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RNA Crossing Membranes: Systems and Mechanisms Contextualizing Extracellular RNA and Cell Surface GlycoRNAs

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Abstract

The subcellular localization of a biopolymer often informs its function. RNA is traditionally confined to the cytosolic and nuclear spaces, where it plays critical and conserved roles across nearly all biochemical processes. Our recent observation of cell surface glycoRNAs may further explain the extracellular role of RNA. While cellular membranes are efficient gatekeepers of charged polymers such as RNAs, a large body of research has demonstrated the accumulation of specific RNA species outside of the cell, termed extracellular RNAs (exRNAs). Across various species and forms of life, protein pores have evolved to transport RNA across membranes, thus providing a mechanistic path for exRNAs to achieve their extracellular topology. Here, we review types of exRNAs and the pores capable of RNA transport to provide a logical and testable path toward understanding the biogenesis and regulation of cell surface glycoRNAs.

INTRODUCTION

RNA is central to life. It performs sensing, switching, catalytic, scaffolding, translational, and coding functions within the cell. Such a vast repertoire of purposes suggests that RNA could play some role in most aspects of biology writ large. However, the extracellular space is traditionally not considered accessible by or hospitable to RNA molecules, and only more recently has robust evidence challenging this view been obtained. With Flynn et al.'s (41) discovery of the new hybrid between RNA and glycans (glycoRNAs) on the surfaces of living cells as well as other surface RNAs (56), there is a critical need to more broadly examine the possibility and scope of cell surface RNA biology. For example, details remain to be uncovered regarding how glycans are connected to the RNA, what the set of glycoRNA glycans are, what proteins might bind glycoRNAs selectively, how glycoRNAs are fixed on the membrane, how glycans may impact the structure of their RNA templates, and how RNAs that begin their life as nuclear biopolymers end up on the cell surface (**Figure 1**). The last concept, covering a localization change of RNA, is the focus here; answers to this question would shed light on the trafficking of glycoRNAs from their site of synthesis to their eventual arrival on the cell surface.

Observing RNAs outside of cells traditionally poses a number of additional problems and questions, including how they are able to systemically and precisely get outside of a cell (how can RNAs cross membranes?), how they can maintain stability long enough to have a function (why are RNAs outside of cells not rapidly degraded?), and how they can find a molecular partner to confer that function (what functional moieties could RNAs possess to have active roles in extracellular biology?). While answers to these questions are not often discussed together, as they concern aspects of RNA biology that are less well studied, in this review we highlight the fact that there are indeed many examples across various biological contexts that suggest evolution has solved these problems many times over.

EXTRACELLULAR RNAs: EXAMPLES AND FUNCTIONS

The vast majority of efforts to study nucleic acids, both RNA and DNA, have typically focused on intracellular activities. However, roles for RNAs in the extracellular space, while having been documented for many decades (11, 66), are more recently being expanded in scope due to higher-sensitivity techniques and more directed funding efforts. Extracellular RNAs (exRNAs), broadly defined as any RNAs found outside of the cell membrane, are most often small RNAs that are proposed to influence processes ranging from immune cell activation to cancer progression (93). Databases such as the exRNA Atlas (86) have begun cataloging these exRNA populations across species and biofluids, and the wide range of RNA types found in the extracellular environment raises questions about their functions. We organize exRNAs into at least three categories related to their topological and membrane constraints, which are summarized in **Figure 2**: (*a*) RNAs inside extracellular vesicles (e.g., exosomes), which are topologically cytosolic; (*b*) free or carrier-bound RNAs outside of vesicles, which are topologically extracellular or luminal but not constrained by a membrane; and (*c*) cell surface–tethered RNAs, which are topologically extracellular but anchored by some means to the plasma membrane. The last category would include more recently discovered RNAs, including glycoRNAs (41). Here, we explore the recent literature on these three categories, examining what RNAs are selected, by what mechanisms they are selected, and what functions (if any) they are known to have.

Vesicular Extracellular RNAs

Vesicles that can be transported from one cell to the extracellular space and eventually toward other target cells are broadly defined as extracellular vesicles (EVs). EVs include exosomes,

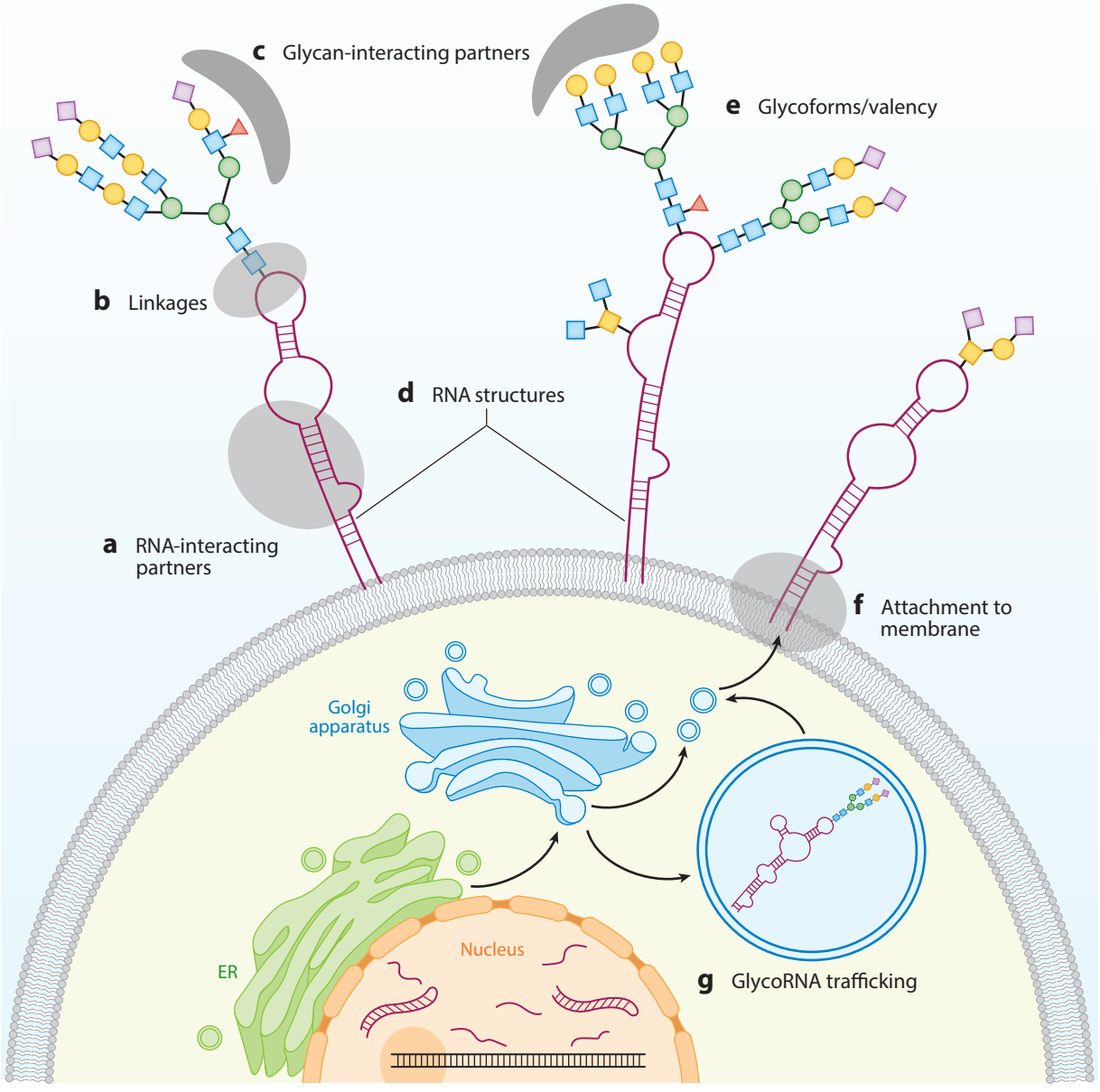


Figure 1

Features and open questions surrounding cell surface glycoRNAs. GlycoRNAs have access to and are positioned on the surfaces of living cells; however, these transcripts begin their life in the nuclei of cells. Many aspects of glycoRNA biology are not yet understood, including (*a*) what proteins or other factors bind cell surface glycoRNAs, (*b*) what the molecular linkage is between glycans and RNA, (*c*) whether there are glycoRNA-specific binding proteins, (*d*) whether cell surface glycoRNAs adopt unique RNA structures compared with the intracellular pools, (*e*) the scope of glycans and glycoforms conjugated to RNA, (*f*) how cell surface glycoRNAs anchor to the membrane, and (*g*) how RNAs are selected and transported into the luminal spaces where glycosylation can occur. Abbreviation: ER, endoplasmic reticulum.

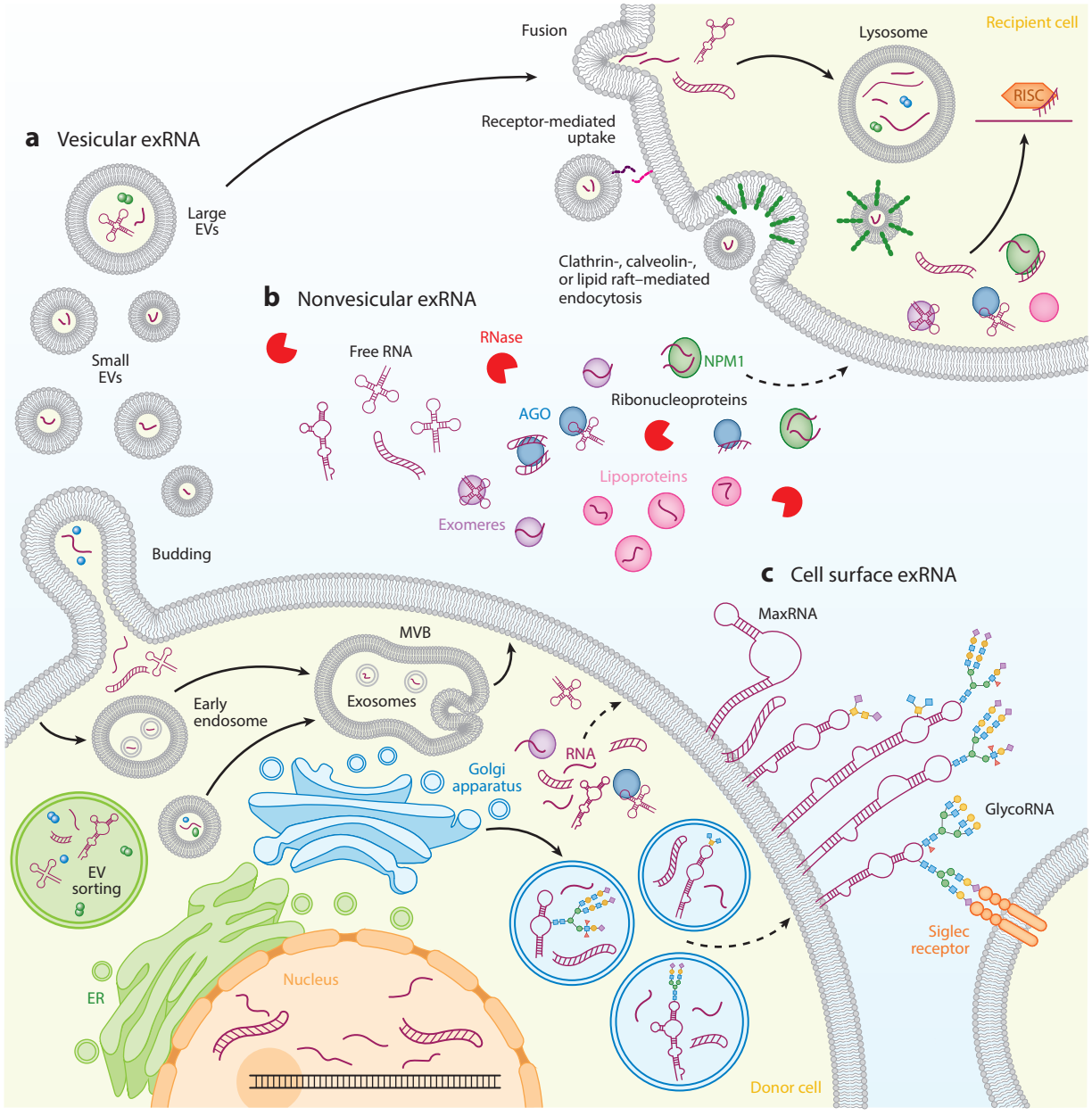


Figure 2

Types of exRNAs. The three main categories of exRNAs are (*a*) vesicular, (*b*) nonvesicular, and (*c*) cell surface membrane-tethered exRNAs. These exRNAs can be released from a donor cell to the extracellular environment, where they must be protected from degradation by RNases and can subsequently interact with recipient cells. Vesicular exRNAs can be selectively or nonselectively sorted into a variety of EVs that are released by mechanisms such as budding and MVB fusion with the plasma membrane. Vesicular exRNA can enter recipient cells through varying mechanisms of endocytosis. Nonvesicular exRNAs can exist as free RNAs or bound to carriers such as exomeres, RNA-binding proteins, or lipoproteins that protect them from RNase degradation. The mechanisms of release and uptake of nonvesicular exRNAs remain to be determined. Cell surface membrane-tethered exRNAs, such as glycoRNAs and maxRNAs, are presented on the cell surface. GlycoRNAs have been reported to interact with Siglec receptors. Abbreviations: ER, endoplasmic reticulum; EV, extracellular vesicle; exRNA, extracellular RNA; maxRNA, membrane-associated extracellular RNA; MVB, multivesicular body; RISC, RNA-induced silencing complex.

microvesicles, apoptotic bodies, and many other subpopulations; these EVs contain many types of cargo, including protein, RNA, and DNA (94). Here, we focus on the RNA contents of EVs, which have been the subject of intense study and have drawn increasing interest with the application of sequencing technologies, uncovering a vast array of RNAs contained in EVs. Cataloging efforts such as the Vesiclepedia Database (63) list more than 27,000 mRNAs and 10,000 noncoding RNAs (often as fragments) that have been identified as EV cargo; some EVs contain many RNA molecules, while others contain very few, if any. A stoichiometric analysis of exosomes from diverse biofluids estimated that, on average, each exosome contains less than one molecule of any given microRNA (miRNA), so these exRNAs may be a minority of EV cargo (26); however, biochemical preparation of exosomes can make counting the number of individual vesicles in a sample imprecise. Major members of EV RNA pools do include tRNAs, tRNA-derived small RNA fragments (tRFs), miRNAs, mRNAs, rRNAs, Y RNAs, Piwi-interacting RNAs, spliceosomal RNAs, long noncoding RNAs, and other small RNAs (129). A defined process, comprising the selection of contents, packaging into a vesicle, and vesicle release from the source cell, is the typical path for the generation of vesicular exRNAs.

EVs are formed and released through different cellular pathways, including the formation of endosomes and multivesicular bodies within the cell, leading to the release of exosomes or microvesicles budding from the plasma membrane (1). Additionally, cell death can lead to EVs in the form of apoptotic bodies, and viral infection often leads to the release of cell-derived viral particles containing viral RNA. The sorting of distinct RNA populations into EVs provides evidence for some selective mechanisms of exRNA release, but it is important to note that differential EV composition does not necessarily imply active selection. One mechanism of exRNA sorting into EVs may be at the level of RNA sequence, as is the case with sumoylated hnRNPA2B1-mediated binding of miRNAs through a GGAG motif exosomal targeting sequence (131). Similarly, two motifs in miR-122 mediate its sorting into EVs by binding with the Lupus La protein (116). Several RNA motifs are enriched in EVs and the extracellular environment in general, including A/G-rich, AU-rich, and C-rich sections, all of which bind to different RNA-binding proteins (RBPs) (36). This motif enrichment is a possible indication that cells possess selective methods of RNA secretion. Furthermore, electrostatic interactions likely play an important role in the association of certain RNA sequences with membranes and lipid raft regions during the processes of EV sorting and membrane surface presentation of RNA. RNA sequence and structure possess varying affinities to lipids, possibly allowing them to exist as part of lipid rafts or other islands within membranes (59). Moreover, RNA modifications, such as miRNA 3′ end uridylation versus adenylation (67), seem to allow for differential sorting and stability in the extracellular environment.

RNA is not the only cargo in EVs, which contain a diverse set of proteins such as RBPs. In this context, RBPs have characterized roles in RNA sorting into EVs, a topic that has been reviewed previously (36). A general concept from work in this area is that RBPs are able to select particular sequences for vesicle inclusion; however, roles for both inclusion and exclusion of RNAs have been ascribed to particular RBPs (e.g., hnRNPA2B1, ANXA2, YBX1, and AGO2 proteins) (76, 81, 95, 131). All of these mechanisms are possible due to the topological arrangement of the EV cargo, namely, that they are contiguous with the cytosol and nucleus (86, 93). Thus, conceptually, when EVs are transported from one cell to another, a fusion event with a target cell results in a transfer of cytosolic/nuclear components from the source cell. This configuration also means that the surface of an EV is topologically contiguous with (although physically disconnected from) the external surface of the plasma membrane. The surfaces of EVs are well characterized to contain cell surface markers and transmembrane receptors, which are thought to participate in EV targeting (98). There have also been reports that the surfaces of EVs maintain a glycocalyx (65, 92), or the dense outer layer of glycosylated biopolymers that surround the plasma membrane. Together, EVs are

a regulated, physical container of cytosolic and nuclear biopolymers, including RNA, that can transit the extracellular space to distal tissues and cells. The lipid bilayer affords the packaged RNA stability outside of the cell as well as targeting or signaling molecules in the form of its own surface membrane proteome.

Nonvesicular Extracellular RNAs

Nonvesicular secreted RNAs that exist in biofluids, without the protection of a membranous EV, are a growing class of RNAs that are truly in the extracellular space, from a topological perspective. This class of exRNAs can be associated with nonvesicular carriers, such as lipoproteins, RBPs, or exomeres, or can freely circulate without the help of another carrier molecule (55, 86, 122). Nonvesicular exRNA populations consist largely of tRNAs and tRNA halves, Y RNAs, miRNAs, whole ribosomes, rRNAs, and mRNAs (86). The composition of exRNAs varies widely across different biofluids and disease states (32, 86, 154). For example, more Y RNAs are found in the blood, while more tRNA-derived fragments are found in urine and bile (47). Here, we highlight some key and recent work that describes what constitutes this class of exRNAs and concepts around how and where nonvesicular exRNAs are generated.

In contrast to EVs, less is known about the biogenesis of nonvesicular exRNAs. It is possible for nonvesicular exRNAs to be released in a selective or nonselective manner. An example of nonselective release would be via shedding of RNAs from dead cells into the culture media or serum, while a selective process could, for example, leverage precise RBPs. Whether one or both of these mechanisms are operable in vitro and in vivo is strongly debated (124). For example, studies have demonstrated that some miRNAs previously identified as exRNAs released by cells were more likely to be contaminants from fetal bovine serum or other cell culture reagents (120). However, nonselectivity does not preclude these types of exRNAs from having important biological roles. Release of exRNA due to cell death may be used as a form of healthy cellular communication or during disease, for example, in the hypoxic centers of solid tumors or during a lytic virus infection, where rates of cell death are much higher, enabling the body to sample those diseased cells (124). However, inhibiting RNA degradation in the extracellular environment results in similar RNA profiles inside and outside of the cell, suggesting that exRNA release could be primarily nonselective, after which differential exRNA stability leads to populations that are different from the intracellular populations (123). In addition to release from the cells of interest, in some contexts much of the exRNA (in some cases up to 80%) can come from exogenous origins, including the microbiome, parasites, or dietary sources (43). There are both selective and nonselective means for exRNAs to exit cells, and defining this path for new exRNAs is an important part of characterizing the full context of the biology of those exRNAs.

Once outside the cell, biopolymers are susceptible to degradation. The human genome contains many genes encoding secreted RNases (49, 78), thus establishing a potentially inhospitable environment for exRNAs. It is worth noting that extracellular biopolymer catabolic activity is not limited to RNA, as there are many examples of secreted proteases (84). In the context of nonvesicular exRNAs, binding partners (including protein chaperones) offer a simple and effective shield to secreted RNases. One of the most well-studied ribonucleoprotein complexes is with AGO2 (10). miRNAs have exceptional stability in serum compared with other types of RNA, and these miRNAs are often bound to AGO2 (126). NPM1 has been proposed as another carrier of exRNA that could work to protect miRNAs from degradation (138). exRNAs, including miRNAs, tRNAs, and Y RNAs, have also been observed to form complexes with lipoproteins such as low- and high-density lipoproteins (5). Precisely and characteristically cleaved tRFs, including 5′ halves of $tRNA^{Gly}$ and $tRNA^{Glu}$, have been identified as some of the most abundant small RNAs in the extracellular space and can stably exist without association with any EV of a protein carrier

(121). These tRNAs are proposed to form dimers that protect them from degradation by single-stranded RNases, highlighting the importance of intermolecular interactions in exRNA stability. Finally, not only do exRNAs undergo degradation, but some amount of functional exRNA processing also seems to occur outside of the cell. In particular, tRNAs and Y RNAs are thought to exit the cell in their full form, where they can then be cleaved by RNase 1 into smaller fragments that are commonly observed in exRNA populations (87). As a class, nonvesicular exRNAs provide an excellent example of topologically extracellular RNAs that have been observed across many biological and experimental systems. Expanding on their functional roles (discussed in part below) and developing more facile techniques to isolate and characterize them will enable deeper insights into this pool of biopolymers in the extracellular space.

Cell Surface–Tethered Extracellular RNAs

Membrane association of RNA, beyond the RNA being encapsulated or free of membranes, offers another physical configuration for RNA biology to occur. RNA–membrane association is a common feature across many biological pathways, and RNA–lipid interactions have been known for nearly 40 years (17). The utility of this feature could be imagined in the context of an RNA associating with one type of cellular membrane and therefore establishing a biased localization pattern within a cell. In the context of translation, initiation often occurs in the cytosol, followed by mRNA trafficking to the surface of the endoplasmic reticulum (ER) with a ribosome and nascent polypeptide chain via the signal recognition particle (SRP) pathway (82, 132, 134). Ribosomes translating on the ER produce the secretory and integral membrane proteins of our cells (12, 132, 133). Once synthesized at the ER, secretory and membrane proteins are translocated into the ER lumen and integrated into the ER membrane, respectively. Protein folding and maturation in the ER are essential for cell physiology. One arm of the unfolded protein response in the lumen of the ER (135) results in activation and clustering of a protein called IRE1. IRE1 is an ER-tethered, cytosol-facing RNase (28, 109) that cleaves an intron from the mRNA encoding the transcription factor XBP1, leading to a culmination in the production of spliced XBP1 and an increase in ER-folding and degradation activities (2, 148). Activated IRE1 also cleaves ER-localized mRNAs through regulated IRE1-dependent decay (53, 54). The IRE1-based signaling provides another example of membrane-associated RNA biology.

Interestingly, mitochondria are also a destination for mRNAs. Studies using *Saccharomyces cerevisiae* have revealed two mechanisms for how mRNAs are trafficked to mitochondria. First, mRNAs are recruited to the surfaces of mitochondria by Puf3, an RBP localized on the outer membranes of mitochondria (103). Second, mRNA localization to the mitochondria requires translation of the N-terminal mitochondrial targeting sequence associating with the import complex embedded in the mitochondrial membrane, which is a Puf3-independent pathway (45). Newer unbiased tools have been developed to define where RNAs localize inside of cells (33, 38, 71), with applicability to membranous organelles. APEX-seq has been directly used to define plasma membrane–associated RNAs, characterizing 75 RNAs to be in physical proximity to the plasma membrane but not the outer mitochondrial membrane (OMM) or ER membrane (146). One of these 75 RNAs, the long noncoding RNA PMAR72, shows affinity to sphingomyelin and localizes to distinct islands in the membrane, suggesting a possible role for RNAs in cell membrane lipid rafts. Together, these results highlight some exciting concepts related to the utility of RNA biology on membranes, and importantly, the above examples have a common topology: The RNAs being studied are contiguous and accessible to the cytosol.

RNAs configured on the luminal side of cellular membranes are less well described, although there are some historical and more recent examples that make this position of increased interest. In principle, a lumen-facing RNA would be topologically contiguous with the extracellular space

and thus could be considered an exRNA; membrane-associated extracellular RNAs (maxRNAs) are one such class of RNAs (56). In an effort to understand whether cells present RNAs on the plasma membrane, Huang et al. (56) developed a technique named Surface-seq that uses cell membrane–coated nanoparticles for the differential analysis of RNAs inside and outside of the cell. By focusing on long noncoding RNAs, they identified 17 long noncoding RNA maxRNA candidates that consistently appeared on the outer cell membrane. They further showed that these maxRNAs differ across cell types. For example, out of human peripheral blood mononuclear cells, monocytes displayed the most maxRNAs, suggesting that maxRNAs play a role in cell–cell contacts. Furthermore, blocking certain maxRNAs inhibited monocyte–endothelial cell interactions, and they found that genes such as *FNDC3B* and *CTSS*, which are known to be involved in cell adhesion, were responsible for maxRNA production.

GlycoRNAs are a different example of exRNAs tethered to the cell surface. Flynn et al. (41) found that a select group of small noncoding RNAs, including tRNA, small nuclear RNA, small nucleolar RNA, and Y RNA, are glycosylated with sialic acid containing glycans. Chemical, genetic, and mass spectrometry analysis demonstrated a dependency of glycoRNAs on the N-glycan biosynthesis pathway. Importantly, glycoRNAs gain access to the external surfaces of living cells through an as yet undefined pathway. On the surface, specific members of Siglec proteins, a family of immunomodulatory receptors (41), bind in a manner fully or partially dependent on RNA. RNA-dependent binding suggests that some signaling through Siglecs could be mediated by recognizing cell surface glycoRNAs. Mechanisms for how maxRNA or glycoRNA could associate with the external surface of the plasma membrane have not been defined. One possibility is that these RNAs directly contact the membrane, and there have been many reports of this in the literature (60, 73). Alternatively, there could be protein intermediates acting as anchors to the membrane. Precisely defining how cell surface–tethered exRNAs are affixed to the membrane will provide biophysical as well as regulatory insights into these cell surface ligands.

The Functions of Extracellular RNAs

While there are many proposed functions of exRNAs, their role in biology remains relatively poorly understood. When considering what exRNAs do, it is important to consider the possibility that no obvious phenotypic change occurs when they are released from or delivered to a cell. Furthermore, where to measure the phenotype is not obvious, as the release of exRNAs may affect the donor cell more than the recipient cell (93). Integrating these complexities with the often technically difficult task of studying exRNAs has made it challenging to quickly establish functions of exRNAs, resulting in various criticisms (4), some of which have been reviewed previously (48). However, there are clear examples of exRNAs performing reproducible actions in biology.

In the context of the ability of exRNA to transmit information from one cell to another, assuming enough exRNA is even delivered, many vesicle-contained exRNAs will be degraded in the cellular lysosome or released shortly after uptake by a recipient cell (111), reducing the likelihood that those transcripts have time to effect a function. However, there is evidence to support the role of exRNAs in meaningful intercellular communication and regulation of gene expression. Extracellular miRNAs have received the most attention and are therefore better understood than other exRNA populations, and the proposed functions of extracellular miRNAs in intercellular communication have been previously reviewed (150, 155). The possible roles of extracellular miRNAs range from the communication between embryonic stem cells to the antigen-driven fine-tuning of the immune response between T cells and antigen-presenting cells, and it is likely that other types of exRNAs play important roles in intercellular communication as well. Neurons release RNAs at synapses, and the RNA contents change with neuronal excitation (63, 93). Astrocytes have been shown to take up miRNAs released by nearby neurons (85). miRNAs from the extracellular

environment are often delivered to recipient cells with components of the RNA-induced silencing complex (RISC), suggesting that exRNAs can participate in traditional RISC functioning in recipient cells (46). Extracellular mRNAs can also be delivered to recipient cells and translated into their corresponding proteins (100). In addition to local intercellular communication, EVs carrying exRNAs might travel to distant tissues. Exosomal miRNAs from adipose tissue have been suggested to affect the expression of Fgf21 mRNA in hepatocytes (118). tRF exRNAs have also been suggested to play a critical role in the epigenetic inheritance of metabolic states in mouse sperm, where tRFs in the epididymosomes from mature sperm are collected by immature sperm progressing through the epididymal tract (23, 106). This represents not only a case of intercellular communication by exRNAs but also one that impacts subsequent generations. Beyond intercellular communication, like that described for processed RNY5 RNA in cancer cells (19), exRNAs may even allow for communication between animals, such as *Caenorhabditis elegans* (93, 137).

The role of exRNAs in the immune system has been the focus of significant research effort. Vesicular exRNAs in the immune system have been previously reviewed (128). A general functional paradigm established for exRNA signaling is that exRNAs from pathogens activate the immune system, while exRNAs from host cells allow for intercellular communication to coordinate a variety of immune responses. An example of the latter occurs during inflammatory responses or a viral infection, when the levels of specific tRNA halves change (153). Human-derived exRNAs, particularly ribosomal exRNAs, also contribute to T cell activation and leukocyte recruitment to the inflamed endothelium (97). Specific exRNAs, such as mRNAs or ribosomes, function as damage-associated molecular patterns released from dead cells, acting as self-RNAs that stimulate various immune responses through pattern recognition receptors (115, 123). Furthermore, nonvesicular RNAs are known ligands of Toll-like receptors (including TLR3, -7, and -8), highlighting how intercellular communication facilitated by exRNAs could lead to immune responses such as inflammation (25, 30, 37).

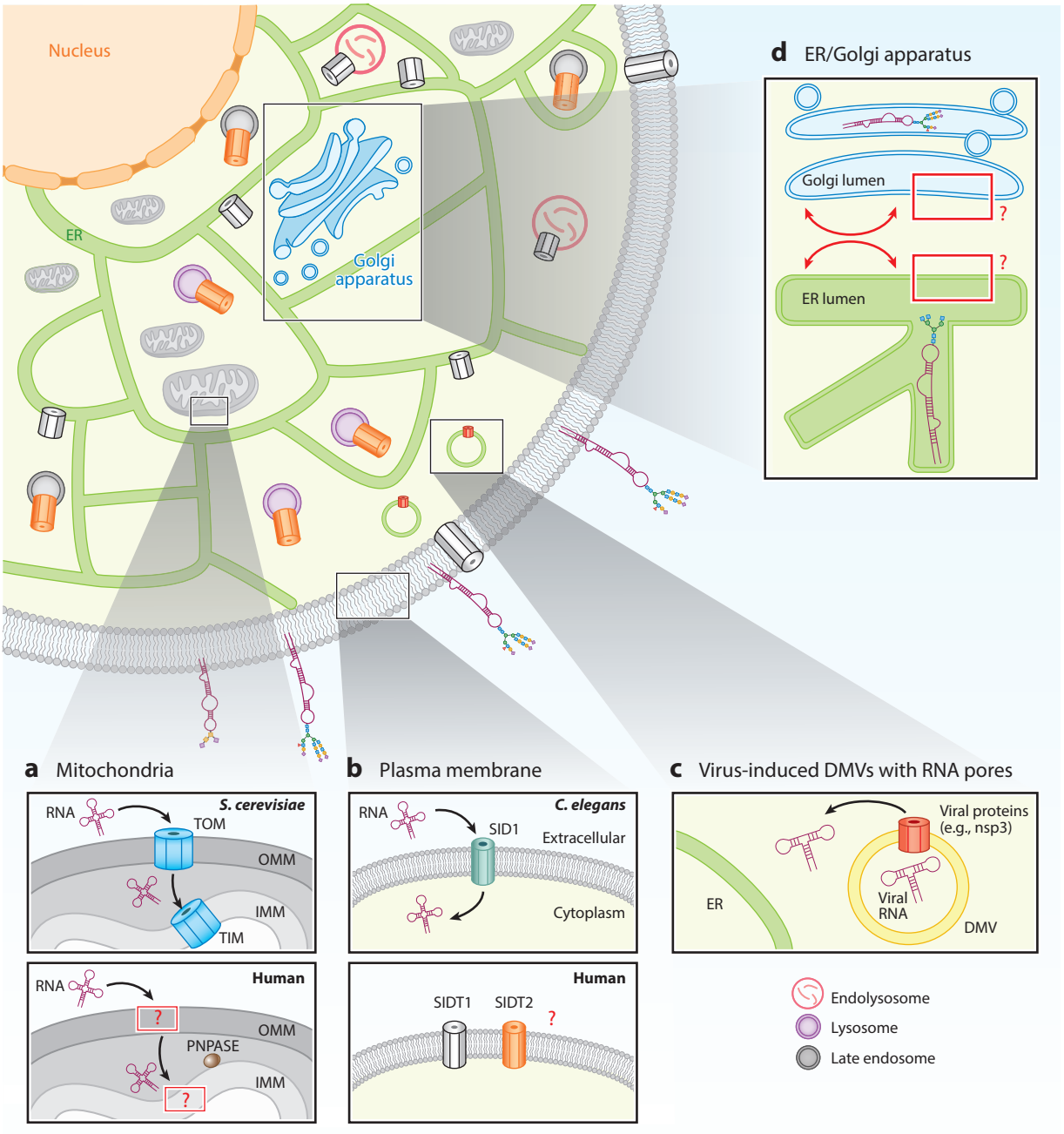
Other important functions of exRNAs likely include the regulation of cellular metabolism, energetics, and homeostasis. Cells may release or take up exRNAs as degradation products for recycling, contributing to metabolic homeostasis. Furthermore, cells likely export excess RNAs to maintain specific levels of gene expression within the cell (18). Circulating exRNAs may also protect cells and their cell surface RNAs from excess RNase activity, keeping these enzymes busy in the extracellular environment (117). Finally, dramatic changes in exRNA populations associated with various cancers, infections, and even aging highlight the likely importance of exRNA functioning in normal physiology. EVs carrying oncogenic miRNAs can drive tumorigenesis by altering the tumor microenvironment and even stimulating metastasis (152). The use of exRNAs as biomarkers in a wide range of diseases has been extensively reviewed (50, 154), and this well-documented change in exRNA associated with disease supports the theory that exRNAs play important roles in healthy cell functioning that are yet to be discovered.

The roles that exRNAs are known to play across biology are growing in scope and function. Our ability to probe exRNAs has been enhanced with higher-sensitivity and lower-cost sequencing technologies coupled with better biochemical isolation and characterization. Of the three classes highlighted above, the majority of our knowledge lies within the EV-contained exRNAs. While our understanding of EV biology is far from complete, a deeper understanding of nonvesicular exRNAs as well as cell surface–tethered exRNAs will expand how we think about RNA's role outside of the cell.

RNA CROSSING MEMBRANES

Eukaryotic cells possess an outer membrane and many internal membrane-bound organelles, both of which consist of semipermeable lipid bilayers (7, 16). The plasma membrane encases the entire

cell and separates the interior of the cell from the outside environment. The internal membranes in the cytoplasm divide the intracellular space into several individual domains with vital functions and dynamic structures, forming subcellular organelles such as the mitochondria, ER, and Golgi apparatus (**Figure 3**). While some classes of small molecules can cross these bilayers passively, many



(*Caption appears on following page*)

Figure 3 (*Figure appears on preceding page*)

RNA translocation across membranes. A complex network of vesicular organelles exists between the nuclear envelope and the plasma membrane of eukaryotic cells. Various mechanisms for transport of RNA molecules have evolved across different species and organelles. Four examples are shown here. (*a*) In *Saccharomyces cerevisiae*, RNA translocation into the mitochondrial matrix is mediated by the TOM/TIM complex, the canonical protein import machinery. In humans, PNPASE is required to transport RNA in the intermembrane space; however, the actual membrane translocation mechanism across the OMM and IMM is unknown. (*b*) In *Caenorhabditis elegans*, SID1 enables transfer of double-stranded RNA across the cell membrane. The human genome encodes SIDT1 and SIDT2, two mammalian orthologs of SID1. The localization of the human proteins varies; SIDT1 occupies the plasma membrane, endolysosome, and ER, while SIDT2 localizes to the lysosome, late endolysosome, and plasma membrane. (*c*) ER-derived DMVs can be generated in the cells infected with SARS-CoV-2. The viral spherules are not fully sealed, as viral proteins like nsp3 can form a crown-like pore embedded in the DMV membranes that allows viral RNA export. (*d*) How endogenous small RNAs could enter the luminal space of the ER and Golgi apparatus is still unknown, but a protein channel would offer a mechanism for selection and transport of the RNA templates for glycosylation and eventual cell surface presentation. Abbreviations: DMV, double-membrane vesicle; ER, endoplasmic reticulum; IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; TIM, translocase of the inner membrane; TOM, translocase of the outer membrane.

molecules both large and small require active transport. Transport across specific membranes is a highly regulated process that can build up electric potential and chemical gradients and concentrate enzymes in specific organelles for enhanced biochemical activity. The presence of glycoRNAs (41) and maxRNAs (56) on the cell surface highlights a requirement for a topological change from where RNA is biosynthesized: The RNA must move from the nuclear/cytosolic space to the luminal side of the cellular membranes. While some exRNAs may be released through ruptured membranes (discussed above), an alternative mechanism is transit through intact membranes. In the context of other biomolecules, including small molecules and proteins, life has evolved numerous strategies to enable precise transport across lipid membranes. Here, we examine the landscape of how biology can pass RNA molecules across cellular membranes in the backdrop of the more recently conceptualized cell surface glycoRNAs.

Extracellular RNA Uptake

There is significant evidence supporting the uptake of exRNA by recipient cells after it has been circulating in the extracellular environment. Vesicular exRNAs have multiple pathways into recipient cells through uptake by the cell membrane (**Figure 2**). Possible mechanisms of EV uptake include endocytosis (e.g., clathrin-, caveolin-, or lipid raft–mediated endocytosis), macropinocytosis, and phagocytosis, all of which have been reviewed previously (1). Fusion of EVs with the cell surface allows for a direct release of EV cargo into the cytosolic compartment. Receptor-mediated uptake is another pathway that utilizes specific exosomal ligands, allowing for more directed targeting (1). Receptor-mediated uptake has also been linked to lipoprotein delivery, such as the scavenger receptor class B type I–dependent pathway for high-density lipoproteins (130). However, it remains to be determined how the uptake of extracellular free RNA or AGO–RNA complexes could occur (77). Nonvesicular exRNAs bound to RBPs, such as AGO2 or NPM1, may be targeted to cell surface receptors that allow for uptake into the recipient cell through interaction with the RBP carrier. Alternatively, perhaps there are cell surface transporters that directly bind RNA.

RNA Transport: Into the Mitochondria

Mitochondria, classically defined as the powerhouse of the cell, have two major membranes. The OMM separates mitochondria from the cytoplasm and fully surrounds the second layer, called the inner mitochondrial membrane (IMM). The conspicuously folded IMM creates a space called the mitochondrial matrix that facilitates ATP production with specialized enzyme complexes.

Mitochondria harbor their own genetic system, and in humans, they retain a circular double-stranded DNA molecule of 16,569 bp (8). Mitochondrial DNA contains only 37 genes encoding 13 core proteins of the oxidative phosphorylation process. However, more than 1,000 proteins are needed for mitochondrial function, and the remainder are encoded in the nuclear genome, subsequently synthesized using cytosolic ribosomes, and finally imported into mitochondria for the optimum homeostasis and activity (140). The primary import gate of preproteins destined for the mitochondria is the translocase of the outer membrane (TOM) complex (88) (**Figure 3**). This complex consists of seven subunits: a core component (TOM40), which forms two β-barrel channels in the OMM as the principal protein-conducting pore structure (9, 52); three small subunits (TOM5, TOM6, and TOM7), which help with complex stability and assembly; and three receptors (TOM20, TOM22, and TOM70), which associate with substrate selection by recognizing the mitochondrial targeting sequence at the N termini of preproteins (88). Once imported into the intermembrane space of mitochondria between the OMM and IMM, the preproteins cross the IMM through the translocase of the inner membrane (TIM) complex (88). Mitochondrial processing peptidase located in the mitochondrial matrix further cleaves the targeting sequence from preproteins to yield the mature protein, which has diverse functions (14). Altogether, mitochondria have evolved a precise and regulated system to traffic large numbers of membrane-impermeant proteins that are necessary for life into their interior.

Proteins are not the only biopolymers that require mitochondrial import. In addition to the 13 protein-coding genes, the mitochondrial genome encodes only one small rRNA (12S), one large rRNA (16S), and 22 tRNAs (8). However, it has been long understood and observed that many nuclear-encoded noncoding RNAs are inside of mitochondria across various species (35, 83). Major classes of noncoding RNAs include tRNAs (102), miRNAs (68), cytosolic rRNAs (13, 149), and the mitochondrial RNA processing (*MRP*) RNA (20, 70). These small noncoding RNAs need to be imported from the cytoplasm to maintain various mitochondrial functions. There are at least three steps an RNA would need to pass through to arrive within the IMM: passage across the OMM, transit from the OMM to the IMM, and passage across the IMM. While all mitochondria require RNA import, the particular RNAs imported into mitochondria and the related mechanisms are variable across different eukaryotic species. Like protein import, RNA uptake requires ATP and is protein dependent. In *S. cerevisiae*, nuclear-encoded $tRNA^{Lys}$ specifically binds Eno2p and then changes its structure to be recruited to the surface of mitochondria by mitochondrial pre-LysRS (34). The subsequent RNA translocation into the mitochondrial matrix is completed by the TOM and TIM complexes (114) (**Figure 3**). Plant cells also possess another voltage-dependent anion channel–dependent pathway to import RNA into mitochondria (104, 105). The mechanistic basis of mitochondrial import in plants and *S. cerevisiae* is relatively well established, unlike the more poorly characterized process in mammals.

In the context of cell surface RNAs, including glycoRNAs, changing topology with respect to cellular membranes will be a critical step in their eventual surface presentation. Considering how mitochondria accomplish this in mammalian cells could lead to conceptual insights into glycoRNA biogenesis pathways. To our knowledge, the best-characterized molecular mechanism defined for RNA import into the mitochondria in mammalian cells is via the protein PNPASE (136). PNPASE was first identified as an evolutionarily conserved 3′-to-5′ exoribonuclease that regulates small RNAs' turnover with the DNA/RNA helicase SUV3 in mitochondrial matrix (15, 147). Work to directly uncover the role of PNPASE in mitochondrial RNA transport demonstrated that PNPASE selectively mediates the import of *RNase P*, *5S rRNA*, and *MRP* RNAs (136). PNPASE itself possesses an N-terminal mitochondrial targeting sequence, which allows it to traffic through the mitochondrial double membranes into the matrix for maturation via the TOM complex. PNPASE is not a mitochondrial transmembrane protein, and it has been shown to form a trimer,

but this occurs only in the mitochondrial intermembrane domain (22, 99). This localization and organization suggest that PNPASE may operate as an RNA tunnel from the OMM to the IMM (136) (**Figure 3**).

While PNPASE has been directly implicated in mitochondrial RNA import, it does not provide a complete model for the entire process of RNA import. The destination of imported RNAs is the mitochondrial matrix; however, how RNA traffics through the membrane portion of the OMM and IMM remains elusive. While the TOM and TIM complexes are conserved from plants to humans, whether they facilitate mitochondrial RNA import in mammals has not been proven. If this translocation function is not conserved, then that suggests that there are other mammal-specific transmembrane pores to facilitate RNA import. Beyond this, at the transcript level, specific nuclear-encoded tRNAs (such as $tRNA^{Gln}_{UUG}$ and $tRNA^{Gln}_{CUG}$) are detected in human mitochondria but are apparently not impacted in their mitochondrial accumulation in PNPASE-knockout cells (136). However, knockout of PNPASE also causes abnormal mitochondrial crista morphology (136), which raises questions regarding a structural role in addition to an RNA transport role in mitochondrial function. Finally, PNPASE has also been observed to facilitate the release of RNA from mitochondria in response to several stress conditions, including viral infection (29) and mitochondrial outer membrane permeabilization (75). Altogether, the mitochondria provide an excellent example of a membrane-enclosed organelle that requires active and precise translocation of intact RNA molecules for homeostatic function and stress response. Many of the key proteins and complexes are highly conserved and provide opportunities to study them and homologous complexes in the context of topologically extracellular RNAs, potentially including glycoRNAs.

RNA Transport: Double-Stranded RNA Movement from Cell to Cell in *Caenorhabditis elegans*

RNA interference (RNAi) is a fundamental gene-silencing pathway in which noncoding double-stranded RNAs (dsRNAs) mediate sequence-specific targeting of mRNA and change gene expression through various mechanisms, including mRNA degradation and modulation of translation (40, 119). While the molecular mechanisms of RNAi are often studied in vitro or within the context of a cell, there exist examples in nature where RNAi is communicated across cells. Given the inability of RNA to directly pass across intact lipid bilayers, these observations invoke biological processes to take up or transport dsRNA across membranes.

C. elegans has been an ideal model to study many aspects of the RNAi pathway. One feature of RNAi in *C. elegans* is this species' ability to be fed dsRNA and exhibit silencing of genes targeted by those eaten dsRNA molecules (119). These early observations suggested that *C. elegans* can take up intact dsRNAs, import them en masse to the interior of cells, and leverage them in the endogenous RNAi pathway. Genetic screening efforts led to the identification of systemic RNA interference defective proteins 1 and 2 (SID-1 and SID-2) (reviewed in 57). Both of these proteins contain transmembrane domains and are responsible in part for exogenous or environmentally sourced dsRNA in the cytoplasm of animal cells (141, 142) (**Figure 3**). SID-1, which contains 11 transmembrane domains and has its N terminus exposed to extracellular space (39), forms an apparent pore that transports dsRNA through the cell membrane (108). Loss-of-function mutational data show that both the extracellular and transmembrane domains are vital for SID-1 function (39). From a tissue-specificity perspective, SID-1 is widely expressed in all nonneuronal cells (62, 141), whereas SID-2 is largely restricted in its expression to the intestine (142). Moreover, SID-2 is a single-pass transmembrane protein with an intracellular C terminus (142), suggesting that, unlike SID-1, it has no direct ability to form a channel in the membrane to transport dsRNA. Nevertheless, SID-2 imports dsRNA from an acidic extracellular environment through endocytosis (80).

The activity and cooperation of these two proteins enable dsRNA from the environment to have systemic effects—uptake from the intestinal lumen via SID-2 and cell-to-cell transport across membranes via SID-1—that highlight multiple molecular mechanisms for moving RNAs across and around membrane bilayers in *C. elegans*. Beyond *C. elegans*, *Drosophila* models have been used to understand features of the homolog of SID-1 and the systemic RNAi pathway (107). For example, *Drosophila* S2 cells expressing *C. elegans* SID-1 provided evidence that SID-1 alone is not sufficient to limit long dsRNA transport across the plasma membrane, although shorter dsRNAs (less than 500 bp) transferred more quickly (107). Given that SID-1-mediated dsRNA transport is proposed to be a diffusion-limited process (39), a high concentration of dsRNA in the environment would act to accelerate transport rates through the pore formed by SID-1 (107).

SID-1 transmembrane family 1 and 2 (SIDT1 and SIDT2) are two mammalian orthologs of the *C. elegans* SID-1 protein. While the mechanistic understanding of these mammalian orthologs is less developed compared with those of *C. elegans*, in the past 15 years a series of studies have revealed various molecular features of SIDT1 or SIDT2 and began to hint at some functional activities. First, a critical property that has been investigated is where these two proteins localize; defining the membranes that they could create pores in would facilitate inference of what biology they may help to regulate. Studies in mammalian tissue culture settings using overexpressed and exogenously tagged proteins have shown that SIDT1 can localize to the plasma membrane (31), endolysosome (90), and ER (113), while SIDT2 localizes mainly to the lysosome (3, 27, 51), late endolysosome (91), and plasma membrane (112). Functional experiments have shown that, similar to SID-1, both SIDT1 and SIDT2 can facilitate dsRNA transport into the cytoplasm from extracellular space (31, 91, 125, 145). The localization to multiple cellular membranes highlights a possibility that SIDT1 and SIDT2 could participate in pathways such as the innate immune response (91); ingestion of extracellular nucleic acids, including antisense oligonucleotides (112, 113); RNautophagy (defined as a pathway by which RNA is taken up directly into the lysosome for degradation) (3, 51); and Parkinson's disease (42).

Both SIDT1 and SIDT2 have antiviral roles, as deficient mice produce less IFN-β following herpes simplex virus 1 (HSV1) infection (90). Mechanistic work with SIDT2 revealed that the antiviral activity of SIDT2 was in part due to the lack of transfer of extracellular viral RNA into the cell during both HSV1 and encephalomyocarditis virus infection (91). SIDT2 deficiency also leads to impaired lipid homeostasis (24), inhibited insulin secretion (21), lung and gastrointestinal tumors (89), and nonalcoholic fatty liver disease induced by ER stress and defective autophagy (24, 44).

Despite these efforts, our present understanding of SIDT1 and SIDT2 is not complete. For example, many of the published data on the localization of the mammalian proteins leverage overexpression systems; however, examples have shown that overexpressed membrane-targeted GFP fusion proteins can cause mislocalization (74). Defining the subcellular distribution of endogenous SIDT1 and SIDT2 using highly specific antibodies or tagged knock-in systems would provide a deeper understanding of the native biology of these RNA-transporting pores. Furthermore, if there are multiple membranes within which SIDT1 or SIDT2 operate, defining the subset of RNAs trafficked by each into the various compartments will be of great interest.

Examples in Virology

Viruses have long provided a functional and mechanistic window into various aspects of their host's biology. Often classified by the type and structure of their genetic information, many viruses are pathogenic in humans, including positive-sense, single-stranded RNA (+ssRNA) viruses such as dengue virus, Zika virus, and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (96). The life cycles of these viruses are complex, but the infectious genetic material is itself an

mRNA that can be directly used for translation once deposited into the cytosol of a host cell. Exposure to the cytosol comes at the expense of possible detection by innate immune sensors such as RIG-I and MDA5, which, if activated, result in an antiviral response (58). One mechanism that some viruses employ is the development of viral replication organelles (79, 139), which can come from remodeling ER membranes. These organelles offer physical domains for the concentration and assembly of viral factors, virion assembly, and protection from innate sensors of viral infection. While ER membranes are used, many of these structures are not fully enclosed, and thus viral components can more freely exchange with the cytosol as needed for translation on host ribosomes; topologically, the viral RNA is cytosolic.

This is in contrast to viral structures called double-membrane vesicles (DMVs), which can be generated during infection by coronaviruses, enteroviruses, noroviruses, and some flaviviruses. These viruses target diverse membranes to initiate a complex and unique membrane rearrangement through a wrapping process (72). Depending on the family and genus of virus, DMVs can be easily identified by electron microscopy (100–300 nm in diameter) and are often adjacent to various organelles, including the ER, late endosomes/lysosome, and mitochondrial outer membrane (69, 144). Although the enclosed nature of a DMV enables protection from the innate immune RNA sensors (64), it also restricts access of the viral RNA to the translational machinery. To allow exchange between the interior of the DMV and the cytosol, a pore-like complex crossing the membrane is required.

Coronaviruses such as SARS-CoV-2 invaginate the ER and form early DMVs in the host cells (101, 143) (**Figure 3**). Coronaviruses possess a large genome (27–32 kb) and express two large replicase polyproteins that are proteolytically cleaved into 16 nonstructural proteins (nsps), including three transmembrane proteins: nsp3, nsp4, and nsp6 (110). Some of these nsps are sufficient for DMV formation and regulate morphology. For example, nsp3 is a transmembrane protein with two transmembrane domains in the middle region (143). Imaging infected cells with cryogenic electron microscopy revealed that six copies of nsp3 form a 6-nm-wide opening embedded in the DMV membranes, allowing the coronavirus genomic RNA to egress into the cytoplasm (143). The nsp3 protein also possesses a conserved ubiquitin-like domain 1 that binds both viral RNA and nucleocapsid protein on the N terminus in the cytoplasm. Analysis of cryogenic electron microscopy data suggests that the nsp3 oligomeric complex offers an encapsidation site for exported viral RNA (143). Other nsps besides nsp3, including nsp4, also participate in DMV formation (6); however, it is not yet clear whether nsp4 has independent pore-forming capacity.

Other +ssRNA viruses form similar crown-like pores on DMVs to export viral RNA. Chikungunya virus nsp1 assembles a dodecamer and a 7-nm-wide pore on the DMVs invaginated with endosome, lysosome, and plasma membrane (61, 151). Additionally, the Flock House virus replication protein A forms a 12-fold symmetric and 35-nm-wide pore on the DMVs from the OMM (127). Once DMVs are formed, in the context of the SARS-CoV-2 infection, the nsp6 protein acts as an organizer, establishing a physical connection between DMVs and host ER membranes, lipid droplets, and clustering DMVs (101). Taken together, RNA viruses provide many clear examples of the need and then ability to have entire RNA molecules transit across lipid bilayers for critical life cycle activities.

CONCLUSION

A need for new functions can drive biology, through evolution, toward creative and unexpected mechanistic solutions. It is initially difficult to imagine how cell surface RNAs such as glycoRNAs and maxRNAs become positioned on the external surfaces of cells. In this review, we have focused on reported features of biology that surround our first stated question, related to the ability to

systemically and precisely transport RNA outside of a cell. When examining the fields of exRNAs as well as various fields of RNA biology concerned with topological translocation of RNA, it becomes clear that many lines of evidence demonstrate that evolution has solved some of these major problems many times over.

Much of the work on exRNAs has focused on vesicular or EV-contained RNA, with the next-most-studied form being nonvesicular exRNAs. While there is still much to be cataloged and studied functionally for both of these types, cell surface–tethered RNAs—including glycoRNAs, maxRNAs, and possibly other novel forms of cell surface RNAs—are the least well understood. Diving deeper into this class of exRNAs will likely require tools leveraging new chemistries or imaging concepts to look at their localization as well as modification status. Flynn et al. (41) found that cell surface RNAs positioned near ligands of lectins such as WGA or MAAII were exclusively glycoRNAs, while other glycoligands (like those of lectin ConA) on the cell surface were not near detectable RNAs. Future work to understand the relationship between maxRNAs and glycoRNAs will be of interest, as the lectin data presented by Flynn et al. (41) suggested that maxRNAs might be glycosylated or occupy distinct physical locations on the surfaces of cells. More broadly, if only glycosylated RNAs are presented on the cell surface, then glycosylation regulates the levels of surface RNAs, raising the question of how RNA glycosylation is linked to the wider microcosm of cellular pathways. If both glycoRNAs and nonglycosylated RNAs are displayed on the cell surface, then nonglycosylated surface RNAs may interact differently or with separate receptors than glycoRNAs. Alternatively, nonglycosylated RNAs may be more quickly degraded and thus alter intercellular interactions. Precisely defining the requirement of glycans on the cell surface presentation of RNA will be of great interest.

Regarding transport and topological change toward the surface presentation, we favor a mechanism based on cell-intrinsic generation of cell surface glycoRNAs, which would require a mechanism to actively transport whole RNAs across membranes. The three examples we reviewed here show that life has evolved a wide array of mechanisms to move RNAs across various types of membranes with high fidelity. A pore formed by transmembrane proteins on the membrane is a common point of these three RNA import/export events. This inspires us to think about the related RNA import pore that exists on the ER and Golgi membrane for glycoRNA biogenesis. Interestingly, SIDT1, the mammalian ortholog of SID1, is reported to localize to the ER. Carefully examining how and where SIDT1 or SIDT2 could operate in RNA crossing membranes may reveal a role for these RNA channels in glycoRNA biogenesis or trafficking.

Beyond the physical transport mechanism, how that or adjacent machinery selects a subset of RNA molecules for translocation and surface presentation is critical to understand. RNAs targeted for glycosylation are small RNAs, which are highly abundant and constitute more than 90% of the transcriptome. In particular, tRNAs constitute approximately 80% of the transcriptome and display variance in usage in all kingdoms of life and substantial genetic differences in higher organisms. High levels of abundance and variation (in sequence and usage) would allow a relatively small fraction of these small RNAs to be utilized in additional ways. Small RNAs also display high levels of posttranscriptional modifications, particularly complex ones that require multiple enzymatic steps, which provides an additional layer of diversification and regulation. Thus, while a highly efficient and precise sorting or selection mechanism is likely required, small RNAs possess the requisites for further functionalization and usage outside of their canonical roles.

Beyond what might be found in previous reports, other major questions posed in the beginning of this review include how glycoRNAs maintain stability long enough to have a function (why are RNAs outside of cells not rapidly degraded?) and finding a molecular partner to confer that function (what functional moieties could RNA possess to have active roles in extracellular biology?). Regarding the former, we speculate that the glycosylation of RNA may provide significant

protection from extracellular RNases either through direct steric hindrance (i.e., the glycans occlude the RNA from the active site) or through interactions with neighboring biopolymers that prevent RNases from accessing the RNA. Regarding the latter, both the RNA and glycan could provide molecular surfaces for receptor interactions. Studies have shown that certain Siglec proteins can interact with glycoRNAs on the surfaces of living cells, and future work will be critical to establish whether RNA binding to Siglecs can confer a signal transduction event, as is possible from binding other glycoconjugates. There may also be novel RNA-binding receptors that can directly interact with the RNA portion of glycoRNAs for signaling or other biophysical activities between cells, in *cis* or in *trans*. Better understanding which of these pathways control glycoRNAs, or if there are novel trajectories and regulatory strategies that do so, will be a key next step in better defining these biopolymers.

DISCLOSURE STATEMENT

R.A.F. is a cofounder, board-of-directors member, and stockholder of GanNA Bio and is a board-of-directors member and stockholder of Chronus Health.

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