**Miranda et al., 2018 – synthetic Protein**

* **engineered synthetic PPR proteins**, tested their **RNA-binding specificity**

**Fig S1 . Designer Proteins Bind their Target RNA similarily effective**

(A) SDS-PAGE of designer proteins.

* SCD14 (14 repeats), MCD14 (modified sequence), SCD11A (missing 2 C-terminal motifs), SCD11B (missing 2 and shuffled in order) all purified

(B) EMSA - RNA-binding specificity of the SCD11A, SCD11B, SCD14, and MCD14

* Each protein is tested with designed target RNA
* Kd ~20nM for each – bind target similarity

(C) Gel mobility shift at different time points

* **Kd larger at 30min than 4hrs? why? – Was not at equilibrium yet**

**Figure S1: Core Questions & Answers**

**Why was this experiment done?**  
To test the **purity and RNA-binding specificity** of synthetic PPR proteins.

**What hypothesis or research question does this address?**  
Do **SCD11A, SCD11B, SCD14, and MCD14 bind their target RNA with high specificity?**

**How was it done?**  
**SDS-PAGE** checked protein purity, and **EMSA** measured RNA binding.

**What experimental setup, controls, and conditions were used?**

* **Target vs. control RNA** tested for each protein.
* **Kd measured at 30 min (26 nM) and 4 hours (9 nM)**.

**What can we conclude?**

* **All proteins bind specifically**.
* **Binding strengthens over time**.
* **Extra repeats don’t increase affinity much**.

**Fig 1 . Designer Proteins Bind their Target RNA similar affinity**

(A) Design of the Protein – Single Consensus (SCD)

* Uses **PPR10 consensus modules**
* 11A and 14 share the first 11 modules
* 11B shuffles some

(B) Design of the Multi-consensus design (MCD)

* **MCD14 is identical to SCD14** but has **modified PPR motifs** based on natural PPR–RNA correlations.
* **underlined amino acids** differ to fine-tune **binding specificity**.

(C) Gel mobility shift of intended targets

* **Kd smallest in SCD11A**
* **Kd largest in SCD11B**
* **Similar overall according to authors**

(D) RNA sequences for the Bind-n-Seq

* 5’, 3’, mid and randomly all mutated RNA

**Figure 1: Core Questions & Answers**

**Why was this experiment done?**  
To test the **design, binding specificity, and affinity** of synthetic PPR proteins.

**What hypothesis or research question does this address?**  
Do **SCD11A, SCD14, and MCD14** bind RNA as predicted, and how do **motif modifications affect binding**?

**How was it done?**  
• **SCD and MCD proteins** were designed based on the **PPR code**.  
• **EMSA measured binding affinity (Kd)** and confirmed specificity.  
• **Bind-n-Seq design of sequence preferences** using **randomized RNAs**.

**What experimental setup, controls, and conditions were used?**  
• **SCD vs. MCD proteins** tested for **differences in binding affinity**.  
• **Target vs. control RNA** assessed specificity.  
• **Kd measured over time**

**What can we conclude?**  
• **All proteins bind RNA with high specificity**.  
• **SCD11A binds more tightly than SCD14**, despite fewer repeats.  
• **Modifying PPR motifs (MCD14) alters specificity without major loss of affinity**.

**Fig 2/3 – Bind-n-Seq of randomized RNA**

**Fig 2 SCD14 – 7kmers**

(A) Enrichment of differently randomized RNAs

* Most enriched in 3’ randomized RNAs
* Equimolar amounts to start with

(B) Conservation or motifs in 5’ end

* Positional importance of U (2), GA (6,7)

**Fig3 Bind-n-Seq SCD14, SCD11A, SCD11B - 9kmers**

* Similarly U, and GA are concerved

**Figures 2 & 3: Core Questions & Answers**

**Why was this experiment done?**  
To identify **RNA sequences enriched by SCD14 (Fig 2) and SCD11A/B/SCD14 (Fig 3) in Bind-n-Seq**.

**What hypothesis or research question does this address?**  
Do synthetic PPR proteins **bind RNA as predicted**, and how do **mismatches affect binding**?

**How was it done?**

* **Fig 2:** SCD14 tested with **partially randomized RNAs**, 7-mer enrichment analyzed.
* **Fig 3:** SCD11A, SCD11B, and SCD14 tested with **fully randomized RNAs**, 9-mer enrichment calculated.

**What experimental setup, controls, and conditions were used?**

* **SCD14 (Fig 2) used three RNA pools with 5′, middle, or 3′ randomization.**
* **SCD11A, SCD11B, and SCD14 (Fig 3) used fully randomised RNA libraries.**
* **Enriched sequences (>5 SD for Fig 2, >10 SD for Fig 3) were analysed with MUSCLE.**

**What can we conclude?**

* **SCD14 follows the PPR code but shows variation at the 3′ end (Fig 2).**
* **SCD11A/B are more selective, while SCD14 tolerates more mismatches (Fig 3).**

**What do the results tell us about RNA-RBP interactions?**  
PPR binding is **modular and sequence-specific**, but **binding strength varies across different positions**.

**Figure S3: Bind-n-Seq MCD14**

**Similar to Fig2/3 just for MCD14**

(A) 7-kmer enrichment MCD14 using Bind-n-Seq

* Enriched sequences in 3’ randomized

(B) **Sequence logo**

* follows PPR code with some more flexibility

(C) **Mismatch tolerance** analysis

* higher protein concentrations restores binding (less specific/strong)
* MCD14 tolerates transversions (across purine<-> purimidine) differently across positions (mainly in 3’ end)
* similar to SCD14 (Fig 4A).

**Figure S3: Core Questions & Answers**

**Why was this experiment done?**  
To test **MCD14’s sequence specificity and mismatch tolerance** using Bind-n-Seq.

**What hypothesis or research question does this address?**  
Does **MCD14 follow the PPR code**, and which positions are **most tolerant to transversions**?

**How was it done?**

* **Bind-n-Seq** analysed **7-mer enrichment** from **partially randomised RNA pools**.
* **tested mismatch tolerance** at different **MCD14 concentrations**.

**What experimental setup, controls, and conditions were used?**

* **MCD14 tested at 200 nM**.
* **5′, middle, and 3′ randomised RNA pools**.
* **Transversion mismatches assessed at different positions**.

**What can we conclude?**

* **MCD14 follows the PPR code** but has **flexibility at the 3′ end**.
* **5′ positions are highly sensitive**, while **positions 13 and 14 tolerate transversions best**.
* **Mismatch effects vary across the binding site**, confirming **position-dependent binding rules**.

**What do the results tell us about RNA-RBP interactions?**  
PPR proteins bind RNA **modularly**, but **some positions allow flexibility**, affecting **binding strength and specificity**.

**Figure S4 EMSA of best binding one compared to natural (nt)**

(A) SCD14

* EMSA tested how single **mutations in the SCD14 target sequence** affect binding.
* Some mutations, like **14G**, were tested on non-contiguous gel lanes (cut together).
  + What does that mean for the result? Hard to make out with your eye

(B) SCD11A

* EMSA showed **central A-to-U transversions** cause a **stronger binding loss** than those at the **3′-end**.
* Results confirm that **binding affinity is highly position-dependent**​

**Figure S4: Core Questions & Answers**

**Why was this experiment done?**  
To test how **mutations in SCD14 and SCD11A sites** affect binding.

**What hypothesis or research question does this address?**  
Do **specific mutations** weaken PPR binding, and does **position matter**?

**How was it done?**

* **EMSA tested mutant RNAs** with **SCD14 and SCD11A**.
* **Kd values measured binding loss**.

**What experimental setup, controls, and conditions were used?**

* **Protein at 0–128 nM**.
* **Binding shifts and Kd values quantified**.

**What can we conclude?**

* **Central A-to-U mutations disrupt binding more** than 3′-end changes.
* **Gels show subtle shifts, but Kd values confirm affinity loss**.

**What do the results tell us about RNA-RBP interactions?**  
**Binding loss depends on mutation position**, confirming **sequence-specific PPR recognition**.

**Fig. 5/S6 Effect of binding site truncation**

(A) Truncated 5’ end or 3’end using Bind-n-Seq SCD14, SCD11A, SCD11B

* Truncating the 5’ end has severe consequences
* 3’ end seems to increase binding

(B) EMSA confirmation for SCD14

* For -2nt 5’ increased Kd (weaker binding)
* -2nt 3’ smaller Kd (stronger binding)

Fig S6:

(A) Same as 5A but using different concentrations

* Shows same effect or 3’ and 5’ truncations as 5A

(B) EMSA confirmation

* Right panel the free RNA bands are stronger (less bound when 5’ end is missing)

**Figures 5 & S6: Core Questions & Answers**

**Why was this experiment done?**  
To test how **5′ and 3′ binding site truncations** affect **PPR protein binding affinity**.

**What hypothesis or research question does this address?**  
Does **PPR binding require a full-length site**, or can **truncated sequences still support binding**?

**How was it done?**

* **Bind-n-Seq** measured **enrichment of truncated sequences** at **different protein concentrations**.
* **EMSA tested SCD14 (Fig 5B) and MCD14 (Fig S6B)** binding to truncated RNAs.

**What experimental setup, controls, and conditions were used?**

* **Full vs. truncated binding sites** compared.
* **Protein concentrations: 0–128 nM**.
* **SCD14 (Fig 5) and MCD14 (Fig S6) tested separately**.

**What can we conclude?**

* **5′ truncations weaken binding more than 3′ truncations**.
* **MCD14 and SCD14 follow the same trend**, confirming a **5′-anchored binding mechanism**.

**What do the results tell us about RNA-RBP interactions?**  
PPR proteins **bind RNA from the 5′ end first**, with **more flexibility at the 3′ end**.