**McDermott et al., 2019**

* **Engineered PPR proteins** were tested for **in vivo RNA binding** in *Arabidopsis*.

**Fig 2/S2**

**Fig 2: Testing RNA binding in vivo using SCD14 and SCD11**

(A) **Immunoprecipitation** of SCD14 and SCD11

* Western blot confirmed SCD14 and SCD11 pull down from *Arabidopsis* stromal extracts using an anti-FLAG antibody.
* Enriched in pellet versus supernatant (1/2S) with RbcL as a loading control.

(B) **RNA Slot-Blot Hybridisation tagging RNA with probe**

* RNA from immunoprecipitated samples was analysed for psbA RNA enrichment.
* psbA 5′ UTR was enriched in the pellet fraction, confirming SCD binding specificity.

(C) **RIP-Seq Coverage Across the Chloroplast Genome – specificity check**

* RNA coimmunoprecipitated with SCD11 and SCD14 was mapped to the *Arabidopsis* plastome.
* SCD14 specifically enriches psbA, SCD11 also enriches 4.5S rRNA

(D) **RNA Enrichment Across Chloroplast Genes – protein coding only**

* SCD11 and SCD14 show high enrichment

Fig S2: Testing RNA Binding of SCD14 and SCD11 repeats

(A) **Replicate RIP-seq for SCD11 and SCD14**

* Shows the same as 2C

(B) **Replicate RIP-seq for HCF173, CP33C, and SRRP1**

**Fig S2A: Replicate experiments**

**(A) Replicate RIP-seq for SCD11 and SCD14**

* Sequence coverage confirms reproducibility of RNA enrichment across the chloroplast genome.
* Bar graphs show RNA enrichment ratios, validating specific binding of SCD11 and SCD14 compared to controls.

**(B) Replicate RIP-seq for HCF173, CP33C, and SRRP1**

* RIP-seq was repeated using antibodies from different rabbits to confirm binding specificity.
* Data are consistent with Figure 5, supporting the reliability of RNA enrichment patterns.

### **Figure 2 & S2A: Core Questions & Answers**

**Why was this experiment done?**  
To confirm **SCD14 and SCD11 RNA binding** using **RIP-seq**.

**What hypothesis or research question does this address?**  
Do **SCD14 and SCD11 selectively bind chloroplast RNAs** in vivo?

**How was it done?**

* **Immunoprecipitation (IP) with anti-FLAG antibodies.**
* **RIP-seq to map RNA enrichment across the plastome.**

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

* **WT Col-0 as a control.**
* **Negative control IP with a non-binding antibody.**

**What can we conclude?**

* **SCD14 and SCD11 bind specific chloroplast RNAs.**
* **Enrichment patterns are reproducible across replicates.**

**What do the results tell us about RNA-RBP interactions?**  
PPR proteins show **strong, sequence-specific RNA binding** in vivo.

**Fig.3/FigS3 Off-Target study**

**(A) Off-Target RNA Enrichment by SCD14 and SCD11**

* **Certain chloroplast transcripts were enriched more than threefold** in both SCD14 and SCD11 immunoprecipitates.
* **Off-target sites contain partial matches** to the intended binding site, including **transition mismatches**.

**(B) Local Enrichment Profiles for Off-Target Binding**

* **rpl32 and ccsA showed strong enrichment peaks**, aligning with **partial SCD14 binding site matches**.
* **Peak nucleotides marked in red** indicate the position of **highest binding enrichment**.

**(C) Sequence Logo of Off-Target Sites**

* **Off-target sequences share similarities** with motif
* **Logo analysis highlights conserved nucleotides**, suggesting **some tolerance for mismatches**.

**Fig S3: Off Target RNA binding sites:**

**RNA co-immunoprecipitation (RIP-seq) results** for **SCD14 and SCD11**, identifying **off-target RNA binding sites** in the Arabidopsis chloroplast genome.

* **SCD14 binds off-target sites in ndhG, ndhA, psbM, psbH, petL, and ndhK.**
* **SCD11 binds off-target sites in rps15, matK, and ndhF.**
* **Enrichment peaks indicate strong RNA-protein interactions sites**
* **specific sequence motifs found at these off-target binding sites.**

### **Figure 3 & S3: Core Q&A**

**Why was this experiment done?**  
To identify **off-target RNA binding** by SCD14 and SCD11.

**What hypothesis does this address?**  
Do these proteins bind **unintended RNAs**, and why?

**How was it done?**

* **RIP-seq** mapped RNA **binding peaks**.
* **Negative control IP** confirmed specificity.

**What can we conclude?**

* **Some off-target sites match the intended motif**.
* **Mismatch tolerance allows unintended binding**.

**What do the results tell us?**  
PPR proteins **bind selectively but tolerate some sequence variation l;eading to off target binding – This may be undesirable**.

### **Fig. 5/FigS2B/Fig5: RIP-seq mapped RNA - Maize Orthologous RBP that bind psbA**

**Fig.5 Maize Orthologous RBP that bind psbA**

**(A) RNA Coverage Across the Chloroplast Genome**

* **RNA binding sites** for **Zm-HCF173, Zm-CP33C, and Zm-SRRP1**  mapped to the maize chloroplast genome.
* **peaks indicate strong protein-RNA interactions** at psbA.

**(B) RNA Enrichment Ratios**

* **IP vs. control (AtpB antibody) for each gene in the chloroplast -> compared RNA binding specificity.**
* **Mostly very specific. HCF173 has a lot of low baseline binding.**

**(S2B) RNA Enrichment Ratios replicate experiments**

* Replicate data in **Supplemental Figure 2B** shows the same

**Fig.S5 Maize Orthologous RBP antibody validation**

**(A) Antibody Validation for Zm-CP33C, Zm-SRRP1, and Zm-HCF173**

* **Mutant maize lines confirmed specificity** of antibodies against **Zm-CP33C and Zm-SRRP1.**
* **HCF173 antibody was generated** against a specific **protein fragment (aa 364-633).**
* **Ponceau staining confirmed equal protein loading** across samples.

**(B) Immunoprecipitation Validation for RIP-seq**

* **Western blots confirmed IP** for Zm-HCF173, Zm-CP33C, and Zm-SRRP1.

**Figures 5, S2B, and S5: Core Questions & Answers**

**Why was this experiment done?**  
To expand Arabidopsis principle of **RNA targets to Zm-HCF173, Zm-CP33C, and Zm-SRRP1** in maize chloroplasts. Maize is an established model.

**What hypothesis does this address?**  
The function of proteins identified in Arabidopsis can be applied as a generic tool in other plant species.

**How was it done?**

* **RIP-seq mapped binding sites** (Fig 5).
* **Replicate experiments confirmed reproducibility** (Fig S2B).
* **Antibody and IP validation ensured accuracy** (Fig S5).

**What can we conclude?**

* **These proteins bind distinct chloroplast RNAs**.
* **RIP-seq is reproducible and specific**.
* **Validated antibodies confirm successful protein capture**.

**What do the results tell us?**  
These RNA-binding proteins **regulate chloroplast gene expression** with **clear target specificity**.

### **Figure 6: High-Res Rip-Seq for Pinpointing interaction sites**

**(A) RNA Binding Sites Across the Chloroplast Genome**

* **RIP-seq mapped RNA coverage** for **SRRP1**, **HCF173 and CP33C** at high resolution.
* **Achieved by partially digesting RNA (mostly that is not bound by protein)**
* **Binding sites show strong peaks**, indicating **specific interactions with psbA mRNA** **for HCF173 and CP33C NOT SRRP1**

**(B) RNase I Treatment to Identify Protected RNA Regions**

* **RNase-resistant peaks indicate direct protein-RNA interactions**.
* **HCF173 and CP33C protect specific psbA mRNA regions**, confirming **binding specificity**.

**(C) HCF173 Footprint on psbA 5′ UTR**

* **HCF173 binds a conserved region in the psbA 5′ UTR**, stabilising its structure.
* **Predicted secondary structure** suggests **a conserved RNA folding pattern** across species.

### **Figure 6: Core Questions & Answers**

**Why was this experiment done?**  
To determine **precise binding sites** of **SRRP1, HCF173 and CP33C** on **psbA mRNA**

**What hypothesis does this address?**  
Do **HCF173, CP33C, and SRRP1 directly bind psbA**, and **which regions are protected from RNase degradation**?

**How was it done?**

* **RIP-seq before and after RNase I digestion** to detect **protein-protected RNA sites**.
* **Multiple sequence alignment** to assess **conserved RNA structures**.

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

* **RNase-treated vs. untreated samples** to identify **protected RNA footprints**.
* **Control antibody used** to confirm specificity.

**What can we conclude?**

* **HCF173 & CP33C protect distinct psbA RNA regions**, confirming **direct binding**.
* **SRRP1 does not show psbA enrichment after RNase treatment**, suggesting **weak, transient, or indirect binding**.

**What do the results tell us?**  
Chloroplast **RNA-protein interactions vary in strength and function**, with **some proteins (HCF173, CP33C) strongly stabilising RNA**, while others (SRRP1) may interact transiently or indirectly. **SRRP1 likely plays a different role in chloroplast RNA regulation than HCF173 and CP33C**.