

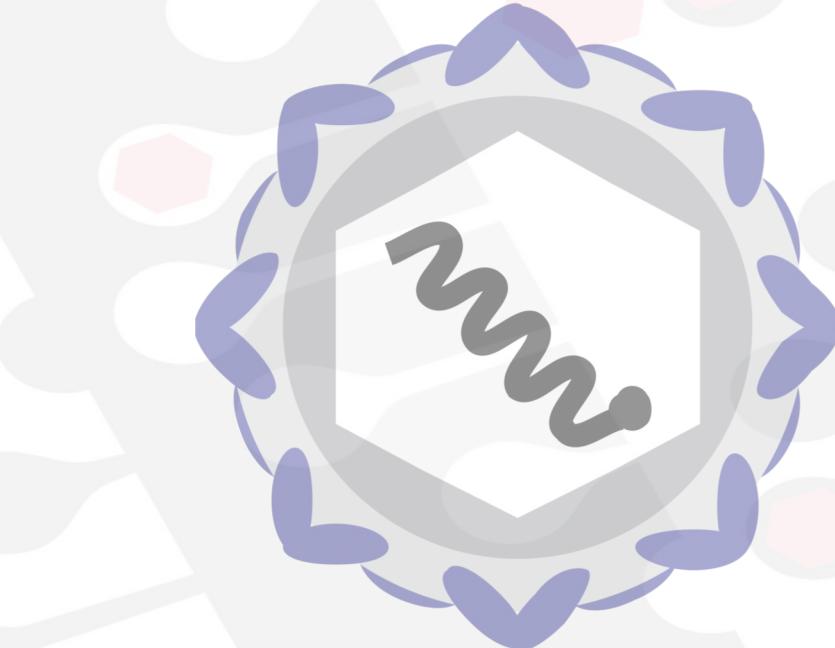
# **RNA modifications: $\text{m}^6\text{A}$ on viral RNA genomes**

**Stacy M Horner, PhD**

Associate Professor

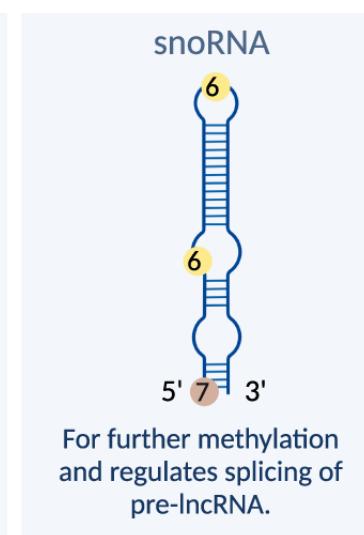
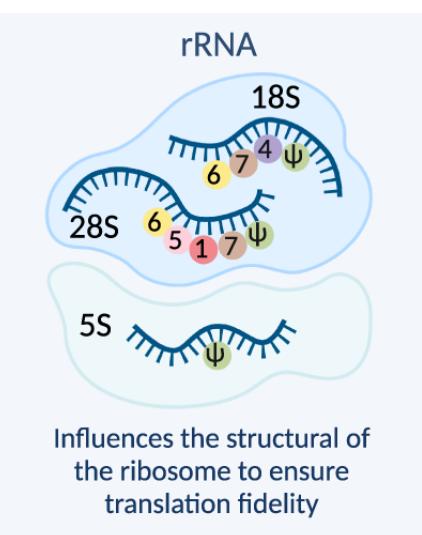
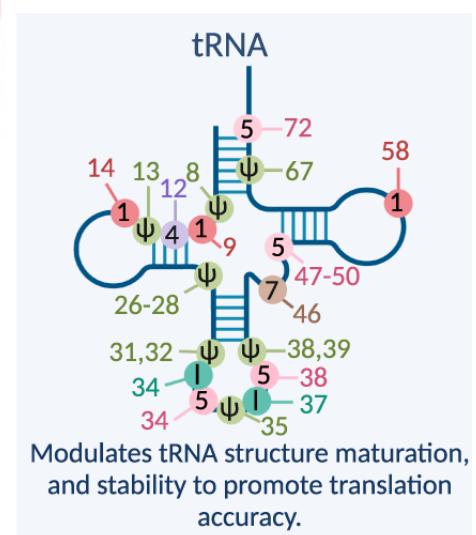
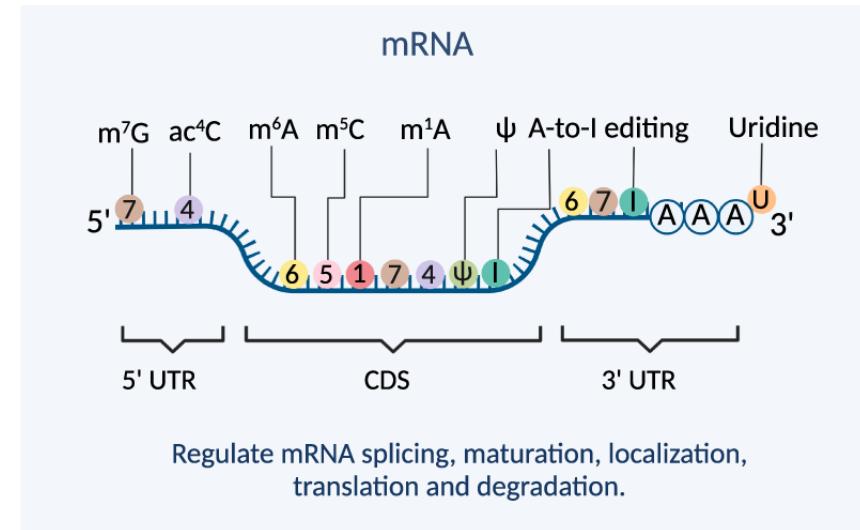
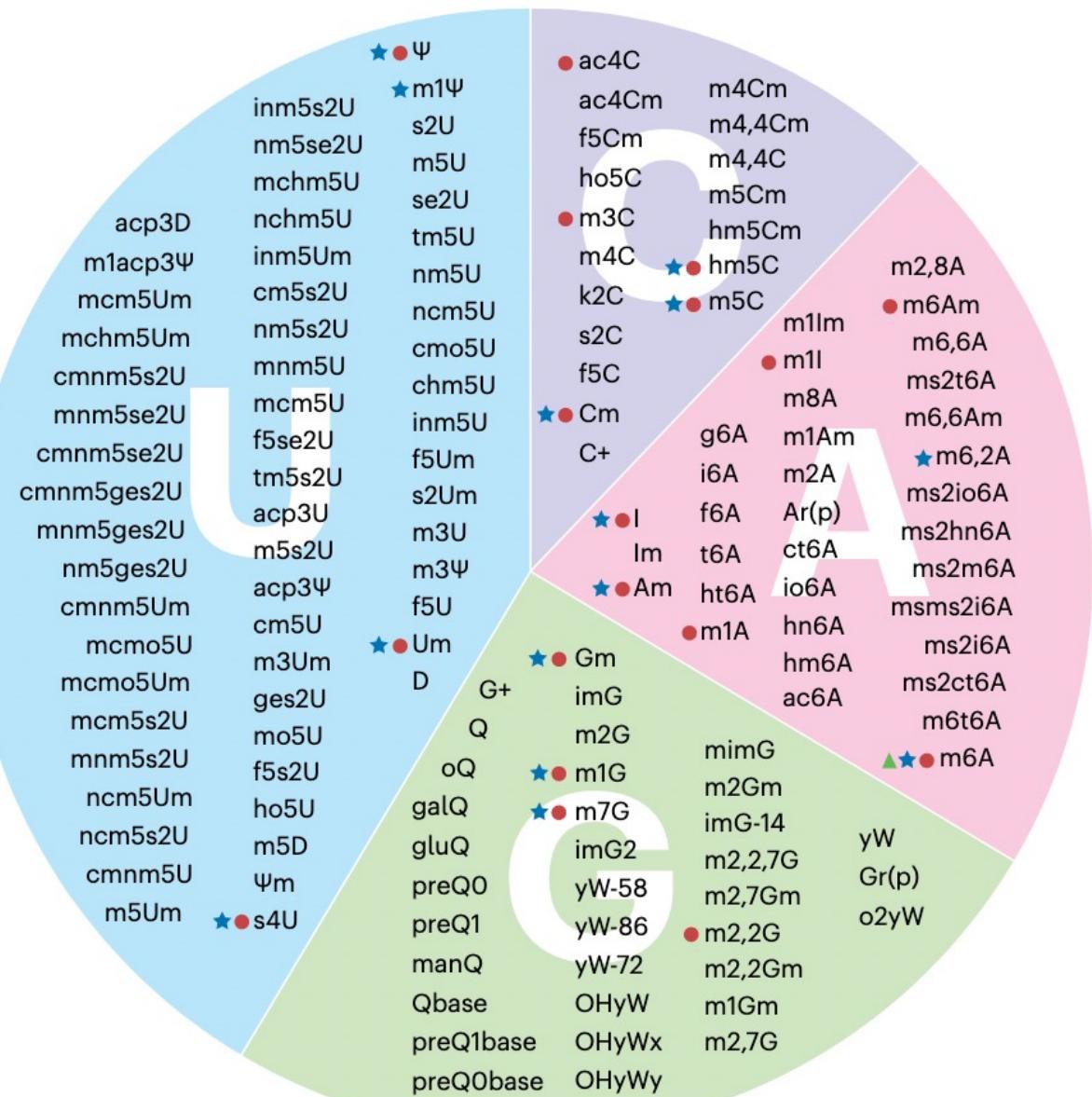
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@DukeRNACenter

# RNA modifications diversify and expand the genetic code

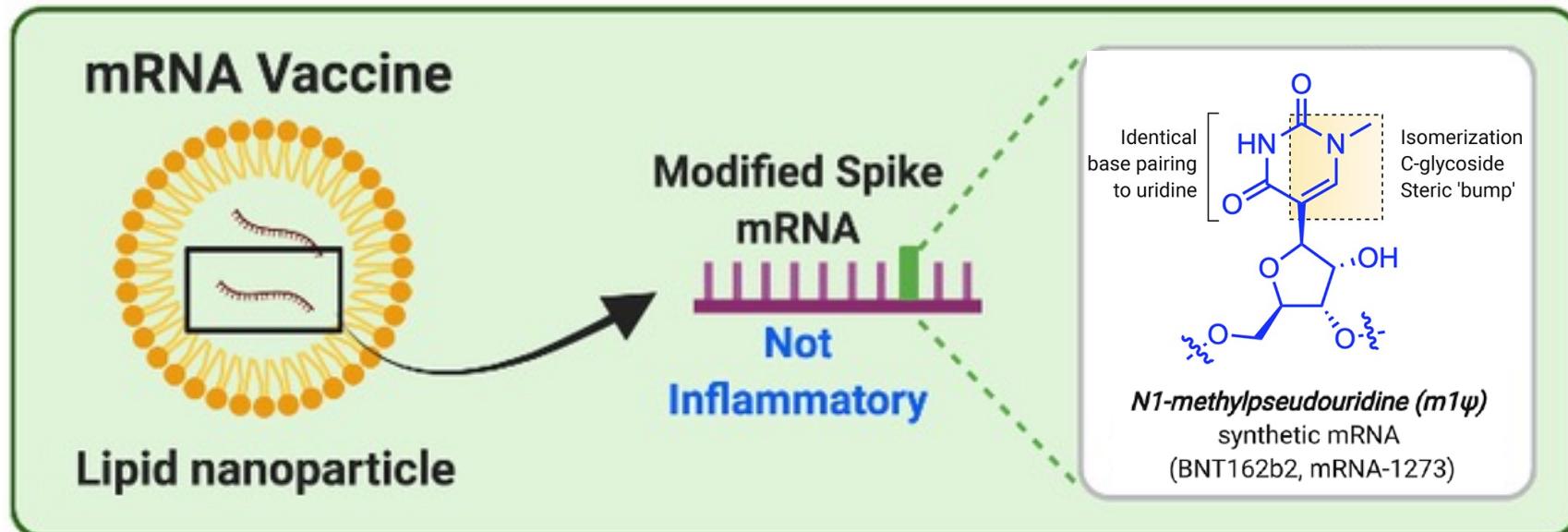
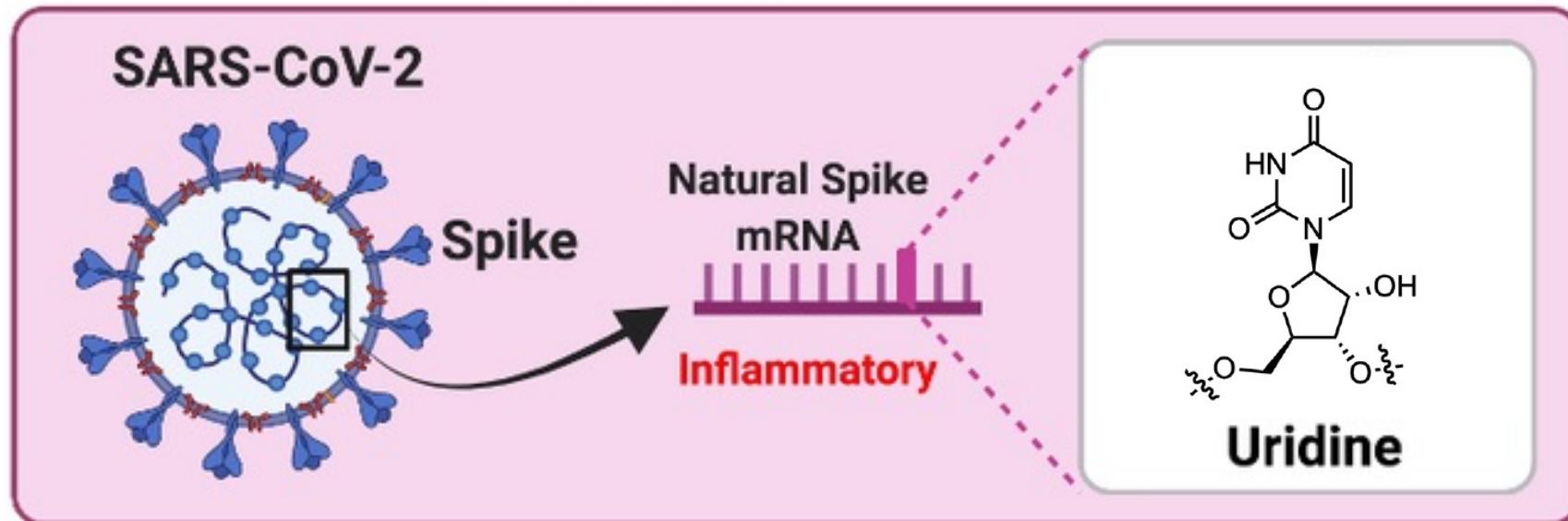


# Roles for RNA modifications and machinery in cancer

- Acute myeloid leukemia
- Hepatocellular cancer
- Glioblastoma
- Breast cancer
- Lung cancer

Gene symbol	Type of enzyme	Cancer type	Role	Main target	Expression in cancer	Refs
<b>m<sup>6</sup>A</b>						
METTL3–METTL14	Writer	AML	Oncogene	MYC, SP1, SP2	Upregulated	37–39
		Lung cancer	Oncogene	EGFR	Upregulated	40
		Hepatocellular cancer	Oncogene	SOCS2, SNAI1	Upregulated	41,42
		Hepatocellular cancer	Tumour suppressor	miRNAs	Downregulated	43
		Endometrial cancer	Tumour suppressor	PHLPP2	Mutated or downregulated	44
		Glioblastoma	Oncogene	SOX2	No change	45
		Glioblastoma	Tumour suppressor	ADAM19	No change	46
FTO	Eraser	Cutaneous melanoma	Oncogene	PDCD1, CXCR4, SOX10	Upregulated	50,51
		AML	Oncogene	ASB2, RARA	Upregulated	52
ALKBH5	Eraser	Glioblastoma	Oncogene	FOXM1	Upregulated	58
		Breast cancer	Oncogene	NANOG	No change	59
YTHDC2	Reader	Colorectal cancer	Oncogene	HIF1A	No change	60
YTHDF2	Reader	AML	Oncogene	TNFRSF1B	Upregulated	61
IGF2BP1	Reader	Hepatocellular cancer	Oncogene	SRF	No change	65
<b>m<sup>5</sup>C</b>						
NSUN1	Writer	Lung and prostate cancer	Oncogene	rRNA	Upregulated	76,77
NSUN2	Writer	Oesophageal squamous cell carcinoma	Oncogene	NMR (ncRNA)	Upregulated	79
		Bladder cancer	Oncogene	HDGF	No change	80
<b>m<sup>1</sup>A</b>						
ALKBH3	Eraser	Prostate and hepatocellular cancer	Oncogene	tRNA	No change	90,93
		Breast and ovarian cancer	Oncogene	CSF1	No change	92
<b>m<sup>7</sup>G</b>						
METTL1	Writer	Lung cancer	Tumour suppressor	Pri-let7	No change	98
		Glioblastoma	Oncogene	Unknown	Upregulated	104
<b>γ-Phosphate methylation</b>						
MEPCE	Writer	Breast cancer	Oncogene	7SK	No change	111
<b>α-Phosphate methylation</b>						
BCDIN3D	Writer	Breast cancer	Oncogene	Pre-mir-145	No change	113
<b>Pseudouridine</b>						
PUS10	Writer	Prostate cancer	Tumour suppressor	Unknown	No change	125
DKC1	Writer	Head and neck squamous cell carcinoma and breast cancer	Tumour suppressor	rRNA	Mutated or downregulated	127,128
		Lung cancer	Oncogene	TERC	Upregulated	130
<b>Uridylation</b>						
TUT1	Writer	Breast cancer	Tumour suppressor	BiK	Downregulated	133
		Osteosarcoma	Tumour suppressor	mir-24, mir-29	Downregulated	134
TUT4 and TUT7	Writer	Various	Oncogene	Pre-let7	No change	138–140
DIS3L2	Reader	Wilms tumours	Tumour suppressor	let-7, IGF2	Mutated	142,143

# RNA modifications can facilitate immune evasion



# RNA modifications can facilitate immune evasion

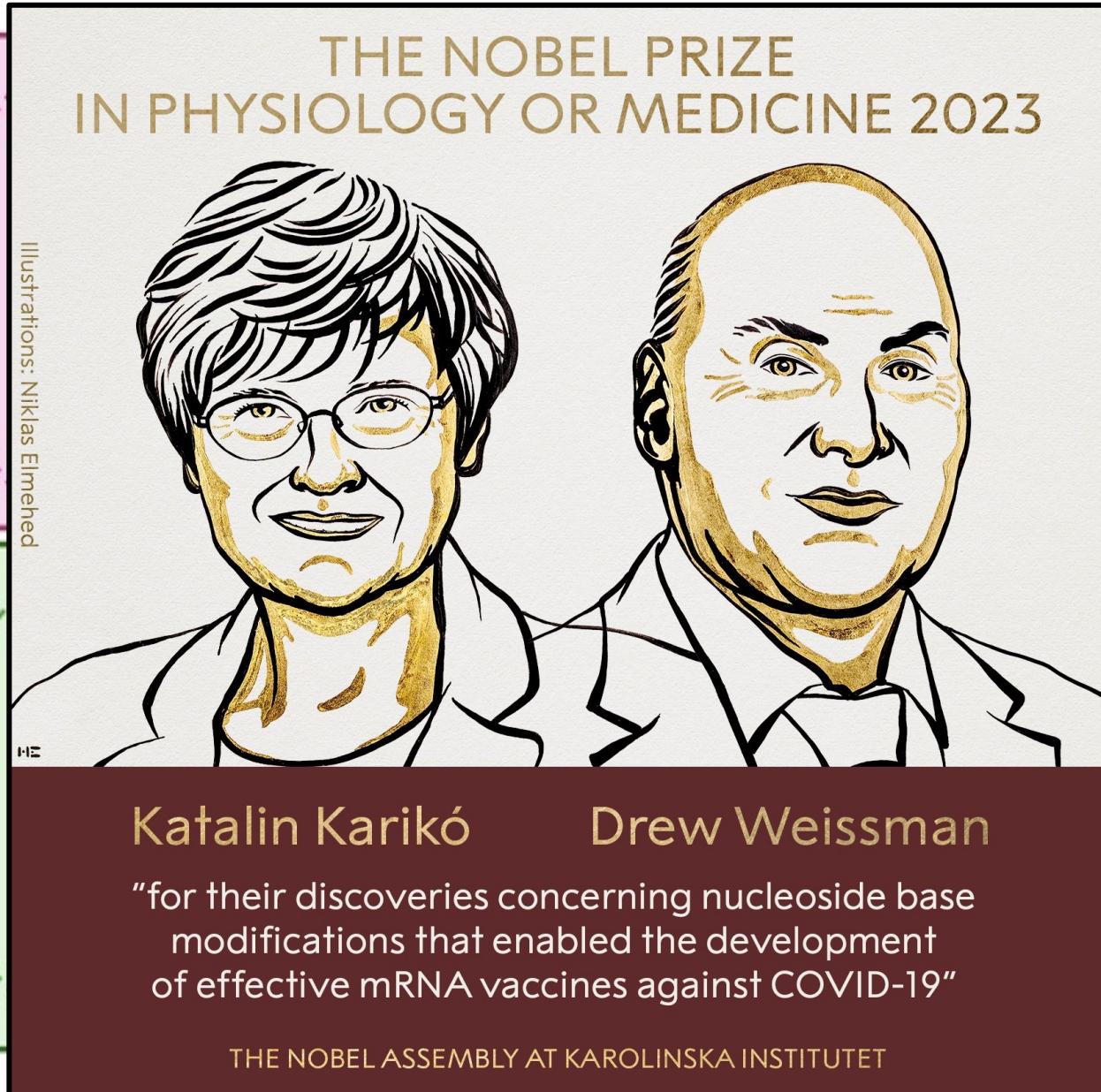
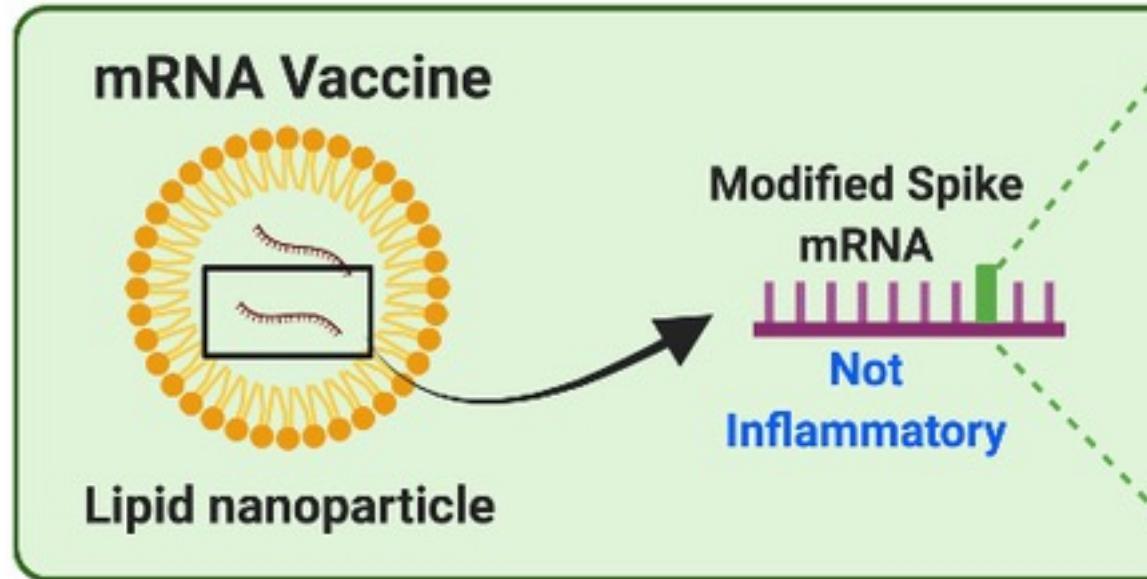
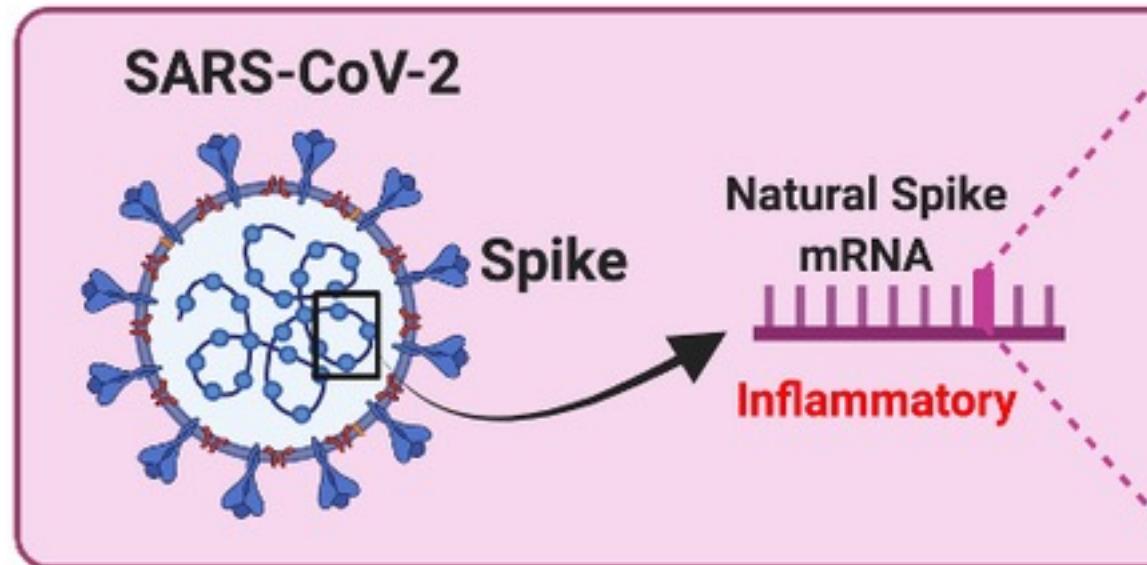
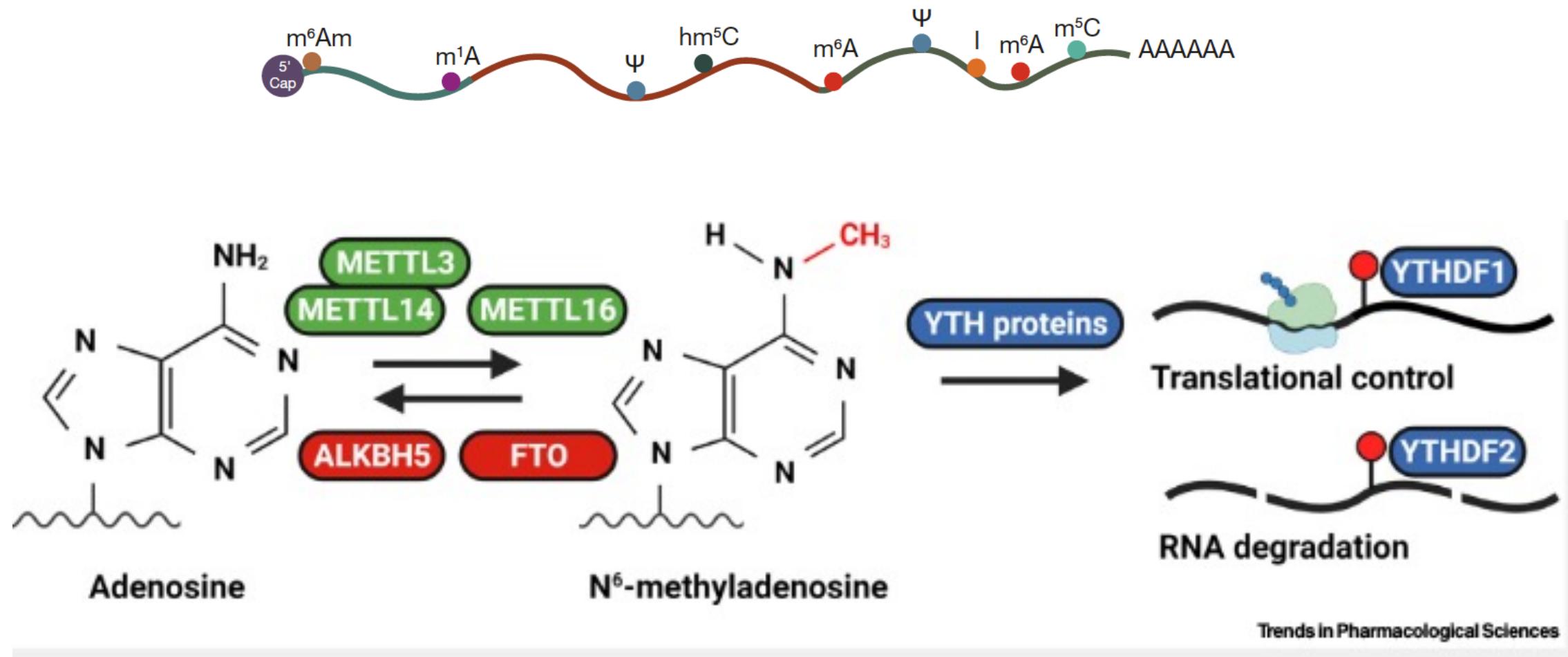


Table 1 | Companies targeting RNA epigenetics

Company	Named targets	Likely lead indication	Estimated phase I trial start date
STORM Therapeutics	METTL3, other methyl transferases	AML <sup>a</sup>	2021 <sup>a</sup>
Accent Therapeutics	METTL3, ADAR1	AML <sup>a</sup> , NSCLC <sup>a</sup>	2021 <sup>a</sup> , 2022 <sup>b</sup>
Gotham Therapeutics	METTL3, undisclosed 'reader', undisclosed 'eraser'	AML <sup>a</sup>	2021 <sup>a</sup>
EPICS Therapeutics	Undisclosed RNA modifying enzymes	Cancer	ND
Twentyeight-Seven Therapeutics	Undisclosed RNA modifying enzymes	Cancer	ND
Korro Bio	ADAR1	ND	ND

AML, acute myeloid leukaemia. <sup>a</sup>For METTL3 inhibitor. <sup>b</sup>For ADAR1 inhibitor. ND, no data available.

# $\text{m}^6\text{A}$ is a reversible mRNA modification with a dedicated machinery



$\text{m}^6\text{A}$  controls RNA-protein interactions  
YTHDF proteins “read”  $\text{m}^6\text{A}$

PMID: 38103979

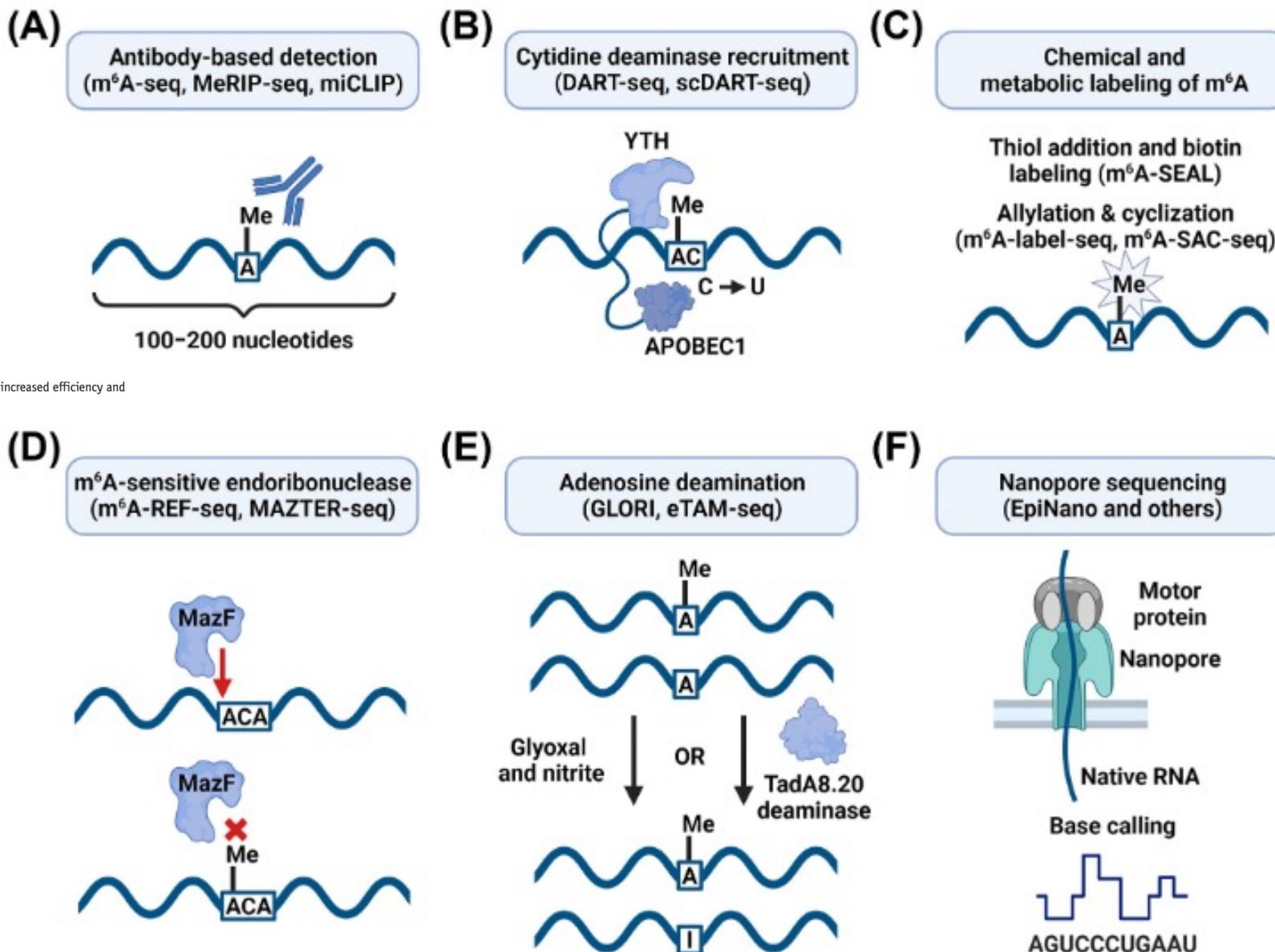
# How to identify and map m<sup>6</sup>A?

## MeRIP-seq

Method of the Year 2016:  
Epitranscriptome analysis

Chemical modifications on ribonucleotides are being profiled with increased efficiency and appreciated as important regulatory features.

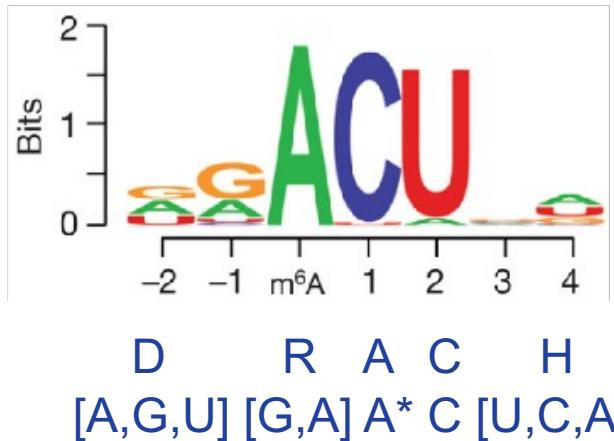
NATURE METHODS | VOL.14 NO.1 | JANUARY 2017 | 1



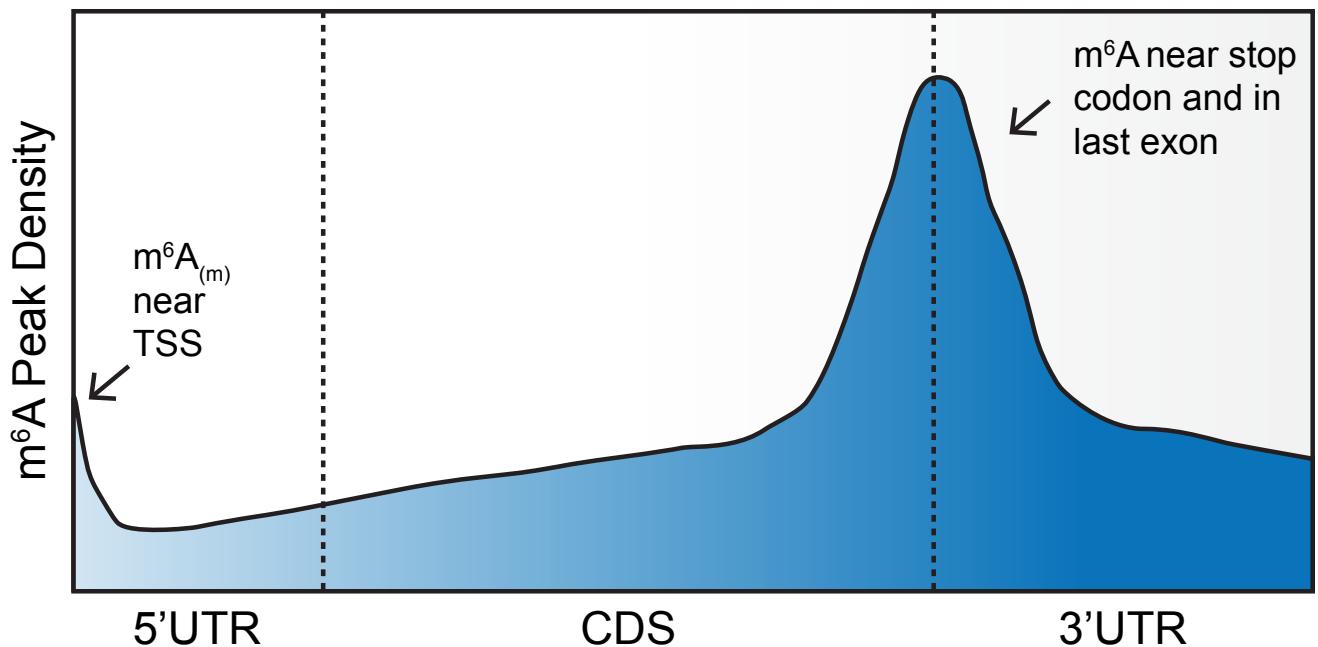
# $\text{m}^6\text{A}$ -mapping studies reveal its distribution

## Consensus Motif

DRA $^{\text{m}}\text{CH}$



## Metagene analysis of m<sup>6</sup>A distribution



What determines which DRACH sites are m<sup>6</sup>A-modified?

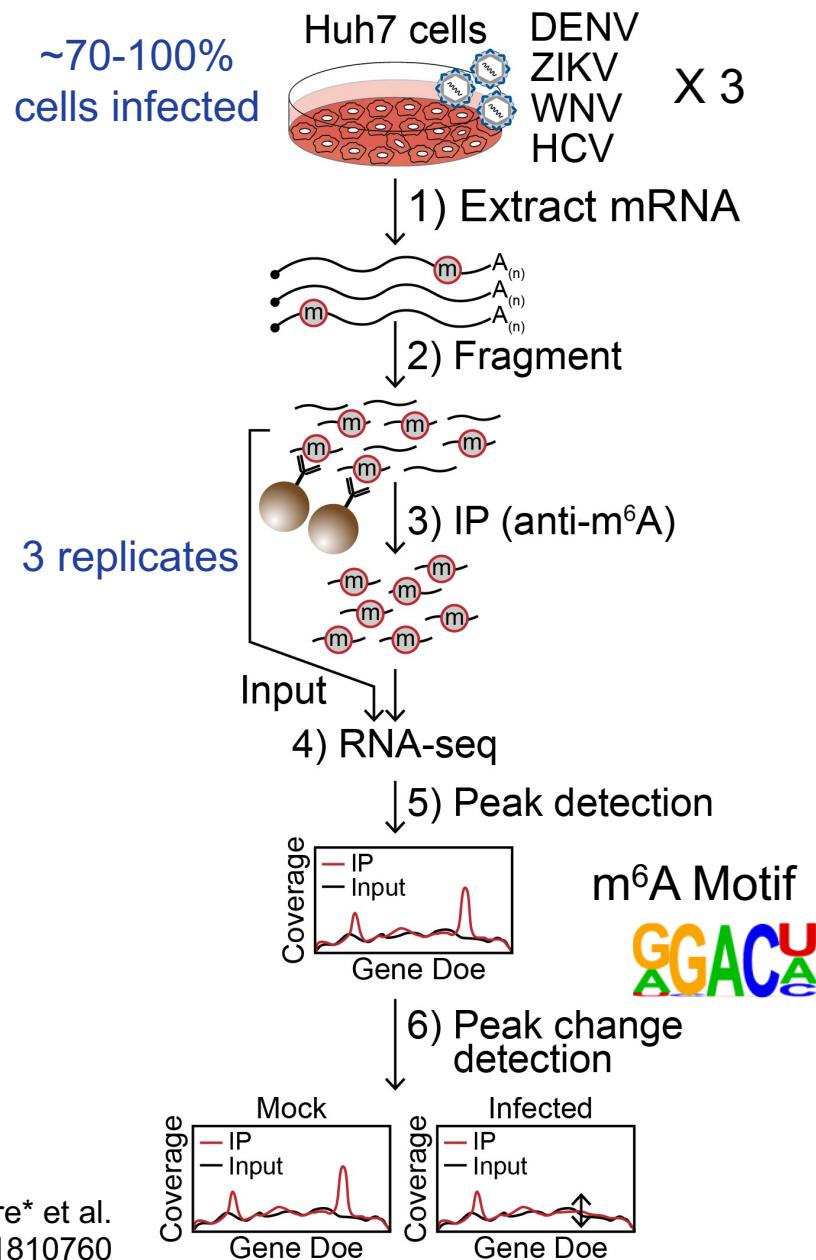
- The EJC limits m<sup>6</sup>A modification within exons.
- RNA binding proteins and specific histone modifications promote.

The methylation level of individual m<sup>6</sup>A sites varies

Meyer et al., 2012; PMID: 22608085  
Dominissini et al., 2012; PMID: 22575960  
Meyer and Jaffrey, 2014; PMID: 24713629

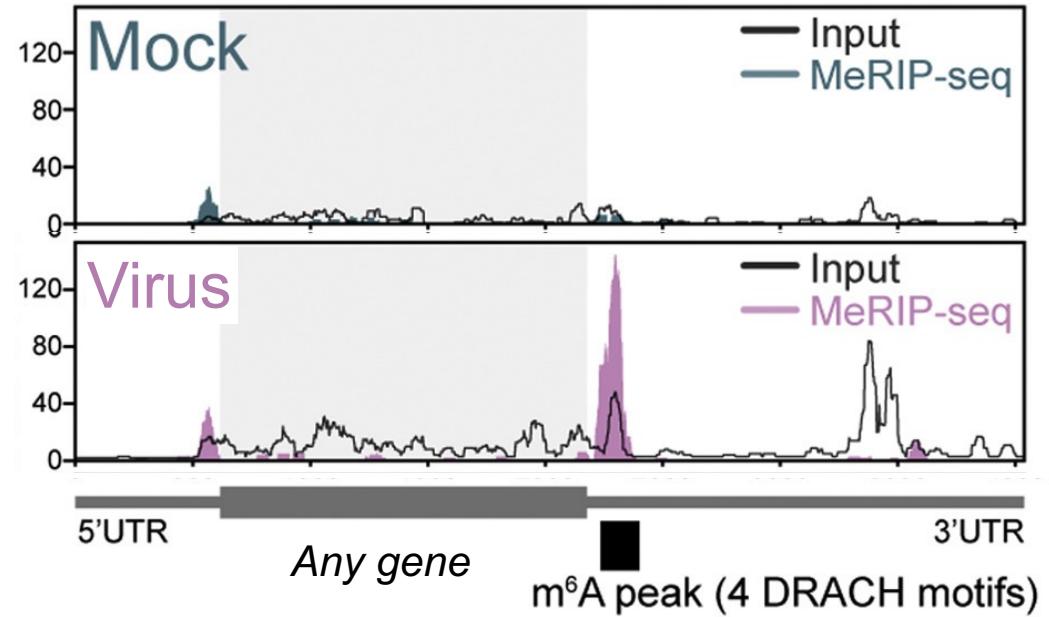
Yang et al., 2022; PMID: 36550132  
Uzonyi et al., 2023; PMID: 36599352  
Liu J. et al., 2023; PMID: 31949099  
Liu C. et al., 2023; PMID: 36302990

# Defining virally-induced changes in the host m<sup>6</sup>A epitranscriptome



Developed a new model for m<sup>6</sup>A peak change detection:

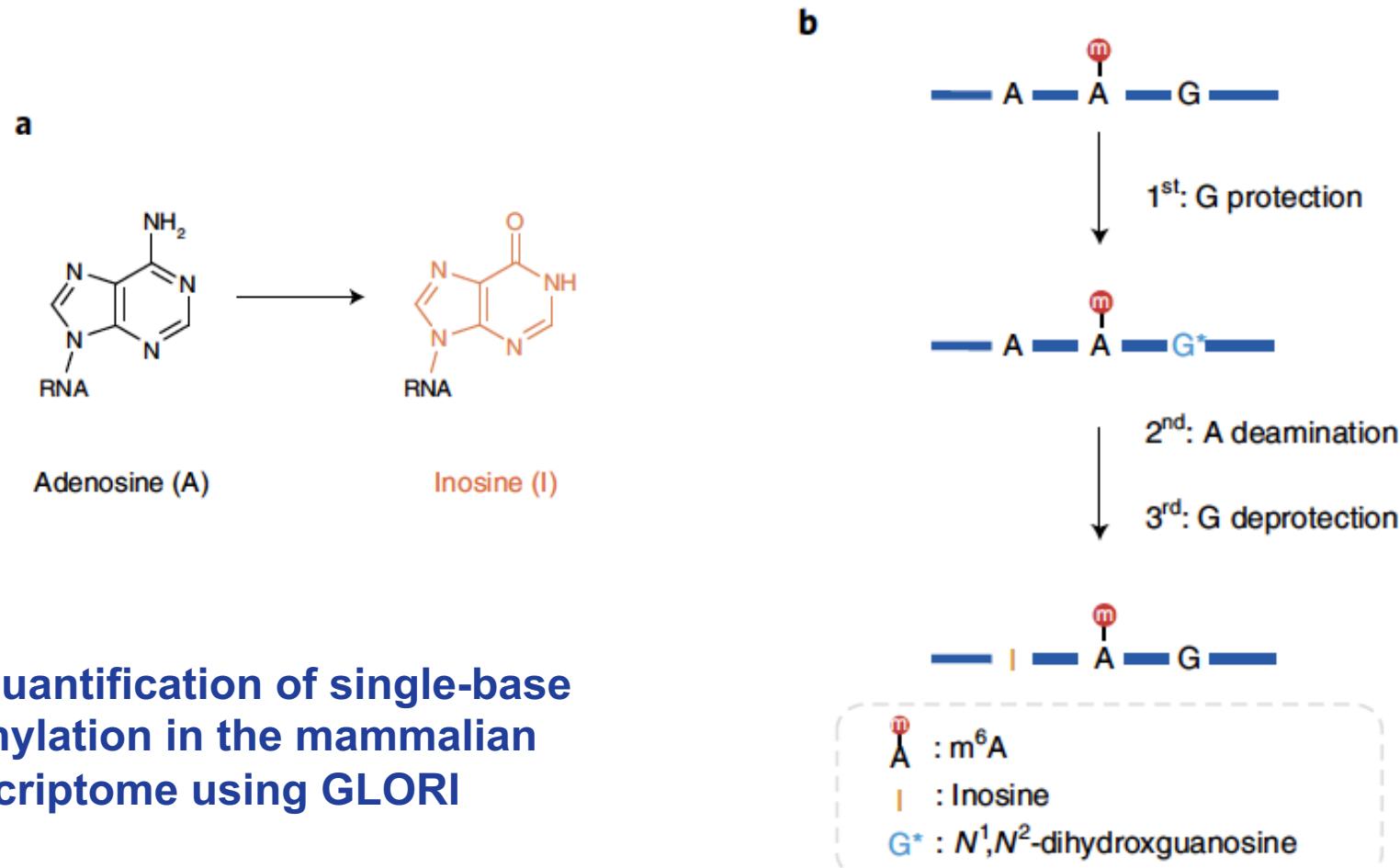
- Normalizes peak changes by gene expression.
- Uses well-calibrated statistical models to identify changes.



The enrichment of specific RNA fragments indicates the presence and location of m<sup>6</sup>A

# New methods define limits of antibody-based m6A mapping strategies

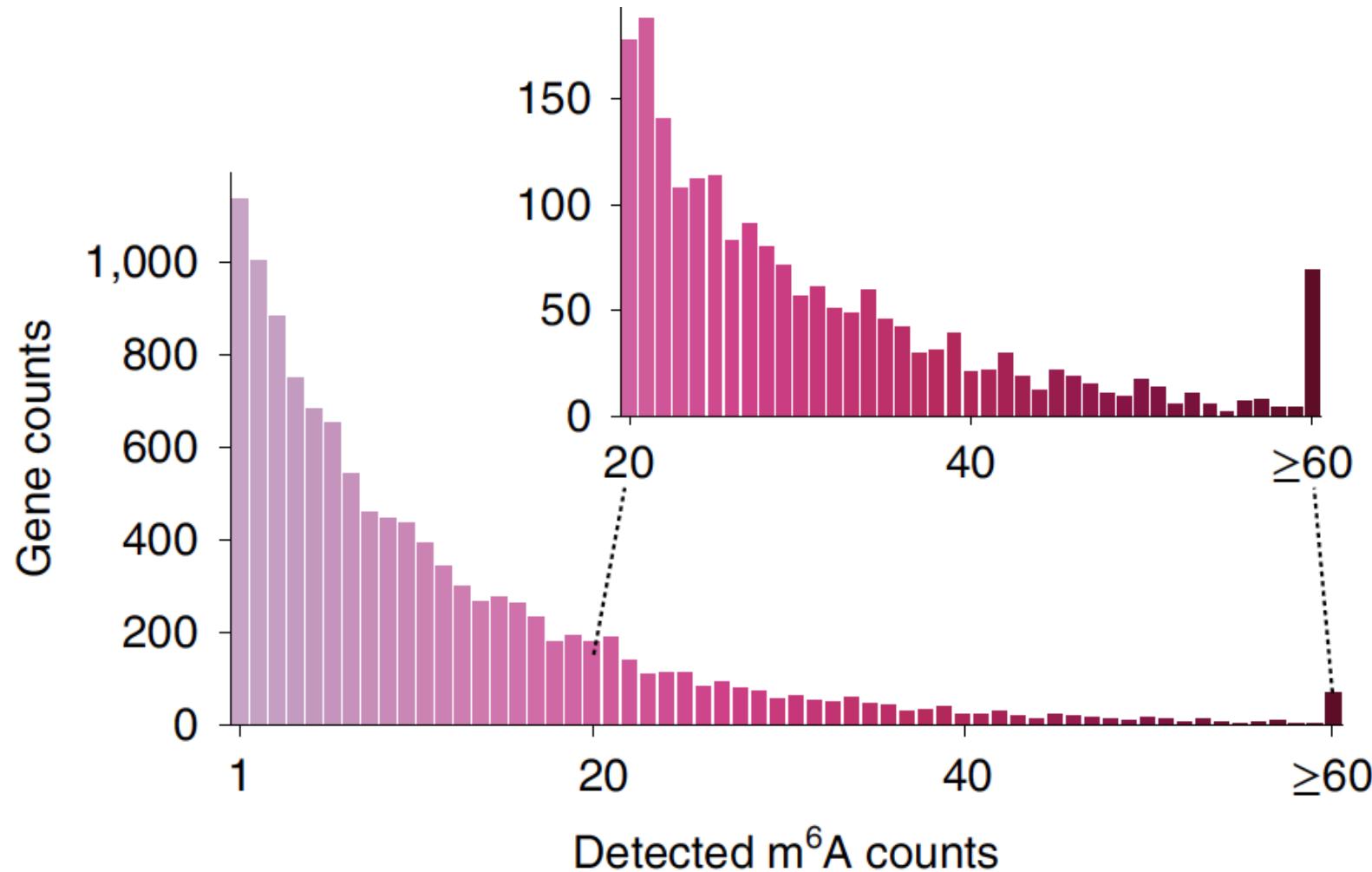
## GLORI (Glyoxal and nitrite-mediated adenosine deamination)



Absolute quantification of single-base  
m<sup>6</sup>A methylation in the mammalian  
transcriptome using GLORI

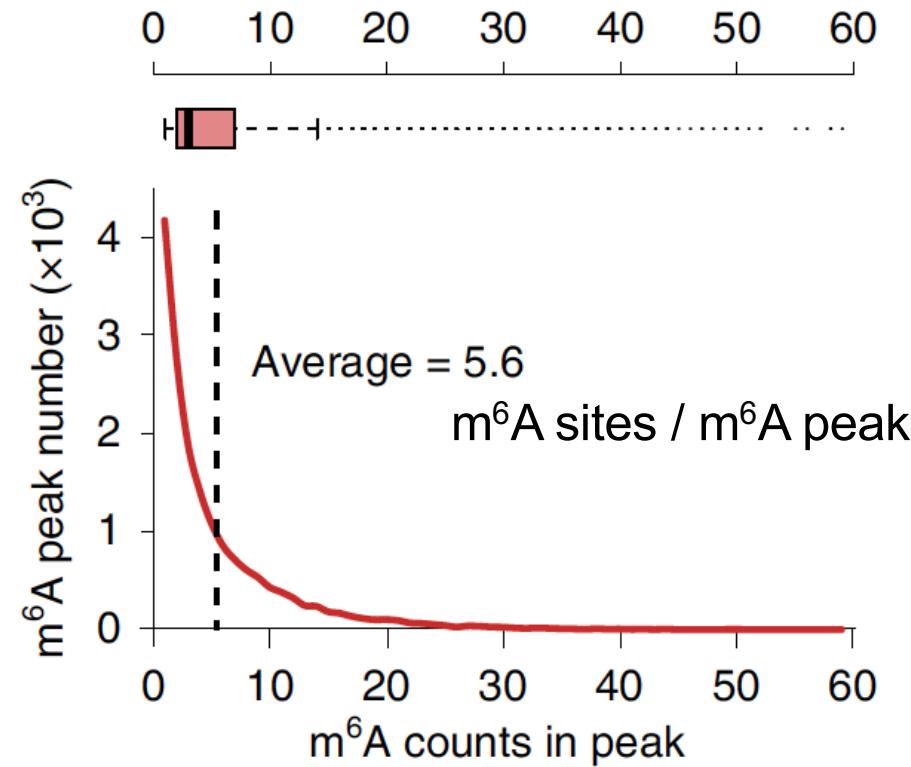
PMID: 36302990  
PMID: 38253658

# Most m<sup>6</sup>A-modified transcripts have multiple m<sup>6</sup>A sites

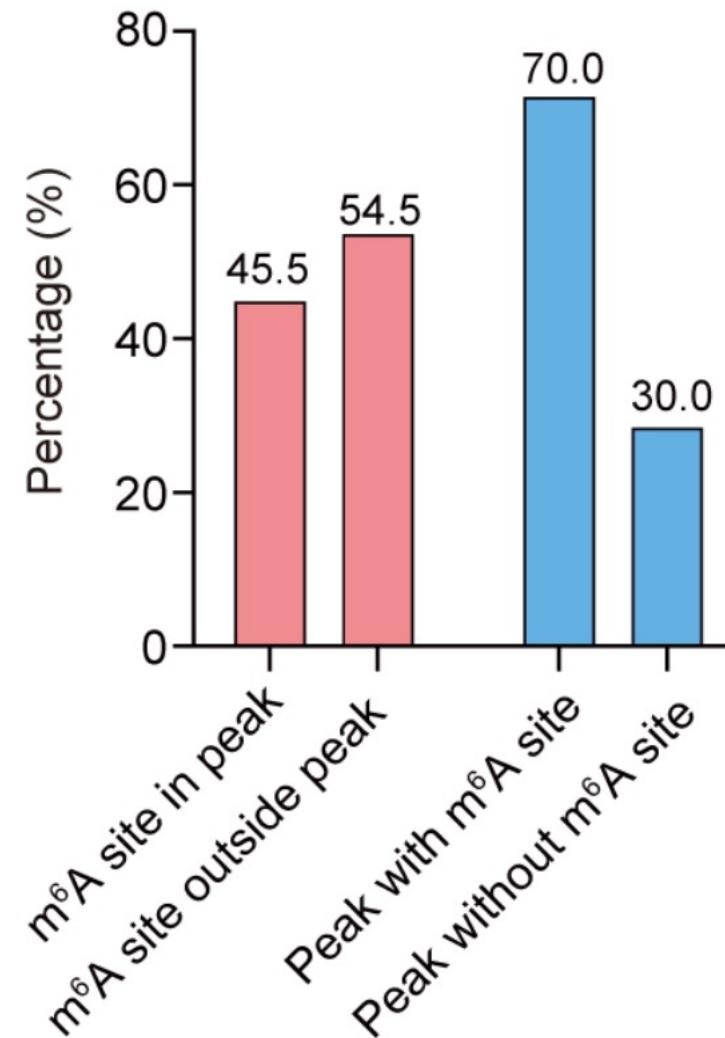


- 86% contain multiple m<sup>6</sup>A sites
- average, ~10 m<sup>6</sup>A sites were found per gene
- ~2,000 genes (17% of all m<sup>6</sup>A-modified genes) >20 m<sup>6</sup>A sites
- SPEN, BSN, and ALMS1 transcripts: >100 m<sup>6</sup>A sites.

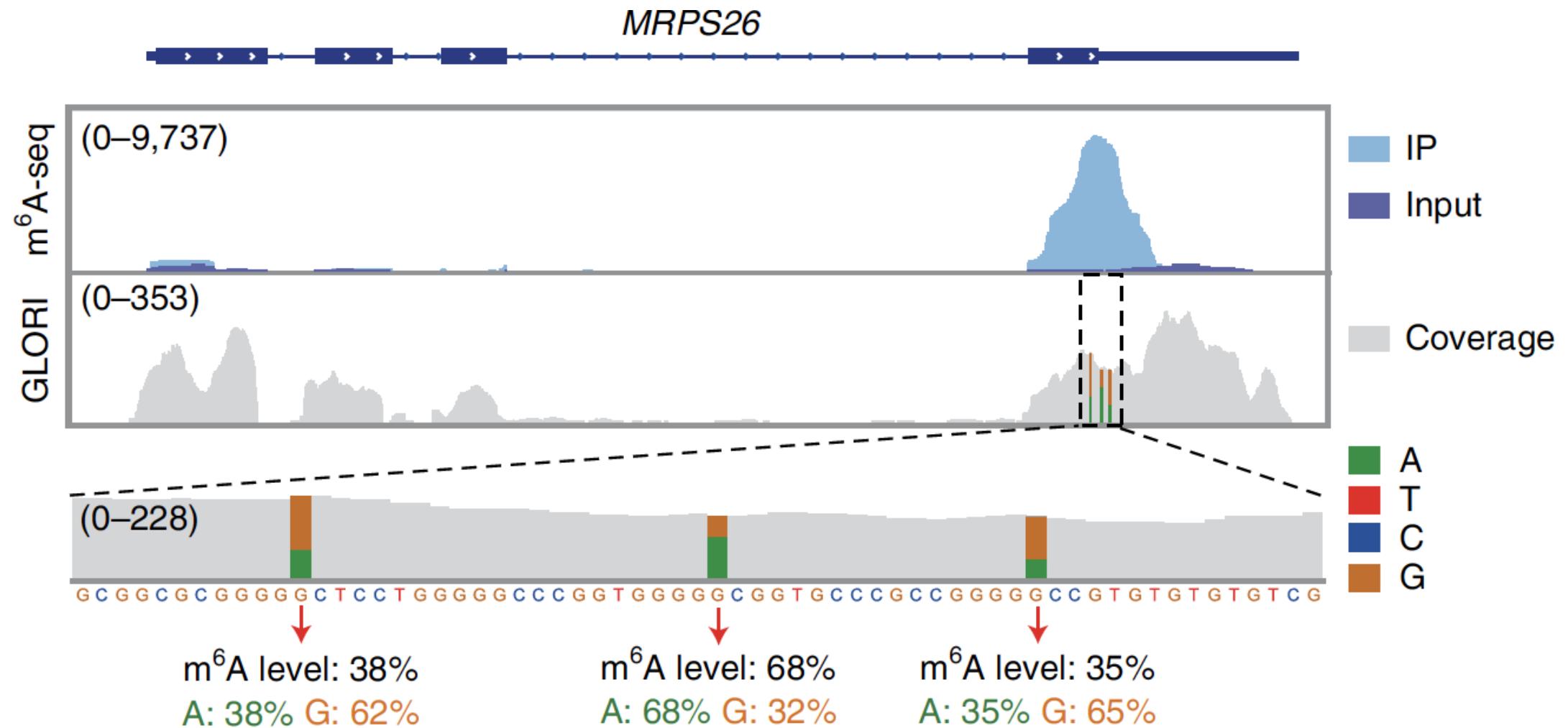
# The distribution of m<sup>6</sup>A sites within peaks (or not)



- 45% of m<sup>6</sup>A sites within a peak
- 30% of peaks don't have an m<sup>6</sup>A site



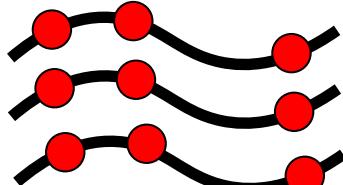
# The modification ratio of sites within a peak can vary



# Challenges to mapping RNA modifications

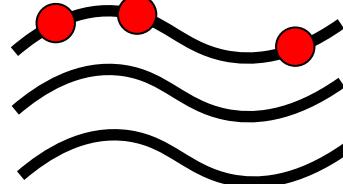


1



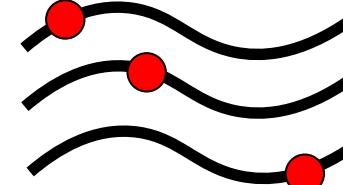
Does m<sup>6</sup>A mark  
every RNA copy?

2

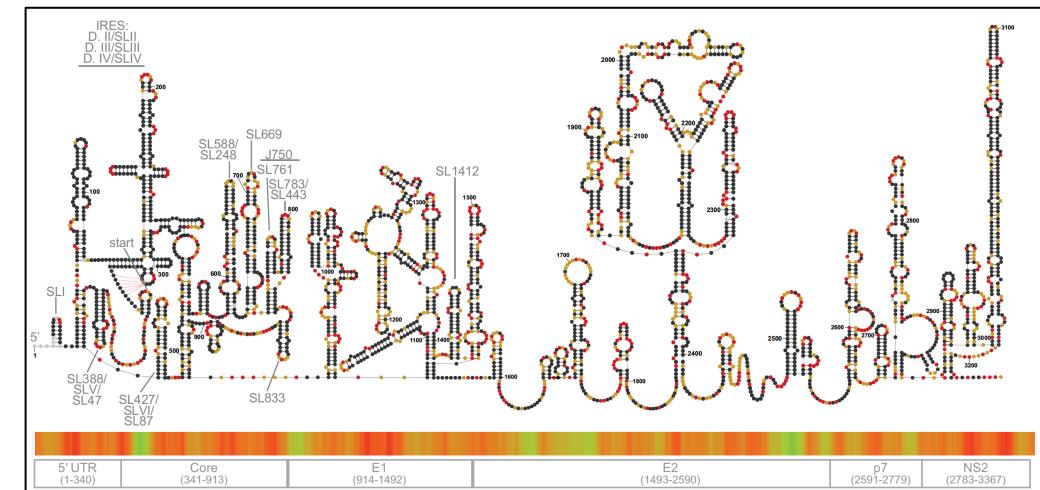


Is m<sup>6</sup>A heterogenous  
across an RNA pool?

3



Could m<sup>6</sup>A tag RNAs for  
specific functions?



4

m<sup>6</sup>A antibodies don't always interact well  
with heavily structured RNA

Liu B., et al., 2018. PMID: 30018356  
McIntyre, A. B. R. et al., 2020. PMID: 32313079  
Sun, H. et al., 2021. PMID: 34362929

PERSPECTIVE

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## Challenges to mapping and defining m<sup>6</sup>A function in viral RNA

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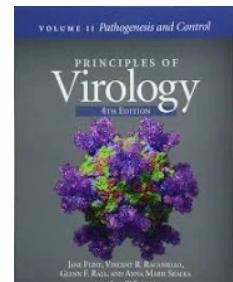
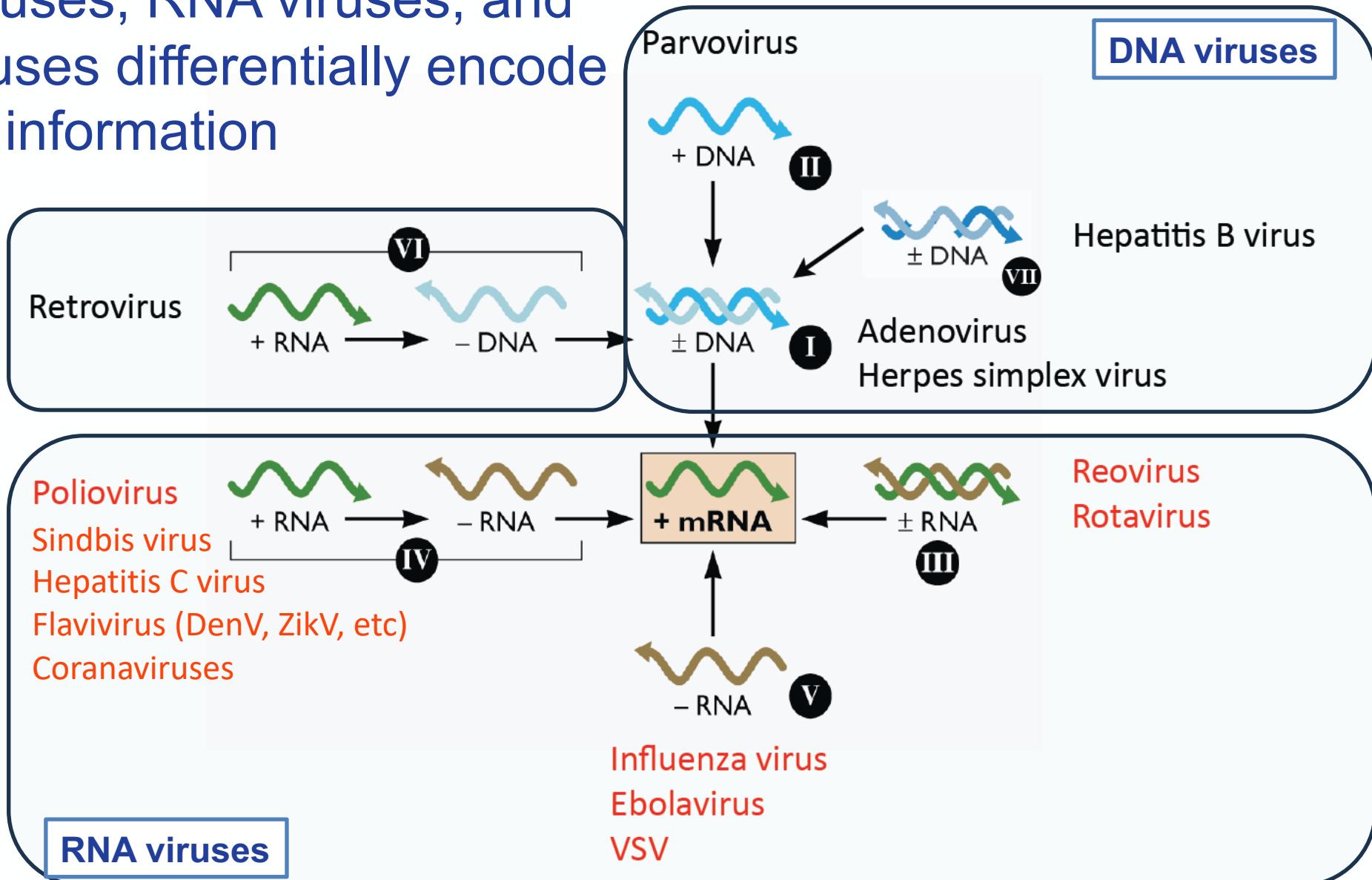
STACY M. HORNER<sup>1,2</sup> and MATTHEW G. THOMPSON<sup>1</sup>

<sup>1</sup>Department of Integrative Immunobiology, Duke University School of Medicine, Durham, North Carolina 27710, USA

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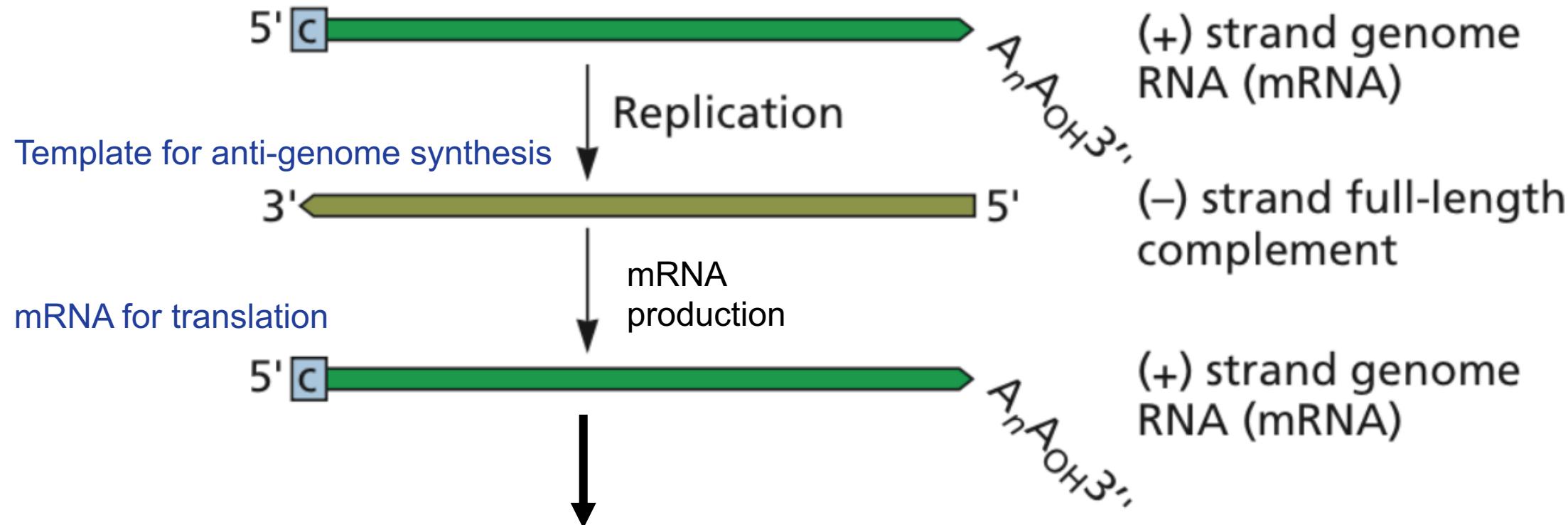
### How best to construct m<sup>6</sup>A maps in viral RNA molecules?

# DNA viruses, RNA viruses, and retroviruses differentially encode genetic information



# Positive-strand RNA virus genomes have multiple roles

Flavi- and picornaviruses



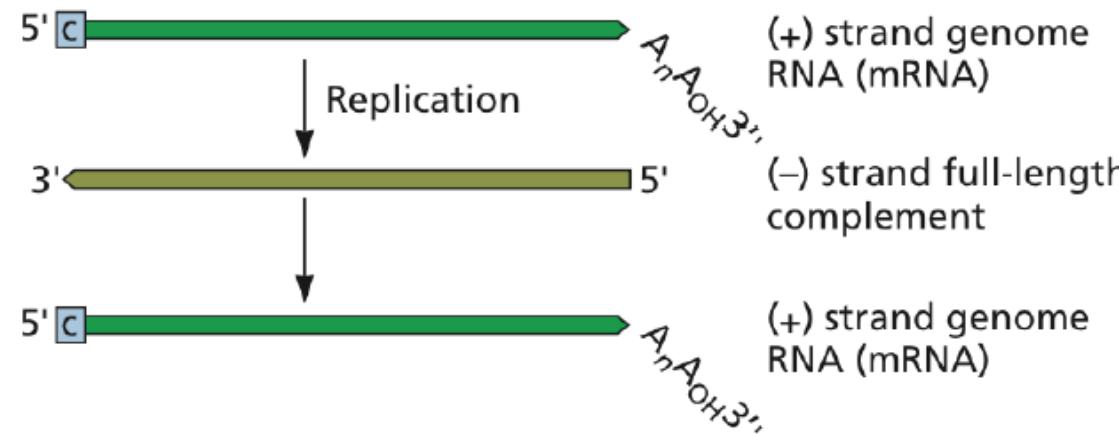
This molecule gets packed into the virion to make progeny virions

# Different RNA molecules can be present in positive-strand RNA viruses

Same template for replication and mRNA synthesis

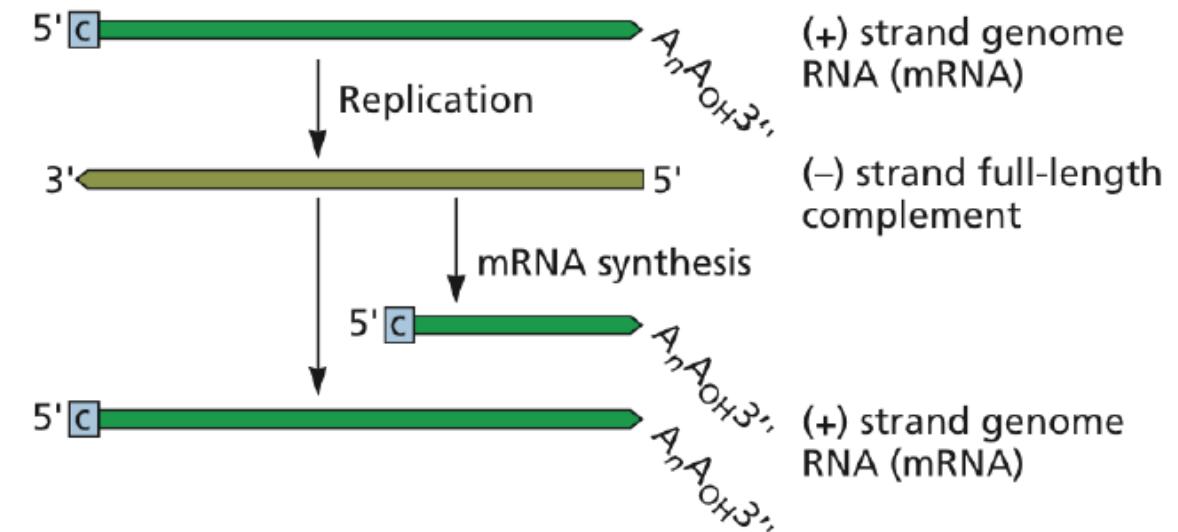
## (+) strand RNA viruses

Flavi- and picornaviruses



Subgenomic mRNAs in addition to full length mRNAs

Alphaviruses (*Togaviridae* - Sindbis, SFV, Chik)



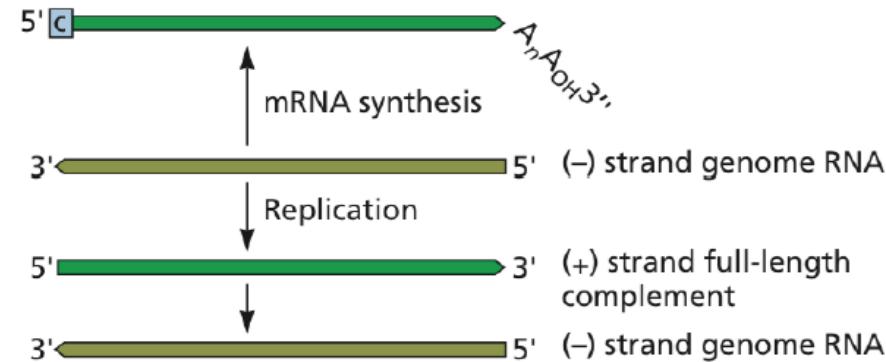
Subgenomic mRNAs allow for viral proteins to be produced in different amounts.

# Negative-strand RNA virus genomes can be segmented or non-segmented

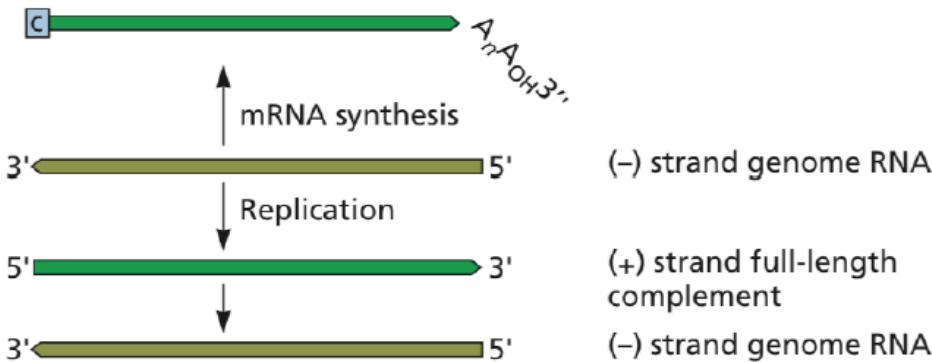
These viruses produce three major molecules: genome, anti-genome, and mRNA

## (-) strand RNA viruses

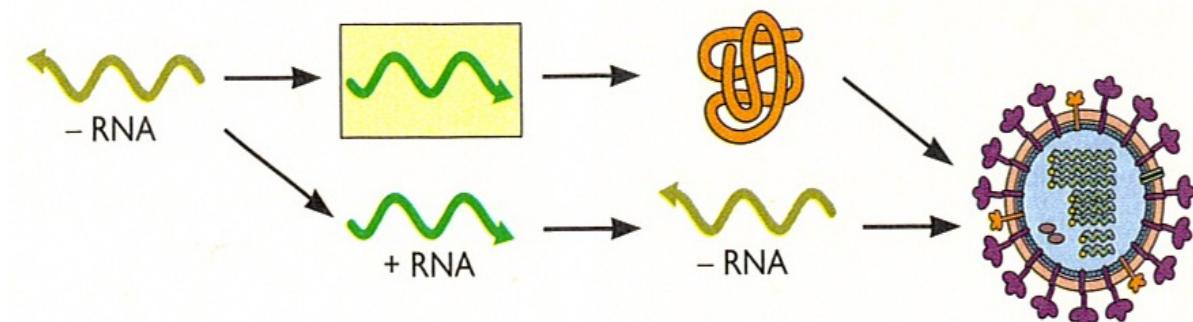
Unimolecular



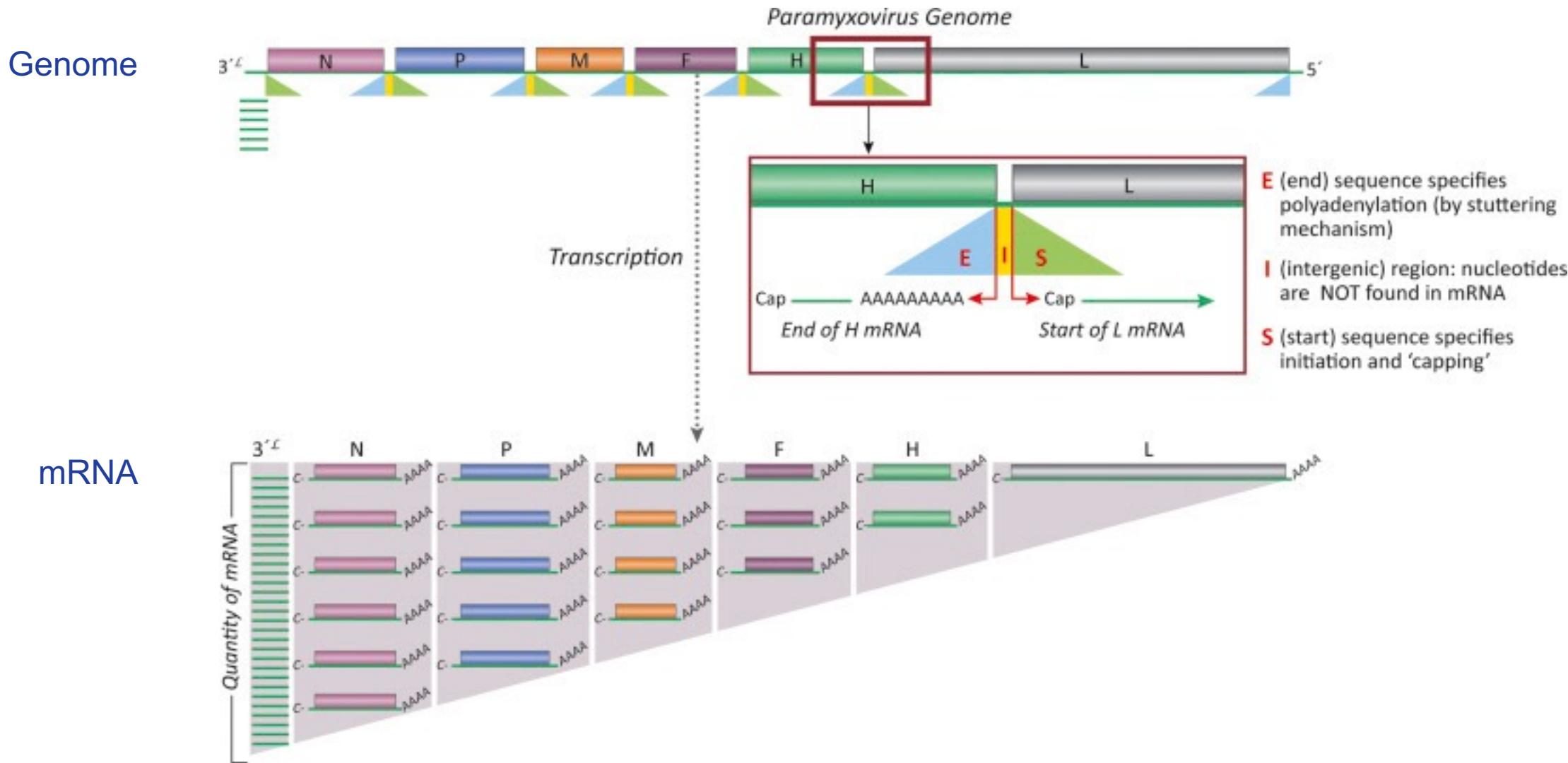
## Segmented



## A ss (-) RNA: Orthomyxoviridae, Paramyxoviridae, Rhabdoviridae



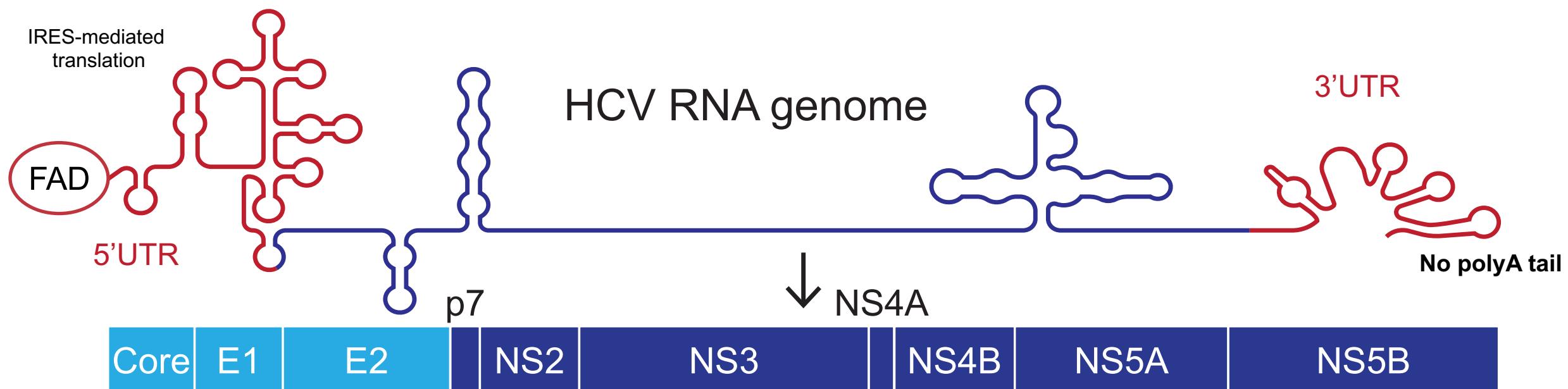
# Negative-strand RNA virus molecules can have different features and exist in non-stoichiometric amounts



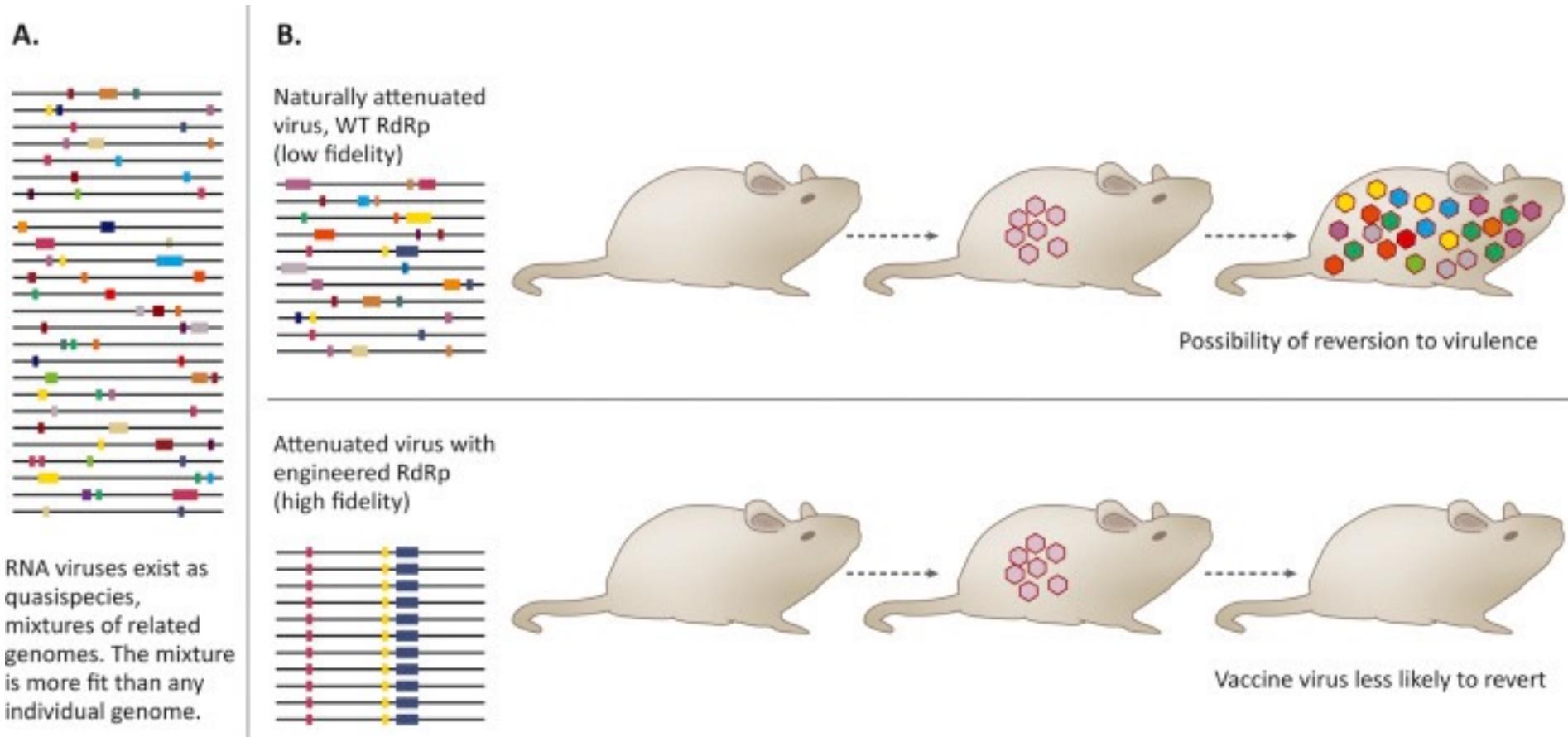
# Viral RNA molecules don't always look like cellular RNA molecules

## Hepatitis C virus (HCV)

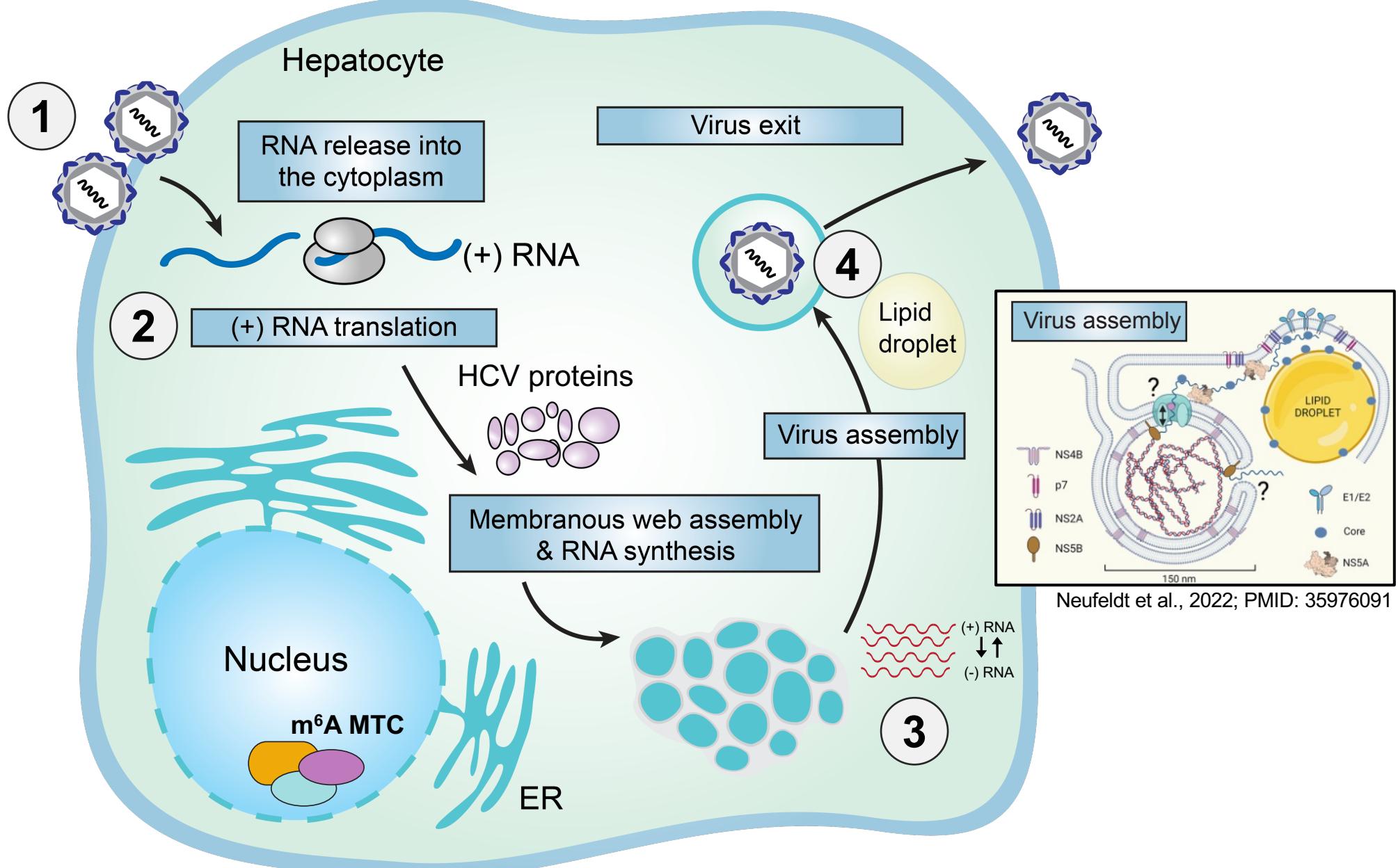
- Positive sense, single stranded RNA genomes (9600 nucleotides long).
- IRES-mediated translation drives expression of 10 viral proteins.
- No polyA tail, no m7G cap
- Uses a viral protein for transcription (NS5B: RNA-dependent RNA polymerase)



# RNA virus populations, called quasispecies, encode genetic diversity



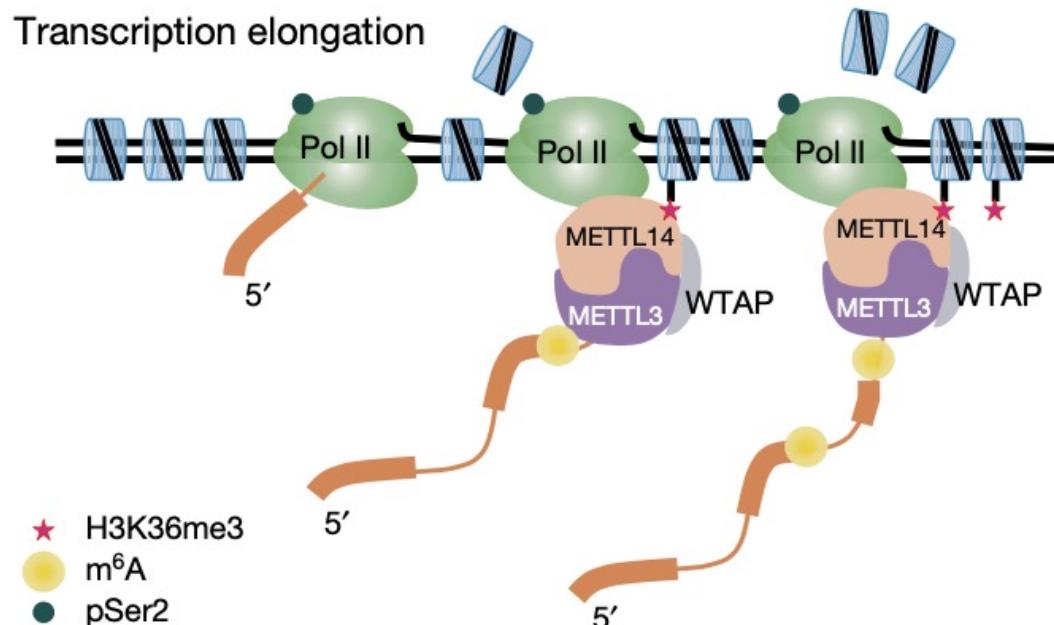
# Viral RNA genomes can have different roles in viral lifecycles and exist in different subcellular locations



# Viral lifecycles may appear incompatible with RNA modifications

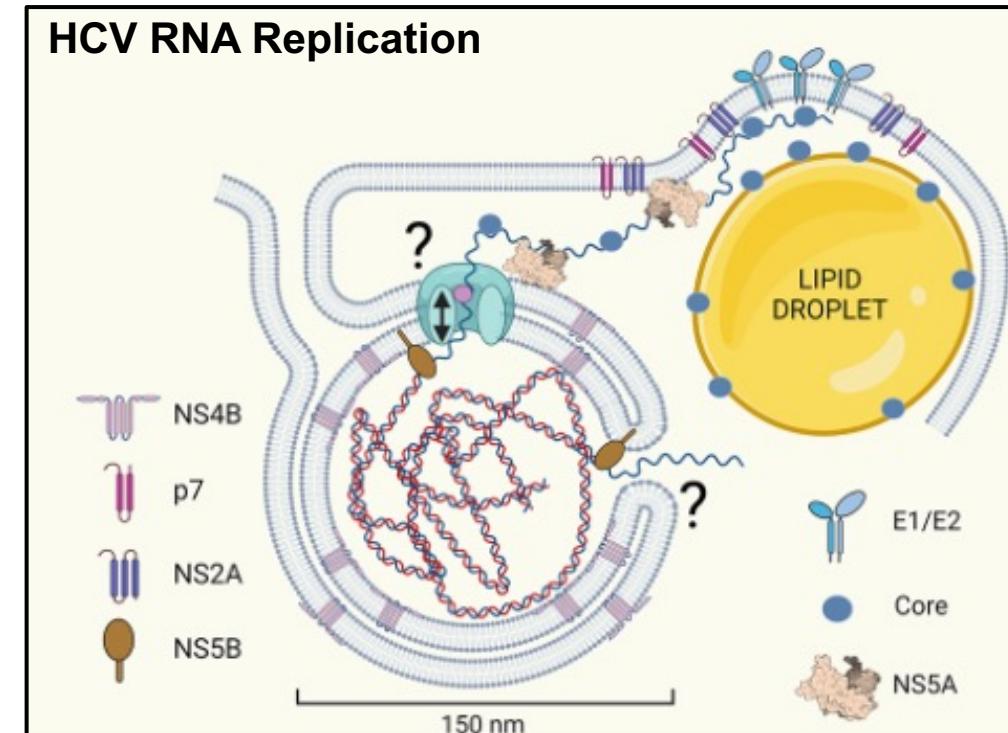
## Canonical mRNA m<sup>6</sup>A methylation

- Occurs within the **nucleus**
- mRNAs are modified **co-transcriptionally** by writer complex interaction with RNA polymerase II

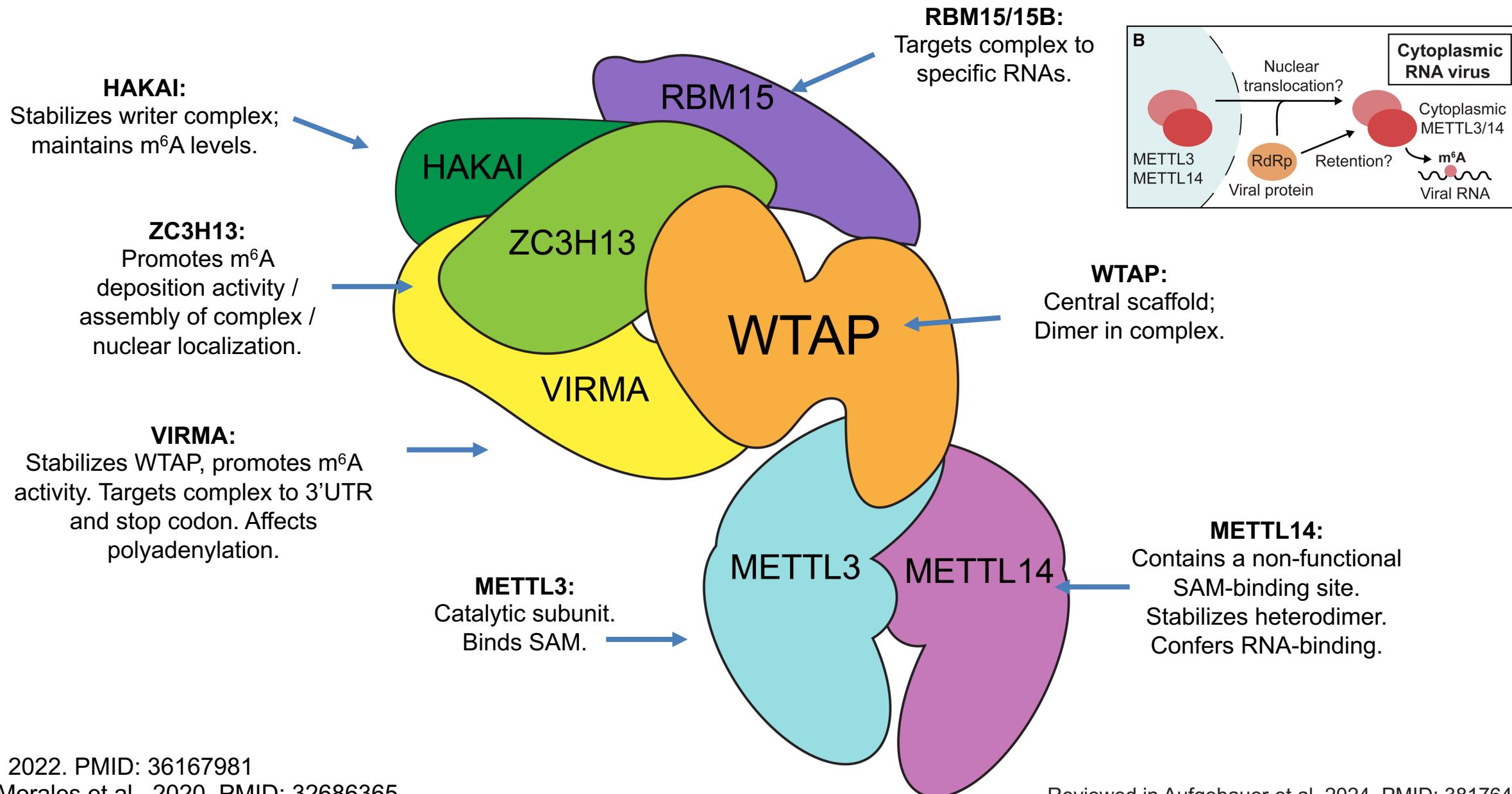


## HCV lifecycle

- Occurs within the **cytoplasm**
- HCV RNA replication is mediated by the **viral RNA-dependent RNA-polymerase NS5B**



# Viruses can relocalize m<sup>6</sup>A machinery to target viral RNA



# Challenges to mapping and defining m<sup>6</sup>A function in viral RNA

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STACY M. HORNER<sup>1,2</sup> and MATTHEW G. THOMPSON<sup>1</sup>

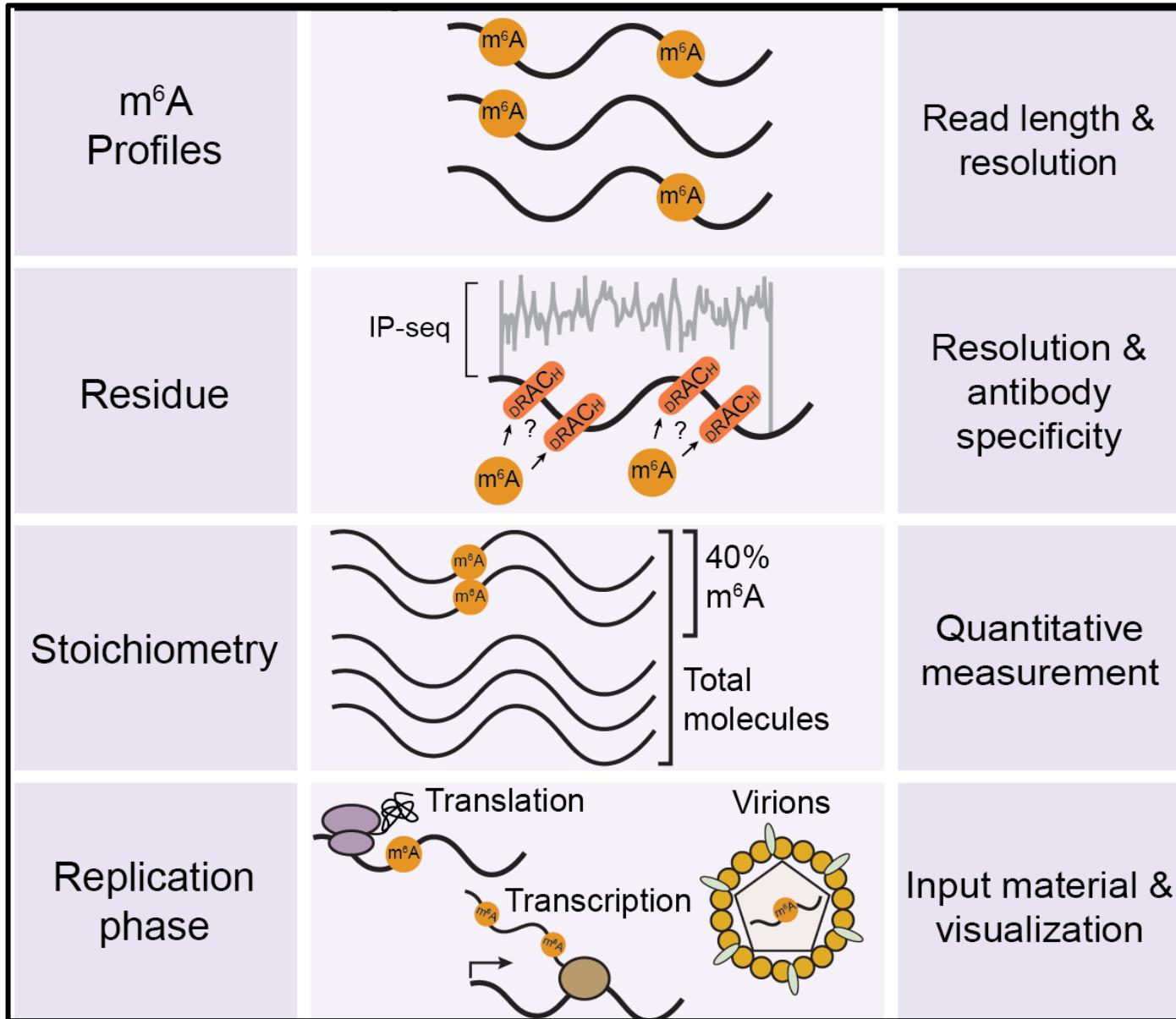
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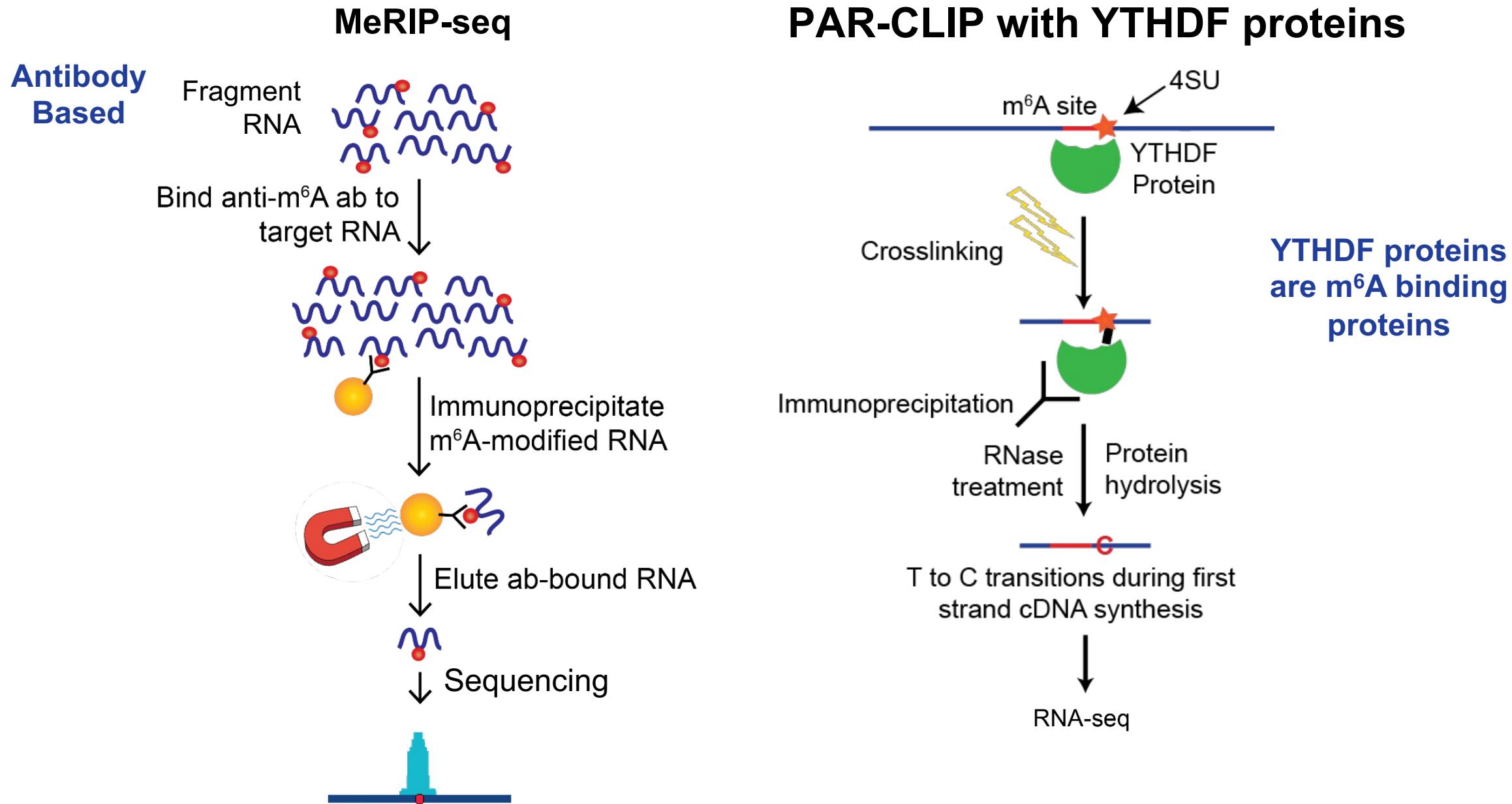
## What do we want for m<sup>6</sup>A viral RNA maps?

- Full-length viral RNA molecules , isoform-specific
- Single-nucleotide resolution
- Specific to viral lifecycle stages
- Virus-relevant cell types

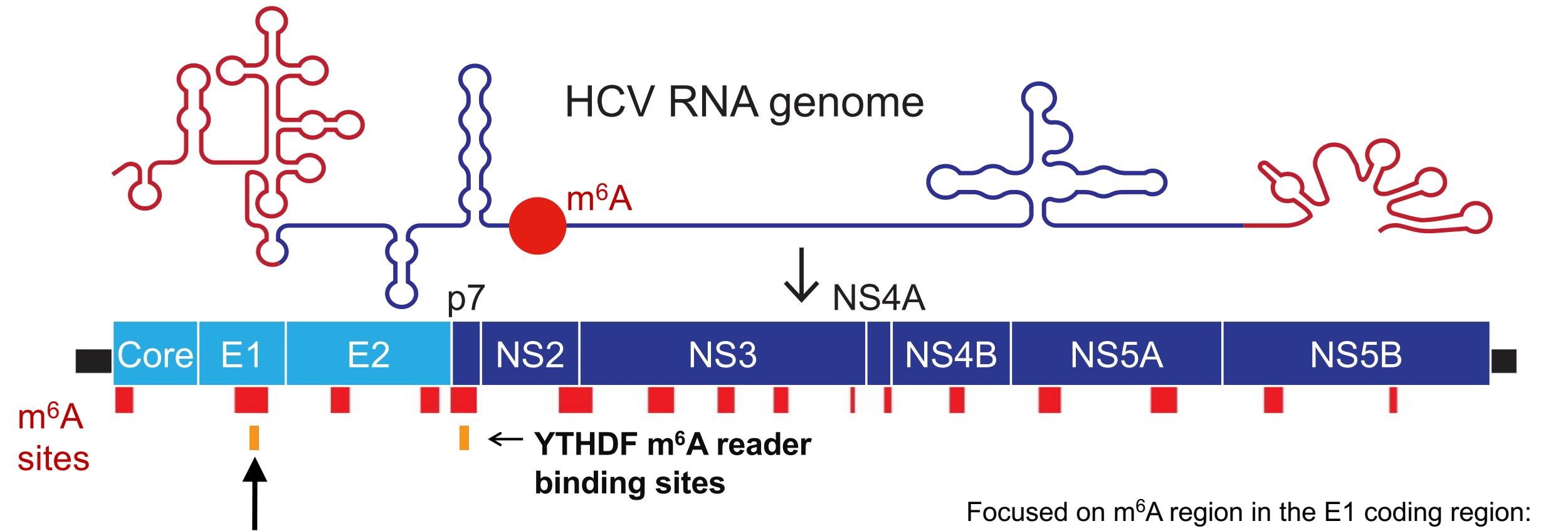
# Mapping m<sup>6</sup>A sites on viral RNA genomes



# Combine two indirect m<sup>6</sup>A-mapping approaches



# $\text{m}^6\text{A}$ is present across the HCV RNA genome



**What is the function of the  $\text{m}^6\text{A}$  site in E1?**

Made mutations to inactivate  $\text{m}^6\text{A}$

Regulates viral RNA packaging

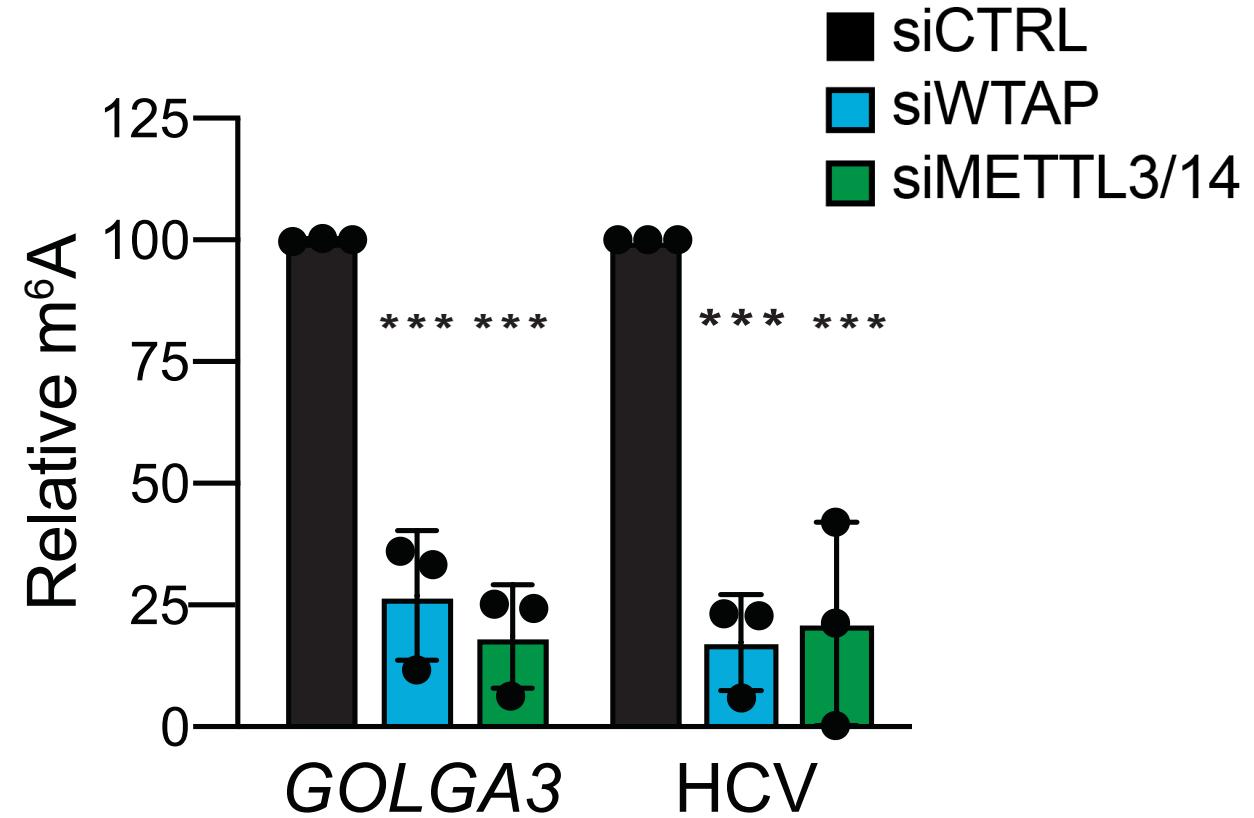
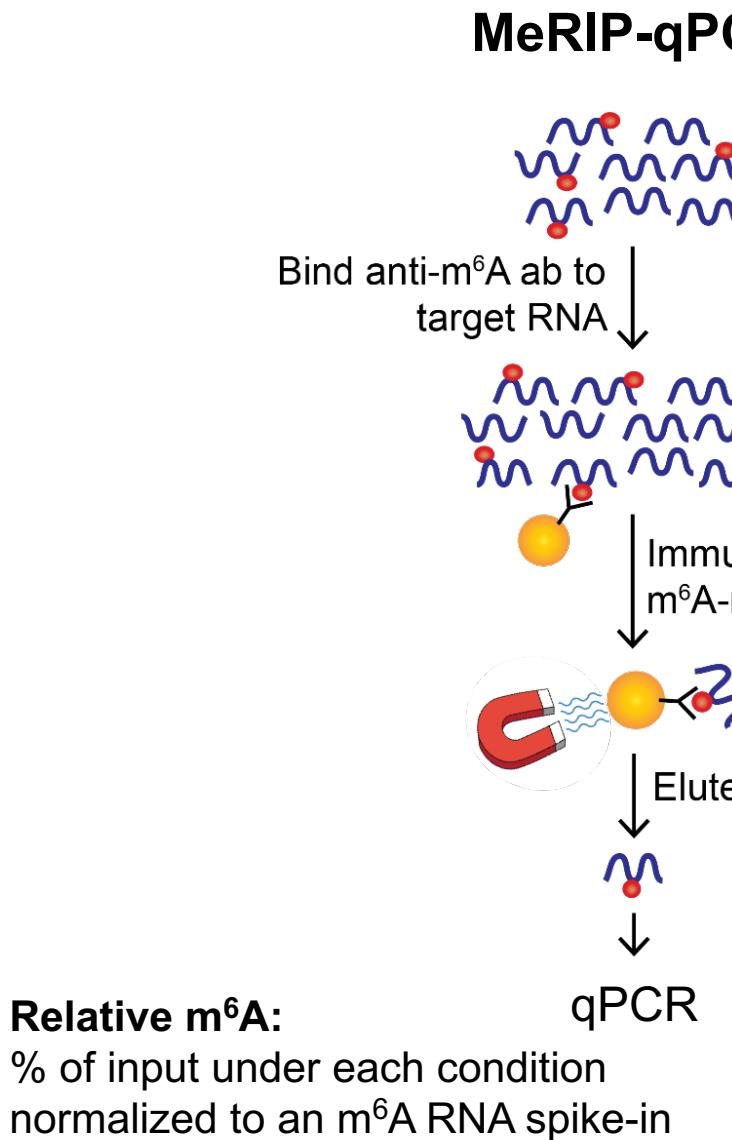
Focused on  $\text{m}^6\text{A}$  region in the E1 coding region:

- Strong enrichment by MeRIP-seq
- Bound by “reader” proteins
- No known RNA structures that could be disrupted
- 4 potential  $\text{m}^6\text{A}$  sites within this region

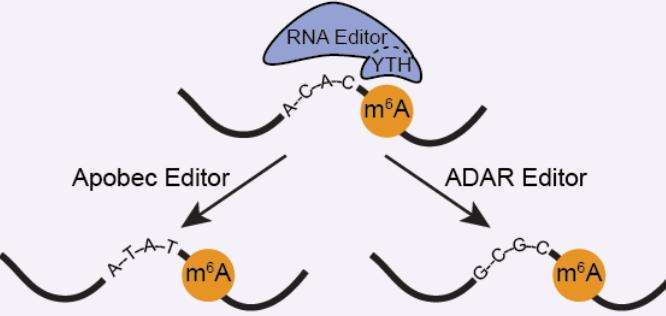
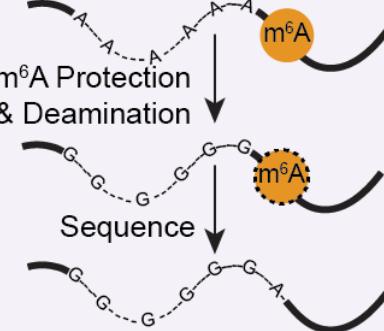
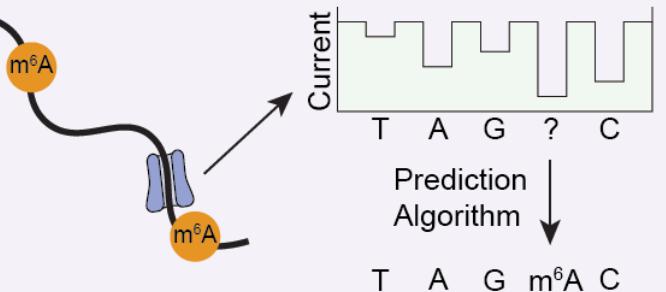
$\text{m}^6\text{A}$  sites mapped via **meRIP-seq** and  
**PAR-CLIP** for YTHDF  $\text{m}^6\text{A}$  binding proteins

# Control experiment

## METTL3 and WTAP are required for m<sup>6</sup>A on a viral RNA



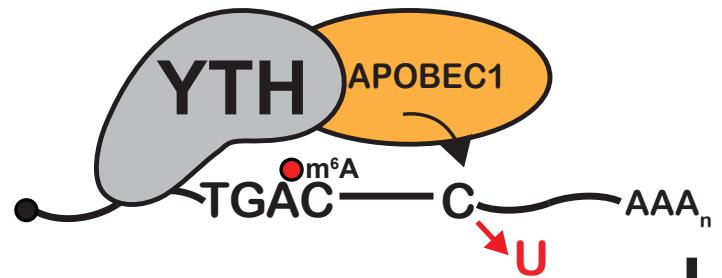
# Possible methods to map m<sup>6</sup>A on viral RNA

New Strategies	Advantages	Drawback
Molecular recording via RNA editing	 <ul style="list-style-type: none"> <li>Low input</li> <li>Semi-quant.</li> <li>Easily paired with long-read for isoform calling</li> </ul>	<ul style="list-style-type: none"> <li>Off-target sites</li> <li>False negatives</li> <li>No residue resolution</li> <li>Antiviral properties?</li> </ul>
m <sup>6</sup> A protection -based sequencing	 <ul style="list-style-type: none"> <li>Residue resolution</li> <li>Absolute quantitation</li> <li>Low input</li> </ul>	<ul style="list-style-type: none"> <li>High read depth required</li> <li>Specialized reagents and protocols</li> </ul>
Direct m <sup>6</sup> A calling via long-read RNA sequencing	 <ul style="list-style-type: none"> <li>Residue resolution</li> <li>Full isoform resolution</li> <li>Can call other RNA mods</li> </ul>	<ul style="list-style-type: none"> <li>High input</li> <li>Virus-specific sample prep.</li> <li>Prediction-based</li> <li>(-)m<sup>6</sup>A controls</li> </ul> <p>CoV genome ~30 kilobases</p> <p>HCV genome 9600 nts</p>

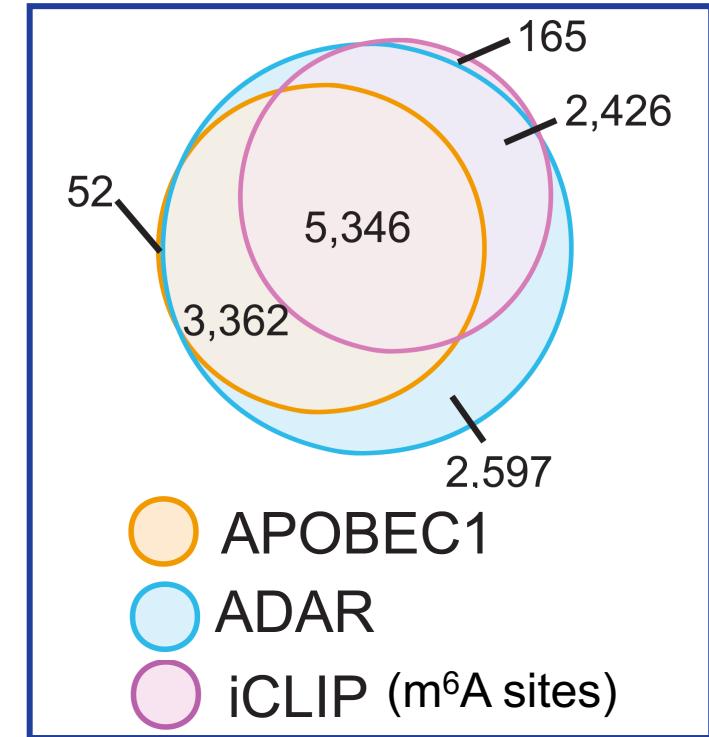
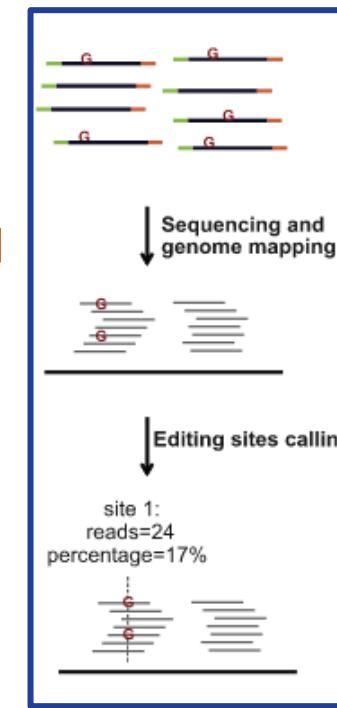
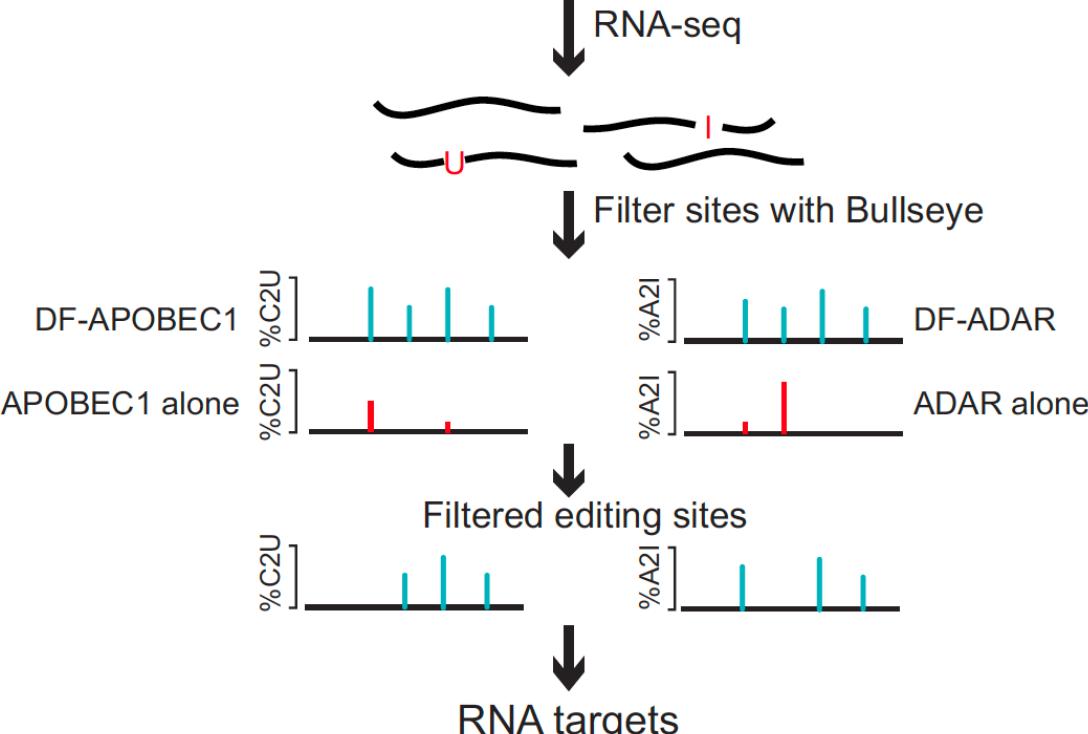
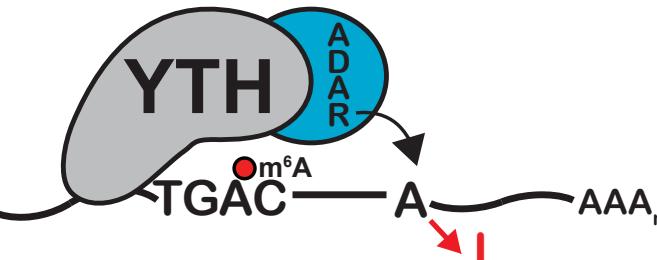
# “Molecular Recording”

## TRIBE and STAMP fuse m<sup>6</sup>A reader proteins to RNA editors

Surveying Targets by APOBEC-Mediated Profiling (**STAMP**)



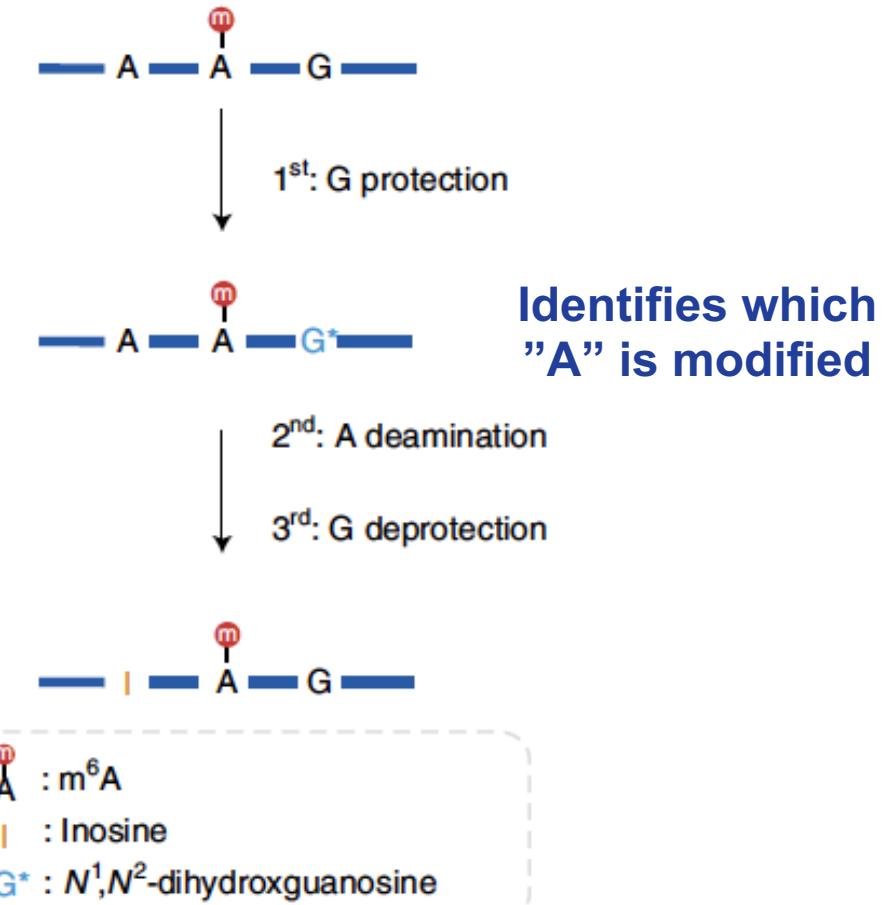
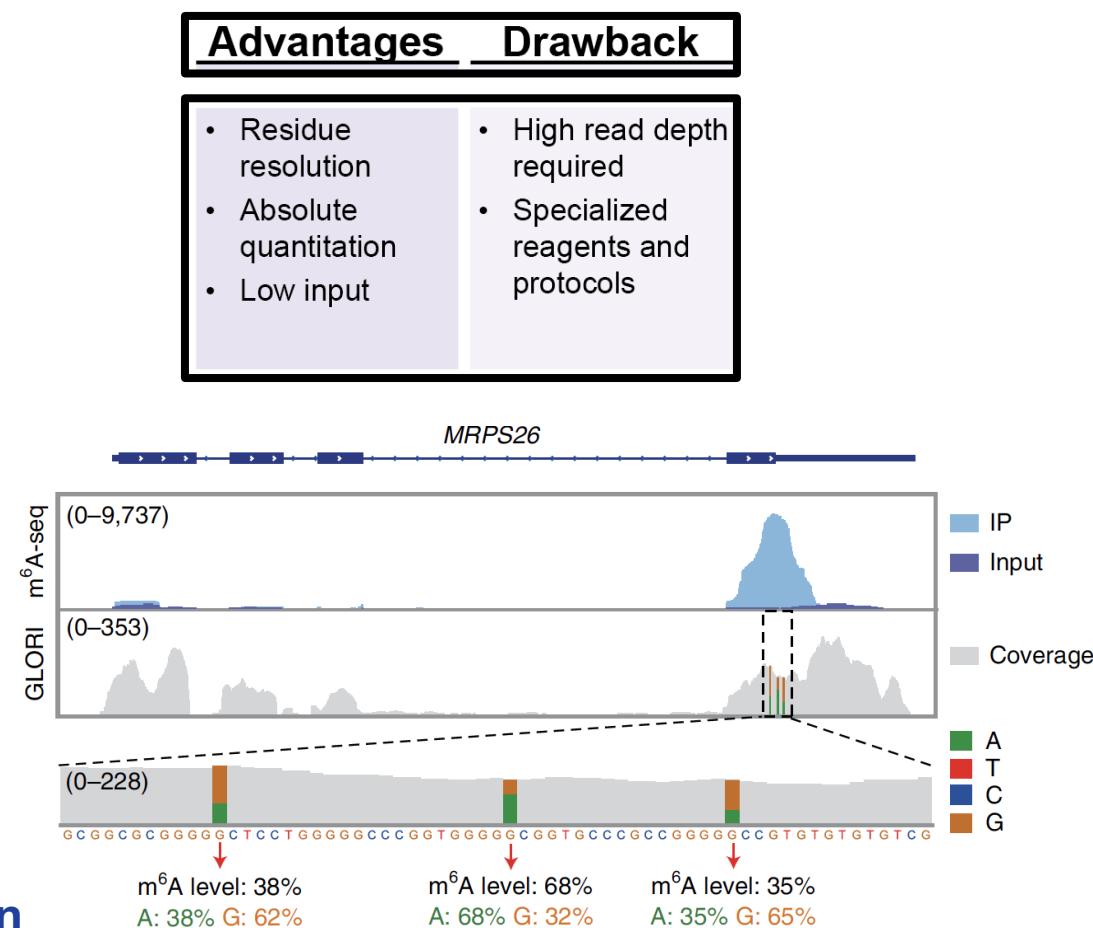
Target of RBPs Identified by Editing (**HYPER-TRIBE**)



- Relative % modification
- Coupled with long read seq, can be single molecule
- Potential for a “kit” (in vitro)

# Absolute quantification of single-base m<sup>6</sup>A methylation in the mammalian transcriptome using GLORI (new method)

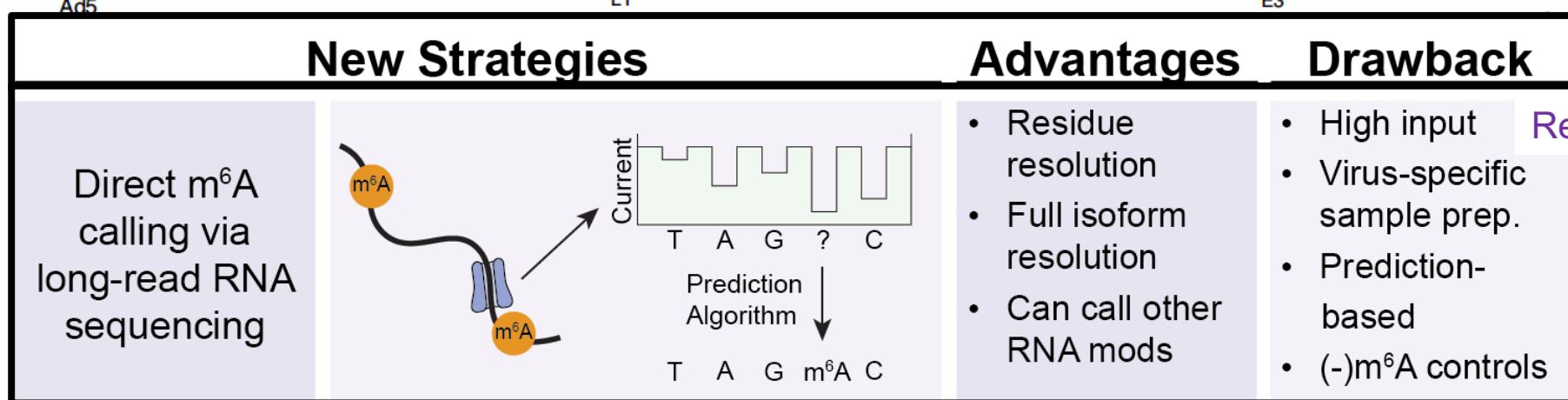
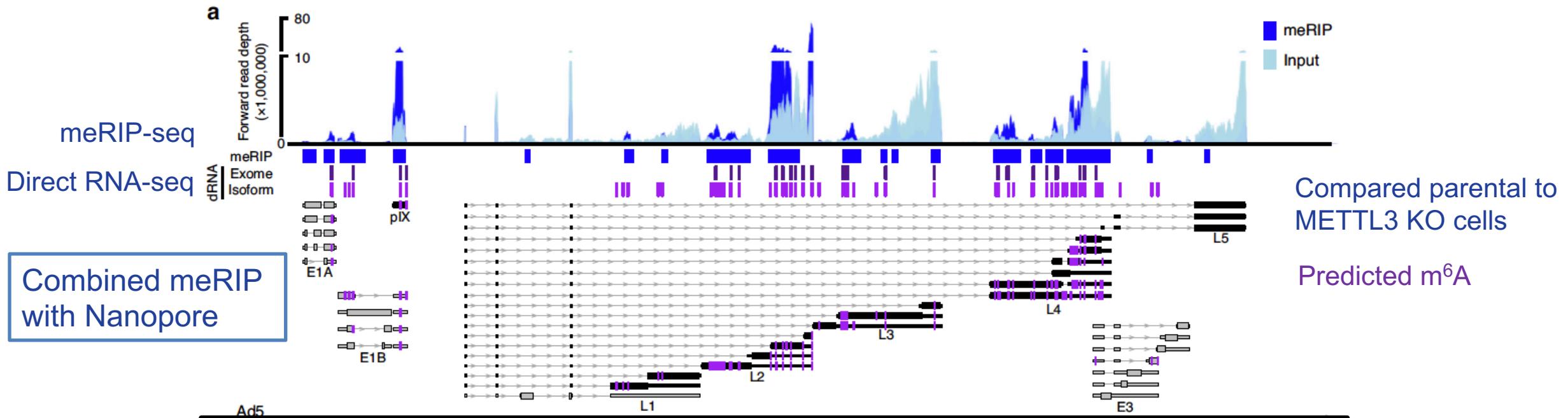
## Glyoxal and nitrite-mediated adenosine deamination



PMID: 36302990

PMID: 38253658

# Combine meRIP with Nanopore to map m<sup>6</sup>A on adenovirus RNA molecules (DNA virus) Reveals transcript-specific METTL3-dependent m<sup>6</sup>A modifications



PMID: 33243990

# Summary

- 1 RNA modifications provide a layer of regulation to the genetic code
- 2 Many methods have been developed to map m<sup>6</sup>A, limitations exist
- 3 Viral RNA molecules are unique from cellular mRNA molecules
- 4 Things to think about when mapping m<sup>6</sup>A in viruses:
  - Full-length viral RNA molecules
  - Single-nucleotide resolution
  - Specific to viral lifecycle stages
  - Virus-relevant cell types
- 5 Combining and integrating data from multiple methods will be needed to define m<sup>6</sup>A function in a viral RNA molecule