The “dark proteome”: Discovering new protein-coding genes in non-canonical open-reading frames

Technical Journal Club

Lukas Frick

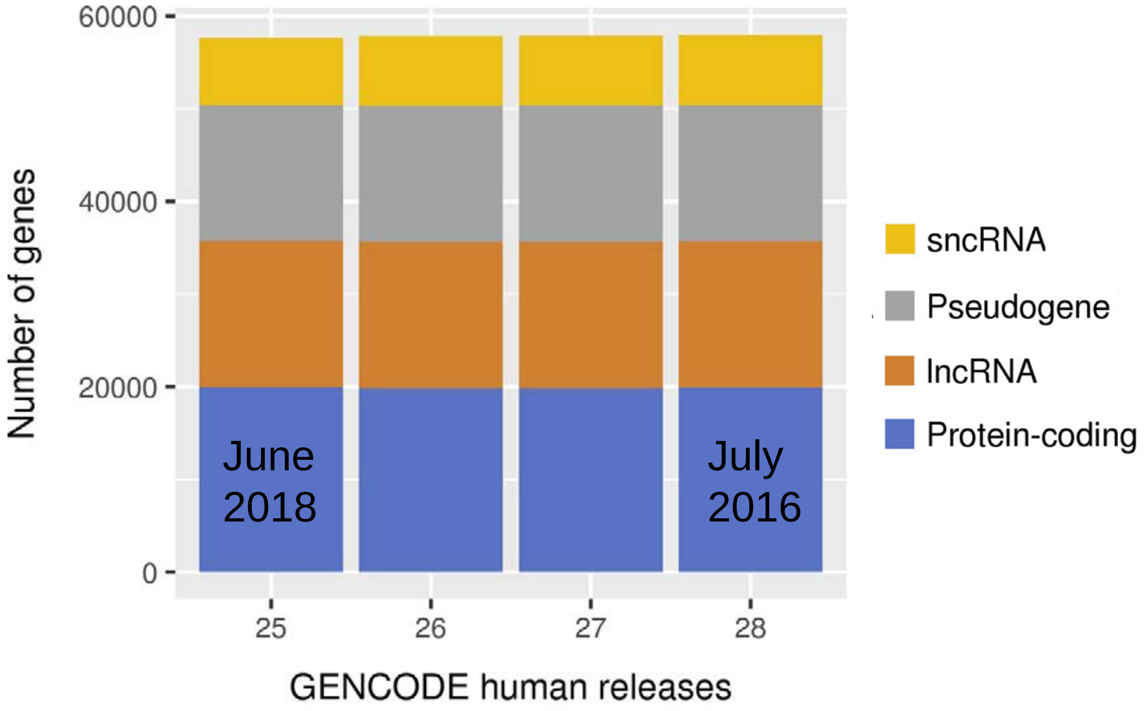
9 June 2020

How many protein-coding genes exist?

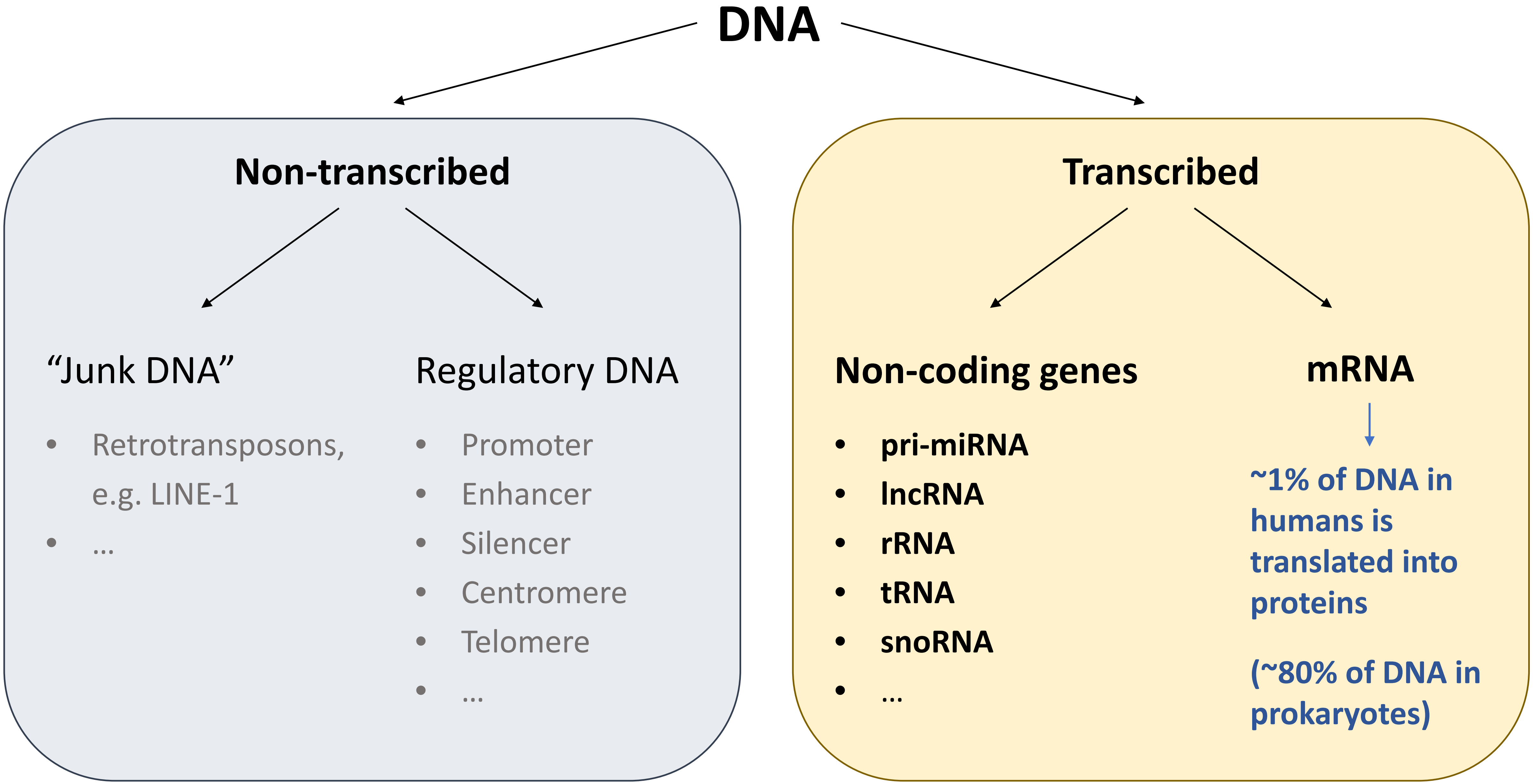
- There are **~20’000** protein-coding genes in the human genome...

  ... but the exact number is unknown!

- The catalog of protein-coding genes is derived mainly from the analysis of **coding sequences**
  - bioinformatics pipelines ± manual review
  - The algorithms have **blind spots**!



GENCODE reference annotation for the human and mouse genomes, *Nucleic Acids Research, 2018*



DNA

Non-transcribed

"Junk DNA"

- Retrotransposons, e.g. LINE-1
- …

Regulatory DNA

- Promoter
- Enhancer
- Silencer
- Centromere
- Telomere
- …

Transcribed

Non-coding genes

- **pri-miRNA**
- **lncRNA**
- **rRNA**
- **tRNA**
- **snoRNA**
- …

mRNA

~1% of DNA in humans is translated into proteins

(~80% of DNA in prokaryotes)

Only the **CDS** (coding DNA sequence – a subset of the exonic sequence) **is translated** into protein

pre-mRNA

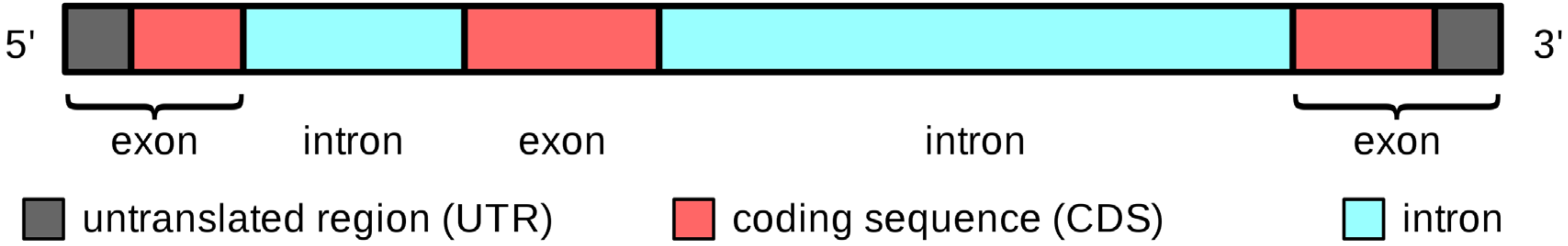


Image: Wikipedia

The simplest way to find potential protein-coding sequences is to look for (long) **open reading frames (ORFs)**

```
1. ATG CAA TGG GGA AAT GTT ACC AGG TCC GAA CTT ATT GAG GTA AGA CAG ATT TAA
2. A TGC AAT GGG GAA ATG TTA CCA GGT CCG AAC TTA TTG AGG TAA GAC AGA TTT AA
3. AT GCA ATG GGG AAA TGT TAC CAG GTC CGA ACT TAT TGA GGT AAG ACA GAT TTA A
```

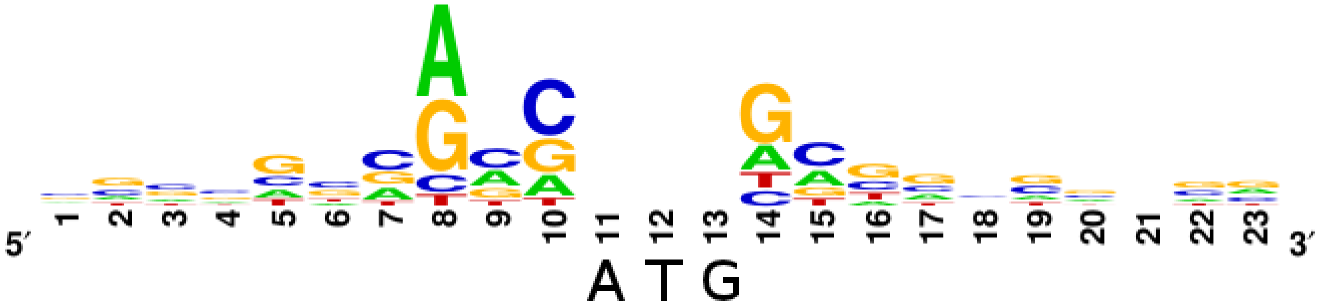
An open reading frame is a continuous stretch of codons that begins with a **start codon** and ends with a **stop codon**.

AUG

UAA / UAG / UGA

CUG / UUG / GUG are non-canonical

In addition to the start codon, the surrounding nucleotides, i.e. the **Kozak sequence**, determine the initiation of translation



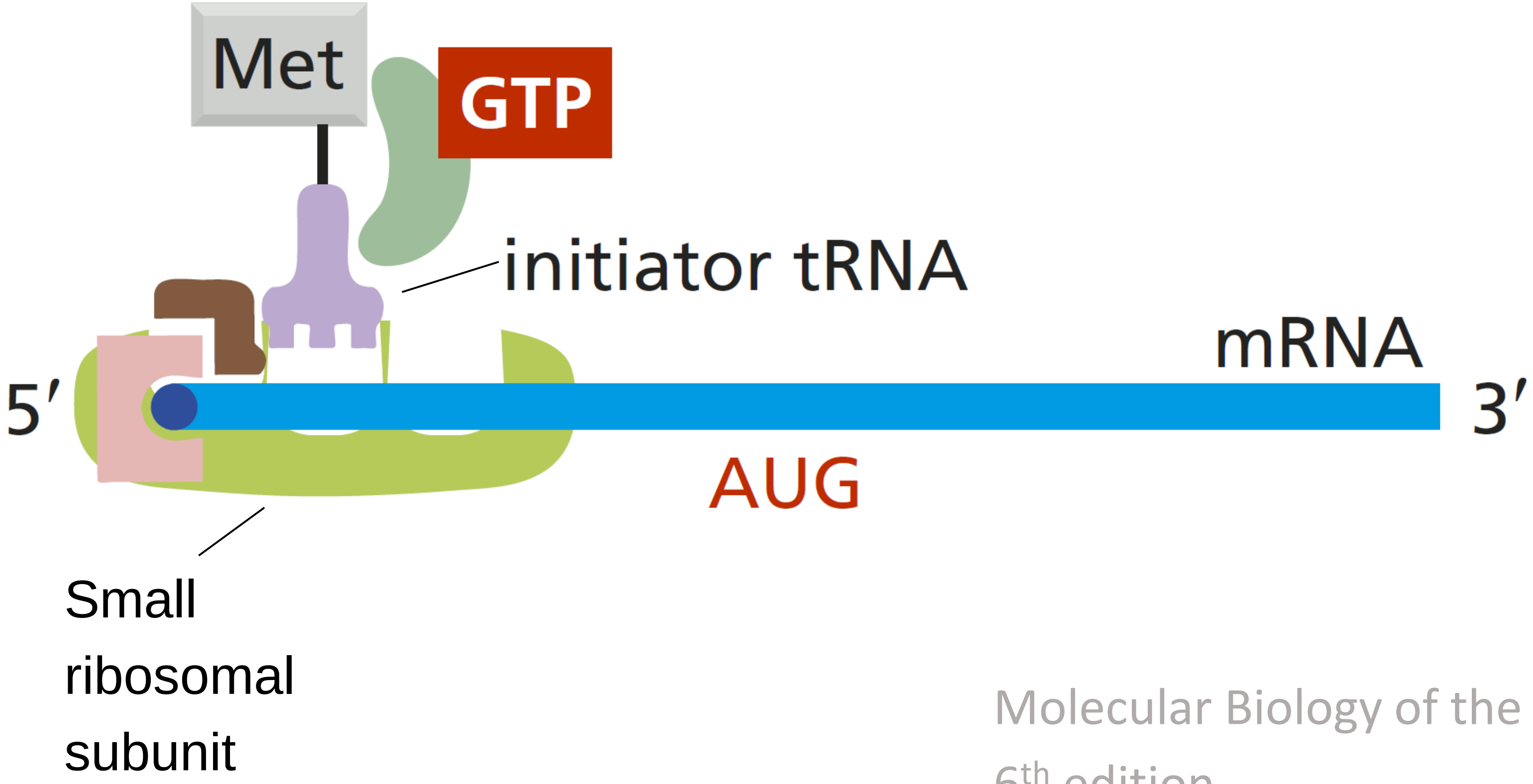
consensus recognition site

5′-ACC<u>AUG</u>G-3′

If a start codon deviates from this sequence, it may sometimes be skipped → **leaky scanning** by the ribosome!

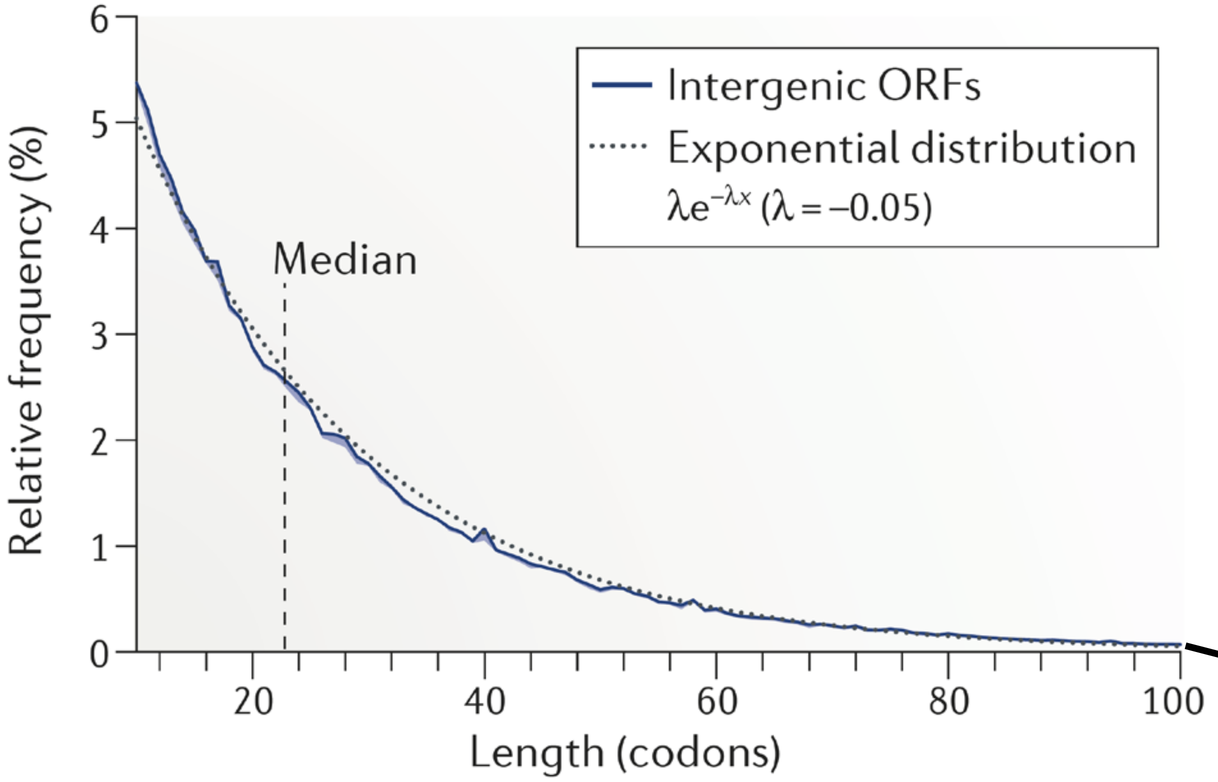
In ~90% of cases, translation begins at the first AUG start codon

In eukaryotes, the ribosome binds to the 5’ cap of mRNA and starts scanning.



Molecular Biology of the Cell
6th edition

In a random DNA sequence, the median ORF is 23 codons long



3/64 triplets are stop codons

= 5% probability

Very few ORFs ≥ 100 amino acids in size are expected by chance!

Among the millions of small ORFs in our genome, only a tiny fraction code for proteins.

Couso and Patraquim, 2017

Criteria for identifying canonical protein-coding ORFs

1. Length > 100 amino acids
2. Canonical start codon & Kozak motif
3. **Homologies** to known proteins
4. ± Mass spectrometry confirmation

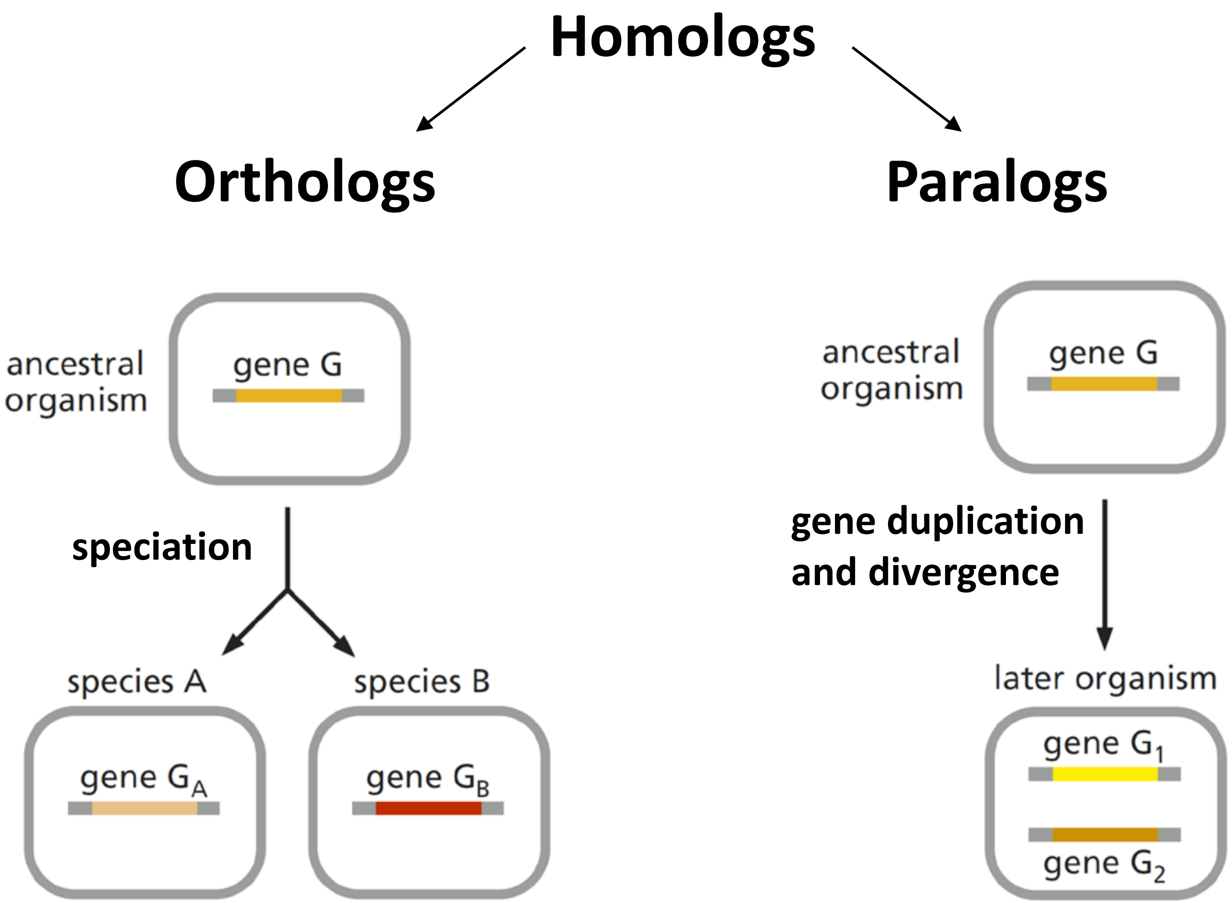
MS is poorly suited for…

- Newly identifying proteins
- Detecting low-abundance proteins

Bioinformaticians rely heavily on evolutionary relationships to known protein-coding genes

Sequence preservation among organisms

PhyloCSF score, Lin et al, 2011



Similarity to known protein domains

When comparing human and mouse sequences, a large fraction of **synonymous substitutions** indicates a protein-coding gene!

	AGA									UUA					AGC					
	AGG									UUG					AGU					
GCA	CGA						GGA			CUA				CCA	UCA	ACA			GUA	
GCC	CGC						GGC		AUA	CUC				CCC	UCC	ACC			GUC	UAA
GCG	CGG	GAC	AAC	UGC	GAA	CAA	GGG	CAC	AUC	CUG	AAA		UUC	CCG	UCG	ACG		UAC	GUG	UAG
GCU	CGU	GAU	AAU	UGU	GAG	CAG	GGU	CAU	AUU	CUU	AAG	AUG	UUU	CCU	UCU	ACU	UGG	UAU	GUU	UGA
Ala	Arg	Asp	Asn	Cys	Glu	Gln	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Trp	Tyr	Val	stop
A	R	D	N	C	E	Q	G	H	I	L	K	M	F	P	S	T	W	Y	V	

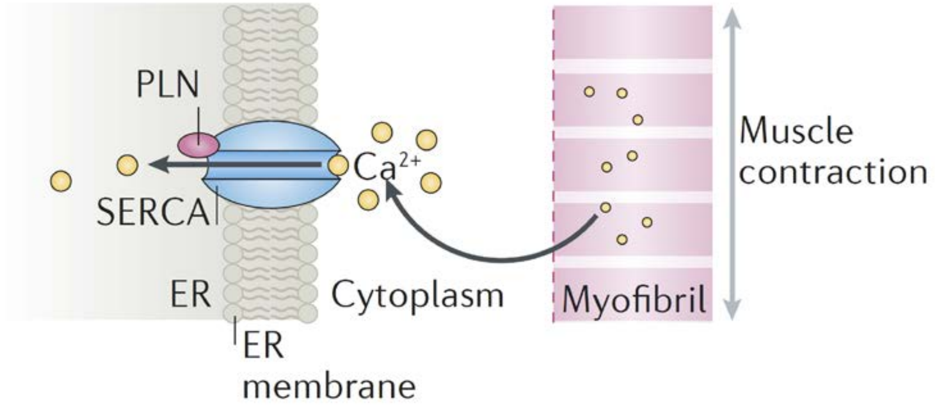
Non-coding sequences / RNA genes will accumulate mutations that **do not conserve** the amino acid sequence!

The existing pipelines have a bias against **small, new** and **non-canonical** ORFs!

“Functional small ORFs are often not annotated because they have not been experimentally corroborated, and they have not been corroborated because they are not annotated…”

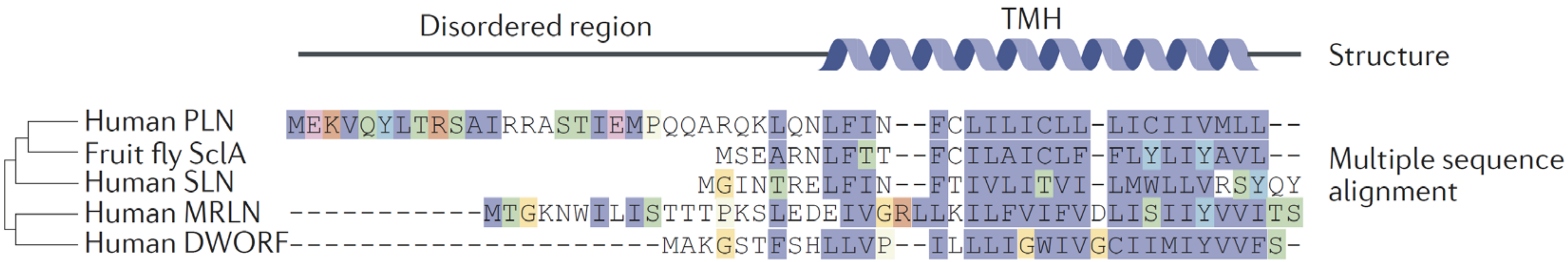
Couso and Patraquim, 2017

Because of their small size, microproteins usually have regulatory functions



Example:

Phospholamban (52 aa) and **myoregulin** (46 aa) inhibit SERCA, which pumps Ca^{2+} back to the sarcoplasmic reticulum to terminate muscle contraction.



Disordered region
TMH
Structure
Human PLN MEKVQYLTRSAIRRASTIEMPQQARQKLQNLFIN--FCLILICLL-LICIIVMLL--
Fruit fly SclA MSEARNLFTT--FCILAICLF-FLYLIYAVL--
Human SLN MGINTRELFIN--FTIVLITVI-LMWLLVRSYQY
Human MRLN -----------MTGKNWILISTTTPKSLEDEIVGRLLKILFVIFVDLISIIYVVITS
Human DWORF---------------------MAKGSTFSHLLVP--ILLLIGWIVGCIIMIYVVFS-
Multiple sequence alignment

Phospholamban and myoregulin are paralogs.

Couso and Patraquim, 2017

Localization of newly discovered non-canonical ORFs

1. On **lncRNAs** (long non-coding RNAs)

2. On transcribed **pseudogenes**

3. On **mRNAs** (near canonical ORFs)
   - Upstream ORFs (uORFs)
   - Downstream ORFs (dORFs) (rare)

True lncRNAs often have regulatory functions (transcription, heterochromatin…)

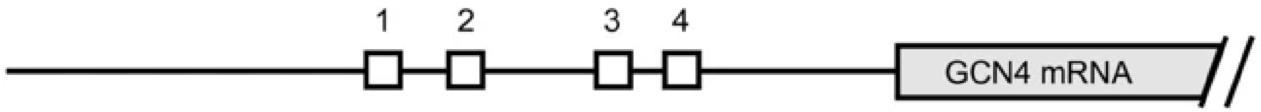
Pseudogenes usually arise from the duplication of a gene, followed by the accumulation of damaging mutations in one copy

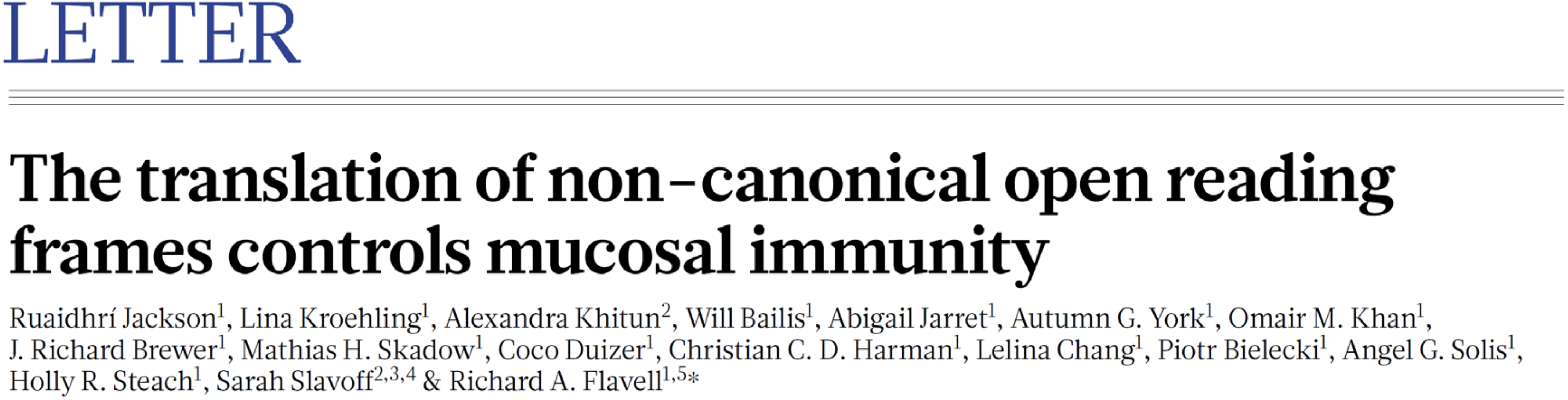
uORFs sometimes **compete with** and inhibit translation of **the canonical ORF**

- Classic example:
  - The **yeast Gcn4 gene** has 4 uORFs that normally inhibit its translation
  - In conditions of starvation, the ribosome skips the uORFs

    → Gcn4 is translated instead
  - The **amino acid sequences of the translated Gcn4 uORFs are irrelevant**

    → do not code for functional proteins





LETTER

The translation of non-canonical open reading frames controls mucosal immunity

Ruaidhrí Jackson[1], Lina Kroehling[1], Alexandra Khitun[2], Will Bailis[1], Abigail Jarret[1], Autumn G. York[1], Omair M. Khan[1], J. Richard Brewer[1], Mathias H. Skadow[1], Coco Duizer[1], Christian C. D. Harman[1], Lelina Chang[1], Piotr Bielecki[1], Angel G. Solis[1], Holly R. Steach[1], Sarah Slavoff[2,3,4] & Richard A. Flavell[1,5]*

Nature, 2018

The authors use mouse models of colitis (e.g. colon infection with *Salmonella typhimurium*) to study the mucosal immune system.

While **RNA-seq** offers a global view of **transcription**, the authors wanted to acquire a global view of **translation** in their colitis model.

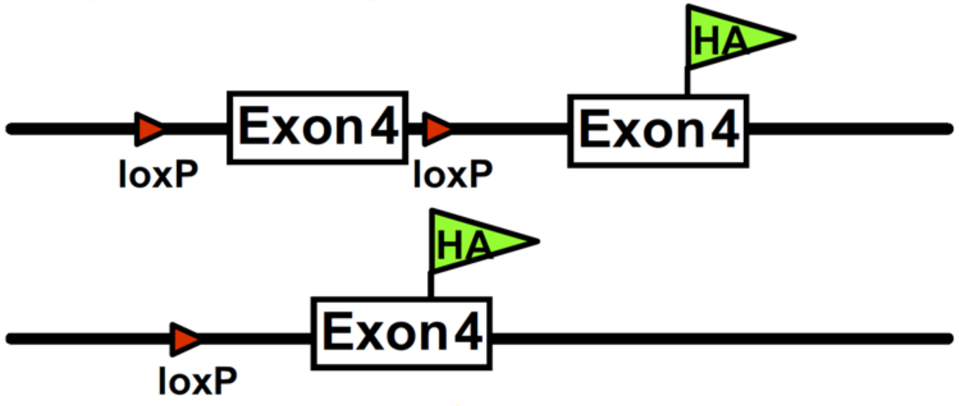
They used two complementary strategies to identify RNAs that are being translated:

1. **RiboTag RNA-seq**
2. **Ribosome profiling**

RiboTag RNA-seq employs Cre mouse lines to enrich for mRNAs from a specific cell type

Modified RPL22 locus

After crossing to Cre Mouse

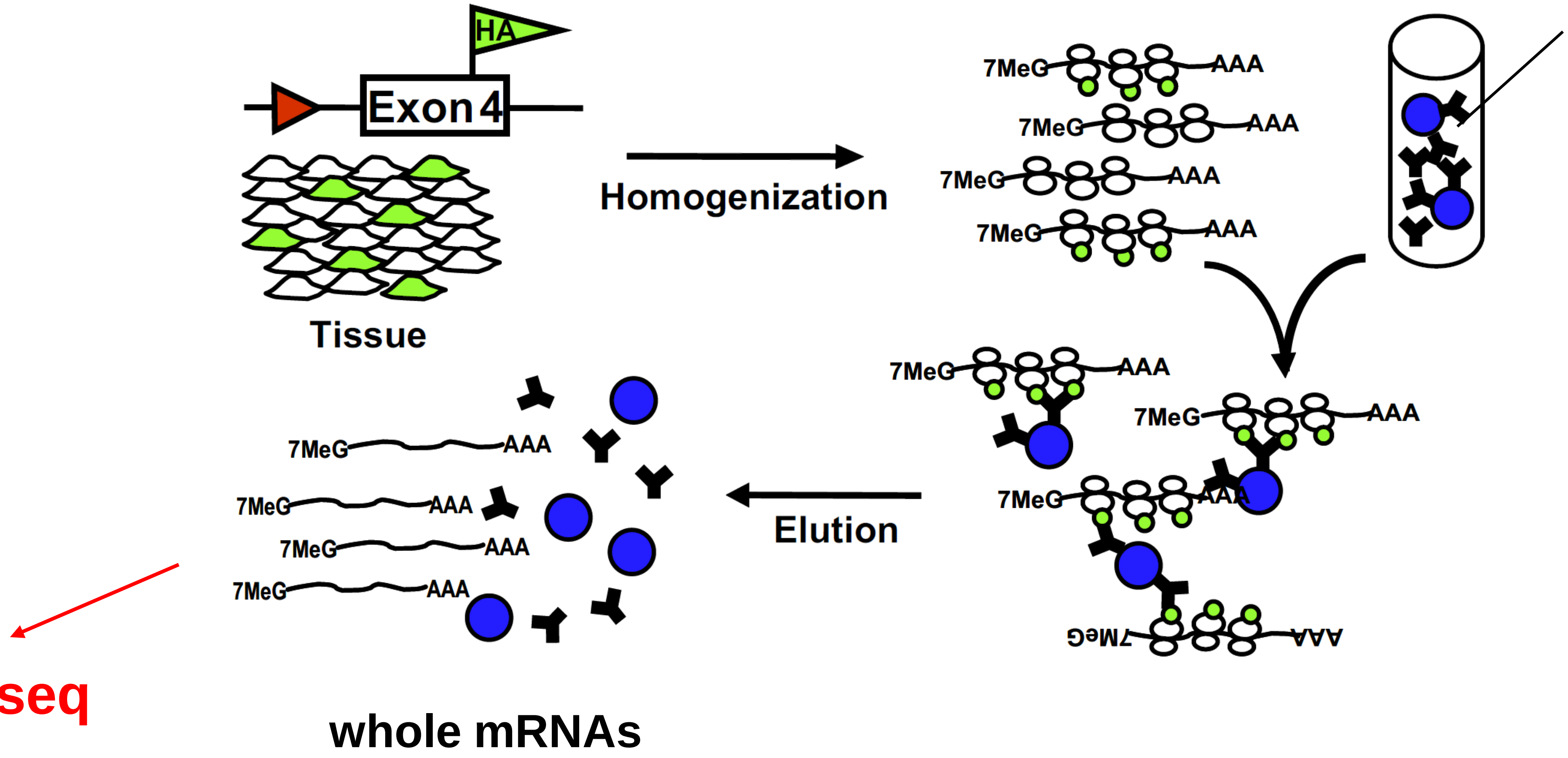


Sanz et al, PNAS, 2009

A transgenic mouse expresses Cre recombinase only in a cell type of interest, e.g. LysM-Cre mice in bone-marrow derived macrophages

If Cre is expressed, the **RPL22 ribosomal protein** is altered: The original exon 4 is excised, and a **HA-(hemagglutinin)-tagged** version of exon 4 is transcribed instead.

RiboTag RNA-seq uses **anti-HA antibodies** to select for ribosome-bound mRNAs from a specific cell type

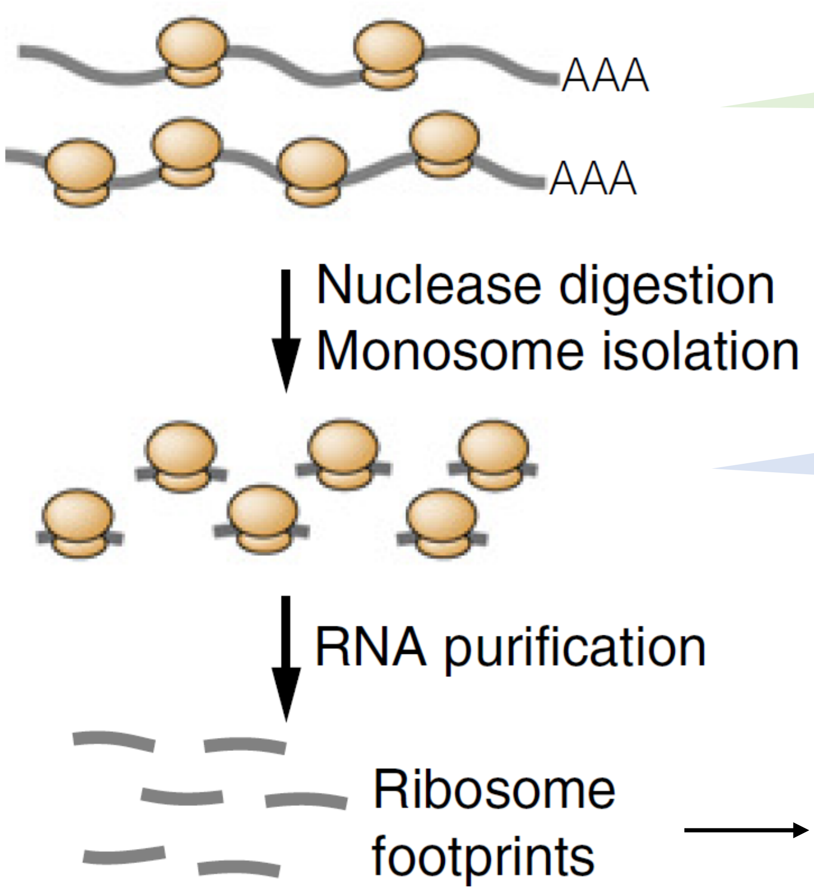


Anti-HA antibodies coupled to magnetic beads

RNA-seq
qPCR

Sanz et al, PNAS, 2009

Ribosome profiling = ribosome footprinting = Ribo-Seq allows for the specific identification of only **translated sections** of mRNA



Ribosomes are immobilized using cycloheximide (a toxin)

After **RNAse** treatment, only **ribosome-protected fragments** remain (**24 – 31 nucleotides**)

→ **Library construction** → **Deep sequencing**

Note: **RiboTag RNA-seq** (or similar systems) **can be combined with ribosome profiling** into one workflow

(but this was not done by the authors).

With RiboTag RNA-seq, they found **many** differentially expressed **ribosome-associated transcripts** that mapped to **non-coding genes**!

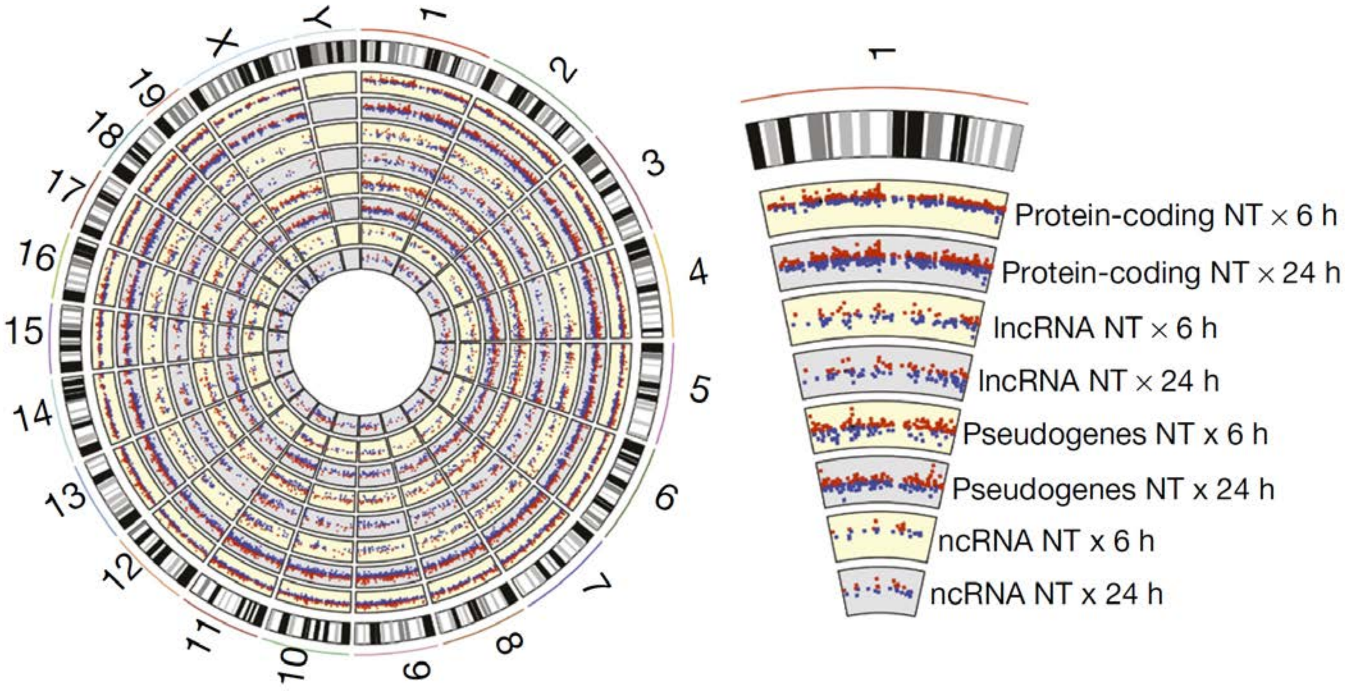


Fig. 1a

Upregulation with LPS

Downregulation with LPS

Bone marrow derived-macrophages were generated from **RiboTag^{LysM} mice** and stimulated with 1 ng/ml bacterial lipopolysaccharide (LPS) for 6 or 24 hours *in vitro*.

With RiboTag RNA-seq, they found **many** differentially expressed **ribosome-associated transcripts** that mapped to **non-coding genes**!

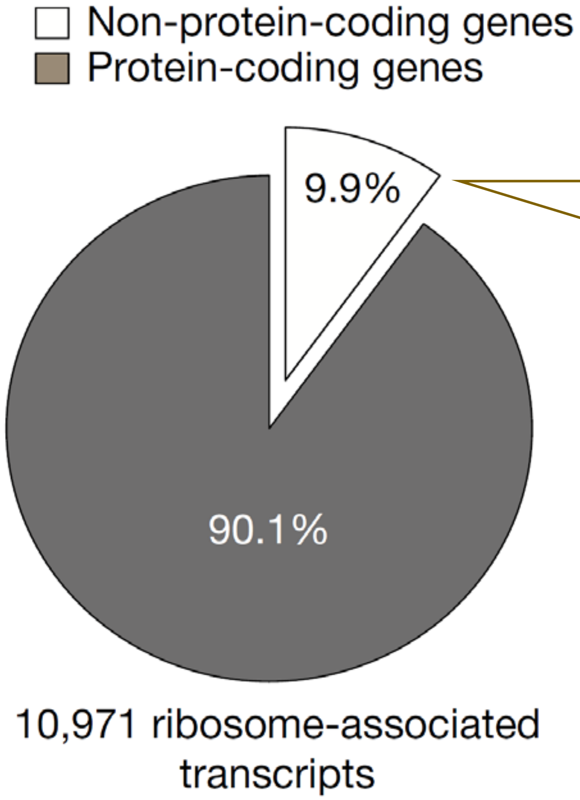


Fig. 1b

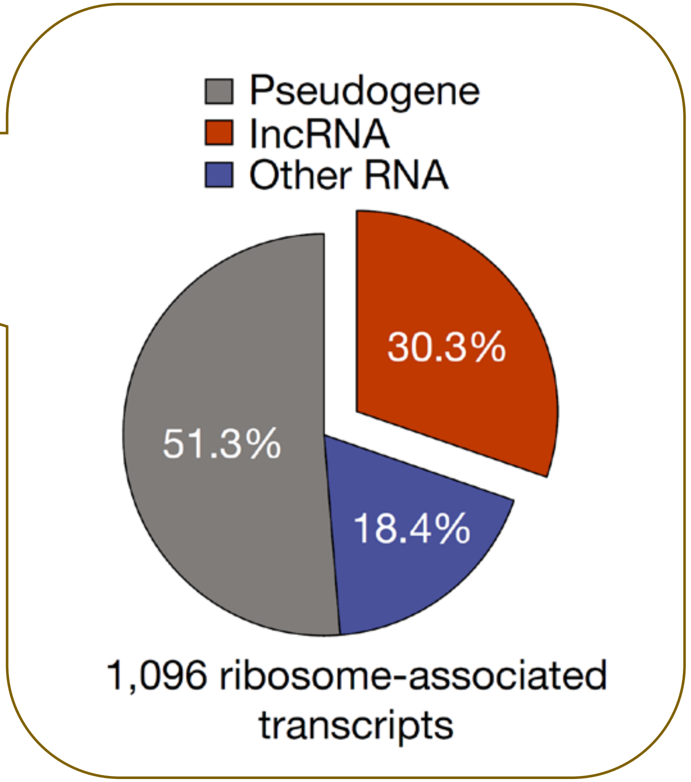
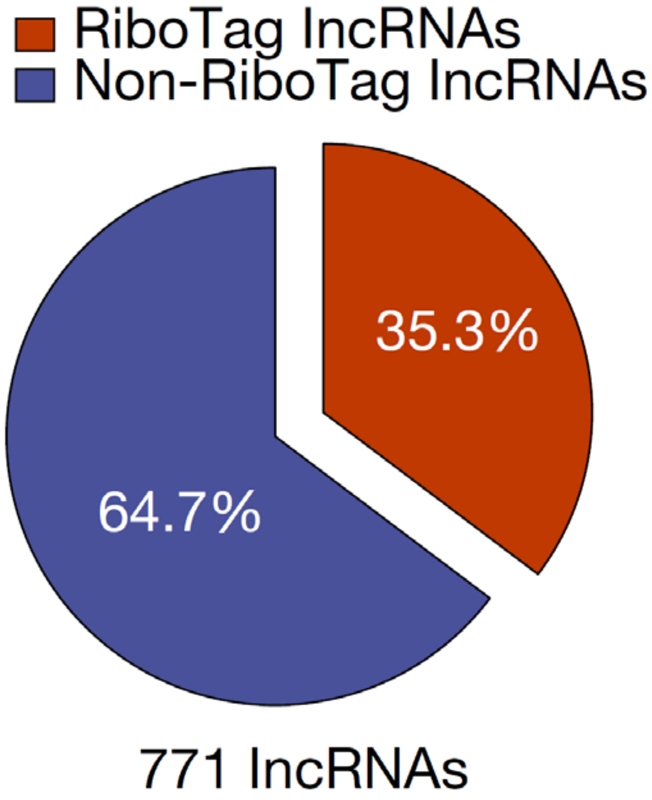


Fig. 1c

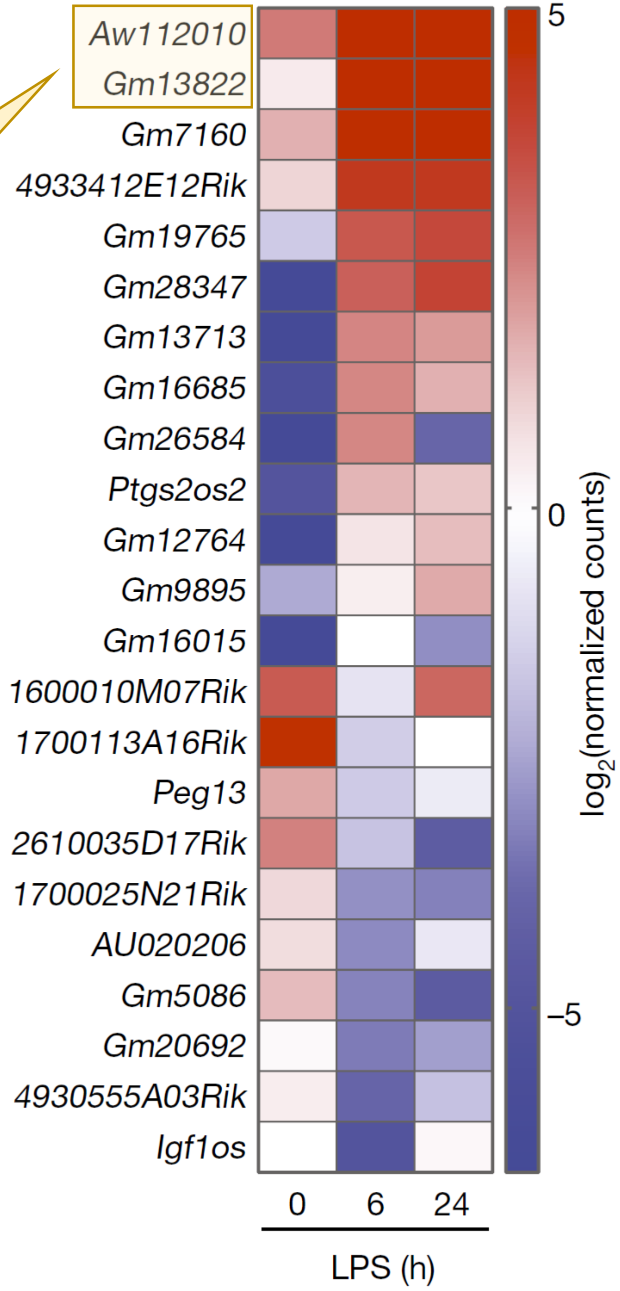
Comparison with paired RNA-seq data indicates that **one third** of expressed **lncRNAs** associate with ribosomes

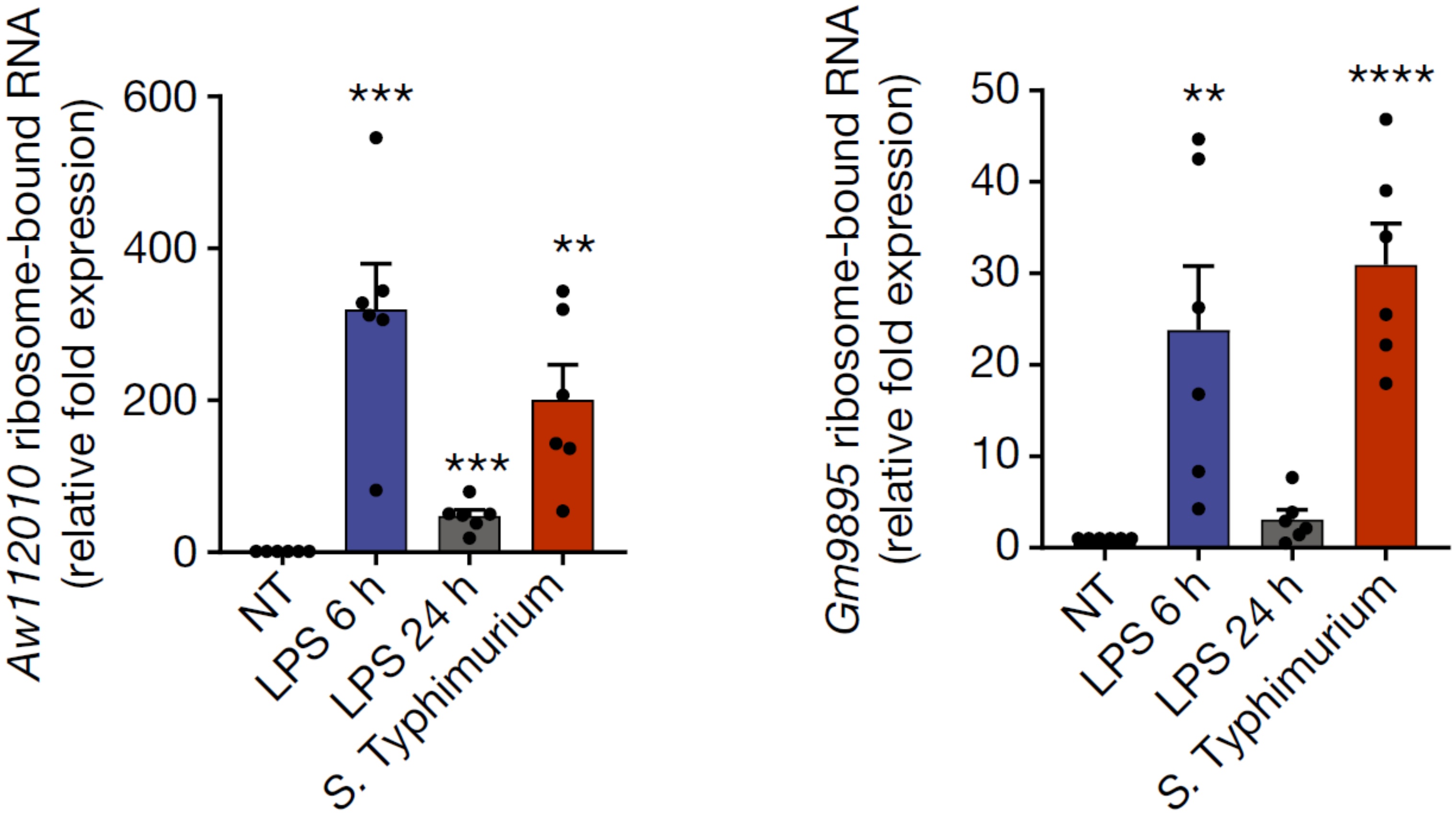


Two top upregulated ribosome-associated "lncRNAs" were examined in more detail:

Aw112010

Gm13822

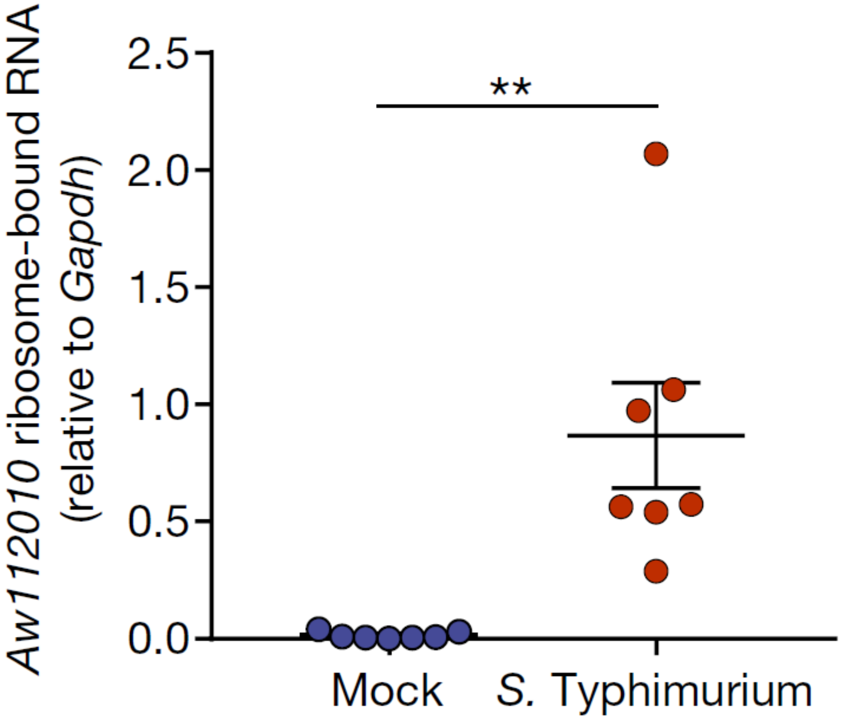


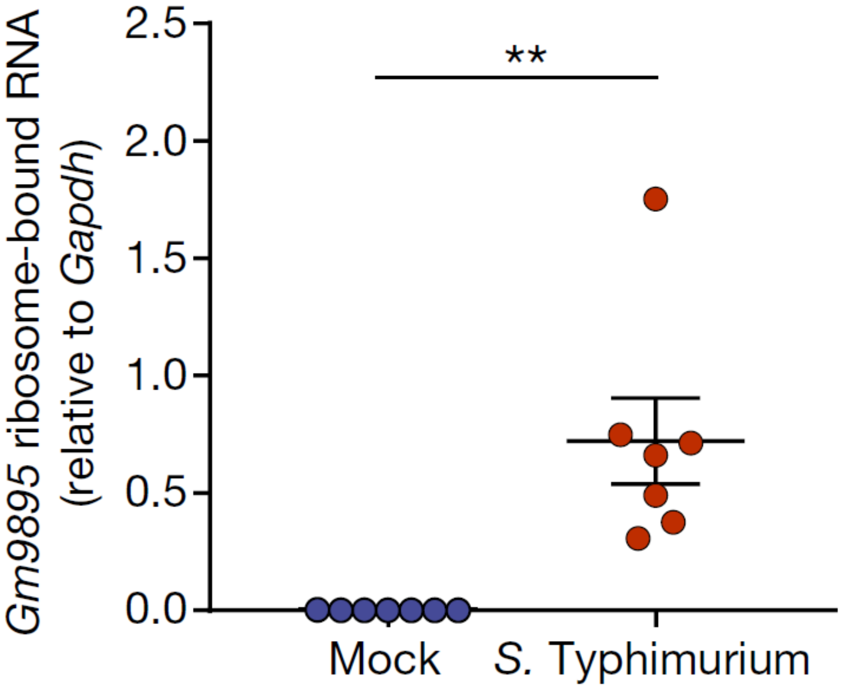


Bone-marrow derived macrophages were stimulated with LPS or infected with *Salmonella typhimurium*.

Ribosome-bound mRNA was measured by qPCR.

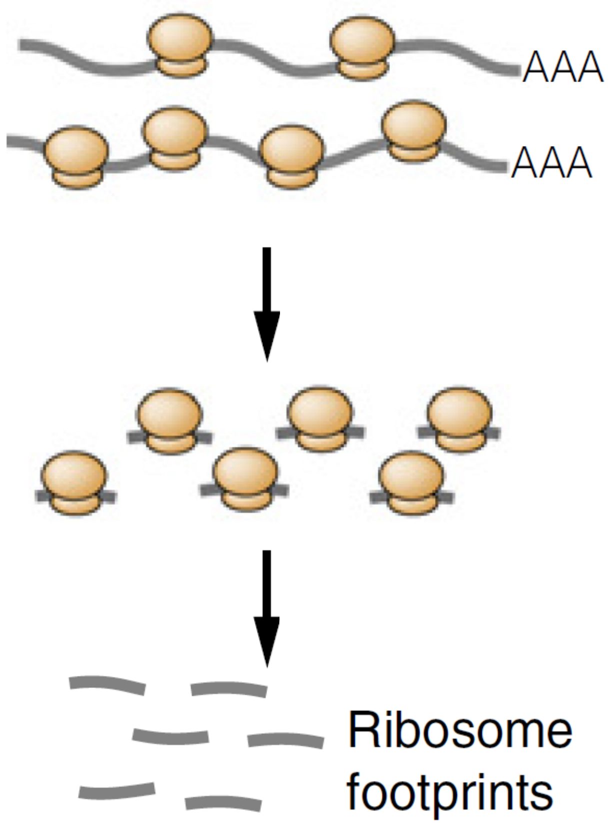
The two ribosome-bound RNAs were **induced in colonic macrophages *in vivo*** 24h after infection with *S. typhimurium*





Colon samples were washed, homogenized, and incubated overnight with HA beads.

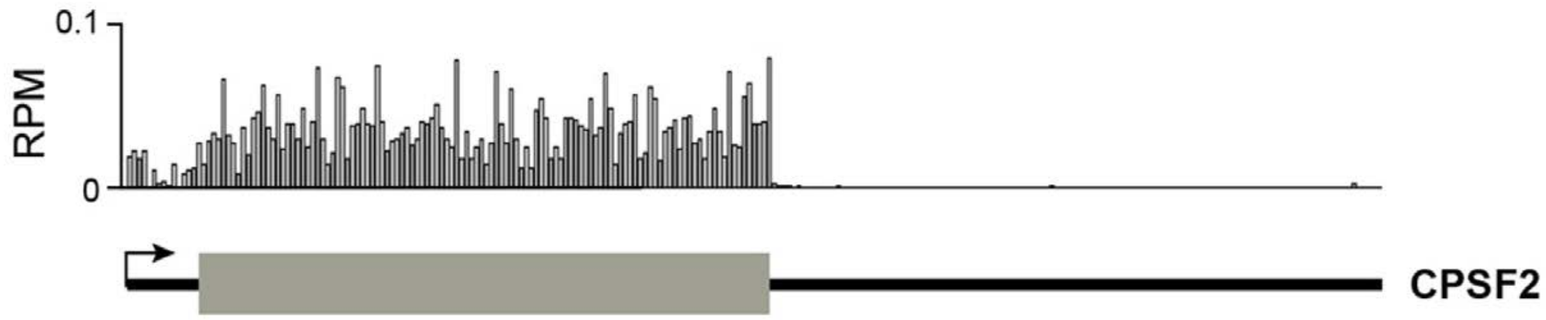
Next, the authors used **ribosome profiling** to corroborate that lncRNAs are truly being translated



They made heavy use of bioinformatics scores and algorithms to decide, for each ORF, whether translation was taking place.

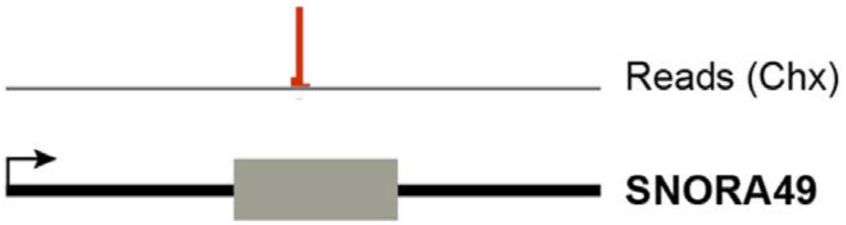
(Ribosome footprints can be artefacts, e.g. represent noise or protection by non-ribosome RNA-binding proteins.)

A high Percentage of Maximum Entropy (PME) value (a **homogenous footprints** profile) indicates translation



Protein-coding gene

Homogeneous spread of reads indicates translation



Non-coding small nucleolar RNA

Single Ribo-Seq peak, very inhomogenous

Ji et al, eLife, 2015

Many lncRNAs had high PME values.

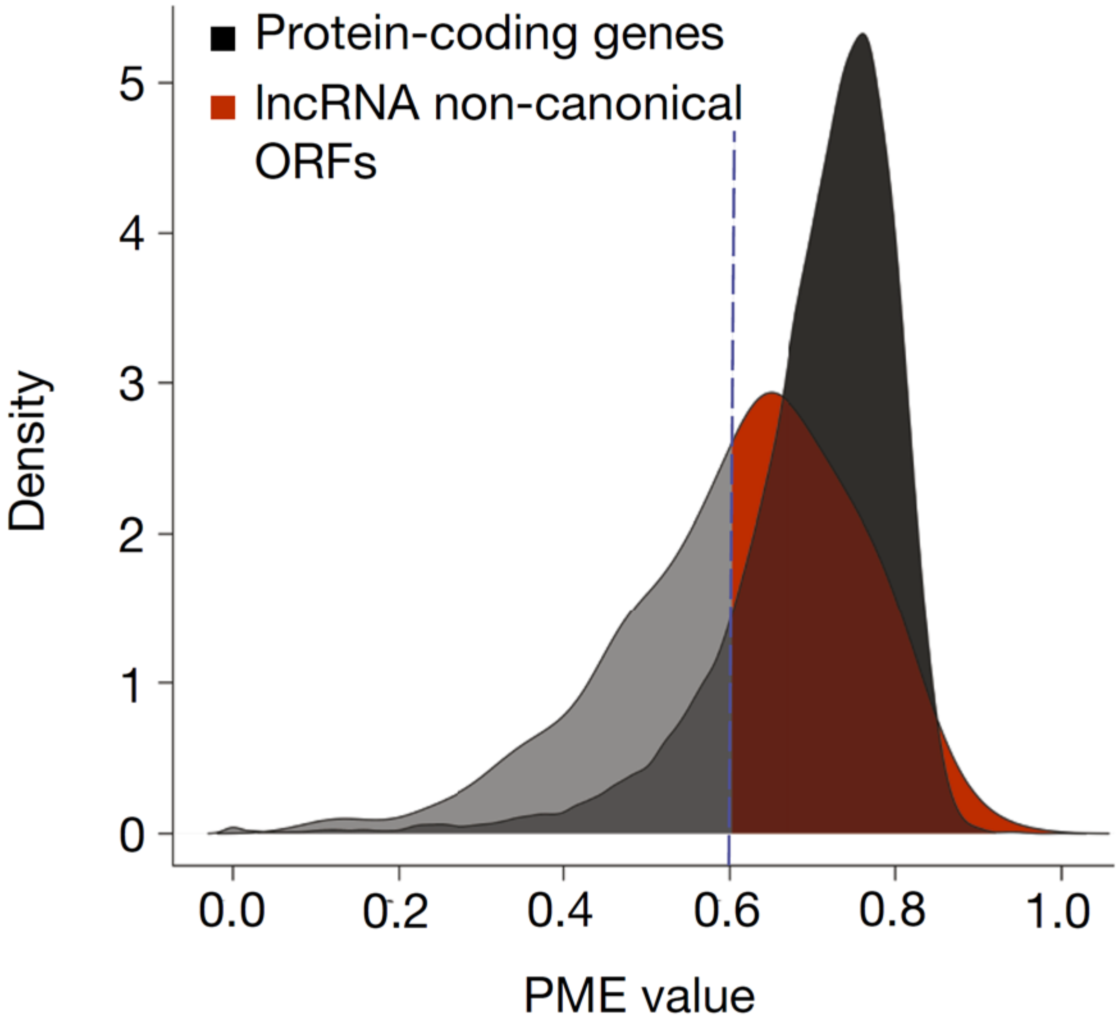
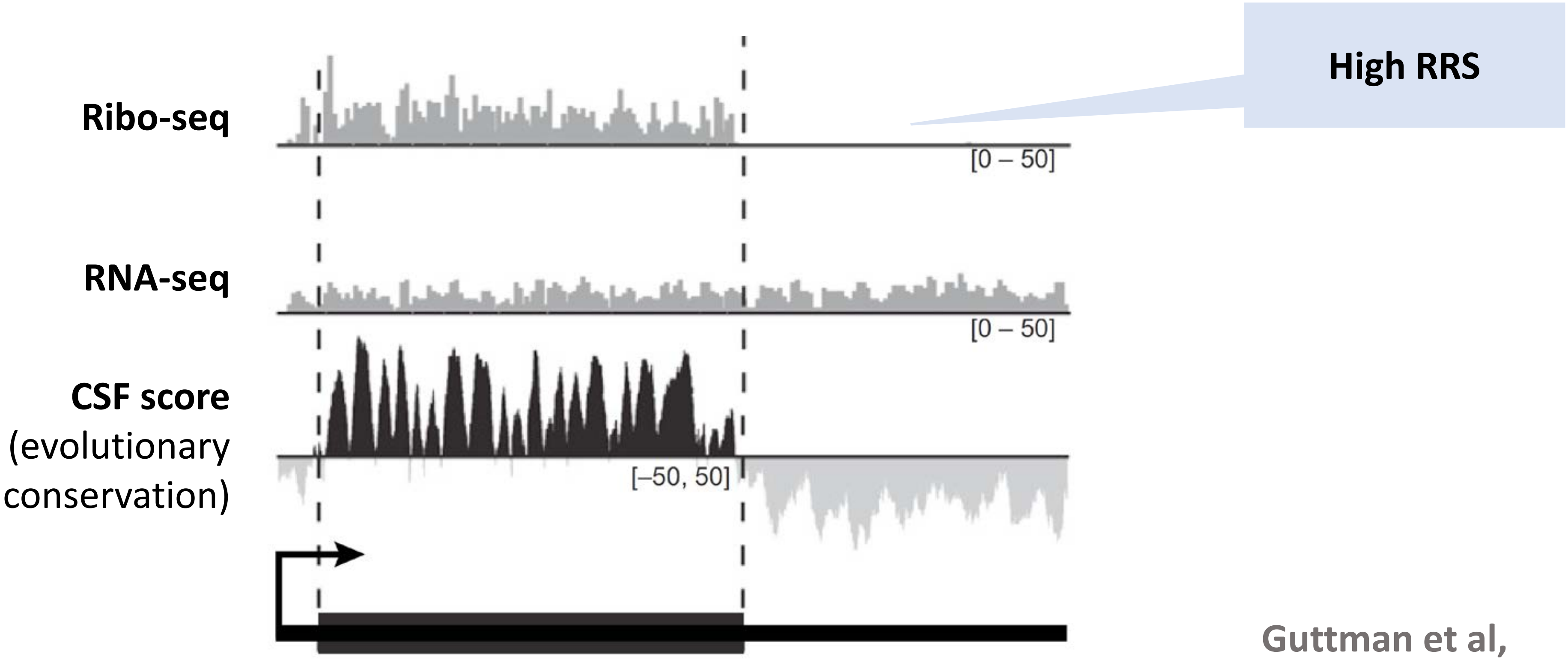


Fig. 2a

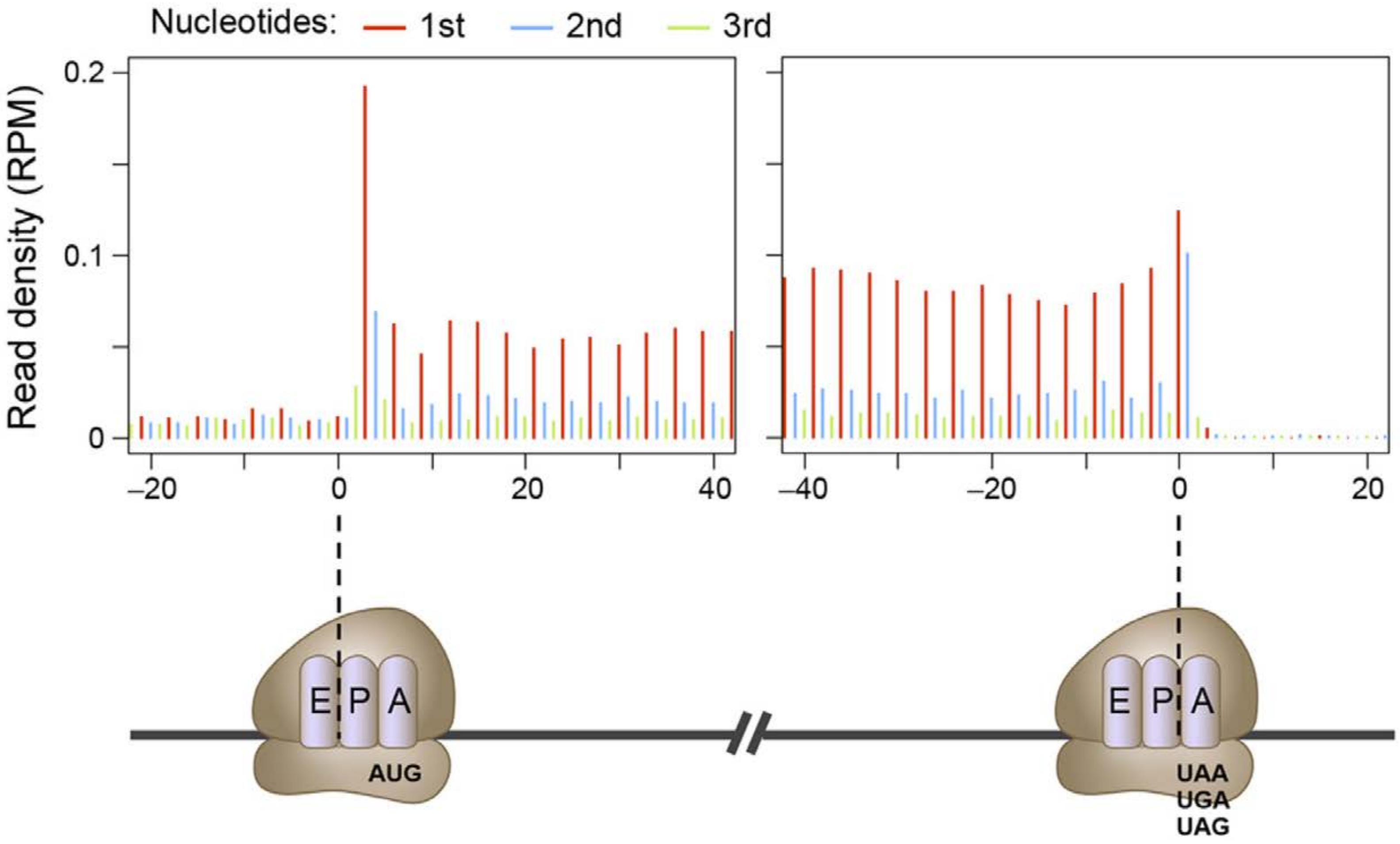
A high **ribosome release score** (RRS) – the ratio of footprints in the **coding region vs. 3’ UTR** – also indicates translation



STAT3 gene

Guttman et al, Cell, 2013

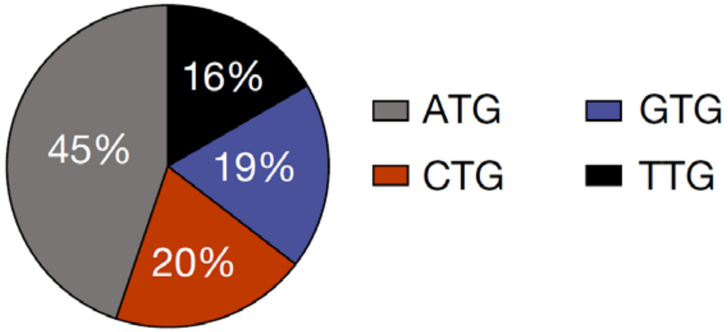
Three-nucleotide periodicity is also a strong indicator of translation!



- Ribosome profiling offers single-nucleotide resolution.
- The ribosome moves in 3-nucleotide jumps.
- Footprints of a given size often have the same offset to the P site (11 nt for a 24 nt footprint)
  - → can be aligned
  - → typical pattern in coding sequences

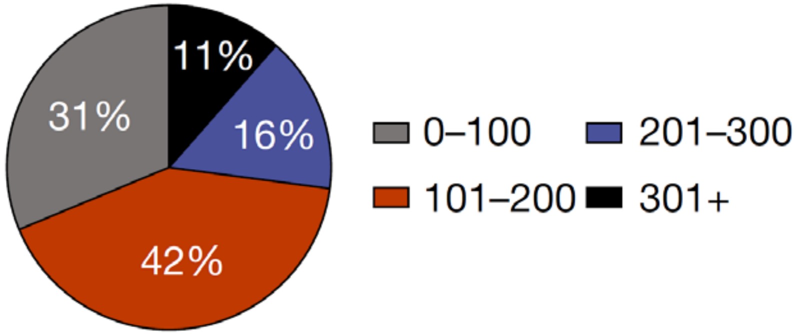
The authors used two tools (RibORF and RiboScan) plus a ribosome release score ≥ 7 to identify **96 translated lncRNAs.**

Aw112010 was among them.



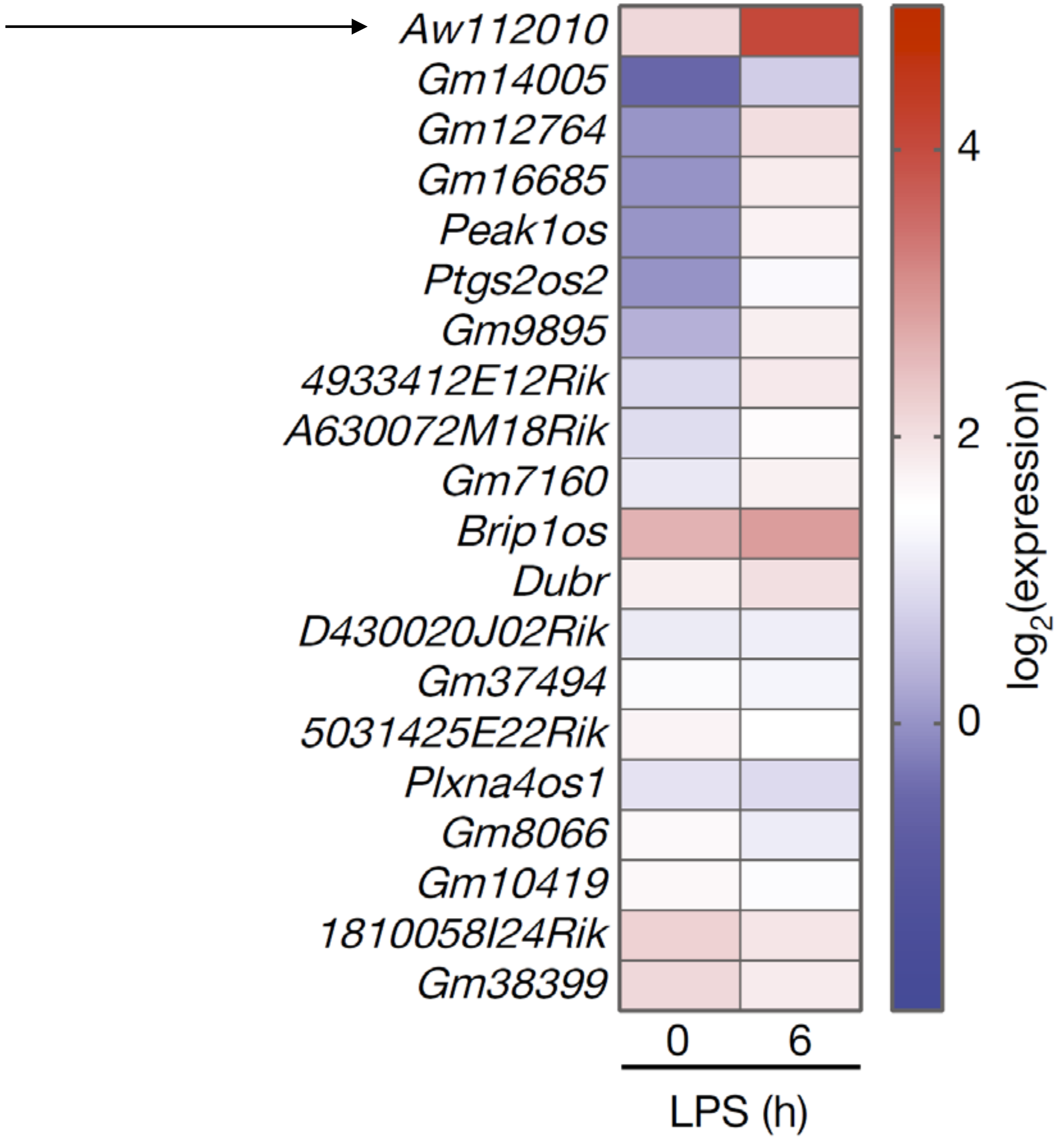
96 non-canonical ORFs

55% used non-canonical start codons



96 non-canonical ORFs

73% were smaller than 100 amino acids

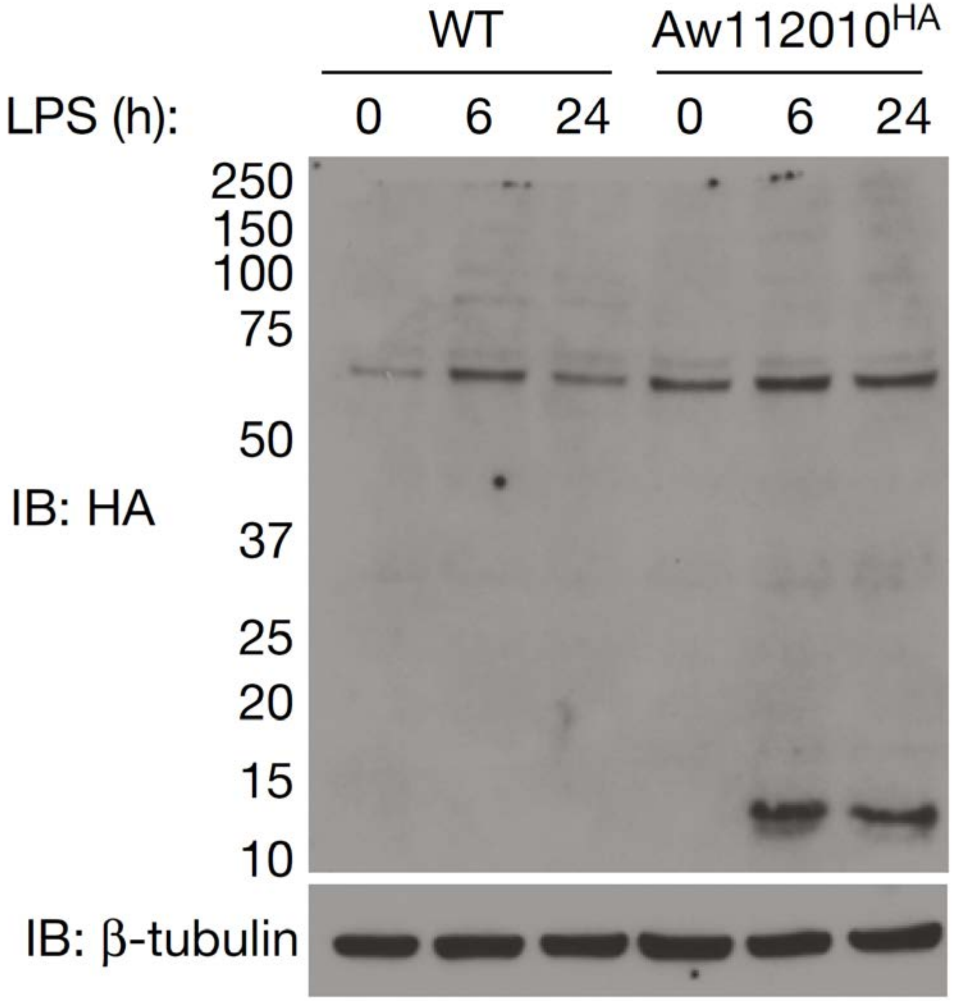


Ribosome profiling revealed ***Aw112010*** as the top differentially translated gene **upregulated after LPS** stimulation of wild-type bone-marrow derived macrophages.

Does *Aw112010* really produce a protein?

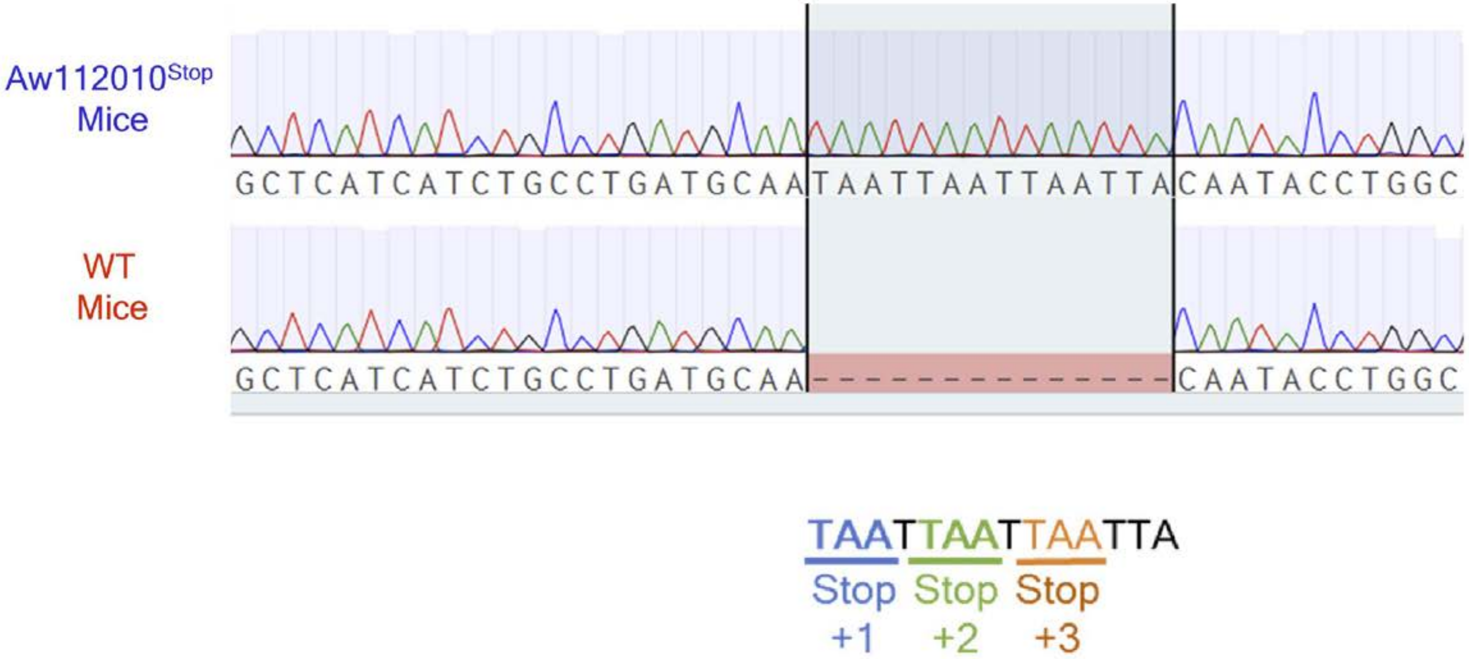
No antibodies for *Aw112010* exist, so an epitope-tagged Aw112010^{HA} knock-in mouse was generated using CRISPR-Cas9.

Mass spectrometry also confirmed expression of the protein.



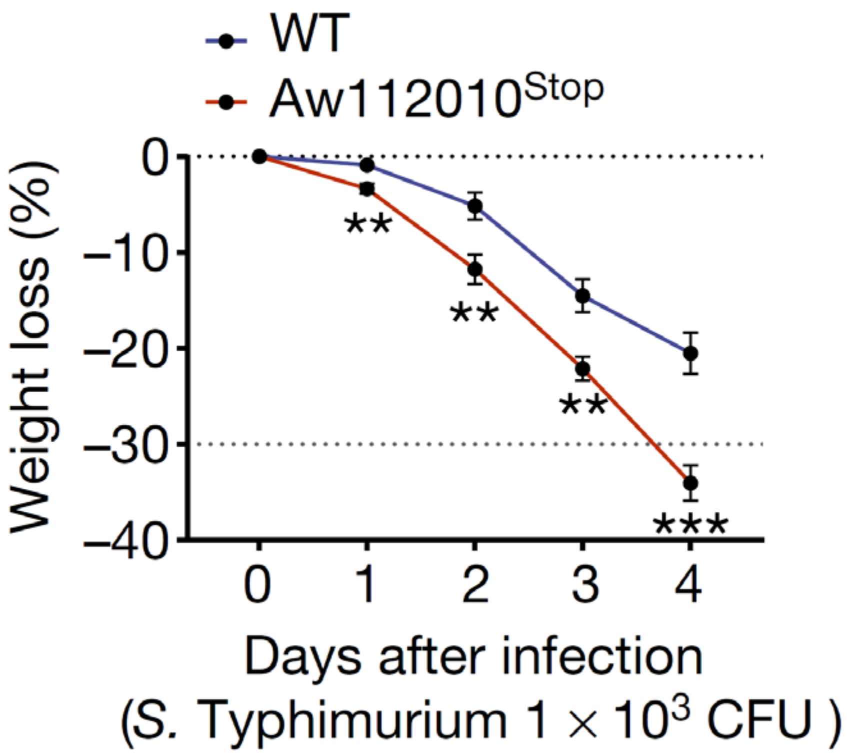
Aw112010-HA protein is induced by LPS stimulation

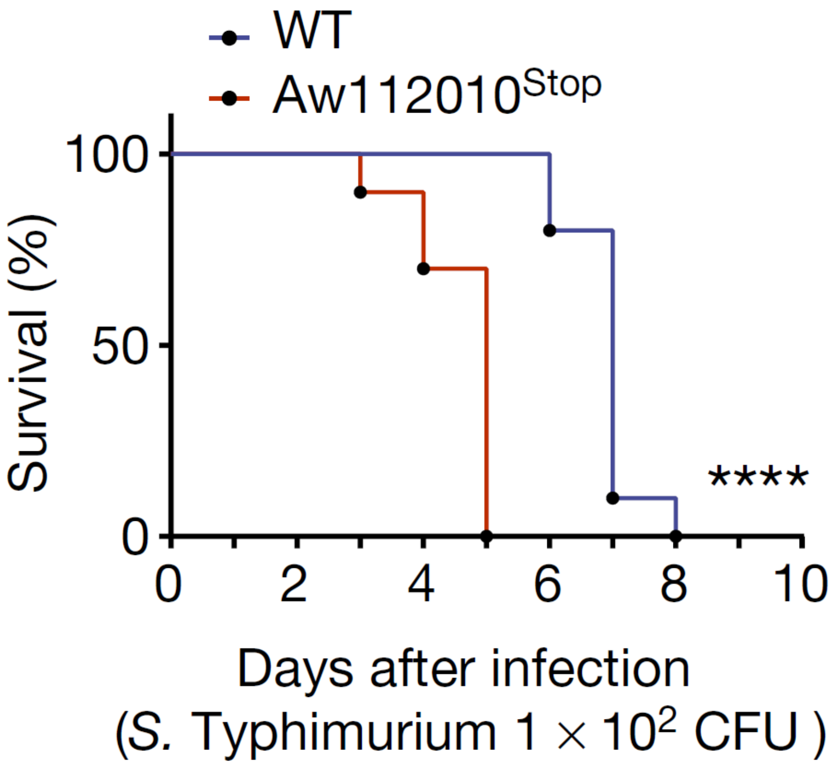
To abolish translation of *Aw112010* and prove its functional relevance, the authors created **Aw112010$^{\text{Stop}}$ mice**



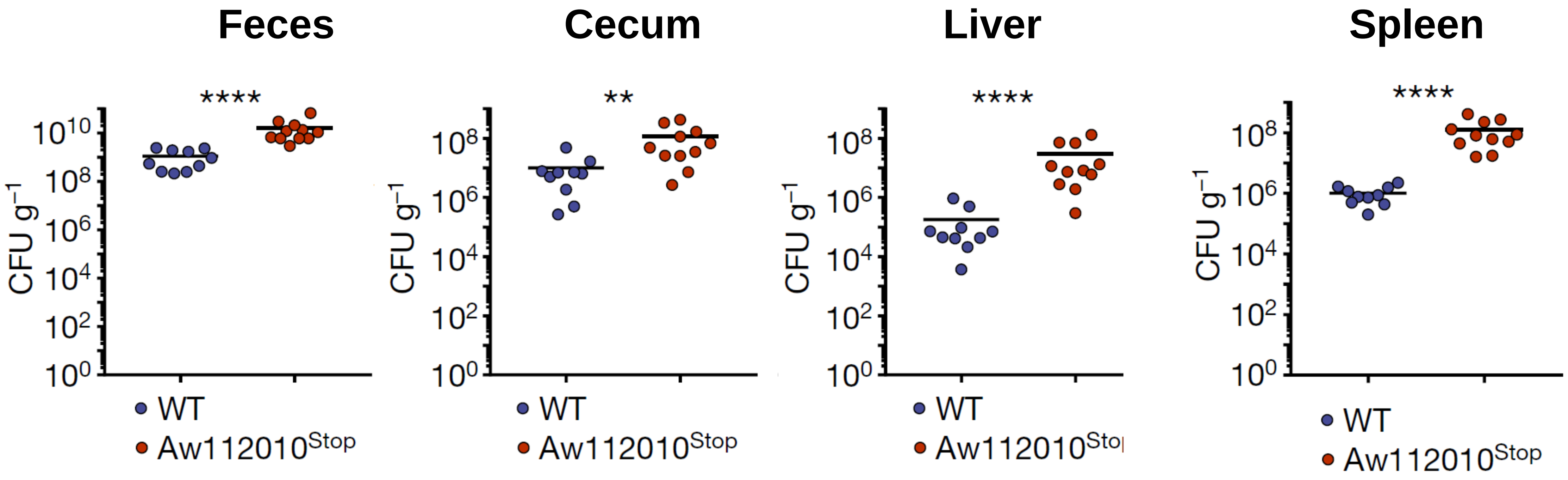
Frameshifting stop insert
(14 nt)

Aw112010$^{\text{Stop}}$ mice developed more severe infectious colitis





Aw112010^{Stop} had a higher bacterial load of *S. typhimurium*

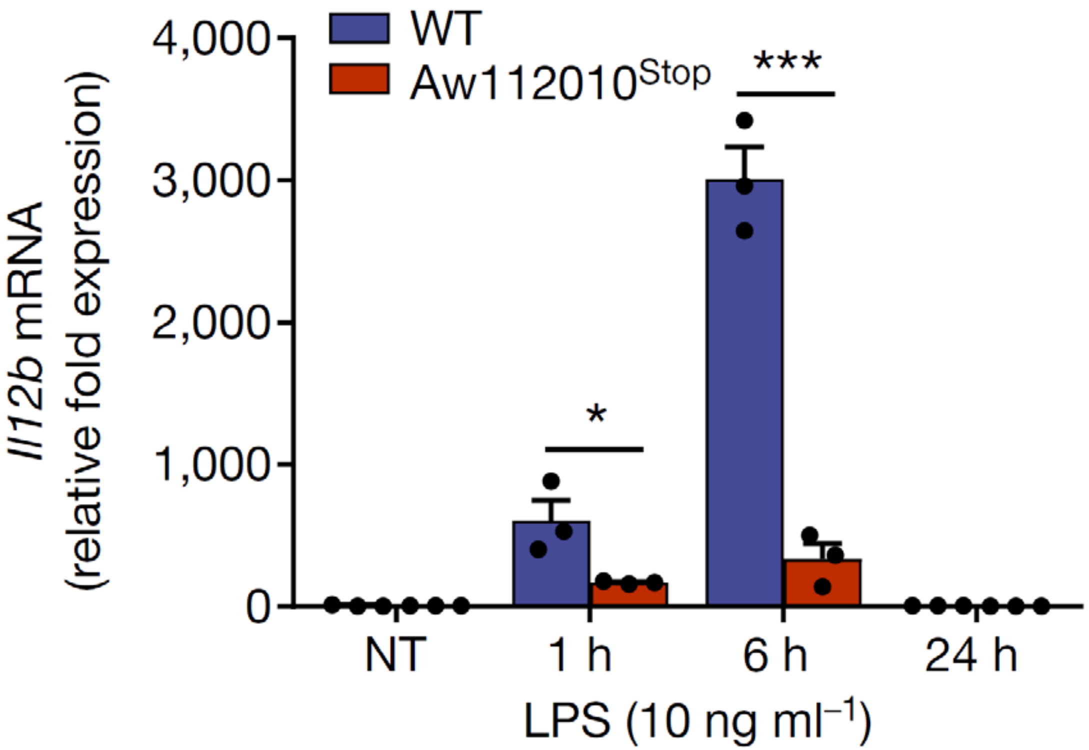


What is Aw112010's mechanism of action?

Phagocytosis, phagosome acidification, intracellular killing, and pyroptosis were unaltered in Aw112010^{Stop} macrophages.

However, production of **IL-12 and IL-6** was impaired! (IL-10 was unaltered)

The cytokine IL-12 is crucial for defense against salmonella.



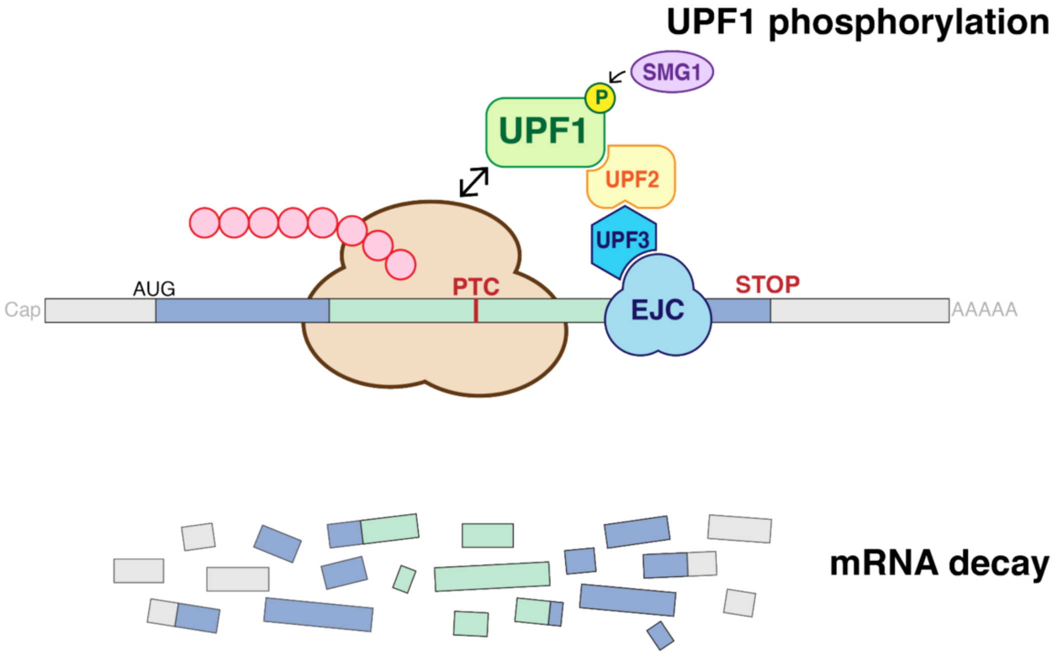
Objection: This still does not prove that the protein product of *Aw112010* accounts for the phenotype of Aw112010$^{\text{Stop}}$ mice.

The lncRNA itself might perform the function.

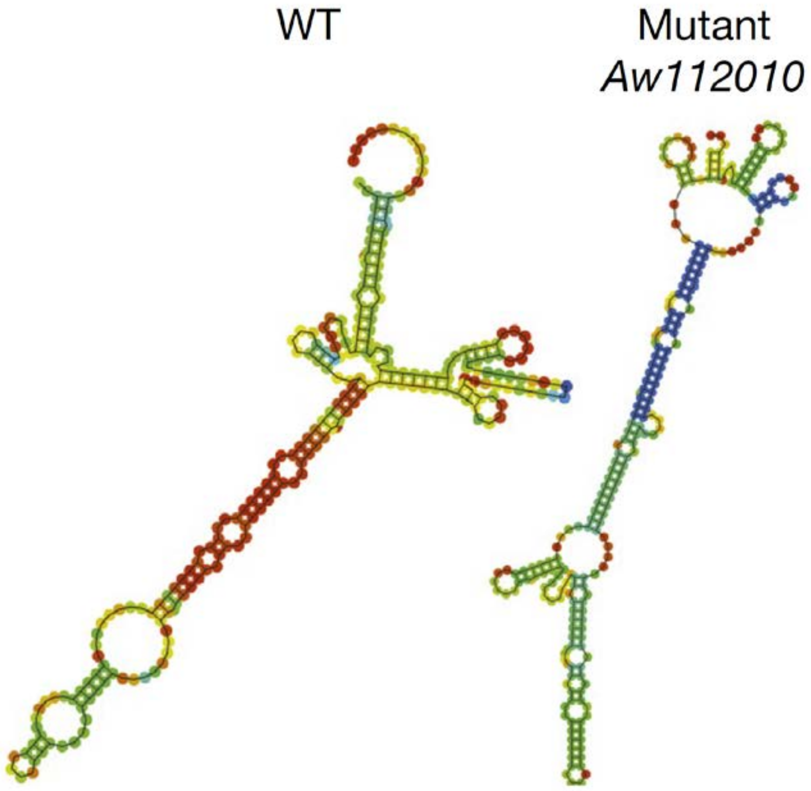
Indeed, the authors found that the altered Aw112010$^{\text{Stop}}$ transcript is subject to nonsense-mediated decay (NMD), which leads to rapid destruction of the RNA.

Nonsense-mediated decay is a mechanism of the cell to protect against **nonsense mutations** (premature stop codons)

- After splicing, exon junctional complexes (EJC) are placed on exon-exon junctions.
- In the first ever round of translation, these are removed.
- In subsequent rounds of translation, encountering an EJC triggers nonsense-mediated decay.

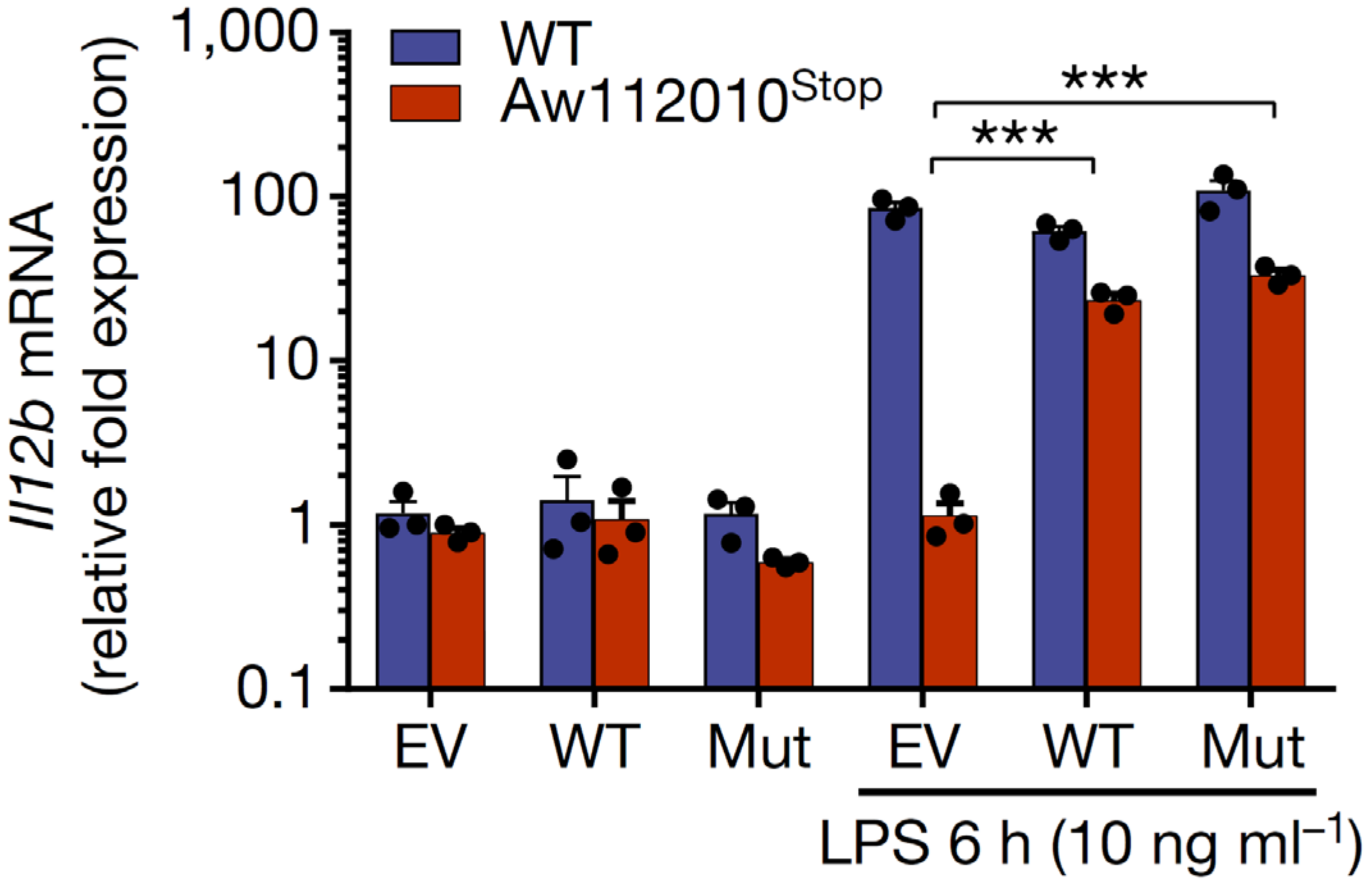


The authors created a very different version of *Aw112010,* where the nucleic acid sequence is heavily mutated, but the amino acid sequence remains the same



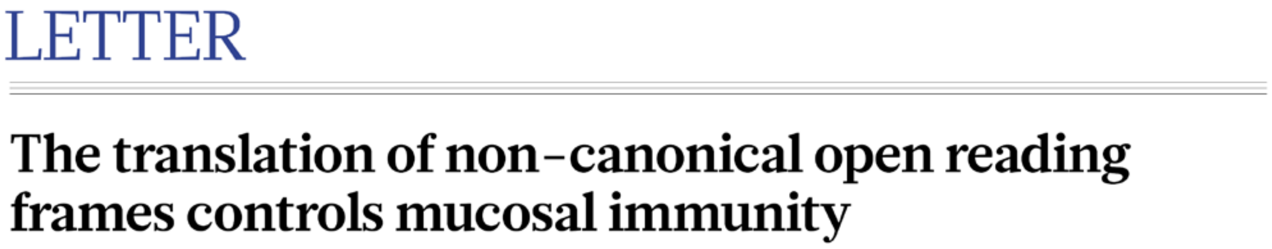
The predicted RNA secondary structures are very different

Re-introduction of both the wild-type and heavily mutated *Aw112010* into Aw112010^{Stop} mice rescued IL-12 production



Plasmids expressing Aw112010 or the empty vector (EV) were delivered to bone marrow-derived macrophages by electroporation.

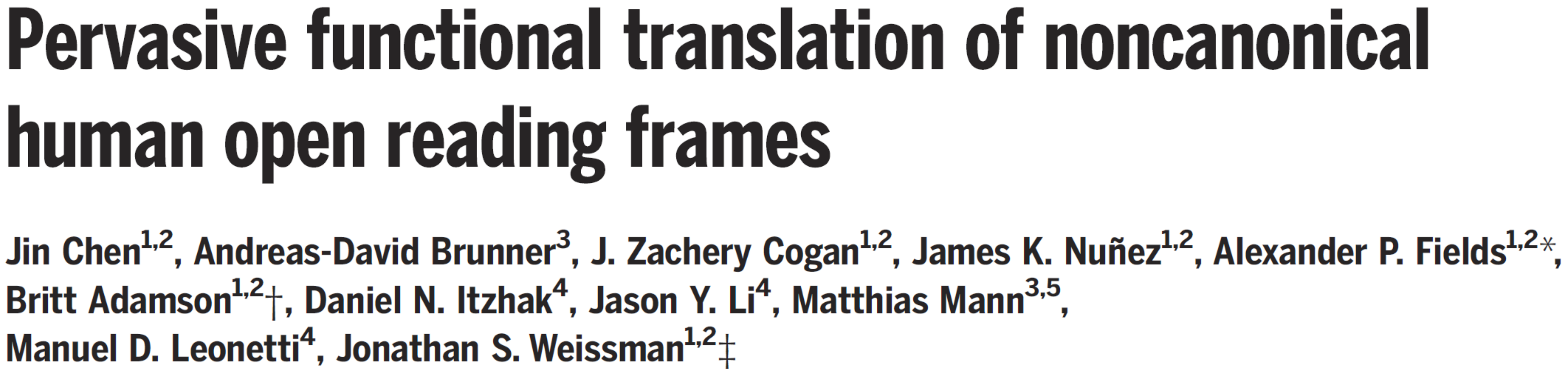
Conclusions



LETTER

The translation of non-canonical open reading frames controls mucosal immunity

- **Profiling translation in specific situations** (e.g. infection, LPS stimulation) can lead to the discovery of previously unknown, functional proteins.
- Various bioinformatics tools exist for **re-analyzing Ribo-Seq** data to identify translated ORFs *de novo*.
- Some open questions remain:
  - What are the functions of the other translated lncORFs?
  - What role does *Aw112010* play in humans?



Pervasive functional translation of noncanonical human open reading frames

Jin Chen[1,2], Andreas-David Brunner[3], J. Zachery Cogan[1,2], James K. Nuñez[1,2], Alexander P. Fields[1,2]*, Britt Adamson[1,2]†, Daniel N. Itzhak[4], Jason Y. Li[4], Matthias Mann[3,5], Manuel D. Leonetti[4], Jonathan S. Weissman[1,2]‡

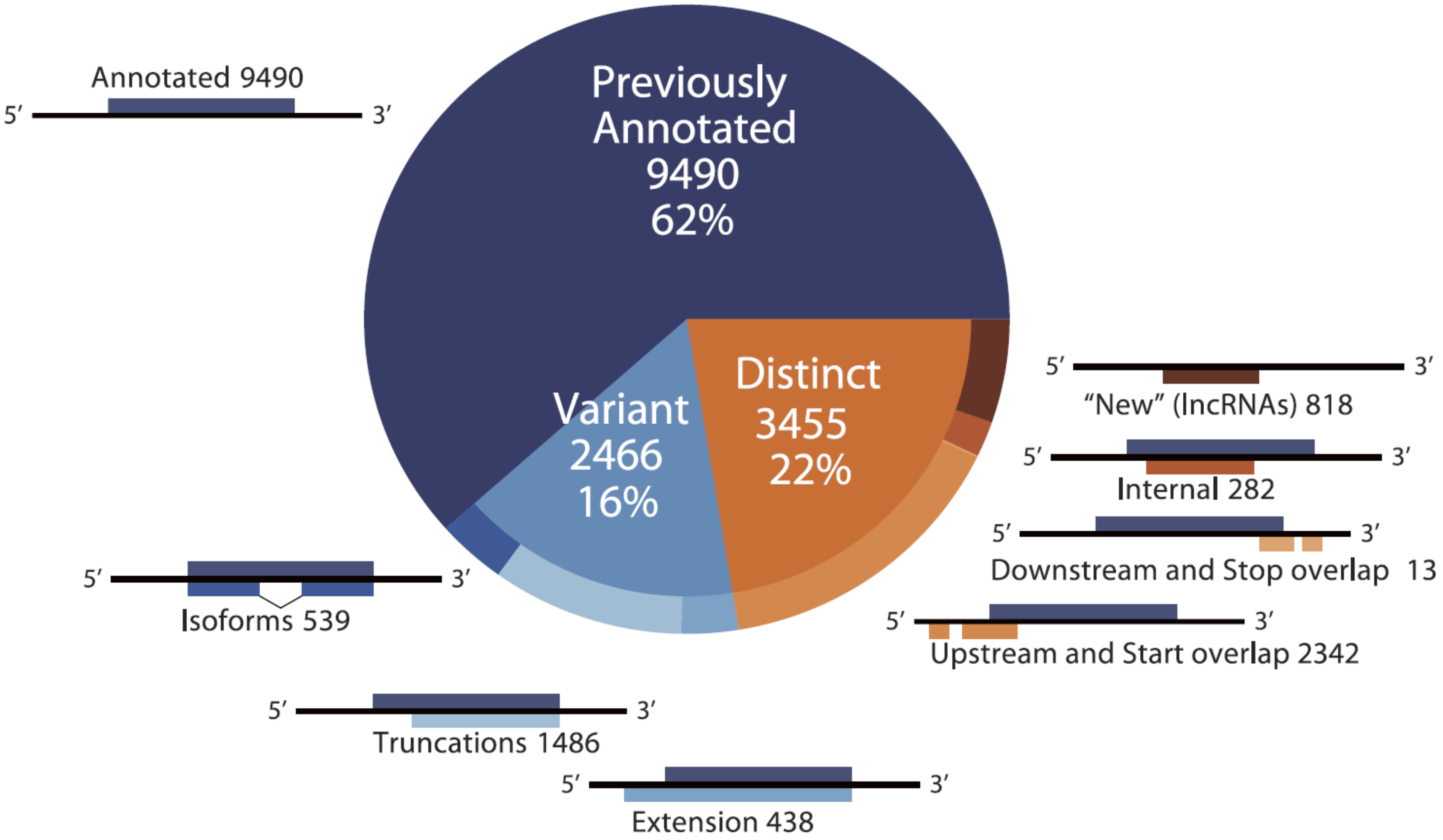
Science, March 2020

The authors aimed to obtain a **global view** of functional non-canonical ORFs.

1. First, they generated a large **Ribo-Seq** dataset, and used ORF-RATER to identify potential ORFs.
2. A specialized **CRISPR-ko library** was then constructed to target 2353 non-canonical ORFs.

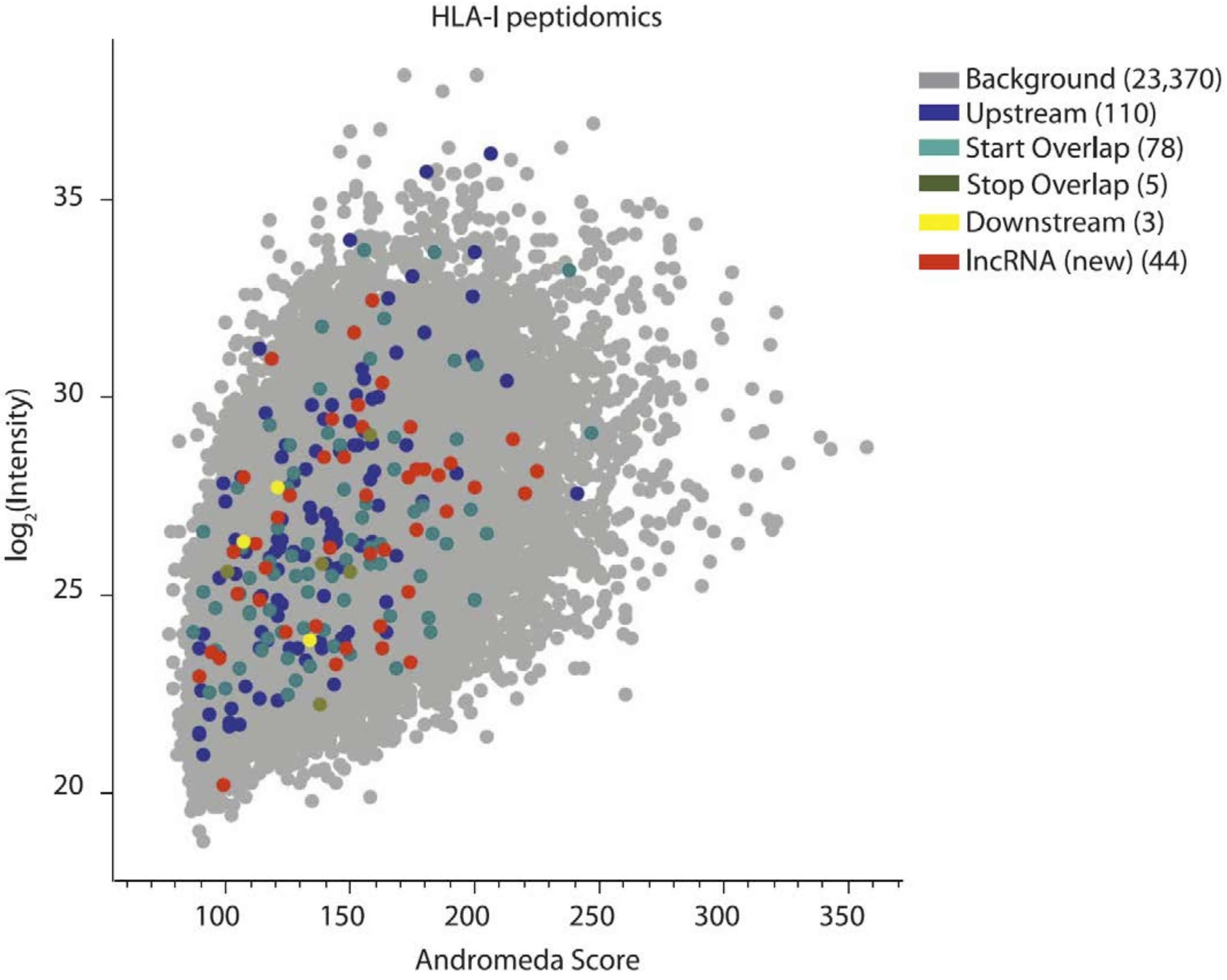
   → A **pooled screen** identified >500 ORFs whose knockout caused a **fitness defect**.
3. Selected hits were validated.

Analysis of Ribo-Seq data using ORF-Finder identifies 38% new ORFs



Data from human induced pluripotent stem cells (iPSCs), iPSC-derived cardiomyocytes, and human foreskin fibroblasts (HFFs) were pooled

HLA-I peptidomics (mass spectrometry of peptides eluted from HLA-I) confirms 240 non-canonical peptides



Design of a CRISPRko library targeting non-canonical ORFs

12044 ORFs

Select high confidence ORFs identified from ribosome profiling (ORF-RATER score > 0.8, length ≥ 10 aa)

↓ 2596 ORFs

Design sgRNAs targeting each ORF

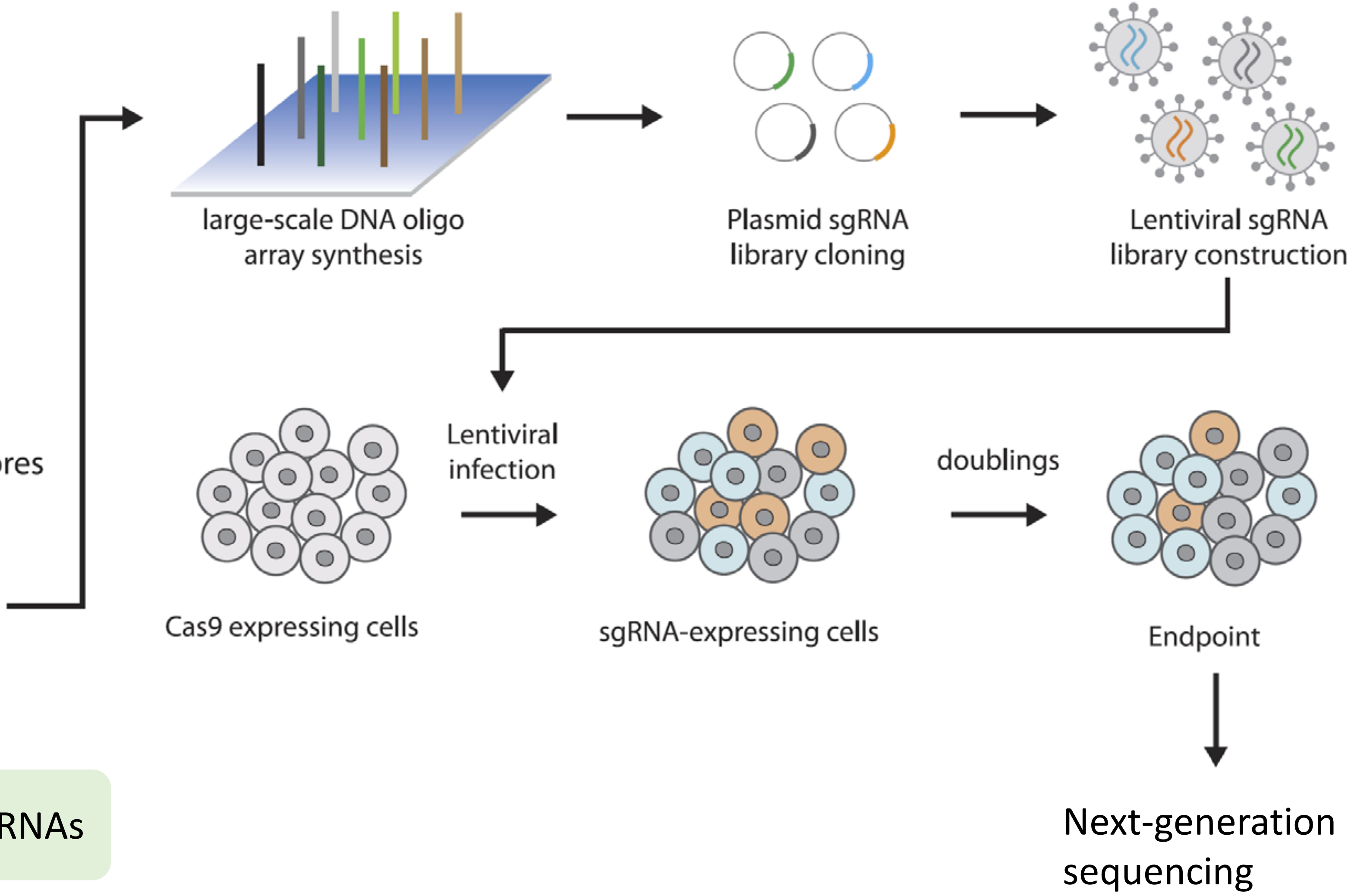
↓

Select top 10 sgRNAs based on on-target scores

↓

Filter sgRNAs with off-targets

2353 ORFs


large-scale DNA oligo array synthesis
Plasmid sgRNA library cloning
Lentiviral sgRNA library construction
Cas9 expressing cells
Lentiviral infection
sgRNA-expressing cells
doublings
Endpoint
Next-generation sequencing

GuideScan was used to choose the sgRNAs

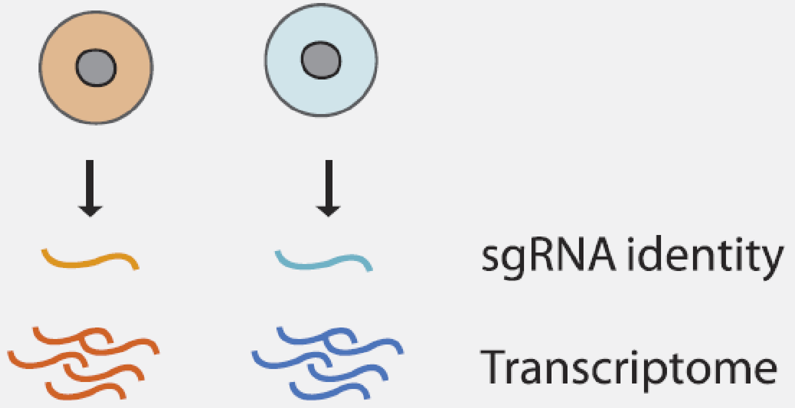
The endpoints included cell fitness/growth, as well as transcriptional changes (Perturb-Seq)

Growth

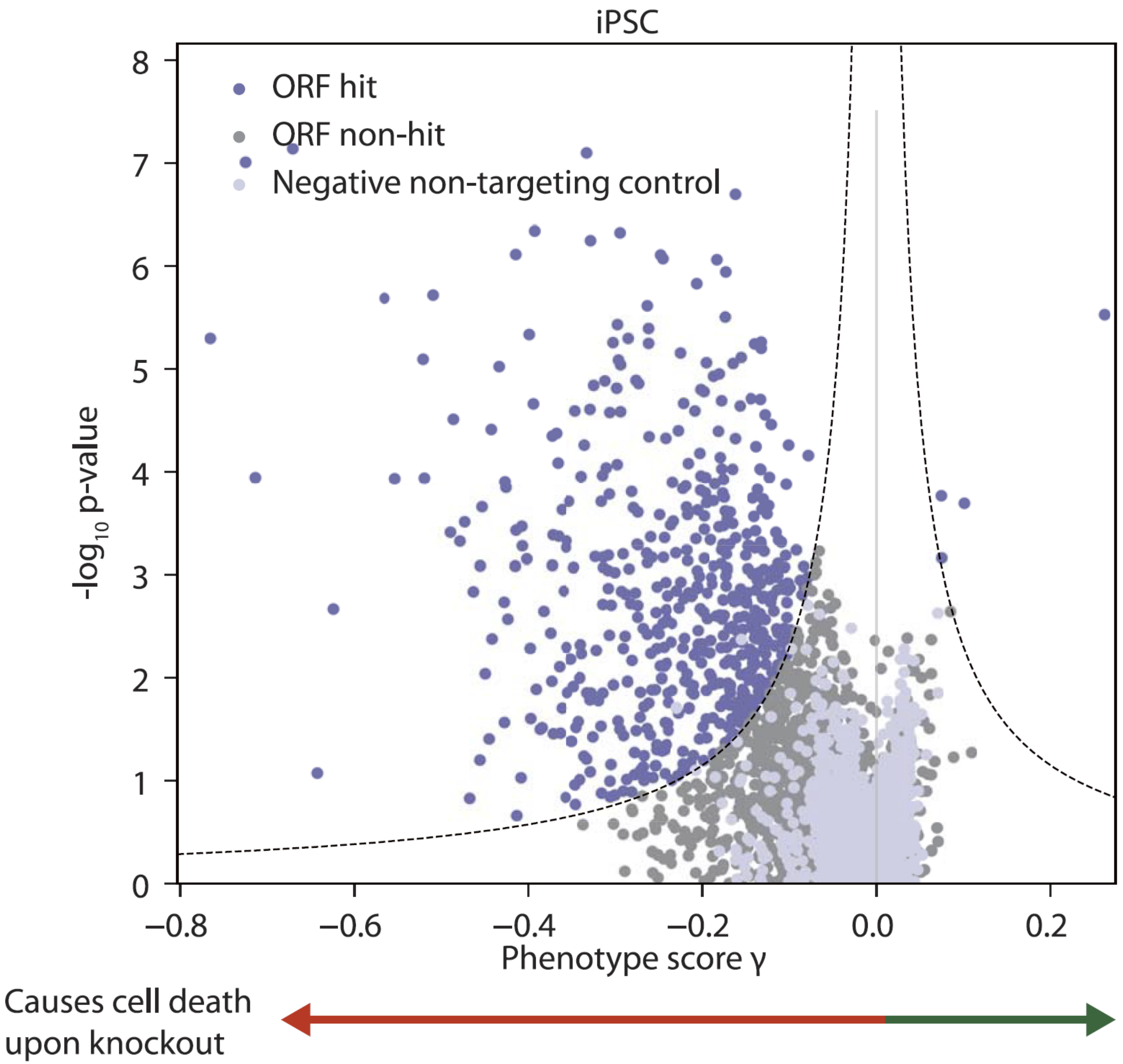
$$\frac{\log_2 \text{sgRNA enrichment}}{\text{cell doublings}}$$

$$= \text{growth phenotype } (\gamma)$$

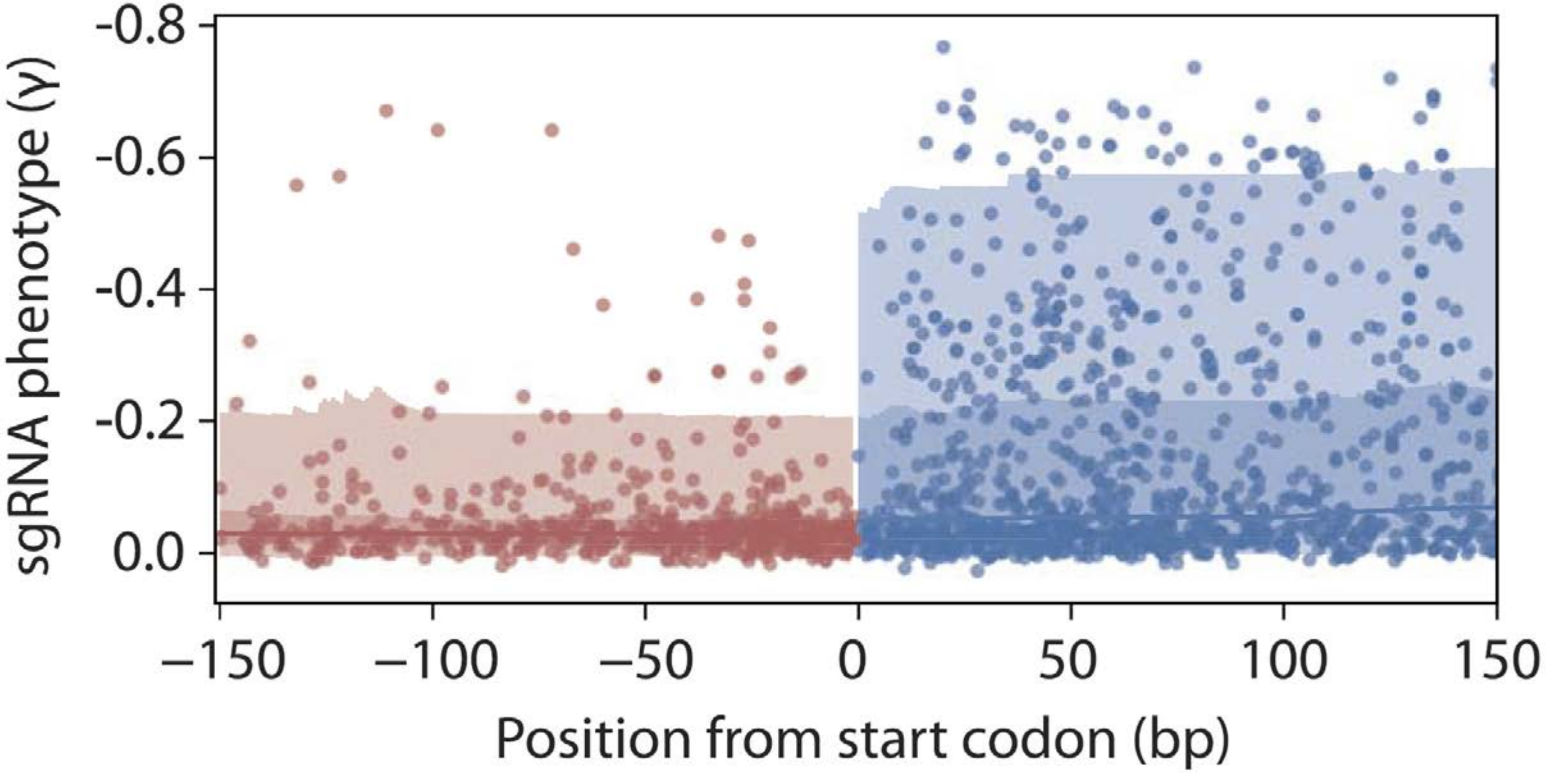
Single-cell RNA-seq (Perturb-Seq)

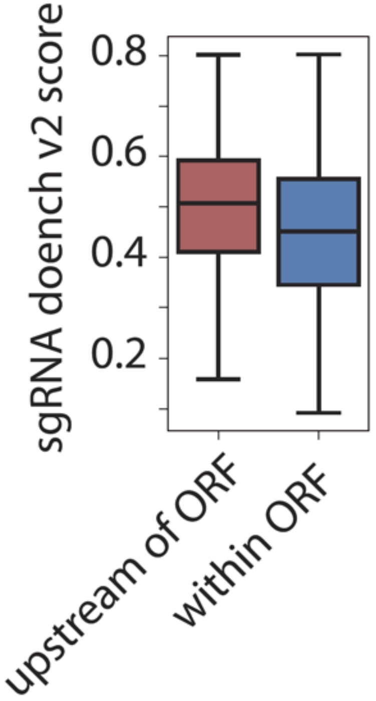


> 500 ORFs were found to influence fitness in iPSCs!



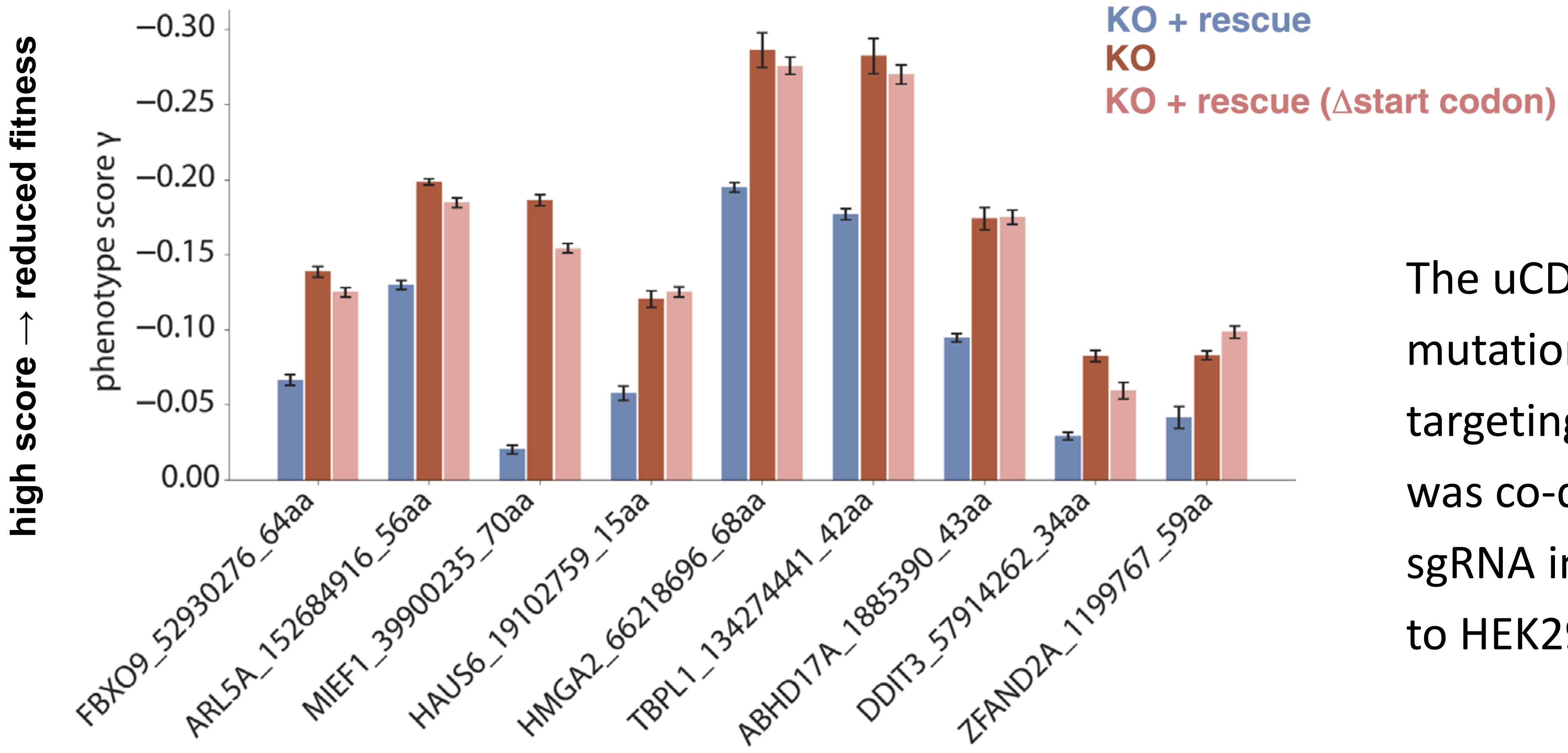
Guides targeting the ORFs showed much higher fitness effects than control guides





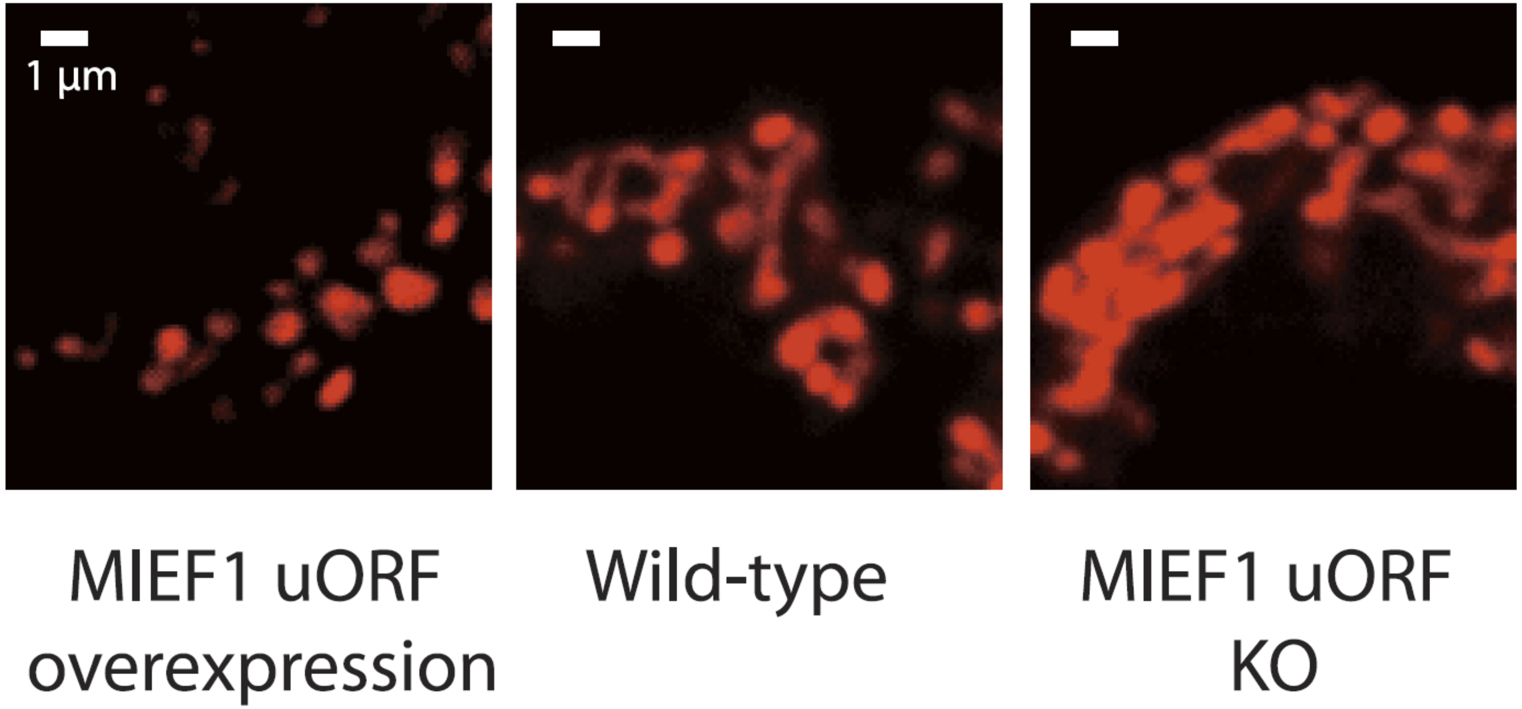
predicted efficiency scores were the same

Selected upstream ORFs were confirmed by ectopically expressing a transcript that encodes **only the uORF peptide**



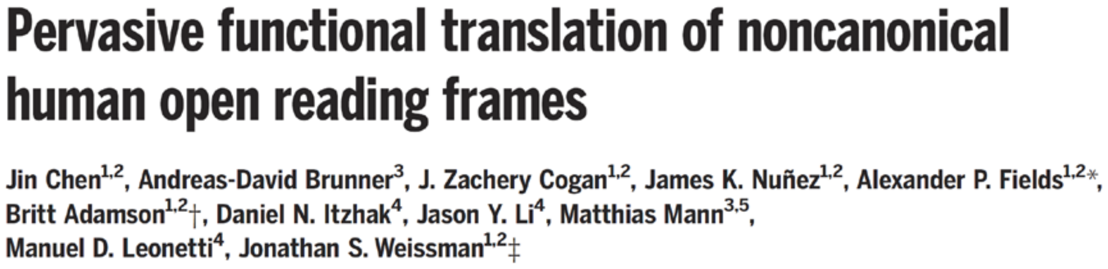
The uCDS (with synonymous mutations to prevent targeting by the sgRNA) was co-delivered with the sgRNA in a lentiviral vector to HEK293 cells.

Example: Overexpression of the MIEF1 uORF increased mitochondrial *fission*, whereas its knockout increased *fusion*



MIEF1 = Mitochondrial Elongation Factor 1

Conclusions



Pervasive functional translation of noncanonical human open reading frames

Jin Chen[1,2], Andreas-David Brunner[3], J. Zachery Cogan[1,2], James K. Nuñez[1,2], Alexander P. Fields[1,2]*, Britt Adamson[1,2]†, Daniel N. Itzhak[4], Jason Y. Li[4], Matthias Mann[3,5], Manuel D. Leonetti[4], Jonathan S. Weissman[1,2]‡

- A CRISPR screen demonstrates that hundreds of non-canonical ORFs have significant fitness effects.
    - ORFs that are dispensible for cell growth or survival, but have specific other functions, are not detected.
- Many upstream ORFs encode for functional proteins.
    - These are sometimes related to the function of canonical ORF.

Thank you for your attention!