$

(n = 4)
A

[hPUM2, nM]

bound RNA

unbound RNA

0x m^6A

A4 m^6A

3x m^6A

Fig S2

B

[competitor RNA, nM]

bound RNA

unbound RNA

0x m^6A

A4 m^6A

3x m^6A

C

Predicted

Observed

$K_D$, nM

3x m^6A

Fig S2
Fig. S3
m6A consensus

<table>
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<tr>
<th>$T_m$ (°C)</th>
<th>62.5 ± 1.1</th>
<th>58.7 ± 1.3</th>
<th>56.4 ± 1.4</th>
<th>57.4 ± 1.3</th>
<th>49.8 ± 1.9</th>
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<td>$\Delta G_{37 \degree C}$ (kcal/mol)</td>
<td>-4.6 ± 0.9</td>
<td>-3.2 ± 0.7</td>
<td>-2.5 ± 0.5</td>
<td>-3.0 ± 0.6</td>
<td>-1.3 ± 0.3</td>
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<tr>
<td>$\Delta \Delta G_{37 \degree C}$ (kcal/mol)</td>
<td>0</td>
<td>1.4 ± 1.1</td>
<td>2.1 ± 1.0</td>
<td>1.6 ± 1.1</td>
<td>3.3 ± 0.9</td>
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Fig. S4
Supplementary Figure Legends

**Fig S1.** Multiple Ψ modifications weaken hPUM2 binding affinity additively. *(A and B)* Representative gel shift assay images from direct binding *(A)* and competition experiments *(B)* of hPUM2 with RNA oligos containing 0, 1 and 3 Ψ modifications (see Methods). For quantification, each lane was divided into two unequal top and bottom portions where the bottom portion corresponded to the migration of free RNA and all material above that band were included in the bound RNA fraction. *(C and D)* Binding isotherms *(C)* and inhibition curves *(D)* measuring hPUM2 affinities to RNA oligos containing 0 and 2 Ψ modifications. Representative curves from two independent replicates shown. *(E and F)* hPUM2 binds with high affinity to the in vitro consensus sequence. hPUM2 affinities to RU13 RNA (sequence shown above each plot in which the consensus 8mer is underlined) were measured directly (binding isotherms, *E*) or via competition (inhibition curves, *F*). Mean and 95% confidence intervals derived from bootstrapping the set of individual $K_D$ values are shown.

**Fig S2.** *(A and B)* Representative gel shift assay images from direct binding *(A)* and competition experiments *(B)* of hPUM2 with RNA oligos containing 0, 1 and 3 m6A modifications (see Methods). For quantification, each lane was divided into two unequal top and bottom portions where the bottom portion corresponded to the migration of free RNA and all material above that band were included in the bound RNA fraction. *(C)* Bar graph comparing predicted (grey) and observed (black) mean $K_D$ values derived from competition assays to the RNA oligo containing 3 m6A modifications. Error bars represent standard deviations. $K_D$ *pred* was calculated for a model assuming independent substituent effects on binding as in Figure 2.

**Fig S3.** Dissociation rate constants *(k_{off})* of hPUM2 to Ψ (top and middle panels; CCUGUAAUAU) and m6A (bottom panels; CCUGUAUAAAAU) containing RNA oligos were measured once each at 25 °C *(A)* and 0 °C *(B)*. *(C)* Bar graph comparing relative $k_{off}$ values measured at 25 °C (grey) or at 0 °C (black)
for Ψ (top) and m₆A (bottom panel) containing RNA oligos. For oligos containing two or more modifications, dissociation rates could not be accurately measured at 25 °C and only limits could be obtained, denoted by upward arrows. For 3x m₆A RNA, no binding could be detected directly so limits could not be obtained. (D) Scatter plot of relative $k_{off}$ values (0 °C) and relative $K_D$ (competition) for Ψ (blue) and m₆A (red) containing RNA. The slopes and Pearson correlation coefficients of the best fit lines to the data are indicated along with line of slope 1 (grey).

**Fig. S4** m₆A destabilizes RNA secondary structure. Nupack web server (Zadeh et al 2011) was used to predict the secondary structure of an unmodified 33mer RNA (UUUCCAAGCACAGUUCGCUUGUAUAAUCUCUU; m₆A positions underlined and hPUM2 consensus sequence in bold). The positions of the m₆A bases in the modified RNA oligos are indicated by red stars while the m₆A consensus sequence (AGACA) and the hPUM2 binding site are highlighted in light yellow and blue shading, respectively. UV melting temperatures ($T_M$) were determined using LIFFT (https://github.com/DasLab/LIFFT) and converted to $\Delta G$ values using standard formulae.
Supplementary Note S1:

We identified in vivo hPUM2 targets containing RNA modifications using RMBase, release version 1.2.0 ([http://mirlab.sysu.edu.cn/rmbase/](http://mirlab.sysu.edu.cn/rmbase/)). RMBase is an online database that cross-references RNA modification sites identified from sequencing datasets against RBP target sites identified via CLIP-Seq experiments (Sun et al 2015). Below is a list of examples of putative hPUM2 target sites (in bold) whose binding may be affected by the Ψ or m6A RNA modification contained within them (underlined and in red). These sites were chosen based on the following two criteria:

   a) presence of a recognizable 8mer binding motif (beginning with a ‘UGUA’ sequence)
   b) presence of a modification site within this 8mer sequence

The sites shown below include a number of non-canonical binding sequences. Human Pumilios have been shown to recognize and bind non-canonical sequences (Cheong and Hall, 2006; Lu and Hall, 2011; Wang et al, 2002; Gupta et al, 2008). In addition, we also have evidence of hPUM2 binding the 8mer sequences shown below (unpublished results).

**m6A modifications:**

ID: m6A_site_2540
Gene name: RPS6KA1
Sequence: GAAAGCGAUUCACUGUAUAAACUUUUUUGAAUUGAAAAAAU


ID: m6A_site_2965
Gene name: SRSF4
Sequence: GUAGAACUGUCACUGCUGUACAUUUAAACUCCCCUAUUG
ID: m6A_site_8897  
Gene name: CHTOP  
Sequence: AGUCAACACAUCUGUAAAUACCUCUGAGAUCAACAGAUGAGA

ID: m6A_site_10274  
Gene name: UCK2  
Sequence: GGCAUGCUCAUCUGUAUACUGUUUCUCCUAGACAAUACU

ID: m6A_site_10661  
Gene name: GAS5  
Sequence: GACCUGAAUGAGCAGUAUAGGUAAACUGAAGAC

ID: m6A_site_21942  
Gene name: QSER1  
Sequence: CACAAAACCAGAAUACUGUACAUUAGUAAAGAGGUCUGG
Ψ modifications: The Ψ site in TAF5 shown below does not fit the PUS7 or PUS4 recognition motif and is likely modified by other guide RNA independent pseudouridine synthases such as PUS1 or by snoRNA-guided Dyskerin (Carlile et al 2014, Schwartz et al 2014). We did not find any other examples of a hPUM2 binding site containing a Ψ within it in the RMBase database, though the number of
identified Psi sites is likely an underestimate (Carlile et al 2014; Schwartz et al 2014; Lovejoy et al
2014) and there may be instances of modified bases in hPUM2 binding sites that have not yet been
identified.

ID: PseudoU_site_711
Gene name: TAF5
Sequence: CUUUGUUGCAUUUUUGUACAGUUUUUAUUUUUGAUAUCUU

Using RMBase, we also identified examples of putative PUF binding sites (bold) in yeast mRNA that
appear to be pseudouridinylated outside the Pus7p consensus motif (UGUAR).
ID: PseudoU_site_86
Gene name: CSG2
Sequence: CUAUCUGACCUUUAUGUAUUUGCUUCGUGGCUCUUGG

ID: PseudoU_site_1720
Gene name: EGT2
Sequence: AUUCUGUUCUGAAUCUGUAUUAGCUCUCCAUACCUGGCAA

ID: PseudoU_site_1676
Gene name: ERG2

Sequence: AGGCUGGAUCCAUGUAUGUUGCCAUUCGGGUGUUUGGACA

http://mirlab.sysu.edu.cn/rmbase/detailResultTable.php?db=sacCer3_mseqbase&table=sacCer3_all_PseudoU_site&modID=PseudoU_site_1676