# Examining the evidence for extracellular RNA function in mammals

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Abstract | The presence of RNAs in the extracellular milieu has sparked the hypothesis that RNA may play a role in mammalian cell–cell communication. As functional nucleic acids transfer from cell to cell in plants and nematodes, the idea that mammalian cells also transfer functional extracellular RNA (exRNA) is enticing. However, untangling the role of mammalian exRNAs poses considerable experimental challenges. This Review discusses the evidence for and against functional exRNAs in mammals and their proposed roles in health and disease, such as cancer and cardiovascular disease. We conclude with a discussion of the forward-looking prospects for studying the potential of mammalian exRNAs as mediators of cell–cell communication.

Extracellular RNAs
(exRNAs). RNAs that are transcribed within a donor cell that are released into the extracellular space.

Extracellular vesicles
Spheres of phospholipids and proteins secreted from the cell, including small and large extracellular vesicles, that contain various molecular cargoes such as RNA and proteins.

Ribonucleoprotein
(RNP). A complex consisting of an RNA-binding protein that is bound to an RNA that is often recognized by an RNA-binding domain.

Lipoproteins
Particles of proteins and lipids that bind RNA, which are divided into different subtypes based on density, such as high-density lipoprotein and low-density lipoprotein.

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*"Extraordinary claims require extraordinary evidence."* Carl Sagan

Multicellular organisms are composed of diverse cell types that rely on receiving signals from other cells, which can occur locally or over vast distances. This cell-to-cell communication is the essence of multicellular life and occurs through different types of molecule. Peptide hormones, growth factors, cytokines and neurotransmitters are secreted by cells and communicate potent physiological responses by binding to cognate receptors on target cells. The discovery of extracellular RNAs (exRNAs) in mammalian body fluids has led to the hypothesis that exRNAs play a role in cell–cell communication[1–3].

We define exRNAs as RNAs that undergo transcription within a cell, the 'donor' cell, that releases the RNA into the extracellular environment. 'Recipient' cells are defined as the cells that take up exRNAs from the extracellular space. In non-mammalian systems, such as plants and nematodes, small RNAs readily spread throughout the organism and function within recipient cells[4–7]. Although mammals produce exRNAs, the current evidence for exRNA function in mammals is limited in scope or has important caveats to consider. Importantly, recipient cells may simply degrade or release most, if not all, exRNAs, and the presence of exRNAs does not imply inherent functionality. Indeed, extraordinary experimental rigor is required to demonstrate potential biological relevance of exRNAs.

For insight into the idea of RNA as a form of cell–cell communication, we first discuss the principles of functional mobile small RNAs in non-mammalian organisms. Next, we explore ongoing efforts to catalogue the diverse roster of mammalian exRNAs. We review the evidence for various carriers of exRNAs, including extracellular vesicles, ribonucleoprotein (RNP) complexes and lipoproteins, and scrutinize the current evidence for exRNA function in mammals in health and disease. We conclude by reflecting on the experimental evidence needed to definitively answer whether exRNAs facilitate cell–cell communication in mammals.

## Discovery of exRNAs

***Mobile small RNAs in non-mammalian organisms.*** The core evidence for functional RNA spreading in plants comes from experiments in which plant tissue, with and without active small RNA-mediated gene silencing, was grafted together, revealing a 'spread' of the silencing signal organism-wide (that is, systemically)[4–6]. These silencing small interfering RNAs (siRNAs) are required for post-transcriptional gene silencing between cells through RNA interference (RNAi)[7]. This nearly organism-wide spreading of RNAs is facilitated by plasmodesmata channels that bridge cells together, directly connecting the cytoplasm of different plant cells[8,9]. Evidence originally based on enzymatic activity in plant extract revealed the presence of an RNA-dependent RNA polymerase (RdRP) that is able to amplify RNA molecules required for silencing[10]. RdRPs generate a double-stranded RNA (dsRNA) template from target RNAs, which is processed by the endonuclease Dicer into siRNAs, which are now capable of silencing complementary target genes[11]. Considering that this gene silencing can begin in a small number of cells and then spread systemically, there is a mechanism to amplify the silencing RNA signal. Similar to PCR primers amplifying a larger target sequence, RdRPs are able to use a small sequence

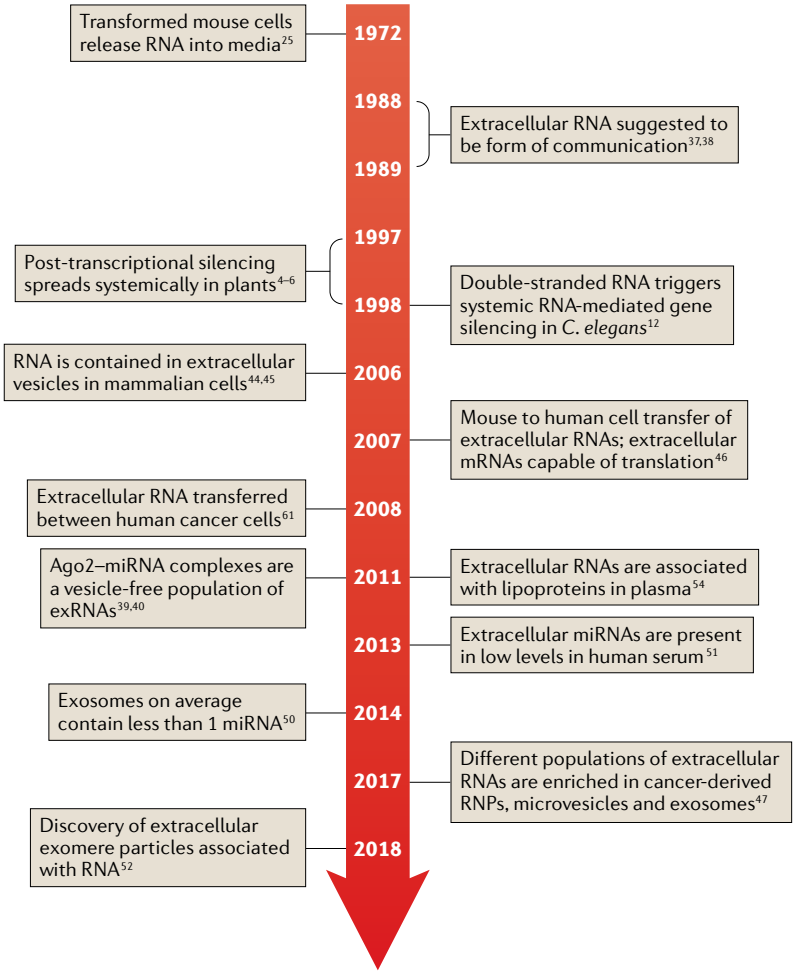


Fig. 1 | **Timeline of exRNA discoveries.** Highlighted research influencing the field of extracellular RNA (exRNA). *C. elegans*, *Caenorhabditis elegans*; miRNA, microRNA; RNP, ribonucleoprotein.

to generate dsRNA (and thus siRNAs) spanning a full sequence (a property named 'transitivity')[5,10].

These findings in plants were revealed nearly concurrently with similar evidence in *Caenorhabditis elegans*, where small RNAs spread systemically to carry out silencing in response to injection or feeding of dsRNA[12]. Similar to the amplification of silencing seen in plants, mutations to the *C. elegans* RdRP enzyme Ego-1 quell RNA-induced silencing[13]; additionally, biochemical and reporter approaches revealed that RdRP amplification occurs in *C. elegans* and is transitive, similar to the mechanism in plants[14]. However, unlike plants, which have channels directly connecting the cytoplasm of cells, mobile cell–cell spreading of small RNAs in *C. elegans* depends on dsRNA transporters, systemic RNA interference defective 1 (SID-1) and SID-2, to import and export dsRNA to and from the extracellular space[15,16]. Evidence suggests an antiviral or anti-transposable element role for this cell–cell spreading of RNA in plants and in *C. elegans* (reviewed in REFS[17,18]). Of note, given our definition of exRNAs, which requires a donor cell to transcribe and release the exRNA, and as the majority of these data are based on artificially injected dsRNA (or transgenes), many of these studies do not directly demonstrate in vivo exRNA function in non-mammalian systems.

Whether similar processes occur in other organisms is an ongoing investigation. For example, some fungi, such as *Neurospora crassa*, possess a conserved RdRP, suggesting that other organisms are capable of amplifying large quantities of small RNAs through RdRP activity[19]. Other organisms, such as *Drosophila melanogaster*, have developed non-RdRP-based strategies for organism-wide RNA-based antiviral responses[20–22]. Instead of RdRP amplification, reverse transcriptase activity converts viral RNA into DNA, which then serves as a template for antiviral RNAs. Similar to the systemic spread in *C. elegans* and plants, these antiviral RNAs seem to spread organism-wide, albeit in extracellular vesicles[23]. The evidence for functional transfer of these *D. melanogaster* RNAs was obtained in studies using vesicles from Argonaute 2 (Ago2)-mutant *D. melanogaster*, which effectively quells antiviral RNA activity. Although the loss-of-function Ago2 mutant eliminates antiviral RNAs, it has the caveat of non-specifically disrupting several other small RNAs, such as microRNAs (miRNAs)[23].

Taken together, the apparent differences between organisms in mechanisms underlying small RNA spreading remind us that small RNAs can spread in a variety of ways but revolve around signal amplification and effective uptake into recipient cells.

***Mammalian cell biology of exRNA.*** Evidence dating back to the 1970s shows that RNA is present in media collected from cultured mouse and human cells[24,25] (FIG. 1). Since then, ample evidence from modern high-throughput RNA sequencing experiments suggests that exRNAs are present within many human bodily fluids, including but not limited to blood, urine and saliva[1–3]. Mammals lack the proteins that are necessary for cell–cell RNA signalling in plants and *C. elegans*, such as a RdRP[21,26]. If exRNAs function in mammals, it is likely through fundamentally different mechanisms from those in plants and nematodes.

The mammalian genes *SIDT1* and *SIDT2* encode proteins that bind to long dsRNA and seem to be important for an effective antiviral response[27,28]. However, these proteins do not import RNA from the extracellular environment as the *C. elegans* homologues SID-1 and SID-2 do[27,28]. Overexpression of SIDT1 and SIDT2 in mammalian cells enhances the transfer of dsRNA analogues from the endosome to the cytoplasm, suggesting that some endocytosed RNA may avoid destruction by the endosome–lysosome pathway by transport from the endosome to the cytoplasm[27,28]. However, the evidence supporting SIDT1/SIDT2-mediated RNA transport used an artificial chemical analogue structurally similar to dsRNA (polyinosinic:polycytidylic acid), or artificially introduced siRNAs, neither of which was transcribed in the donor cell and thus does not fit the definition of an exRNA[29]. Silencing *SIDT1* in human cells reduces exRNA-reporter readouts in recipient cells, albeit in vitro[30]. These data indicate that mammals do not use SIDT1/SIDT2 transporters to move RNA between cells in a manner analogous to SID-1/SID-2 in *C. elegans*.

RNA interference
(RNAi). A process resulting in small RNAs binding to complementary RNA sequences to suppress their translation or direct their degradation.

microRNAs
(miRNAs). Small non-coding single-stranded RNAs that often regulate gene expression by binding to the 3′ untranslated region of mRNAs to induce translational repression, destabilization or cleavage of the transcript.

### Carriers of mammalian exRNAs

All exRNAs begin their journey through secretion or decomposition of a cell (FIG. 2). Several potential ends to the journey of an exRNA include clearance through the liver, kidney and other tissues — or uptake into recipient cells. Generally, endocytosis of extracellular molecules results in either degradation in the lysosome or exocytosis[31–33] (FIG. 2). Labelling and imaging analyses in immortalized endothelial cells found that most extracellular vesicles within recipient cells co-localize

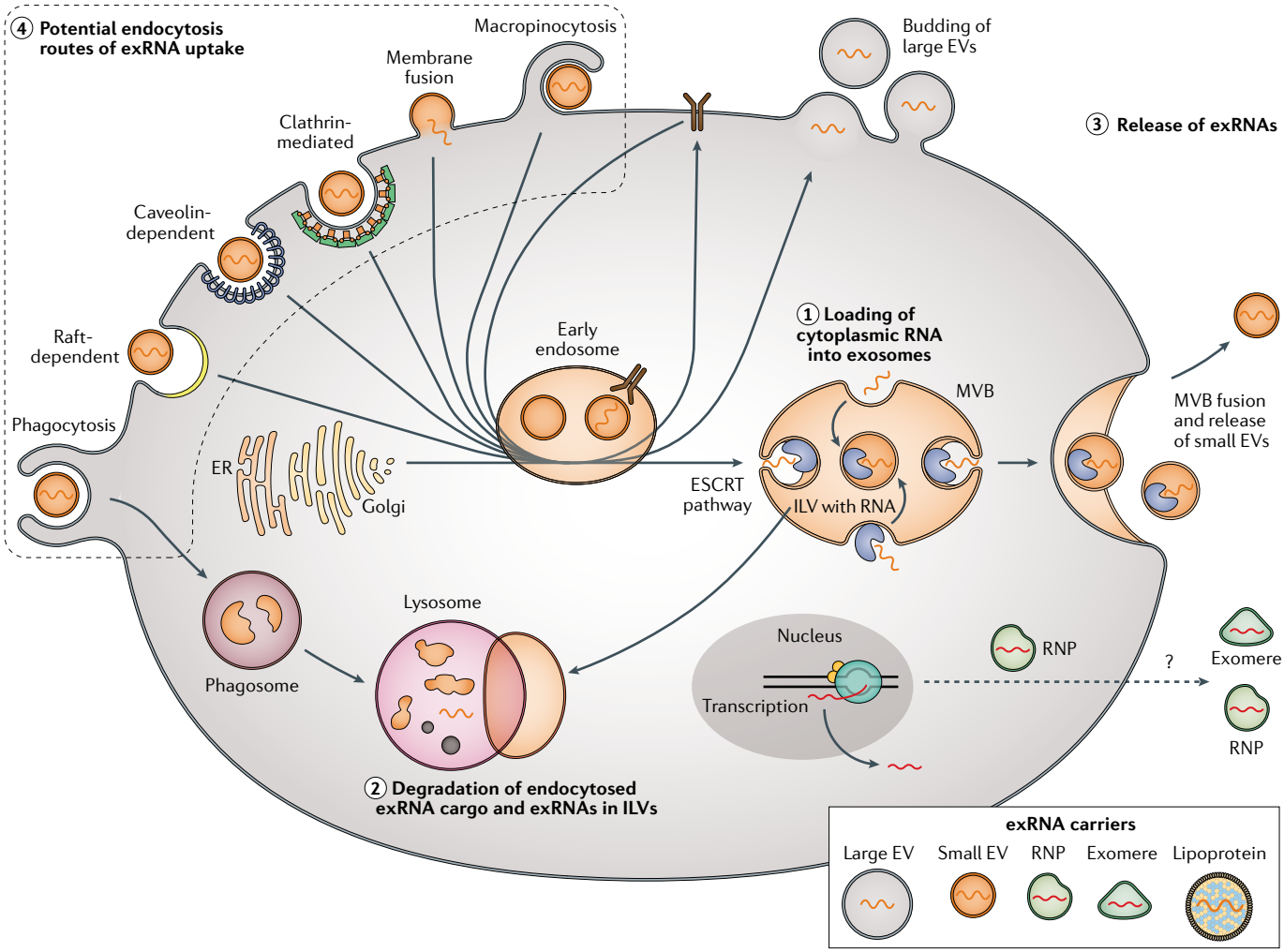


Fig. 2 | **Model of the life cycle of exRNAs.** For extracellular RNAs (exRNAs) found within small extracellular vesicles (EVs), the journey depends on early endosomal maturation into a late endosome, which is also called the multivesicular body (MVB). In addition to molecules taken up via endocytosis, the MVB takes in specific cytoplasmic cargo, including RNA. The targeted RNAs are first bound to the external surface of the MVB, which then undergoes inward budding that results in tiny vesicles contained within the MVB, termed intraluminal vesicles (ILVs)[31,100,101]. A collection of proteins in the MVB membrane — the endosomal sorting complexes required for transport (ESCRT) complex — is responsible for producing some ILVs[31,33,100,101] (1). The biogenesis of ILVs occurs in a series of steps ending with inward scission of the endosome membrane, which takes protein and RNA cargo from the cytoplasm[31,33,100,101]. An alternative syntenin-dependent pathway involving ceramide or phosphatic acid that affects the membrane structure of the endosome can also form ILVs[31,101]. Subtypes of MVBs fuse with the lysosome (2) or plasma membrane, which releases the ILVs (now named small EVs) with exRNA cargo into the extracellular environment (3); different lipid and protein profiles are associated with exosome release versus lysosomal degradation[31–33]. Proteins involved in vesicular fusion are known to regulate MVB fusion[31,101]. Large EVs form via direct budding off the plasma membrane, which seems to encapsulate exRNAs from the cytoplasm. Despite this unique biogenesis, microvesicle budding shares similar molecular mechanisms with ILV formation[100,101]. It remains unclear how, or whether, specific RNAs are localized to the site of large EV budding. Several signalling pathways affect the release of EVs, including Hedgehog, Wnt and thrombin as examples[33,100,102]. The mechanisms underlying the biogenesis of other non-vesicle exRNAs (such as Argonaute 2 (Ago2)–microRNA (miRNA) complexes) remain largely uncharted territory; however, inhibiting neutral sphingomyelinase 2 enzyme decreases miRNA levels in EVs and increases the levels of miRNA secreted with lipoproteins, suggesting that this enzyme is involved in the biogenesis of non-vesicle exRNAs[54,69]. Endocytosis of extracellular molecules can occur through several mechanisms, including clathrin-dependent endocytosis, caveolar endocytosis, micropinocytosis, raft-dependent endocytosis and phagocytosis[101,103] (4). These endocytic pathways could take up extracellular RNA associated with EVs, ribonucleoproteins (RNPs) or lipoproteins. In support of this hypothesis, exRNAs are often found within endosomes, but it is mostly unknown how exRNAs escape the endosome. SIDT2 may transport dsRNA from the endosome to the cytoplasm, although this evidence is based on artificial dsRNA[28]. Further studies investigating the molecular mechanisms underlying mammalian endosomal escape are needed to better understand whether the end of an exRNA's journey includes functional activity. RAB GTPases (indicated by dashed lines) are major regulators of endosomal trafficking[31,101]. ER, endoplasmic reticulum.

Ribonucleases
(RNases). Enzymes that cleave RNA, which are often found in the extracellular environment where they efficiently degrade RNAs that are not protected by other factors such as extracellular vesicles or proteins.

with lysosome markers[34]. Therefore, it is likely that most (if not all) exRNAs partnered with vesicles, RNPs, lipoproteins or any other molecules are degraded in the lysosome of recipient cells.

***Naked exRNAs are degraded and cause immune activation.*** Cell death and viral replication are both ample suppliers of RNA in the extracellular space. After viral infection, cells secrete dsRNA into the extracellular space, which triggers the mammalian immune response[28]. This innate immune signalling seems to be a general reaction to extracellular 'naked' RNAs, as in vitro transcribed RNA is sufficient to activate toll-like receptor (TLR) signalling in cultured immune cells[35]. By contrast, RNA contained within vesicles does not trigger an immune response; however, these data are based on miRNAs coated with artificial liposomes and not endogenous extracellular vesicles that contain exRNA[36].

Furthermore, the extracellular environment is rife with ribonucleases that readily degrade RNA, suggesting that some exRNAs are resilient against RNase activity[37,38] (FIG. 1). exRNAs themselves are not intrinsically resistant to RNases, as unpartnered, or 'purified', exRNAs are readily degraded by RNase treatment, whereas exRNAs with a protein partner are resistant to degradation[39,40]. Thus, exRNAs may partner with other molecules to prevent triggering an immune response, enhance exRNA stability or for no functional reason.

***The nature of extracellular vesicles that harbour exRNAs.*** Many exRNAs, such as mRNA fragments, miRNA, small nuclear RNA (snRNA), tRNA fragments and Y RNA, exist within extracellular vesicles (FIG. 3). As most exRNA studies focus on exRNAs within extracellular vesicles, the ever-shifting definitions and discoveries of extracellular vesicles influence the exRNA field[41–43]. Indeed, some of the earliest evidence that mammalian cells produce exRNAs came from the real-time (quantitative) PCR amplification of specific mRNAs isolated from extracellular vesicles in human and mouse cells[44,45] (FIG. 1). Since these initial discoveries, a wealth of studies have characterized the constituents, sorting (BOX 1) and potential biological functions of exRNAs within extracellular vesicles.

There are many extracellular vesicles with unique properties (FIG. 3a,b). The terminology for various populations of extracellular vesicles is not standardized, thus interpreting data between studies is difficult[41–43]. Some features such as size, density, protein markers, biogenesis and RNA content help define extracellular vesicle populations[41–43]. Defining extracellular vesicles is an essential pursuit for exRNA studies, and characterizing the myriad of vesicle subpopulations is a challenge still faced by the field of extracellular vesicle biology[41–43].

Although early evidence using quantitative PCR (qPCR) with mRNA-specific primers showed that RNAs are present in extracellular vesicles[44,45], it was not tested directly whether these were full-length transcripts or mRNA fragments. Similarly, biochemical analysis revealed that extracellular vesicles contain mRNA sequences and abundant small RNAs, such as miRNAs[46]. Subsequent RNA-sequencing-based studies revealed that exRNAs are not usually full-length RNAs, but commonly fragments (such as mRNA fragments) or small non-coding RNAs, such as Y RNAs[47–49]. On average, extracellular vesicles contain very few (if not less than one) molecules of a given RNA, thus raising scepticism that exRNAs can be robust messengers of cell–cell communication[50,51].

***Ribonucleoprotein and lipoprotein carriers.*** In addition to exRNAs within extracellular vesicles, exRNAs associate with proteins to form RNP complexes (FIG. 3d). For example, miRNAs co-precipitate with extracellular Ago2, which was one of the first indications that not all exRNAs exist within extracellular vesicles[39,40]. Recent evidence suggests that exRNAs bind to small nanoparticles made of protein and lipids, called exomeres[52,53] (FIG. 3e). Two alternative methods have identified exomeres, starting with chromatography-based separation[52] followed by a high-speed ultracentrifugation approach[53]. Lastly, both high-density lipoproteins (HDLs) and low-density lipoproteins (LDLs) co-purify with miRNAs, tRNAs, ribosomal RNAs (rRNAs), small nucleolar RNAs (snoRNAs), snRNAs and Y RNAs[54,55] (FIG. 3f,g). Although reconstituted HDL can bind to miRNAs when injected in vivo[54], it is unclear how exRNAs physically associate with lipoproteins.

## Extracellular RNA function in mammals

***Evidence of function for vertebrate exRNAs.*** One of the first experiments to test exRNA function found that media-derived RNA can enhance DNA synthesis when applied to genomic DNA in vitro[24]. A significant challenge to directly testing the potential function of exRNAs is controlling for the confounding effects of other carrier molecules or experimental manipulations. Although there was an increase in DNA synthesis[24], other factors from the cells could have enhanced DNA polymerase activity; alternatively, applying this highly concentrated RNA to DNA could have non-physiological effects. The most convincing evidence of function would rule out the effect of other molecules while being within the physiological range of exRNA levels seen in biofluids. Besides testing for a function carried out by the exRNA itself, it is vital to consider that cells may release exRNAs into the extracellular space as a way to purge 'cellular waste', which is an idea that has been around since 1983 as a potential reason for extracellular vesicle production[56]. Most importantly, it is necessary to keep in mind that there may be no biological function carried out by mammalian exRNAs.

After uptake, distinguishing an endocytosed exRNA from RNA transcribed in the recipient cell is immensely challenging, but can be solved with careful experimental design. Recent experimental evidence using genetic ablation of the exRNA loci within recipient cells convincingly demonstrated the transfer of extracellular snoRNAs between cultured cells and between mice[57]. In vivo evidence of snoRNA transfer comes from parabiosis experiments, in which the circulatory systems of mice in which the snoRNA has been deleted and control mice are linked[57]. In support of functional snoRNA transfer from wild-type to deletion mice, parabiosis was

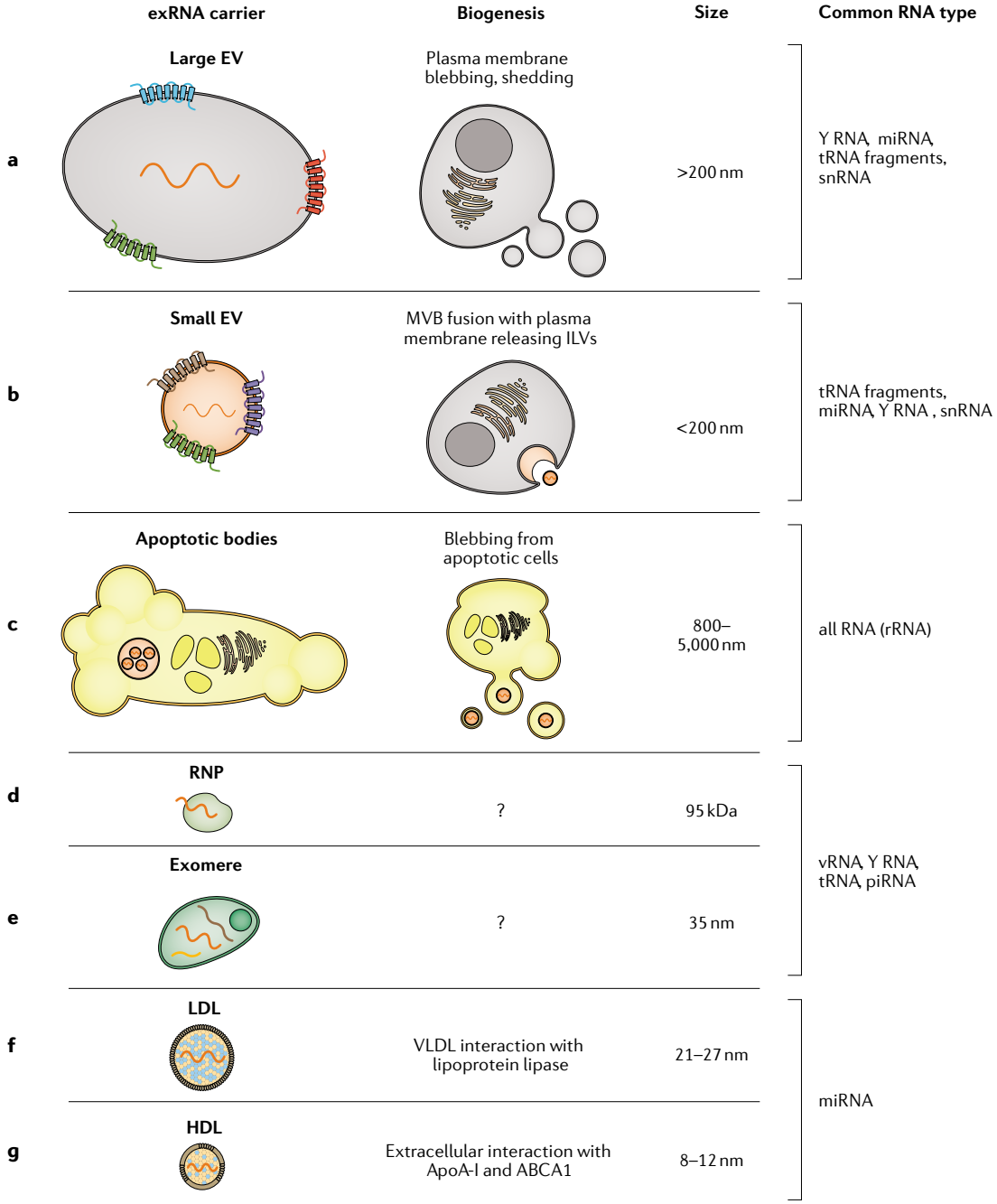


Fig. 3 | **Mugshot of exRNA carriers. a** | Large extracellular vesicles (EVs) are bigger than other EVs (>200 nm in size)[41,42] and form via budding directly off the membrane. **b** | Small EVs (<200 nm) are formed within multivesicular bodies (MVBs) and are released upon MVB fusion with the cell membrane[41,42]. **c** | Apoptotic bodies are large vesicles that bleb off cells undergoing apoptosis that range from ~800 to 5,000 nm in size[41,104]. **d** | Various ribonucleoproteins (RNPs) are associated with extracellular RNAs (exRNAs). Argonaute 2 (Ago2) is found within the extracellular fluid associated with microRNAs (miRNAs) and is relatively small compared with other exRNA carriers[39,40]. Vault proteins are also found in the extracellular space associated with vault RNAs (vRNAs)[41]. **e** | Exomeres are small particles associated with lipids, protein, DNA and RNA that are not contained within EVs[41,52,53]. **f** | Low-density lipoproteins (LDLs) are formed outside the cell and contain ApoB[105]. **g** | High-density lipoproteins (HDLs) are relatively small particles (8–12 nm) formed outside the cell that include several different apolipoprotein proteins (ApoA, ApoC and ApoE)[105]. RNA composition approximated from multiple profiling experiments obtained from various cell lines[41,47,104]. ILV, intraluminal vesicle; piRNA, PIWI-associated RNA; rRNA, ribosomal RNA; snRNA, small nuclear RNA; VLDL, very-low-density lipoprotein.

able to rescue a phenotype — loss of 2′-*O*-methylation of rRNAs — observed in snoRNA-deletion animals[57]. Although promising, these data are not conclusive because parabiosis between animals transfers many molecules other than extracellular snoRNAs, which might have caused the observed changes in methylation. Future studies can design experiments that ensure exRNAs are responsible for functional activity, such as

Box 1 | **Sorting of RNAs into extracellular vesicles**

Sorting of RNA into extracellular vesicles may depend on non-templated additions based on profiling evidence that found that microRNAs (miRNAs) with 3′ uridylated ends are more enriched in extracellular vesicles, whereas miRNAs that stay in the cell are enriched for 3′ end adenylation[106]. RNA levels may also affect sorting, as increasing (or inhibiting) miRNA biogenesis affects the numbers of miRNAs sorted into extracellular vesicles more dramatically compared with the levels remaining in the cell[34]. Moreover, addition of a mRNA target for a particular miRNA reduces the respective levels of miRNAs in extracellular vesicles, which implies that unused miRNAs (those not bound to target mRNAs) are more likely to be found in extracellular vesicles[34].

There is some evidence that proteins may mediate exRNA sorting into extracellular vesicles, as manipulating heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1) via knockdown or overexpression affects the exRNA levels detected in extracellular vesicles[107]. Additionally, miRNAs within extracellular vesicles are enriched in motifs bound by hnRNPA2B1, although it was not directly tested whether these sequences are required specifically for hnRNPA2B1-dependent exRNA levels[107]. Based on co-precipitation of the RNA-binding protein YBX1 with RNAs, and a reduction of these respective exRNAs in YBX1-deleted cells, YBX1 may be an agent for sorting a variety of small RNAs (miRNAs, tRNAs, Y RNAs, vault RNAs) into extracellular vesicles[94,108]. Similarly, the RNA-binding protein La directly binds to and sorts miRNAs into high-density, but not low-density, vesicles[109].

using cell-type-specific genetic deletion of the exRNA in recipient cells within the same animal[58].

***Non-endogenous exRNA transfer to recipient cells.*** Other evidence of exRNA transfer between mammalian cells includes monitoring exRNAs that cannot be transcribed by the recipient cell[46,59,60]. In a seminal study, Valadi et al. demonstrated that mouse-derived extracellular vesicle mRNAs are translation competent by using an in vitro translation assay, thus begging the question of whether transferred extracellular mRNAs make protein that functions in recipient cells[46]. Similarly, treating cells with extracellular vesicles containing luciferase mRNA resulted in luciferase activity in recipient cells[61]. Although these experiments clearly show that the transferred RNA is not from the recipient cell, they do not definitively provide evidence of mRNA translation in the recipient cell, as extracellular vesicles could directly transfer the protein of interest to the receiving cell.

Several studies have monitored extracellular vesicle-mediated transfer of Cre recombinase and mRNA by expressing Cre protein in select donor cells and then tracking Cre-specific reporter levels in recipient cells[59,60]. Injecting extracellular vesicles that contain *Cre* mRNA into mice results in reporter activity in neural cells, which may indicate the transfer and translation of *Cre* mRNA[59]. However, non-specific or 'leaky' expression of Cre could also cause this result. These in vivo experiments are an essential step towards finding a biologically relevant role for exRNAs and do not share some of the same confounding variables as using immortalized cells, such as serum-derived extracellular vesicles that contain RNA[62].

The functional transfer of extracellular mRNA between cells is an exciting idea, although many published studies have limitations. When examining mRNA transfer, it is plausible that the protein of interest is co-sorted into the extracellular vesicle along with the mRNA, thus confounding results. One approach might be to create a system to prevent translation of the exRNA specifically in the donor cell, which would ensure that any observed protein in recipient cells is not derived from extracellular vesicles. Also, new methods to visualize translation of transcripts have been developed in recent years[63–66], thus allowing the exciting possibility of monitoring the translation of transferred mRNA within the recipient cell.

Several recent studies have utilized the CRISPR–Cas9 genome editing system to study exRNA transfer between cells[67,68]. One study used donor cells stably expressing single guide RNAs (sgRNAs) to measure sgRNA-specific reporter activity, the Stoplight sensor, within recipient cells[67]. After transwell separation or treatment with high levels of concentrated donor cell extracellular vesicles, Stoplight-expressing recipient cells showed sgRNA-dependent GFP fluorescence activity[67]. These data support a model for exRNA activity transferred between cells. However, under optimized conditions the activity was only present in a scant 0.03% of cells[67]. These low numbers under non-physiological conditions raise questions around the potential biological relevance of mammlian exRNAs.

***exRNAs of the mammalian immune system.*** Extracellular vesicles contain many small RNAs, including miRNAs, which are well-known regulators of gene expression. Some evidence suggests that purified vesicles that contain miRNAs can downregulate reporters that include the miRNA target site[69]. However, because these findings were obtained through overexpression of miRNAs in donor cells, they do not reflect the low levels of exRNAs observed in endogenous extracellular vesicles. Other early experiments found that treating cells with extracellular vesicles caused a dramatic increase in active miRNAs within the recipient cell[70]. An important caveat is that treating cells with these vesicles could be sufficient to cause miRNA transcription within the recipient cells. These data have inspired many similar studies searching for functional transfer of extracellular miRNAs in a wide range of cell types and biological states.

On account of the antiviral role that mobile RNAs have in plants and *C. elegans*, and the plethora of exRNAs secreted from immune cells in mammals, there is a collection of literature examining the potential function of exRNAs in the mammalian immune system[71–75]. Data derived from profiling the miRNA content of vesicles secreted from a variety of immune cells suggest that T cell-derived extracellular vesicles transfer miRNAs to B cells, which is dependent on triggering close cell–cell interaction, the immune synapse[72]. Quantification of extracellular miRNA activity in recipient cells, as read out by transfected reporters, suggests that the immune synapse is necessary for the transfer of miRNA[72]. However, these data were obtained by overexpressing miRNAs from transfected plasmids within donor cells, thus enhancing extracellular miRNA levels above lowly endogenous amounts[72]. Given the low levels of exRNAs in vesicles, recipient cells would need to take up a high number of vesicles for the exRNAs to function. Nevertheless, the immune synapse warrants further investigation, as the ample exchange of vesicles may lead to accumulation of physiologically relevant levels of exRNAs in recipient cells.

Several studies indicate macrophage to endothelial cell transfer of exRNAs[34,70]. There is evidence that macrophage-derived extracellular vesicles carrying miRNAs enhance the migration of endothelial cells in vitro[70]. Furthermore, injection of mice with macrophage-derived extracellular vesicles containing miRNAs resulted in increased levels of said miRNA in endothelial cells in vivo[70]. The evidence for functional transfer suffers from the caveat that the recipient cells possess functional miRNA processing machinery as well as the endogenous gene encoding the miRNA. Thus, treating cells with extracellular vesicles could enhance miRNA transcription within recipient cells regardless of exRNA transfer.

To ensure miRNAs are not derived from recipient cells, some studies use the approach of preventing all miRNA production by deleting core miRNA processing machinery, such as the endonuclease Dicer. For example, co-culturing wild-type macrophages with Dicer-null endothelial cells increases miRNA activity in the Dicer-deficient endothelial cells[34]. However, direct incubation of extracellular vesicles with Dicer-null endothelial cells does not increase miRNA activity, which suggests that the increase in miRNA activity is not due to vesicle-mediated transfer of miRNAs[34]. These findings suggest that a non-extracellular vesicle mechanism may underlie the increase in miRNA activity. Future studies should consider using isolated extracellular vesicles, without the entire donor cell present, to corroborate the hypothesis that extracellular vesicles are sufficient to transfer RNA to recipient cells.

***exRNA transfer to hepatocytes.*** In addition to vesicle transfer of miRNAs, some studies have examined whether extracellular lipoprotein–RNA complexes are taken up by recipient cells. Treating hepatocytes with HDL–miRNA complexes results in an increase in miRNA levels and a decrease in target mRNAs in recipient cells[54]. As these studies experimentally incorporated miRNAs with HDL, it is unknown whether lipoprotein–miRNA complexes function within recipient cells under biological conditions. Using an adipocyte-specific deletion of Dicer to prevent adipocytes from secreting extracellular miRNAs, recent evidence suggests that adipocyte-secreted extracellular vesicle miRNAs reduce target-mRNA levels in hepatocytes[76]. Moreover, transplanting adipocytes that express human miRNAs into mice reduces miRNA-specific reporter activity in the liver[76]. One major caveat of these results is that the deletion of Dicer causes many other downstream cell-autonomous effects, which could alter signalling molecules secreted from fat that affect gene expression in the liver. Although the concept of extracellular vesicles educating distant tissues is tantalizing, definitive data are needed before a solid conclusion that exRNA cargo plays a functional role in signalling can be reached.

***exRNAs within the mammalian nervous system.*** Neurons exchange information with other cells in various ways, including neurotransmitter release, gap junctions and potentially through exRNAs. Thorough genetic labelling strategies in vivo show that neurons do indeed secrete extracellular vesicles (containing miRNAs) that are taken up by recipient vascular endothelial cells[77]. However, non-specifically inhibiting extracellular vesicle production is not direct evidence that these miRNAs function in recipient cells. Similarly, treating neurons with mesenchymal extracellular vesicle miRNAs promotes axon growth[78], but there is no direct test of the role of the miRNA. Another study found that injuring peripheral nerves enhances the number of neural extracellular vesicles containing miR-21, which macrophages then engulf[79]. Extracellular vesicles from neurons that overexpress miR-21 induce pro-inflammatory transcripts in macrophages, whereas extracellular vesicles from neurons treated with anti-miR-21 antagonists do not[79]. Although these data indicate that miR-21 levels in neurons affect the macrophage response to neural extracellular vesicles, they do not show directly whether the miRNA within the extracellular vesicles is responsible[79].

Recent studies showed that the protein Arc1, which contains a domain similar to that of retroviral capsid proteins, promotes the packaging of *Arc1* mRNA into extracellular vesicles in human and *D. melanogaster* neurons[80,81]. Arc1 protein–mRNA complexes transfer from motoneurons to muscle cells, and deletion or knockdown of Arc1 in neurons reduces synaptic bouton formation between neurons and muscle cells. However, this effect could be due to the known neuron-intrinsic function of Arc1 (REF.[80]). Similarly, in mammalian cells, ARC protein promotes *ARC* mRNA localization into extracellular vesicles[81]. After incubation with wild-type extracellular vesicles, *ARC*-null recipient neurons are positive for intracellular ARC protein and RNA. Although these data are exciting, whether *ARC* RNA has a non-cell-autonomous function in *D. melanogaster* and mammals remains unclear.

## exRNAs in disease

***exRNAs in cancer.*** Cancer cells produce extracellular vesicles that contain exRNAs, and thus many are excited by the potential of exRNA-mediated regulation of cancer proliferation and metastasis[68,82,83]. There is plentiful evidence that patients with cancer have a different pool of circulating exRNAs compared with those without cancer[48,84]. The aggressiveness of some cancers correlates with exRNA levels[82], although these changes in exRNA levels may be functionally innocuous.

Different cancer subtypes release distinct exRNA profiles, thus reflecting the unique transcriptomes of various cancers or differences in exRNA sorting[61,85]. For example, cancer cells with or without an oncogenic *KRAS* mutation secrete different populations of RNAs in extracellular vesicles[68,85,86]. Although treating recipient cells with extracellular vesicles alters exRNA-reporter activity, some of the RNAs are endogenously expressed within the recipient cell, making it difficult to rule out whether changes in reporter expression are not due to the action of RNA from the recipient cell. Recent work examining KRAS-mutant colorectal cancer cells has overcome this caveat by fusing non-endogenous sgRNA sequences to several exRNAs, including miR-100 and the long non-coding RNA (lncRNA) CRNDE, which triggered reporter activity in recipient cells[68]. However,

the evidence is based on transfection of the sgRNA–miRNA constructs, which enhances levels beyond low endogenous levels[68]. Hence, to date, the evidence for the pathological consequences of these cancer exRNAs remains indirect.

Cancer cells secrete extracellular vesicles that prime sites for metastasis, although whether exRNAs are involved has not been directly tested. For example, extracellular vesicles from breast cancer cell lines that endogenously possess high levels of miR-105 promote metastasis and vascular permeability in mice[82]. Similarly, there is evidence that astrocyte extracellular vesicles promote brain cancer outgrowth[83]. However, whether the RNA is derived from extracellular vesicles is inconclusive because the recipient cells can transcribe the exRNA of interest[83].

Recent work using a mouse model lacking miR-21 expression, which is frequently disrupted in cancer, found that glioblastoma cells transfer miRNAs to astrocytes through extracellular vesicles[58]. After implanting GFP-expressing glioma cells, which highly express miR-21, into the brain of miR-21-null mice, the authors showed that microglia that take up extracellular vesicles exhibit lower levels of miR-21 target transcripts relative to microglia that did not take up these vesicles[58]. Additionally, glioma-derived, fluorescently labelled, injected extracellular vesicles were taken up by microglia of miR-21-null mice[58]. Although this evidence is promising, it remains possible that implanting the tumour or injecting extracellular vesicles could cause gene downregulation in microglia[58]. To determine whether exRNAs play a role in cancer, or other human diseases, more direct evidence is needed that separates the effect of the exRNA from that of the extracellular vesicle.

***exRNAs in cardiovascular disease.*** The development of various cardiac diseases in human — for example, acute peripartum cardiomyopathy, dilated cardiomyopathy and coronary artery disease — correlates with disease-specific changes to exRNA profiles[87–89]. Experiments using a mouse model of peripartum cardiomyopathy showed that endothelial cells release higher levels of extracellular vesicles containing miRNAs, which are taken up by cardiomyocytes[87]. Moreover, extracellular vesicles from endothelial cells impaired metabolic activity in cardiomyocytes in vitro[87]. These functional data use transfection to increase miRNA levels in donor cells to model the levels in vivo, thus making it difficult to determine whether this metabolic effect also occurs in response to endogenous miRNA-containing extracellular vesicles[87].

Evidence from an atherosclerosis model found that endothelial cells secrete extracellular vesicles that cardiomyocytes receive[90]. Injecting endothelial cell-derived extracellular vesicles that contain high miR-143/miR-145 levels protected against atherosclerotic lesions in the aorta of mice[90]. Furthermore, treating endothelial cells with locked nucleic acids to inhibit donor cell miRNAs prevented the protective effect of the extracellular vesicles. However, inhibiting these miRNAs in endothelial donor cells likely changes other properties of endothelial extracellular vesicles[90]. Thus, the evidence collected so far in mice has yet to clarify whether exRNAs play a functional role in human cardiac disease.

Locked nucleic acids
Artifical nucleoside analogues that are more resistant to degradation than endogenous RNA.

## Experimental guidance

As a field, we face immense challenges investigating whether there is a biological role for mammalian exRNAs. To study exRNAs, we must keep abreast of advancements in the fields of the carriers that bind to exRNAs, with an emphasis on extracellular vesicle and RNA isolation techniques[43,91–93]. For example, extracellular vesicles isolated by centrifugation and subsequent affinity enrichment do not have detectable Ago2 present[41,94], whereas extracellular vesicles isolated through centrifugation and density gradient separation stain positive for Ago2 (REF.[95]). The technique to isolate exRNAs is also a crucial variable, as there are differences in exRNA present depending on the RNA isolation method. Recent work has diligently characterized how different RNA isolation methods alter the detectable exRNA populations for various biofluids[92]. The recommended isolation protocols depend on what population of exRNAs (for example, miRNAs, PIWI-associated RNAs (piRNAs) or tRNAs) one wants to isolate and also on the sample type (for example, cell culture medium, plasma, serum or urine)[92]. Thus, one should exercise care when selecting the protocols and kits used to isolate and quantify exRNAs[92].

As we highlight throughout, the presence of exRNA in circulating blood does not connote exRNA functionality. Extracellular miRNA in human serum and plasma is of low abundance and below the amounts needed to exact gene silencing in cells. In fact, the miRNA abundance in blood is at least ~100-fold less than that of circulating peptide hormones[51]. Because mammals do not seem to harbour an RNA amplification system similar to that observed in plants and worms, the levels of circulating miRNA do not seem to be sufficient for silencing gene expression if taken up in recipient cells. A more compelling case could be made to explore exRNAs transferred through cell–cell interaction through trogocytosis, as neighbouring cells might help boost transferred miRNA levels. However, even in this scenario, escape from lipid-bound vesicles would need to occur. Unlike in plants and nematodes, as yet there are no definitive data that show activity of transferred exRNAs in physiologically relevant settings in mammalian recipient cells.

Uncovering the potential function of exRNAs in recipient cells is inherently challenging. Using common genetics approaches such as genetic perturbation, sequencing or even combing through disease-associated genomic variants cannot distinguish the role of an RNA in a donor cell versus a recipient cell. To directly test whether exRNAs are functional, we need creative and specific approaches to overcome these challenges. Researchers should consider various quality-control considerations when designing experiments to test for exRNA function (FIG. 4).

Reducing the number of false-positive 'exRNAs' is fundamental. For example, exRNAs from animal serum in cell culture media, as well as RNA from dead or lysed mammalian cells, can be an abundant source

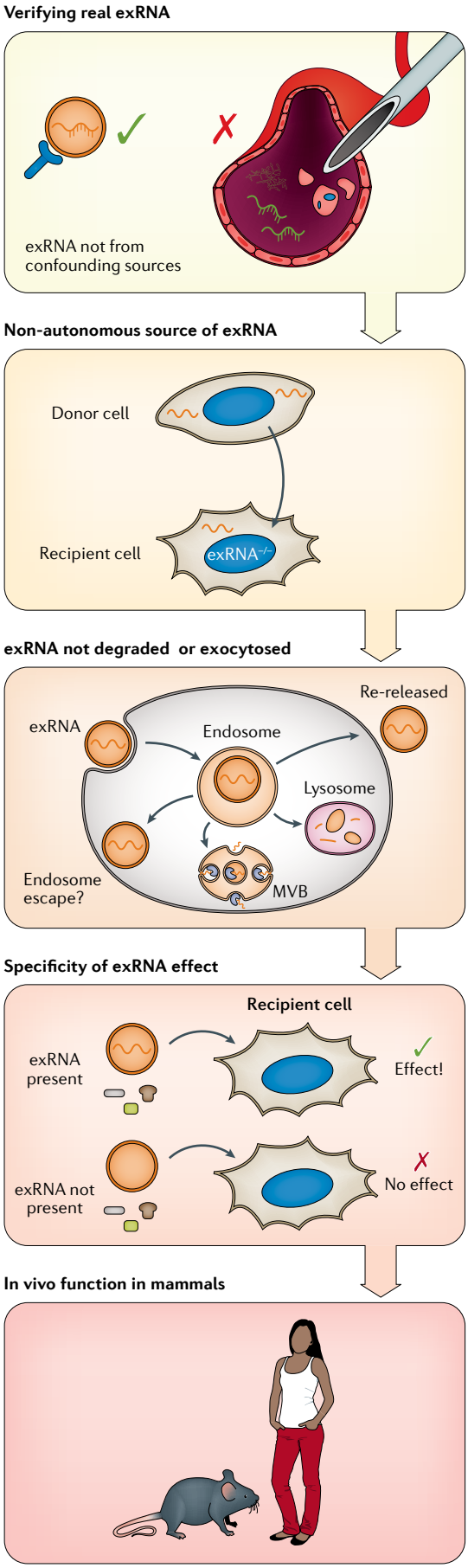


Fig. 4 | **Standards for demonstrating exRNA function.** First, experiments should rule out that the RNA of interest is not a technical artefact and is from the correct biological source. Second, demonstrating that the recipient cell cannot itself transcribe the extracellular RNA (exRNA) of interest must be diligently shown. Third, uptake of an exRNA from the extracellular environment into a recipient cell is not sufficient evidence to conclude that the exRNA is functional, because the lysosome could degrade the exRNA or the recipient cell could exocytose the exRNA back into the extracellular environment. Fourth, the functional consequence of an exRNA must require the exRNA to ensure that the effect is not only from other factors associated with exRNAs (proteins, hormones or lipids). Fifth, researchers must demonstrate a function in vivo to claim that exRNAs are important biologically. MVB, multivesicular body.

of contaminating exRNA[62,96]. As many exRNAs are protected by extracellular vesicles or other carriers, treating samples with RNase is a standard method to reduce contaminating RNA[39,40,46]. Furthermore, performing affinity purification for specific subtypes of extracellular vesicles, RNPs or lipoproteins helps distinguish the specific exRNA carrier[41,94]. Processing of sequencing data to distinguish between mammalian, microorganism-derived or contaminating synthetic nucleotides (such as vectors or primers) in samples is another critical step for the study of exRNAs[55,97].

Similar to eliminating RNA from confounding sources, another step towards demonstrating exRNA functionality in mammals is ensuring the exRNA is not made in the recipient cell (FIG. 4). Even if cells under homeostatic conditions do not express the exRNA of interest, it is plausible that experimental manipulation (such as treatment of the cells with isolated extracellular vesicles) may result in exRNA transcription in the recipient cell. Experimentally controlling for this false-positive exRNA transcribed in the recipient cell should be balanced with how confounding the solution is; thus, the least-invasive and specific experimental strategy to prevent this is ideal. For example, precise genetic removal of the candidate exRNA locus in the recipient cell is a successful strategy to guarantee that the transferred exRNA is from a donor cell[57,58,90]. By contrast, globally inhibiting miRNA biogenesis through genetic ablation of Dicer can cause many non-exRNA-related biological changes that confound data[98]. Verifying that an RNA as a bonafide exRNA is crucial, but much more evidence is required to show function.

Another step towards demonstrating exRNA function is determining whether, and how, exRNAs escape from the endosome to the cytoplasm before degradation in the lysosome (or secretion via exocytosis) (FIG. 4). For example, tracking extracellular vesicles containing exRNAs after endocytosis into recipient cell reveals that most internalized extracellular vesicles are found in the lysosome[34]. RNA proximity labelling techniques may help determine the subcellular location of endocytosed exRNAs[99]. Once it is established that the exRNA has escaped the endosome (or at least lysis), the next step is identifying and validating the role of an exRNA within a cell.

The most substantial evidence would demonstrate that the exRNA is necessary to create or rescue an observed phenotype (FIG. 4). As extracellular vesicles (or other exRNA carriers such as exomeres) contain a myriad of proteins and lipids capable of affecting the recipient cell, it is necessary to demonstrate that the exRNA is essential for the phenotype. For example, if applying an extracellular vesicle that contains a miRNA to target cells results in the downregulation of mRNAs, this is not sufficient evidence that the extracellular miRNA is causing this effect. Experimental approaches that change one variable, such as removing or adding the exRNA, produce more convincing data.

The most challenging step is demonstrating that an exRNA is functional in vivo (FIG. 4). Example experimental approaches that could help untangle potential in vivo functions of exRNAs in mammals include genetically encoded RNA sensors and tissue-specific deletion of RNAs, although new methodological developments are likely necessary. The cornerstone piece of evidence would demonstrate how these low-level exRNAs are taken up in large enough quantities to produce a meaningful biological effect in mammals.

## Conclusions

In sum, the idea that mammalian cells regulate one another at vast distances by secreting RNAs is captivating but remains speculative. For the moment, the extraordinary evidence that definitively establishes exRNAs as a medium of cell–cell communication in mammals remains elusive. However, absence of evidence is not evidence of absence.

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