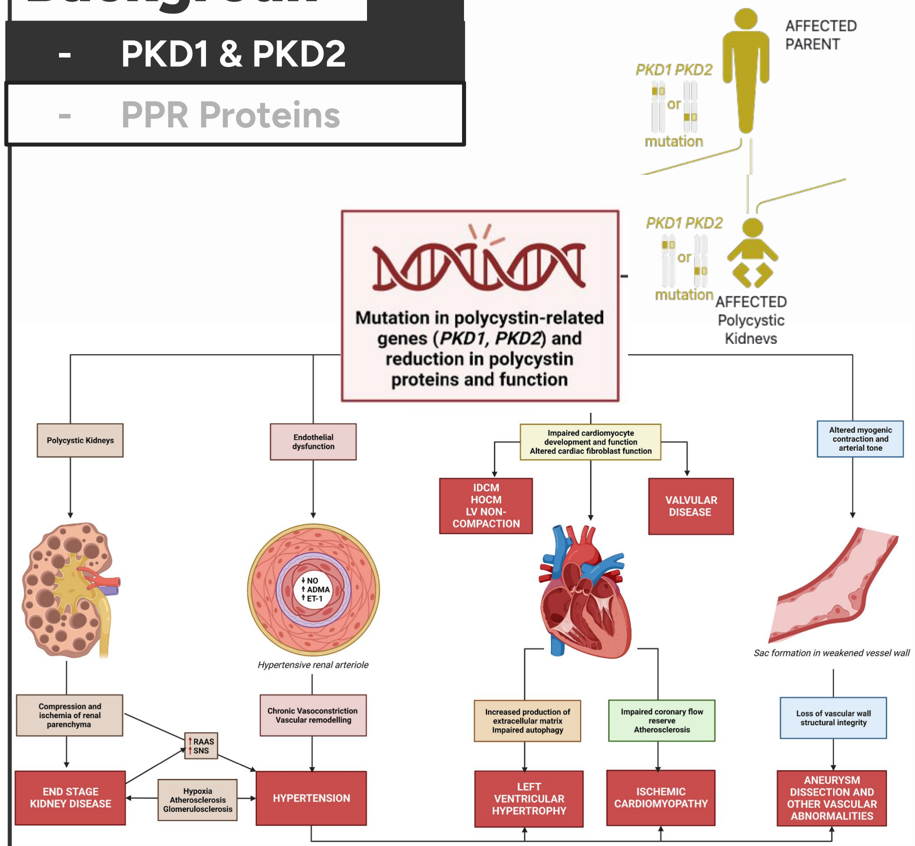
九州大学
KYUSHU UNIVERSITY

Targeted Upregulation of *PKD1/PKD2* with Programmable PPR Proteins

Carl Andersen Tan | Laboratory of Genome Chemistry Engineering

Background 01

- PKD1 & PKD2
- PPR Proteins



PKD1 and PKD2 Mutation Leads to Autosomal Dominant Polycystic Kidney Disease (ADPKD)

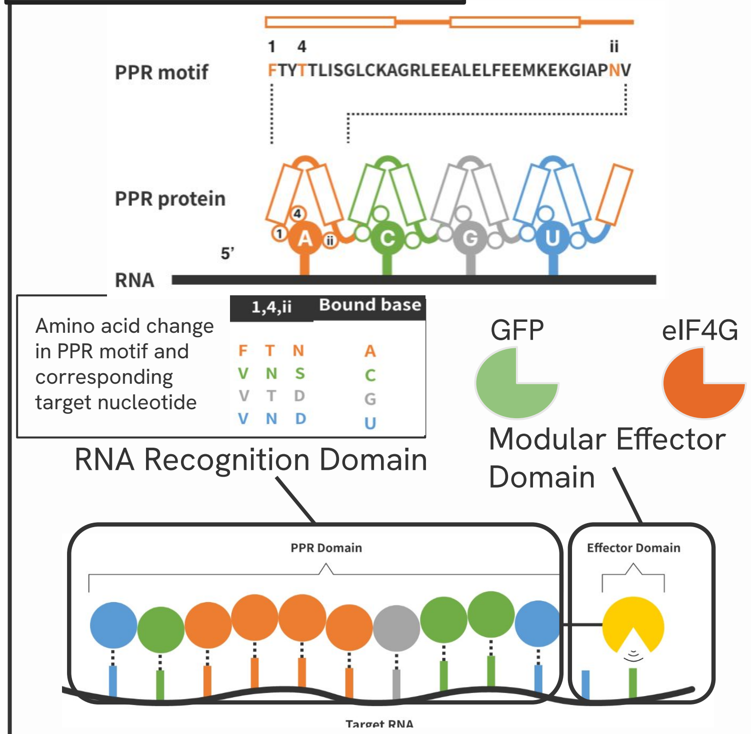
- One of the most common **genetic disorders** globally.
- loss-of-function mutations in either ***PKD1*** **or *PKD2*** cause majority of ADPKD
- PKD1 and PKD2 protein loss/ downregulation leads to ADPKD
- **Haploinsufficiency**– Autosomal Dominant PKD: Disease is the dominant phenotype
- Current treatment slows symptoms rather than targeting root genetic causes

[1] Sagar, P. S., & Rangan, G. K. (2023). Cardiovascular Manifestations and Management in Autosomal Dominant Polycystic Kidney Disease. *Kidney International Reports*.

Background 01

d- PKD1 & PKD2

- PPR Proteins



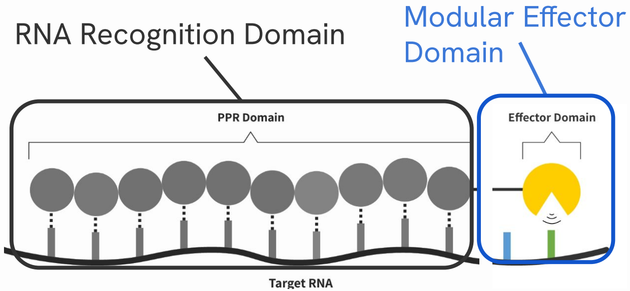
RNA Manipulation Using PPR Proteins

- Pentatricopeptide repeat (**PPR**) proteins are sequence-specific RNA binding proteins.
- PPR proteins are composed of smaller PPR motifs with each motif recognizing a specific nucleotide.
- PPR proteins can be designed to **target specific RNA sequences** with high RNA binding affinity and accuracy
- Effector domains can determine PPR functionality (e.g. GFP=visualisation)

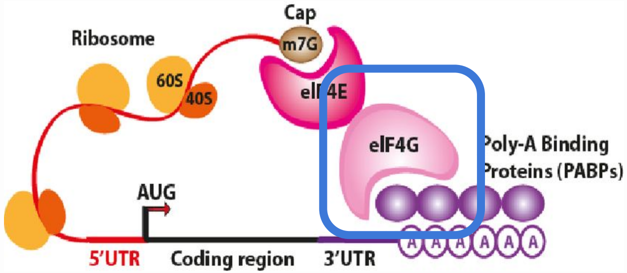
[2]. Yagi, Y., Hayashi, S., Kobayashi, K., Hirayama, T., & Nakamura, T. (2013). Elucidation of the RNA Recognition Code for Pentatricopeptide Repeat Proteins Involved in Organelle RNA Editing in Plants. *PLoS ONE*, *8*(3). https://doi.org/10.1371/journal.pone.0057286

Backgroun d 01

- PKD1 & PKD2
- PPR Proteins



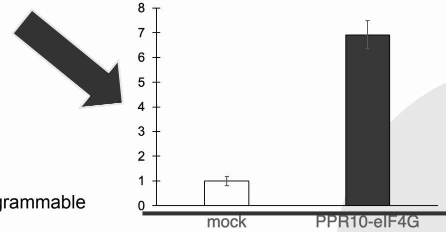
Cap-dependent translational initiation



Upregulation of PKD1/PKD2 by Translational Enhancement Effector PPR-eIF4G

- **eIF4G** (eukaryotic translation initiation factor 4G) is a central scaffolding protein in the translation initiation complex.
- **Improves translation efficiency** of mRNA
- PPR proteins fused to eIF4G can **enhance translation** of endogenous mRNAs.
- Previous research: transfection of p53 targeting-eIF4G-fused PPR constructs led to a ~**7-fold** increase in p53 expression (Ping et al., 2024)

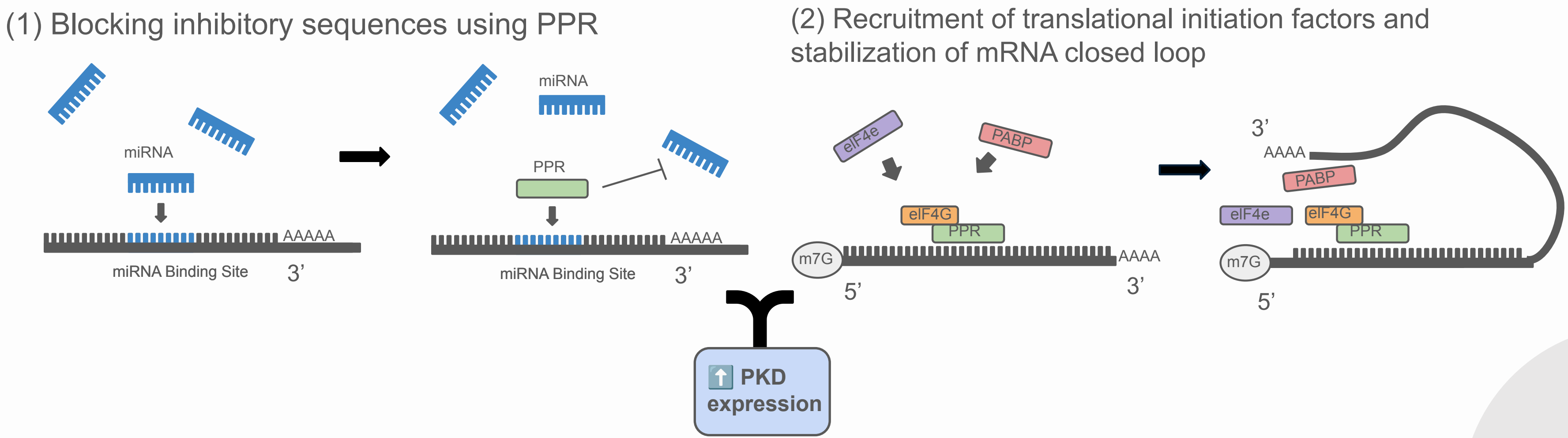
eIF4G is a translational enhancement effector



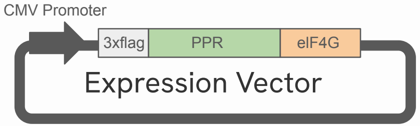
[3] Ping, N., Hara-Kuge, S., Yagi, Y., Kazama, T., & Nakamura, T. (2024). Translational enhancement of target endogenous mRNA in mammalian cells using programmable RNA-binding pentatricopeptide repeat proteins. *Scientific Reports*, *14*(1). https://doi.org/10.1038/s41598-023-50776-zc

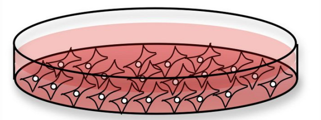
02 Experimental Design:

Demonstrate that treatment of designer PPR-eIF4G proteins can effectively enhance the expression of disease-related genes *PKD1* and *PKD2* in mammalian cells



03 Experimental Flow



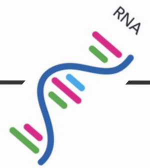


01 Design of PPR Constructs

- **22 (PKD1 targeting) and 16 (PKD2 targeting)** 12-motif PPR proteins was constructed.
- Some positioned on inhibitory sites (**miRNA binding sites/uORFs**)

02 Cell Transfection

- Transfection into **HEK293T** cells and harvested 48 hrs post-transfection
- **3 Biological Replicates**

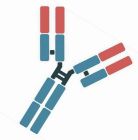


03 qPCR

- PKD1 & PKD2 **mRNA** quantified with qPCR and compared to **mock** (empty vector) transfected controls
- β-Actin as housekeeping gene

04 Western Blot

- PKD1/PKD2 **protein** change was observed Band intensity compared to β-Actin



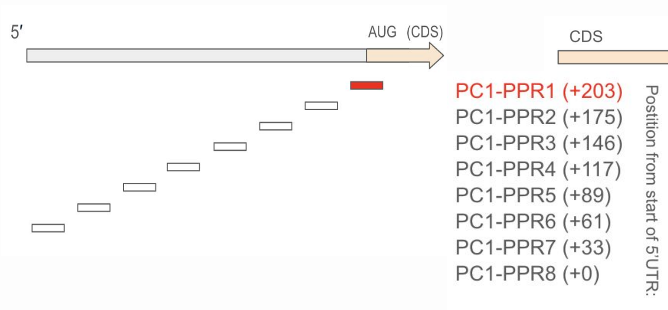
05 ELISA

- **Protein Quantification** of PKD1 and PKD2
- Bicinchoninic acid (BCA) assay used to standardize total protein levels

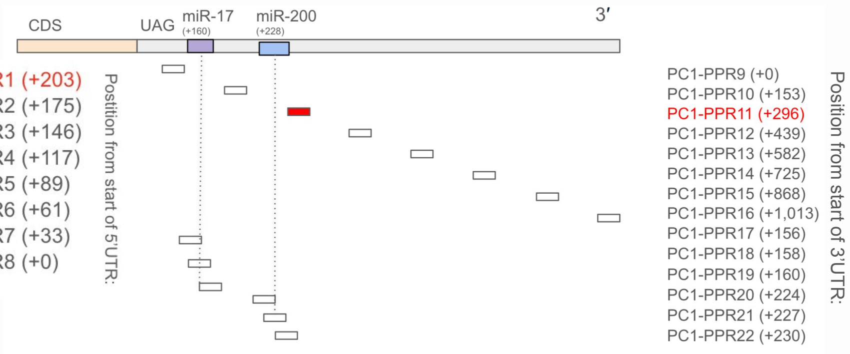
Results 04

- **PKD1**
- PKD2

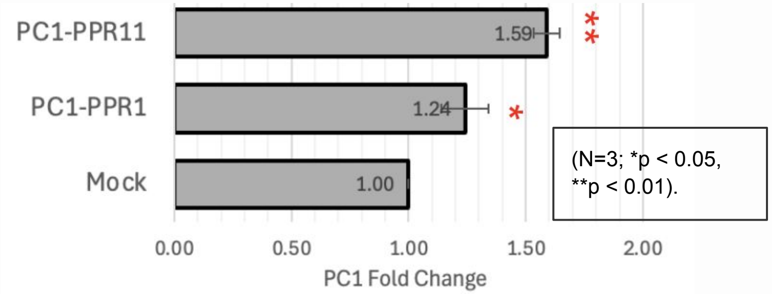
PKD1 mRNA 5′ UTR (214 nt)



PKD1 mRNA 3′ UTR (1,019 nt)



ELISA of PKD1 Protein (PC1) Expression



Upregulation of *PKD1* Protein (Polycystin-1, PC1) fold change compared to mock (empty vector) transfected control

Marked increase in PKD1 Protein Expression (~1.6 fold; $p<0.01$)

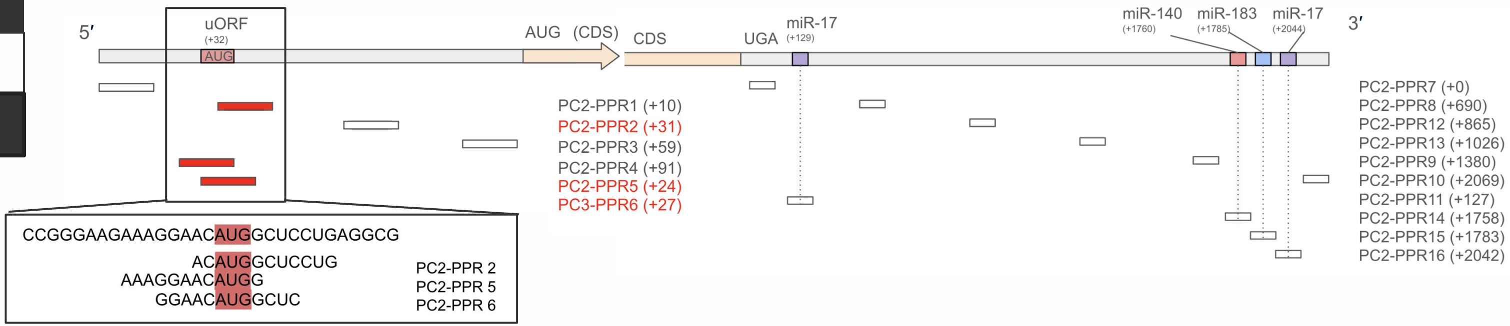
- 3′UTR and 5′UTR targeting constructs significantly upregulated PKD1 expression

Demonstrates eIF4G-mediated translational enhancement independent of inhibitory element blockade

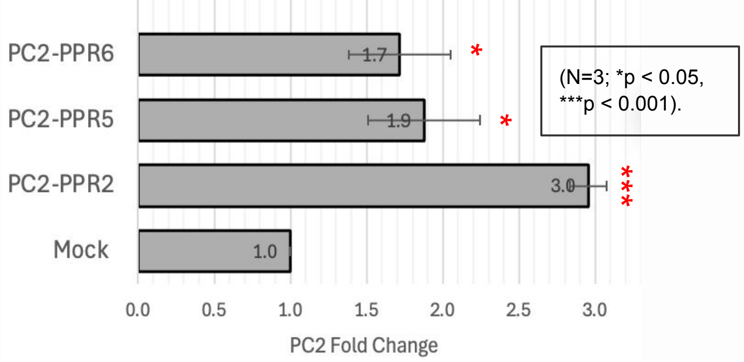
Results 04

- PKD1
- **PKD2**

PKD2 mRNA 5′ UTR (99 nt) / PKD2 mRNA 3′ UTR (2084 nt)



ELISA of PKD2 Protein (PC2) Expression



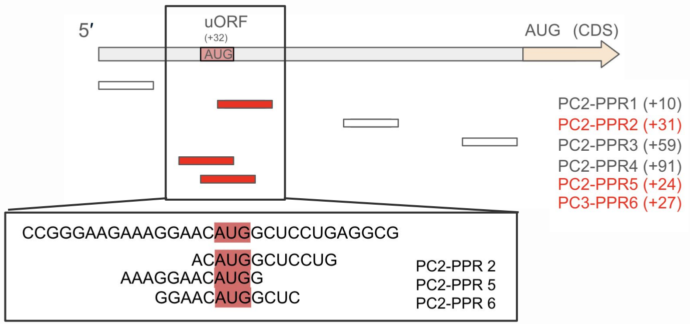
Upregulation of *PKD2* Protein (Polycystin-2, PC2) fold change compared to mock (empty vector) transfected control

Marked increase in PKD2 Protein Expression (~3 fold; $p<0.001$)

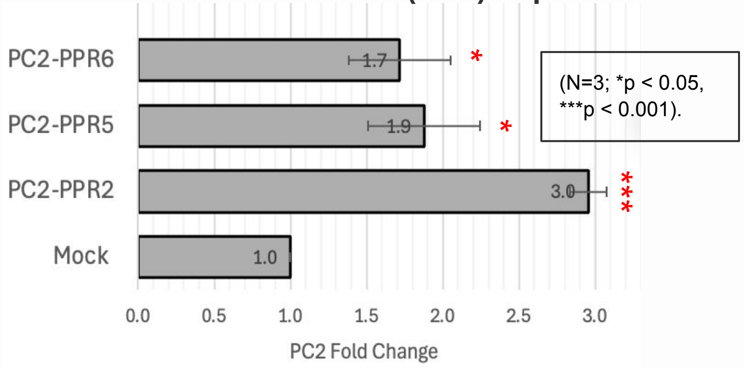
- **uORF blocking** PPR significantly upregulated PKD2 protein expression.
- No change in mRNA level was observed

First successful application of designer PPR proteins to achieve upregulation of PKD1 (1.6-fold) and PKD2 (3-fold) protein expression.

PKD2 mRNA 5′ UTR (99 nt)



ELISA of PKD2 Protein (PC2) Expression



Upregulation of *PKD2* Protein (Polycystin-2, PC2) fold change compared to mock (empty vector) transfected control

Marked increase in PKD2 Protein Expression (~3 fold; $p<0.001$)

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First successful application of designer PPR proteins to achieve upregulation of PKD1 (1.6-fold) and PKD2 (3-fold) protein expression.

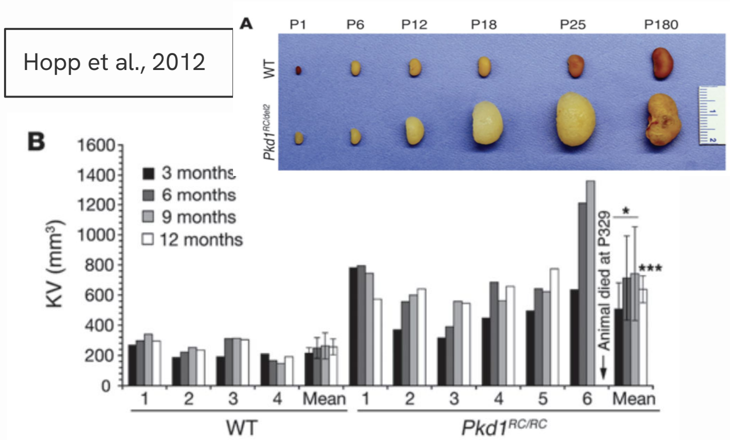
05 Significance

- <u>2-fold increases</u> should suffice to rescue kidney function due to nature of **haploinsufficiency**
- Hopp et al. (2012): **60% difference** in endogenous PKD1 substantially influences ADPKD markers: <u>cyst formation</u>, kidney function and kidney size.
- Li et al. (2015): **2-fold increase of PKD2** led to <u>2-fold increase in lifespan</u>, and restored kidney function in PKD2 hemizygous ADPKD mouse models.

This study's findings: 1.6-fold PKD1 and 3-fold PKD2 protein increase are therapeutically relevant

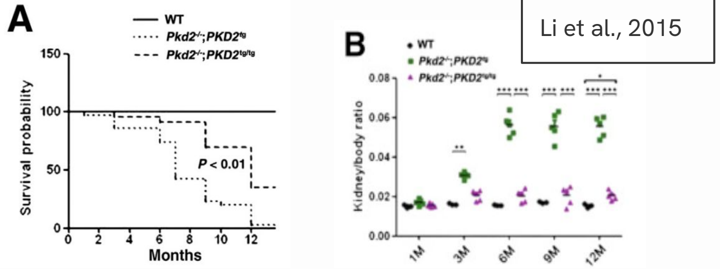
<u>WT (100%) vs. PKD1 hemizygous (40% PKD1)</u>

Hopp et al., 2012



<u>WT vs. PKD2 hemizygous (~50% PKD2)</u>

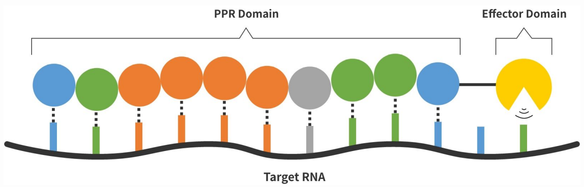
Li et al., 2015



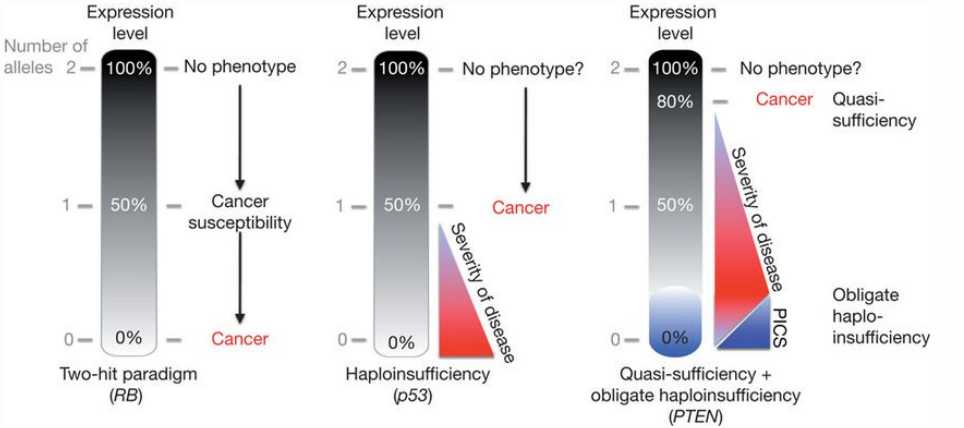
PPR proteins present a viable therapeutic option for ADPKD

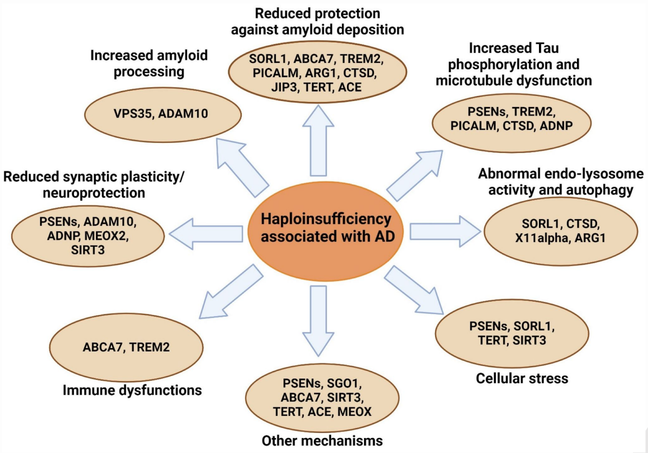
[4] Hopp, K., Ward, C. J., Hommerding, C. J., Nasr, S. H., Tuan, H. F., Gainullin, V. G., Rossetti, S., Torres, V. E., & Harris, P. C. (2012). Functional polycystin-1 dosage governs autosomal dominant polycystic kidney disease severity. Journal of Clinical Investigation, 122(11), 4257–4273. https://doi.org/10.1172/JCI64313
[5]Li, A., Tian, X., Zhang, X., Huang, S., Ma, Y., Wu, D., Moeckel, G., Somlo, S., & Wu, G. (2015). Human Polycystin-2 Transgene Dose-Dependently Rescues ADPKD Phenotypes in Pkd2 Mutant Mice. American Journal of Pathology, 185(10), 2843–2860. https://doi.org/10.1016/j.ajpath.2015.06.014

05 Conclusion



This study highlights the potential of PPR proteins as a novel therapy for haploinsufficiency related disorders such as ADPKD.





Targeted Upregulation of *PKD1/PKD2* with Programmable PPR Proteins: A Versatile Approach for Addressing Haploinsufficiency

Q&A

Carl Andersen Tan | Laboratory of Genome Chemistry Engineering

Supplementary

*Use for Q&A

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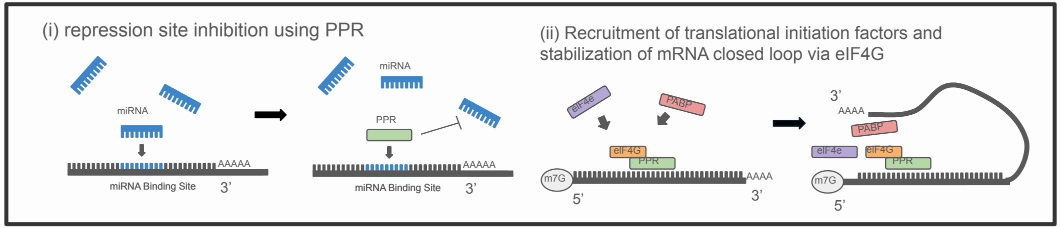
06 Future Research



Determine Individual Influence of Blocking Strategies

All Experiments were conducted with PPR-eIF4G fusion proteins yet blocking only requires PPR. Examining PPR blocking can reveal whether results were due to **either (i) repressional site inhibition or (ii) translational upregulation by effector**

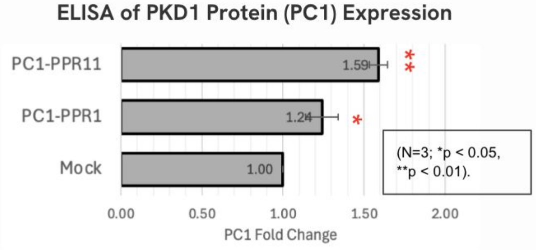
Optimization of PPR Construction and Effector

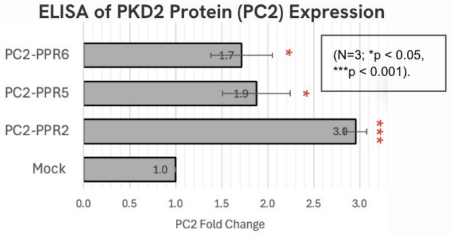
Testing of alternative translational effector domains may identify more potent candidates for gene upregulation (e.g. YTHDF)

Screen for Off-Targets

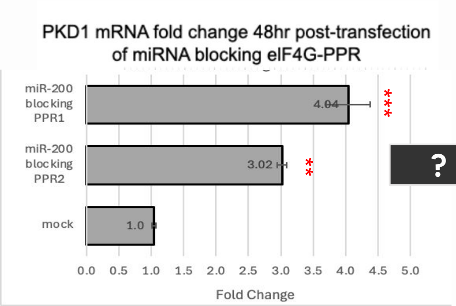
12-motif (12nt) PPR was used. RNA Immunoprecipitation assay (RIP) can be used to check of off-target PPR binding.

06 Future Research: Discrepancy Between mRNA & Protein Levels

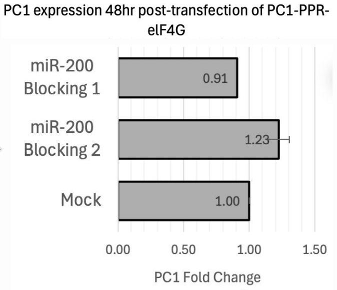




- In two targets, significant increases in PKD1 mRNA was observed but no parallel increase in protein levels?



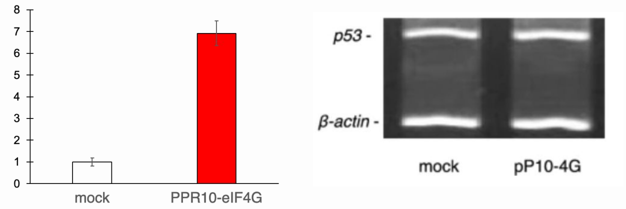
?



(Ping et al., 2024)

Protein Change (ELISA)

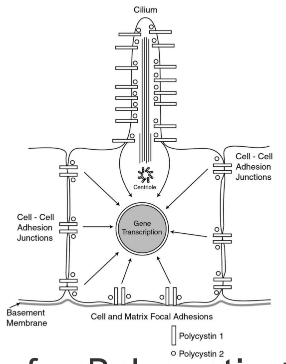
mRNA level (RT-PCR)

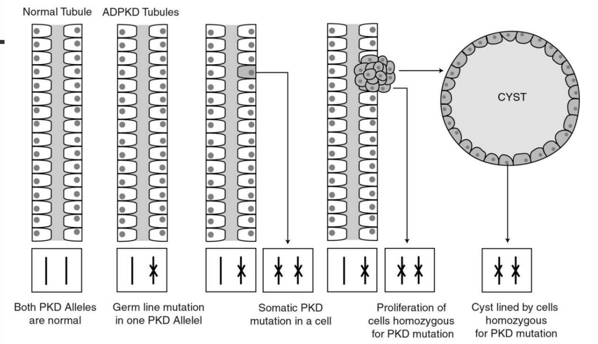


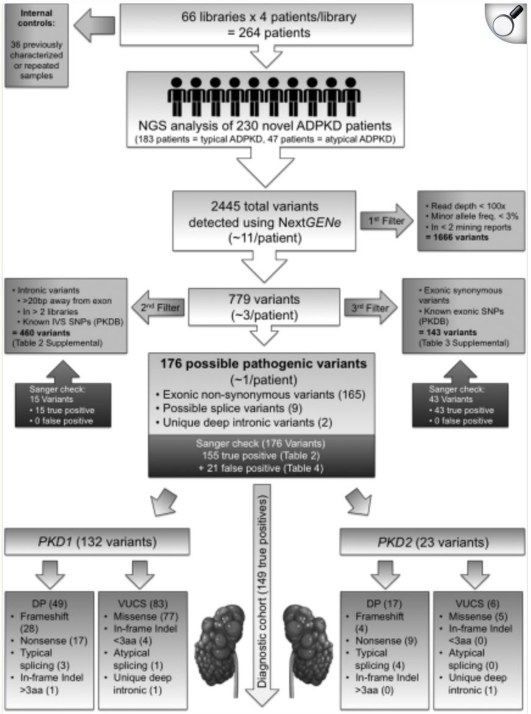
Elucidating this phenomenon can advance understanding of miRNA-RNA and PPR-RNA interactions

? Hypothesis: miR-200 may shift targeting to downstream translational regulators that indirectly modulate PKD1 protein synthesis

More About ADPKD





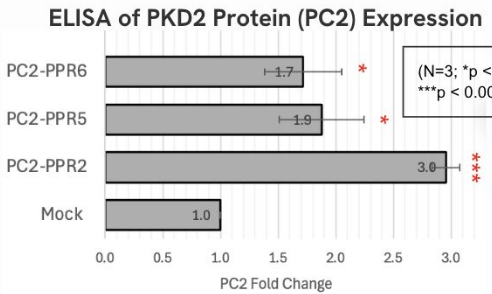


- PKD1 (coding for Polycystin-1, PC1) and PKD2 (coding for Polycystin-2, PC2) are membrane proteins involved in Ca^{2+} signalling in tubular epithelial cells.
- These signals regulate normal proliferation levels.
- Mutations are typically **frameshift or nonsense mutations** in one PKD allele (Rosetti et al., 2012)
- When levels of functional polycystin fall below a threshold, it causes aberrant proliferation, fluid absorption, and ultimately cyst formation
- ADPKD is caused by the decreased expression of functional PKD1 and PKD2, not accumulation of nonfunctional PC1/PC2

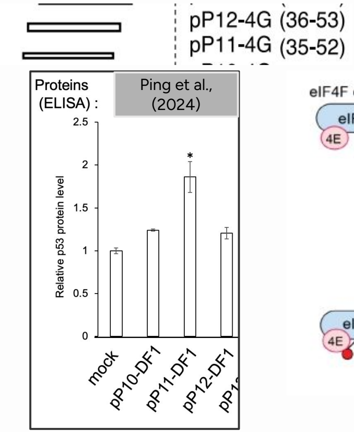
Rossetti, S., Hopp, K., Sikkink, R. A., Sundsbak, J. L., Lee, Y. K., Kubly, V., Eckloff, B. W., Ward, C. J., Winearls, C. G., Torres, V. E., & Harris, P. C. (2012). Identification of gene mutations in autosoma polycystic kidney disease through targeted resequencing. *Journal of the American Society of Nephrology : JASN*, *23*(5), 915–933. https://doi.org/10.1681/ASN.2011101032

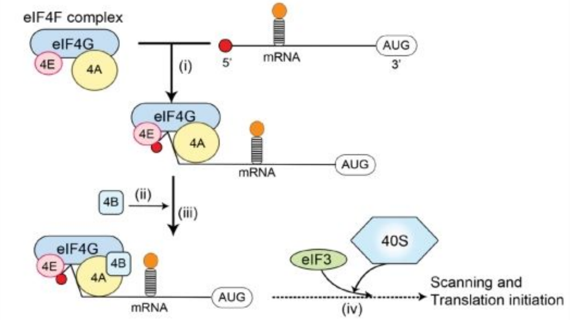
Target Selection

In this study: Translational enhancement was highly dependent on the specific target position .



PKD2 mRNA 5′ UTR (99 nt)





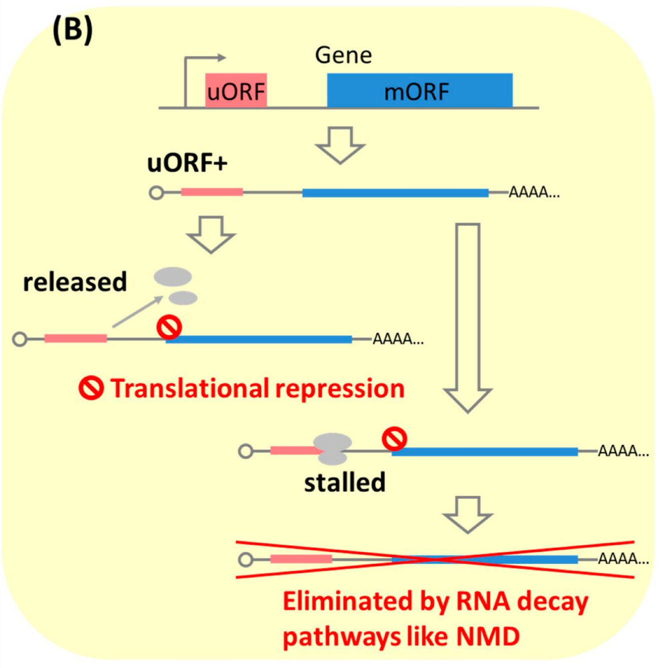
- Small nucleotide shifts (~1nt shift) cause great effects (Ping et al. 2024)
- Enhancement did not correlate with proximity to 5' end or start codon, nor with known internal ribosome entry sites (IRES)
- The presence of known secondary structures did not correlate with translational enhancement.

?

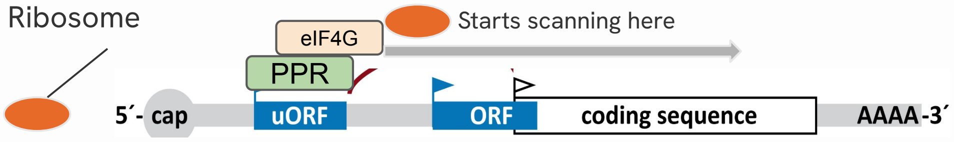
Hypothesis: only certain mRNA positions allow eIF4G to stabilize the translation pre-initiation complex. Moving the PPR-eIF4G by 10–20 nt could disrupt its ability to "reach" critical factors like eIF3 or ribosomes loading site. –<u>Position of PPR may affect translational initiation complex assembly</u>

Mechanism of uORF Blocking

Normal uORF gene repression

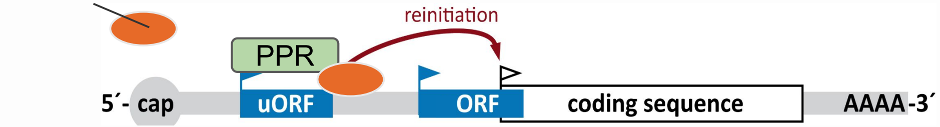


(1) PPR-eIF4G alters ribosome scanning from 5′cap to PPR target



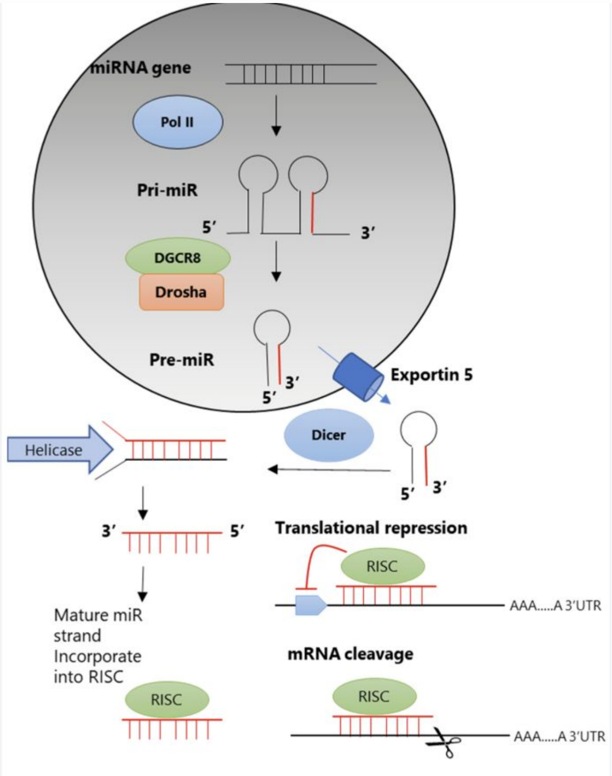
(1) eIF4G interacts with eIF3, which is bound to the 40S ribosomal subunit. This bridges the mRNA to the ribosome. Because PPR-eIF4G proteins were used, scanning could start from PPR target

(2) PPR-mediated "reinitiation"



(2) Ribosome normally scans from 5′cap but due to presence of PPR, it does not go through the uORF and instead directly initiates translation on main ORF

Mechanism of miRNA Blocking



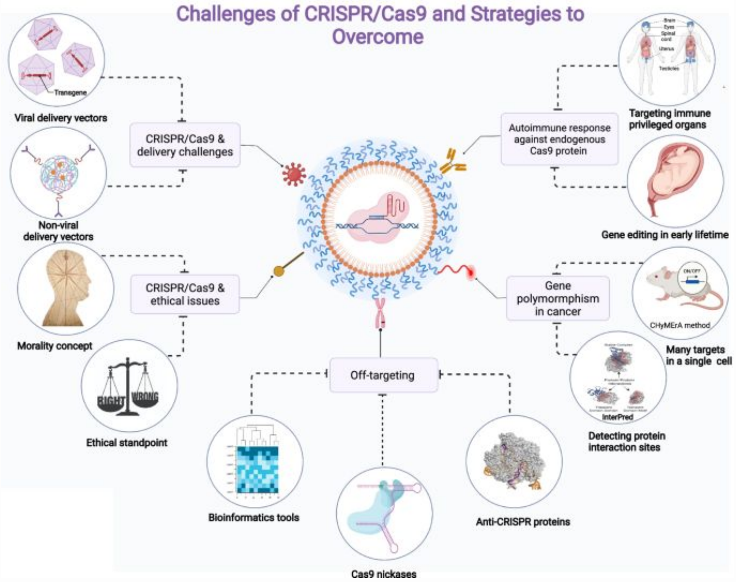
- One strand of the duplex (the guide strand) is loaded into the RISC, primarily containing the Argonaute (AGO) protein.

Mechanism	Description
Translational Repression	Decreases translational initiation efficiency
mRNA Destabilization / Degradation	Recruitment of deadenylases (CCR4-NOT), decapping enzymes → mRNA decay
mRNA Cleavage	AGO2 cleaves the mRNA directly

- Blocking of PPR prevents AGO Protein from associating with mRNA. Since in 3′UTR, it does not interfere with canonical cap-dependent scanning

Gene manipulation Technology

1. CRISPR raises concerns about **off-target and irreversible effects** of genome editing.
2. CRISPR gRNA target site limitations (e.g. GC-rich regions)
3. PPR distinguishes between bases more effectively compared to canonical Watson-Crick base pairing (McDowell, 2022)
4. Limitations to Existing RNA manipulation techniques (ASO, siRNA, PUF, MS2)--Next slide



McDowell, R., Small, I., & Bond, C. S. (2022). Synthetic PPR proteins as tools for sequence-specific targeting of RNA. *Methods*, *208*, 19-26.

Existing RNA manipulation Technology

Nucleotide-Based Techniques:

RNA interference (RNAi) with **siRNAs** and **antisense oligonucleotides (ASOs).**

Limitations:

- Primarily suppress RNA levels
- Potential off-target effects due to weaker canonical Watson-crick base pairing

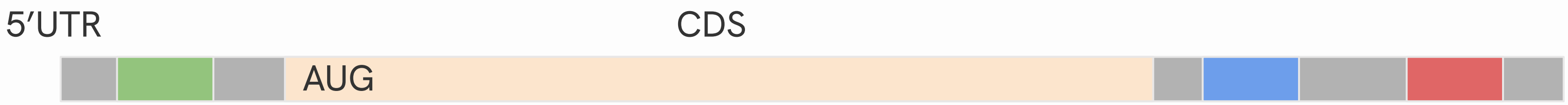
Protein-Based Techniques:

MS2 systems and Pumilio/fem-3 (**PUF**) RNA-binding proteins.

Limitations:

- Limited use in endogenous systems
- Limited programmability and sequence recognition capacity (e.g., PUF proteins bind 8–9 bases).

Special sequences in PKD1 and PKD2



3′UTR

uORF

- Short coding sequence located in the 5' UTR of the **PKD2** mRNA
- Ribosome dissociates from PKD2 mRNA before it reaches the start codon of the main coding sequence.

The FASEB Journal • Research Communication

Translational up-regulation of polycystic kidney disease protein PKD2 by endoplasmic reticulum stress

Jungwoo Yang,[1] Wang Zheng,[1] Qian Wang, Carlos Lara, Shaimaa Hussein, and Xing-Zhen Chen[2]

miR-17

- MicroRNA that has been shown to be upregulated in ADPKD models
- Binds to the 3' UTR of **PKD1 and PKD2** mRNAs, leading to their **degradation**

ARTICLE

Received 7 Jun 2016 | Accepted 22 Dec 2016 | Published 16 Feb 2017 DOI: 10.1038/ncomms14395 OPEN

microRNA-17 family promotes polycystic kidney disease progression through modulation of mitochondrial metabolism

Sachin Hajarnis[1,*], Ronak Lakhia[1,*], Matanel Yheskel[1], Darren Williams[1], Mehran Sorourian[2], Xueqing Liu[2], Karam Aboudehen[3], Shanrong Zhang[4], Kara Kersjes[2], Ryan Galasso[2], Jian Li[2], Vivek Kaimal[2], Steven Lockton[2], Scott Davis[2], Andrea Flaten[1], Joshua A. Johnson[5], William L. Holland[5], Christine M. Kusminski[5], Philipp E. Scherer[5], Peter C. Harris[6], Marie Trudel[7], Darren P. Wallace[8], Peter Igarashi[3], Edmund C. Lee[2], John R. Androsavich[2] & Vishal Patel[1]

miR-200

- miR-200 family members directly bind to **PKD1** 3′-UTR and inhibit expression post-transcriptionally
- Influence downstream negative regulators of PKD1

Original Article

Effect of miR-200 on autosomal polycystic nephropathy in rats

Kebo Bi, Xiaoling Deng, Weihong Yu, Chunlian Yu, Xinglei Cui, Meiyan Yu, Tao Zhang

Department of Nephrology, Weihai Central Hospital, Weihai, Shandong, China

Received November 25, 2015; Accepted January 27, 2016; Epub April 1, 2016; Published April 15, 2016