GlycoRNAs

By;
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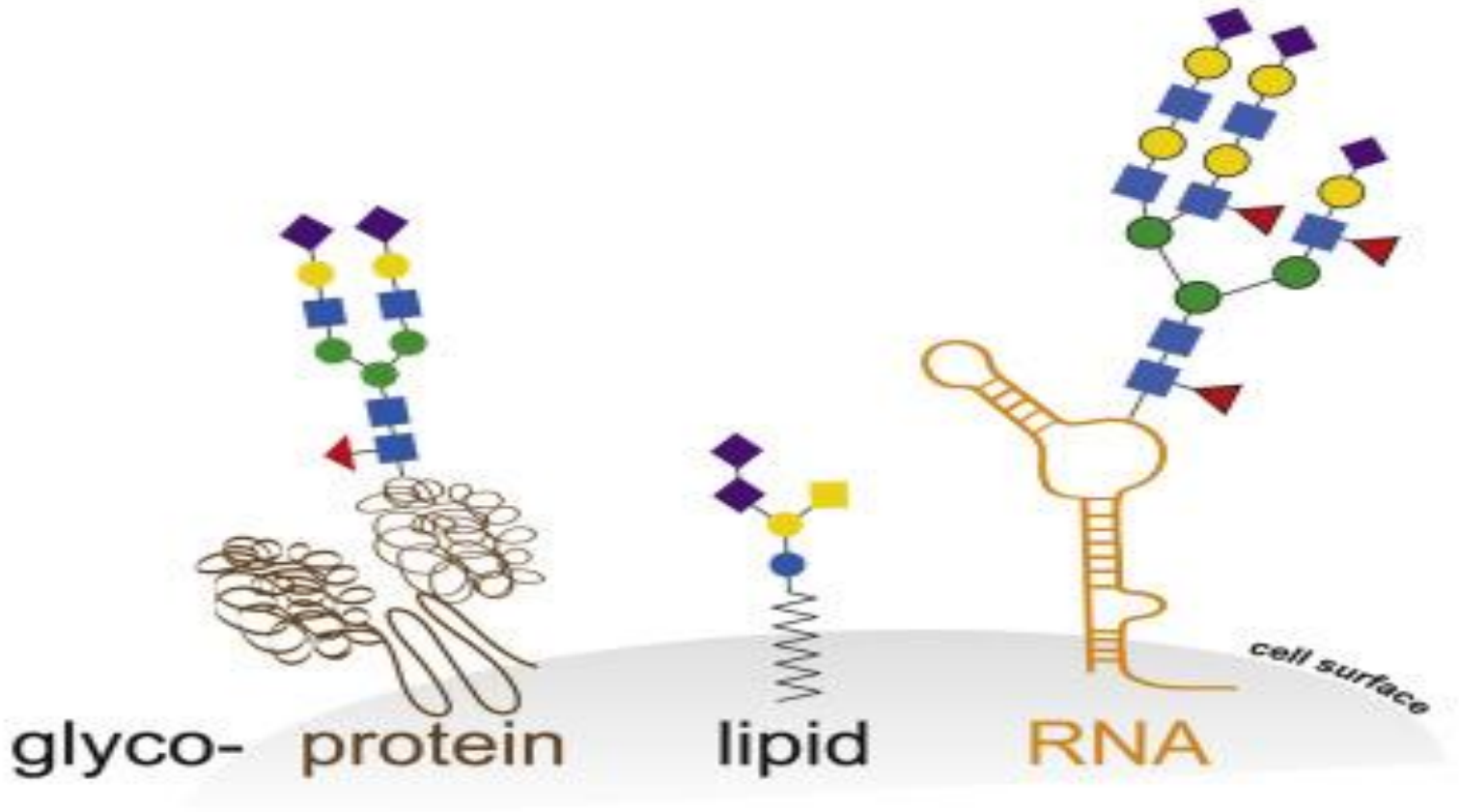
- Glycans modify lipids and proteins to mediate inter- and intramolecular interactions across all domains of life. RNA is not thought to be a major target of glycosylation.
- In 2021an American researches team were challenged this view with evidence that mammals use RNA as a third scaffold for glycosylation. Using a battery of chemical and biochemical approaches, they found that conserved small noncoding RNAs bear sialylated glycans. These "glycoRNAs" were present in multiple cell types and mammalian species, in cultured cells, and *in vivo*.

- GlycoRNA assembly depends on canonical N-glycan biosynthetic machinery and results in structures enriched in sialic acid and fucose. Analysis of living cells revealed that the majority of glycoRNAs were present on the cell surface and can interact with anti-dsRNA antibodies and members of the Siglec receptor family. Collectively, these findings suggest the existence of a direct interface between RNA biology and glycobiology, and an expanded role for RNA in extracellular biology.

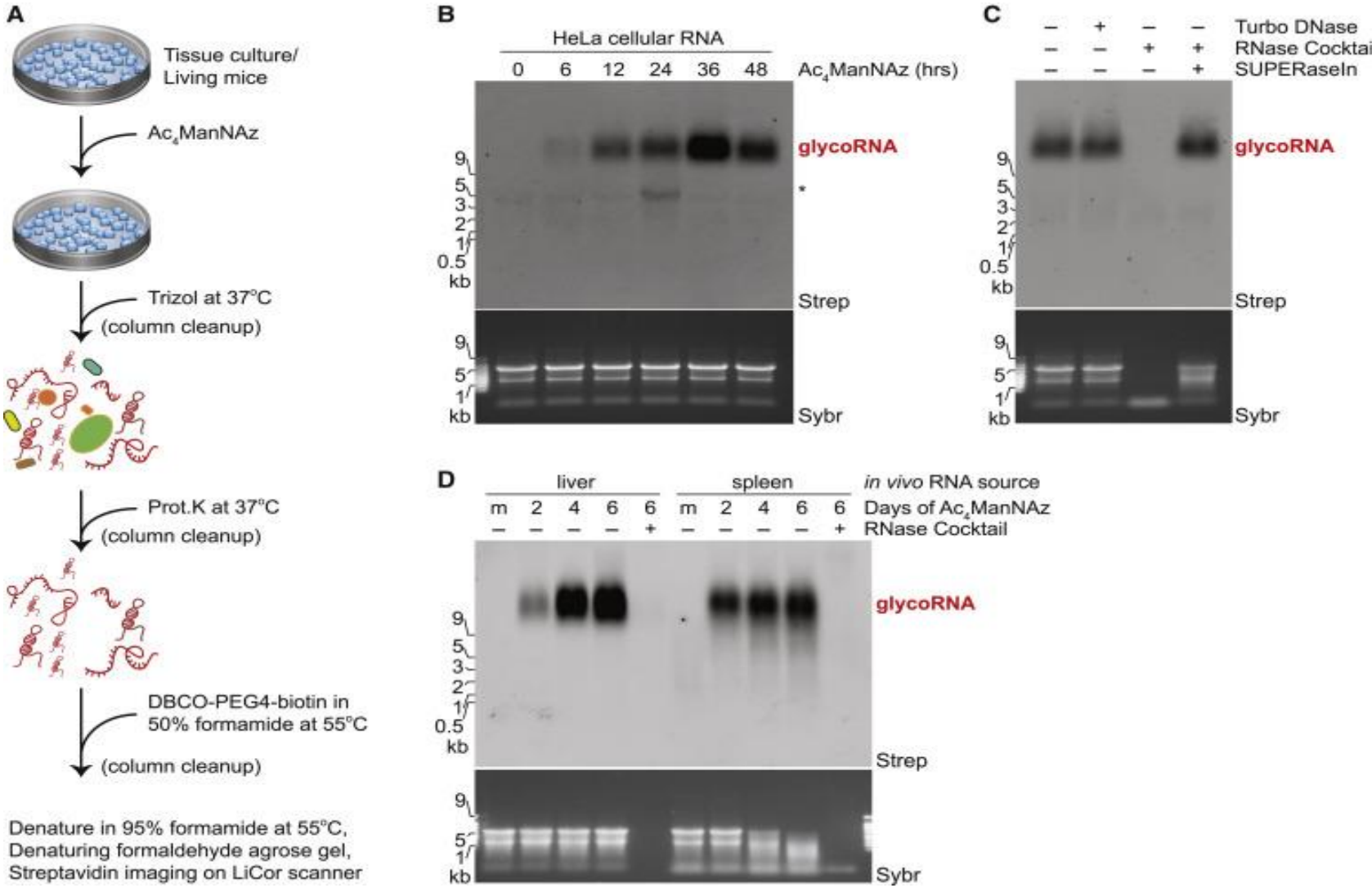

glyco-
protein
lipid
RNA
cell surface

- Glycans regulate a myriad of essential cellular functions, especially in the context of cell surface events. For instance, complex glycans facilitate the folding and purposeful trafficking of proteins and lipids for secretion or membrane presentation.
- Thus, many fundamental processes such as embryogenesis, host-pathogen recognition, and tumor-immune interactions rely on glycosylation . Glycans are present in every cell studied to date across the kingdoms of life and in mammals, are composed of roughly 10 monomeric carbohydrate units.

- In a traditionally adjacent field of study, RNA represents another biopolymer that is central to all known life. Although the building blocks of RNA are canonically limited to four bases, post-transcriptional modifications (PTMs) can dramatically expand the chemical diversity of RNA, with >100 PTMs having been identified .
- It is therefore not surprising that the cellular role for RNA is more complex than that of a simple messenger. For instance, RNAs function as scaffolds, molecular decoys, enzymes, and network regulators across the nucleus and cytosol

- With the exception of a few monosaccharide-based tRNA modifications , there has been no evidence so far of a direct linkage between RNA and glycans in nature.

- To explore the possible existence of RNA modified with sialoglycans (hereafter referred to as glycoRNA), HeLa cells have been labeled with 100 μM Ac_4ManNAz for up to 48 h and then used a rigorous protocol to chemically and enzymatically extract RNA with high purity: RNA is extracted with warm TRIzol (acid phenol and guanidine salts), then ethanol precipitated, desalted via silica columns, stripped of protein contamination via high concentration proteinase K digestion, and repurified over silica columns . To visualize azide-labeled components, copper (Cu) free click chemistry was used by adding RNA samples to dibenzocyclooctyne-biotin (DBCO-biotin) in denaturing conditions (50% formamide) at 55°C, subsequently separated by denaturing gel electrophoresis and analyzed by blotting


A
Tissue culture/
Living mice
Ac4ManNAz
Trizol at 37°C
(column cleanup)
Prot.K at 37°C
(column cleanup)
DBCO-PEG4-biotin in
50% formamide at 55°C
(column cleanup)
Denature in 95% formamide at 55°C,
Denaturing formaldehyde agrose gel,
Streptavidin imaging on LiCor scanner
B
HeLa cellular RNA
0 6 12 24 36 48 Ac4ManNAz (hrs)
glycoRNA
Strep
Sybr
kb
C
Turbo DNase
RNase Cocktail
SUPERaseIn
glycoRNA
Strep
Sybr
kb
D
liver
spleen
in vivo RNA source
m 2 4 6 6 m 2 4 6 6 Days of Ac4ManNAz
RNase Cocktail
glycoRNA
Strep
Sybr
kb

GlycoRNAs are small noncoding RNAs

- Across all cell types and organs tested, glycoRNA was found to migrate very slowly by denaturing agarose gel electrophoresis .The glycoRNA fractionated with the small RNAs but still demonstrated extremely slow migration (high apparent MW) in the agarose gel . It had been speculated that glycoRNA's anomalous migratory behavior is caused by its associated glycans

Label and label-free detection of sialic acid in glycoRNA

- Next, to define the glycan structures on glycoRNAs. To confirm that glycoRNAs are sialylated, an independent method not relying on metabolic reporters had been used . The fluorogenic 1,2-diamino-4,5-methylenedioxybenzene (DMB) probe is used to derivatize free sialic acids for detection and quantitation
- by high performance liquid chromatography (HPLC)-fluorescence

Enzymes and their inhibitors studies

- the effects of glycosylation inhibitors on glycoRNA biosynthesis. Oligosaccharyltransferase (OST) mediates protein N-glycosylation by transferring a 14-sugar glycan to asparagine residues on nascent polypeptides ,and the effect of NGI-1, a specific and potent small molecule inhibitor of OST , on glycoRNA production have been tested . Such treatment caused a dose-dependent loss of glycoRNA labeling with Ac_4ManNAz , suggesting that OST is involved in biosynthesis of glycoRNA-associated glycans.

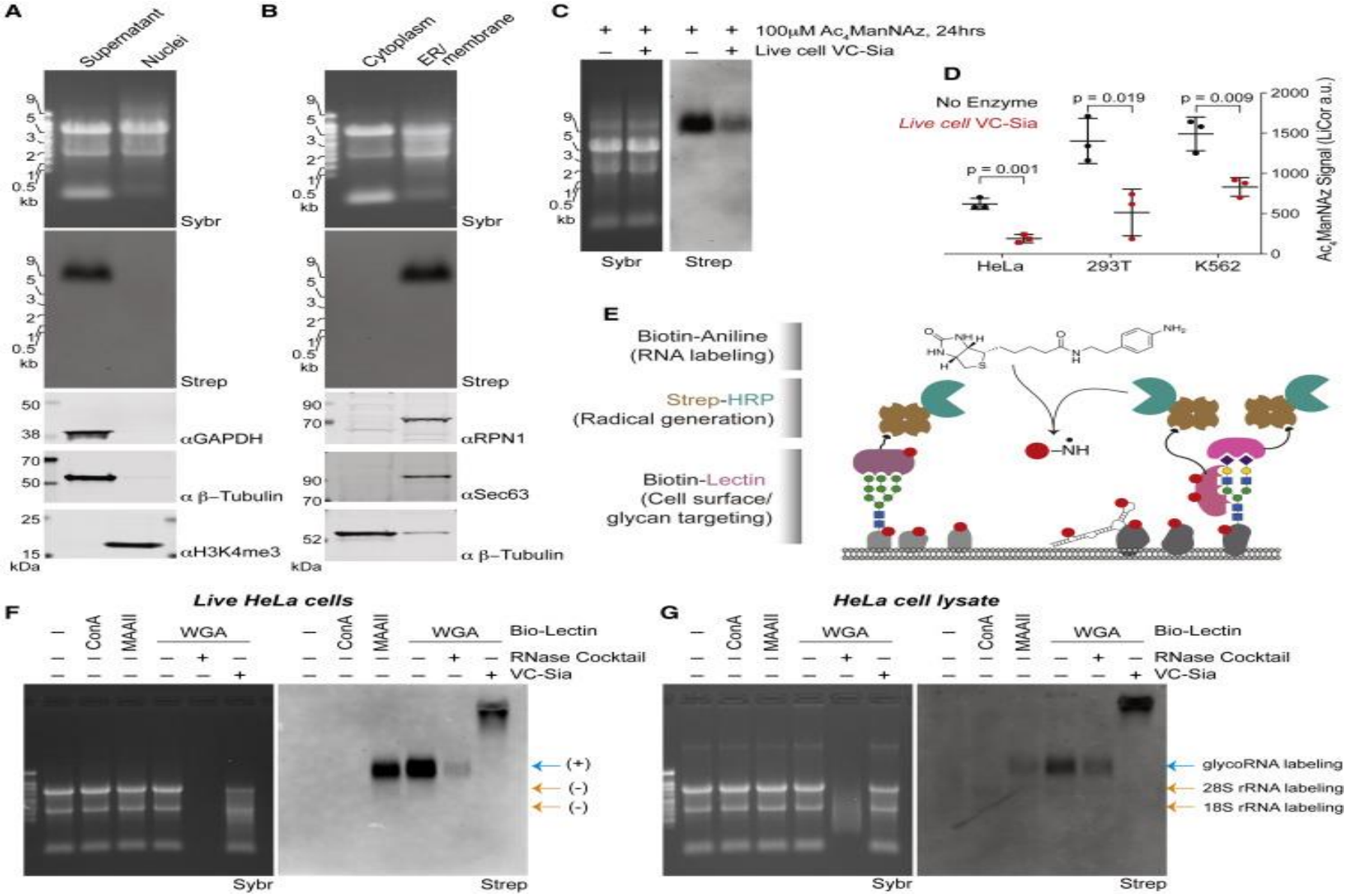
- To further define the glycan structures on glycoRNA, a panel of endoglycosidases was employed . Purified RNA from Ac_4ManNAz-labeled HeLa cells was first exposed to each enzyme and then reacted with biotin for visualization

Mass spectrometry defines distinct compositions of glycans on RNA

- The above data suggest that glycoRNA are modified with complex-type N-glycans with at least one terminal sialic acid residue.
- Further, the set of unique glycans found on RNA was smaller and more constrained relative to those on peptides . When examining the features that distinguished RNA glycans from peptide glycans, we noticed that both 293T and H9 cells had a higher fraction of glycans modified with fucose on RNA as compared to the peptides from these same cells .

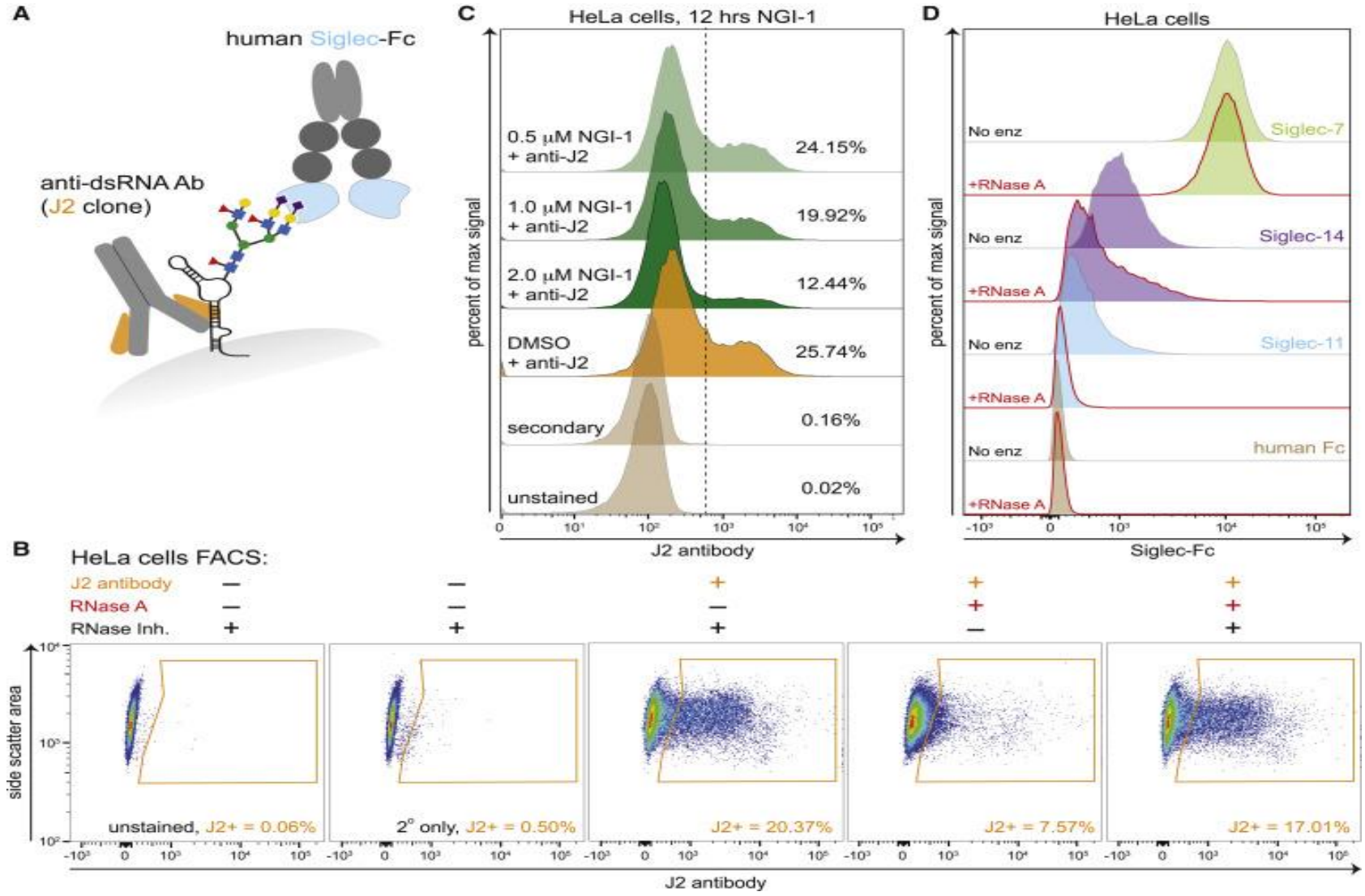
GlycoRNAs are associated with cellular membrane

- Finally, They assessed the subcellular localization of glycoRNA. The biogenesis of sialylated glycans occurs across many subcellular compartments including the cytosol , the nucleus , and the secretory pathway (where sialyltransferases add sialic acid to the termini of glycans). The localization of Y RNAs has been reported to be mainly cytoplasmic with a minor fraction in the nucleus . Other major classes of glycoRNA transcripts such as tRNAs and sn/snoRNAs are classically localized to the soluble cytosol and nucleus, respectively. To determine where glycoRNAs are distributed inside cells, they used two biochemical strategies:
- one that isolates nuclei away from membranous organelles and the cytosol (Gagnon et al., 2014) and
- a second that separates the soluble cytosolic compartment away from membranous organelles (STAR Methods).
- Nuclear RNA from Ac_4ManNAz-labeled HeLa cells yielded no detectable azide-labeled species whereas the membrane fraction exclusively contained the glycoRNA (Figures 5A and 5B). This suggests that glycoRNAs are closely associated with membrane organelles.


A
Supernatant
Nuclei
Sybr
Strep
αGAPDH
α β–Tubulin
αH3K4me3
kDa
B
Cytoplasm
ER/ membrane
Sybr
Strep
αRPN1
αSec63
α β–Tubulin
kDa
C
100μM Ac4ManNAz, 24hrs
Live cell VC-Sia
Sybr
Strep
D
No Enzyme
Live cell VC-Sia
p = 0.001
p = 0.019
p = 0.009
HeLa
293T
K562
Ac4ManNAz Signal (LiCor a.u.)
E
Biotin-Aniline
(RNA labeling)
Strep-HRP
(Radical generation)
Biotin-Lectin
(Cell surface/
glycan targeting)
F
Live HeLa cells
ConA
MAAII
WGA
Bio-Lectin
RNase Cocktail
VC-Sia
(+)
(-)
(-)
Sybr
Strep
G
HeLa cell lysate
ConA
MAAII
WGA
Bio-Lectin
RNase Cocktail
VC-Sia
glycoRNA labeling
28S rRNA labeling
18S rRNA labeling
Sybr
Strep

Siglec receptors and anti-RNA antibodies recognize cell surface glycoRNAs

- Biopolymers localized to the cell surface often participate in molecular interactions with binding partners in *cis* or in *trans* at cell-cell junctions. Because glycoRNAs are present on cell surfaces, they hypothesized that they too could engage in these types of interactions. They assessed if existing reagents to study the biology of cell surfaces, such antibody or recombinant protein-based affinity reagents, might interact with cell surface glycoRNA (Figure 6A).


A
human Siglec-Fc
anti-dsRNA Ab
(J2 clone)
C
HeLa cells, 12 hrs NGI-1
0.5 μM NGI-1
+ anti-J2
24.15%
1.0 μM NGI-1
+ anti-J2
19.92%
2.0 μM NGI-1
+ anti-J2
12.44%
DMSO
+ anti-J2
25.74%
secondary
0.16%
unstained
0.02%
percent of max signal
J2 antibody
D
HeLa cells
No enz
+RNase A
Siglec-7
Siglec-14
Siglec-11
human Fc
percent of max signal
Siglec-Fc
B
HeLa cells FACS:
J2 antibody
RNase A
RNase Inh.
side scatter area
unstained, J2+ = 0.06%
2° only, J2+ = 0.50%
J2+ = 20.37%
J2+ = 7.57%
J2+ = 17.01%
J2 antibody

- Finally, they sought to determine whether glycoRNAs can interact with glycan-binding receptors whose ligands have been assumed, based on convention, to be cell-surface glycoproteins and glycolipids.
- As described above, the N-glycans associated with glycoRNAs are highly sialylated. Thus, they asked whether members of the sialic acid binding-immunoglobulin lectin-type (Siglec) receptor family could recognize glycoRNAs. Notably, with 14 members distributed on all classes of immune cells, the Siglecs are the largest family of sialoside-binding proteins in humans . Their roles in immune modulation are well established and include host-pathogen interactions , cancer immune evasion , and genetic associations with autoimmune disease
- Their data support the hypothesis that cell surface glycoRNAs could be direct Siglec receptor ligands.

Conclusion

- In sum, the framework in which glycobiology is presently understood excludes RNA as a substrate for N-glycosylation. Their discovery of glycoRNA suggests the current view is incomplete and points to a new axis of RNA glycobiology, including as of yet undiscovered biosynthetic and trafficking mechanisms. Further, it highlights the possibility that cell surface glycoconjugates, which mediate and regulate important inter-cellular interactions, may now have an expanded template base to include RNA.