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Constitutive and adaptive sorting of exosomal miRNAs: a novel classification framework and its implications for disease and therapy

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Highlights:

- To our knowledge, this is the first classification of exosomal miRNA sorting into constitutive and adaptive categories.
- The classification reveals that the mechanisms governing adaptive sorting remain almost entirely uncharacterized.
- Provides a theoretical basis for investigating disease pathogenesis and identifying upstream therapeutic targets.

Abstract: Exosomes are nanosized vesicles secreted by cells. By transporting cargo such as proteins, RNAs, and particularly miRNAs, they mediate intercellular and intertissue communication and play important roles in the pathogenesis of multiple diseases. Accordingly, the targeted inhibition of exosomal miRNAs has been explored as a therapeutic approach and has shown some therapeutic potential. However, this approach has considerable limitations. Some researchers have begun to elucidate the mechanisms underlying exosomal miRNA sorting. Based on an analysis of exosomal miRNA-sorting mechanisms, we classify exosomal miRNA sorting into two categories: constitutive sorting, which reflects a cell's intrinsic, stimulus-independent miRNA-packaging preferences, and adaptive sorting, which is dynamically reconfigured in response to physiological or pathological stimuli. Crucially, both categories share a defining feature: abundance independence, meaning that the exosomal enrichment of a given miRNA is decoupled from its intracellular expression level. This classification reveals a striking gap: although the molecular mechanisms of constitutive sorting have been partially elucidated, those governing adaptive sorting remain almost entirely unknown, making adaptive sorting the most pressing frontier in the field. We hope this classification framework will guide future mechanistic studies more precisely, particularly toward the largely uncharted area of adaptive sorting, and ultimately deepen our understanding of disease pathogenesis and open new avenues for more targeted therapeutic strategies.

Keywords: exosomes; miRNA; sorting; constitutive sorting; adaptive sorting; abundance-independence; stimulus-driven

1. Introduction

MicroRNAs (miRNAs) are non-coding RNA (ncRNA) molecules with a length of 20–25 nucleotides (nts) [1]. By participating in post-transcriptional regulatory processes [1], they regulate multiple biological activities, including cell proliferation [2], differentiation [2], migration [3], apoptosis [4], hormone secretion [5], metabolism [6], and immunity [7]. Exosomes are a type of extracellular vesicles (EVs) [3], and the core event in their biogenesis is the formation of intraluminal vesicles (ILVs) within multivesicular bodies (MVBs). Current research on the mechanisms of this process has mainly focused on the Endosomal Sorting Complexes Required for Transport (ESCRT) machinery and ESCRT-independent mechanisms [8]. Whether other mechanisms exist remains unclear and requires further investigation. After ILVs are formed, early endosomes (EEs) gradually mature into MVBs [9]. There are two ultimate fates for MVBs: one is fusion with lysosomes, leading to degradation of ILVs and their contents [10]; the other is fusion with the plasma membrane, leading to the release of ILVs into the extracellular space as circulating exosomes [9]. Exosomes can be secreted under physiological or pathological conditions by almost all cell types and are present in nearly all body fluids, such as blood, urine, saliva and breast milk [3]. Throughout this review, the term ‘exosomes’ refers to small EVs of endosomal origin with a diameter of approximately 30–150 nm, generated through the fusion of MVBs with the plasma membrane. We use ‘exosomal miRNA’ to denote miRNAs associated with or encapsulated within these vesicles. We acknowledge that, as noted in MISEV2023, the unambiguous isolation of exosomes from other small EV subtypes remains technically challenging; where cited studies use the term ‘small EVs (sEVs)’ rather than ‘exosomes’, we have retained the original terminology in those specific citations.

Since Zhang *et al.* discovered in 2010 that exosomal miRNAs can serve as a novel class of functional molecules [11], extensive research has been conducted on the relationship between selectively sorted exosomal miRNAs and various diseases. Research has shown that exosomal miRNAs participate in the regulation of disease-related pathways and serve as potential biomarkers of disease; however, their mechanisms of sorting are still not fully elucidated. A critical obstacle has been the absence of a systematic classification of sorting phenomena: without distinguishing the cell’s intrinsic constitutive sorting preferences from its dynamic, stimulus-driven adaptive sorting responses, mechanistic findings inevitably lack specificity. More fundamentally, no mechanistic framework yet exists to explain how cells reprogram their exosomal miRNA cargo in response to stimuli. This gap leaves the most disease-relevant sorting behaviors unexplained.

In this review, we briefly summarize the basic properties and sorting mechanisms of exosomal miRNAs, while primarily focusing on the characterization and classification of exosomal miRNA sorting phenomena. We propose, for the first time, a classification of exosomal miRNA sorting into two categories—constitutive sorting and adaptive sorting—distinguished by whether sorting operates as a stable cellular default or is dynamically reconfigured by stimuli. Abundance-independence, the decoupling of exosomal miRNA content from intracellular abundance, is a shared property of both categories rather than a classification criterion of either. By establishing this framework, we aim to direct future mechanistic research more precisely—especially toward adaptive sorting, where molecular

mechanisms are almost entirely unknown. And we also aim to provide a conceptual foundation for understanding how exosomal miRNA dysregulation drives disease.

2. Overview of exosomal miRNAs

2.1. Discovery and basic properties of exosomal miRNAs

In 2007, Valadi *et al.* found that RNA within exosomes can be transferred to other cells and exert biological effects [12]. In 2008, Skog *et al.* discovered that exosomes released by glioblastoma tumor cells contain miRNAs [13]. However, neither study explicitly demonstrated that exosomal miRNAs could affect recipient cells. Until 2010, Zhang *et al.* showed that, upon stimulation, cultured THP-1 cells could selectively secrete miRNAs into exosomes and deliver exosomal miRNAs to recipient HMEC-1 cells. These miRNAs bind to the 3′ untranslated region of c-Myb mRNA, reduce c-Myb protein levels in HMEC-1 cells, and promote the migration of HMEC-1 cells [11]. The research team further demonstrated that the elevated level of exogenous miR-150 in the plasma of patients with atherosclerosis could likewise promote HMEC-1 cell migration [11]. Through this experimental study, the fact that monocyte-secreted exosomal miR-150 promotes migration of recipient endothelial cells is shown. Based on this, Zhang *et al.* concluded that exosomal miRNAs can indeed exert functions in recipient cells (Figure 1) [11].

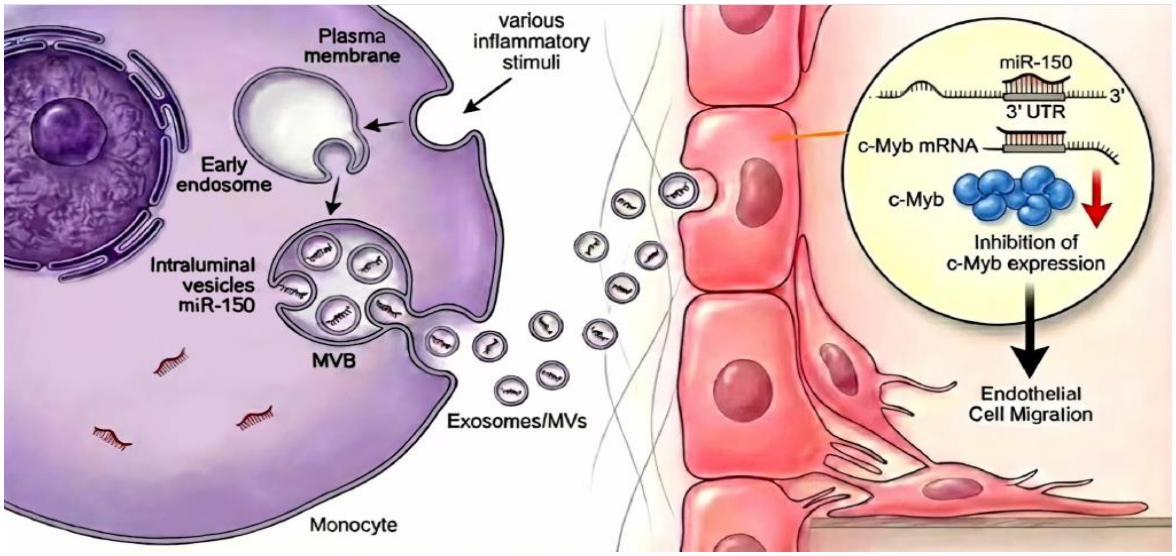

Figure 1. Schematic illustration of the mechanism by which monocyte-derived exosomal miR-150 promotes migration of recipient endothelial cells. Monocytes (THP-1 cells) selectively sort miR-150 into exosomes upon stimulation. The secreted exosomes are internalized by recipient human microvascular endothelial cells (HMEC-1), where exosomal miR-150 binds to the 3′ untranslated region (3′ UTR) of c-Myb mRNA, reduces c-Myb protein levels, and promotes HMEC-1 cell migration. Diagram created with Deepsider.

2.2. Roles of exosomal miRNAs in disease

Based on the fact that exosomes can mediate intercellular or intertissue communication by carrying cargo, exosomal miRNAs play an important role in the progression of various pathological processes

as signaling molecules. Under tumor conditions, exosomal miRNAs can accelerate tumor development by promoting tumor angiogenesis [3] and facilitating tumor immune escape [14], among other pathways. Exosomal miRNAs are also involved in regulating the insulin signaling pathway, significantly affecting the development of type 2 diabetes mellitus (T2DM) and its complications. Changes in the levels of related miRNAs in T2DM patients cause pathological changes [15]. For example, a decrease in exosomal miR-26a impairs insulin sensitivity [16], and the upregulation of miR-27a in obesity disrupts skeletal muscle glucose metabolism, leading to insulin resistance [16]. Changes in the levels of different miRNAs can impact different stages of metabolism, ultimately triggering T2DM. In addition, certain urinary exosomal miRNAs, such as miR-320c [17], miR-6068 [17], and miR-451 [18], also promote the development of diabetic nephropathy. At the same time, exosomal miRNAs are widely involved in the progression of various other diseases, including liver injury [19], and cardiovascular diseases [20].

These findings indicate that exosomal miRNAs are involved in the pathogenesis of many types of diseases and play important roles therein. Therefore, targeted regulation of exosomal miRNA function has become an effective therapeutic strategy for treating these diseases. At present, disease treatment strategies targeting exosomal miRNAs mainly focus on the injection of antisense RNA oligonucleotides to inhibit the function of pathogenic exosomal miRNAs. These approaches have shown certain therapeutic effects. For example, under laboratory conditions, psoriasis has been shown to be alleviated by injection of antisense oligonucleotides against relevant miRNAs to modulate miRNA levels to suppress disease progression [21]. In preliminary non-human primate studies, subcutaneous injection of 8-mer locked nucleic acid inhibitors targeting miR-33a/b increased circulating high-density lipoprotein cholesterol levels by 40% and reduced lipid accumulation [22]. Similar therapeutic strategies have shown beneficial effects in various models of ischemia and atherosclerosis, and the anti-miR-92a locked nucleic acid inhibitor used in these studies has already entered Phase I clinical trials [22].

However, this therapeutic approach has certain limitations. Firstly, injection of antisense RNA oligonucleotides can only be used to treat diseases caused by increased levels of exosomal miRNAs and cannot be applied to diseases caused by decreased levels of exosomal miRNAs. Secondly, such treatment can only passively inhibit the function of miRNAs that have already been secreted, but it cannot actively regulate the amount of miRNAs packaged into exosomes during the sorting stage to treat disease at a more upstream level. In addition, some exosomal miRNAs participate in the regulation of multiple signaling pathways involving many genes [23], meaning that substances used to inhibit miRNA function may theoretically have broad regulatory effects. For example, anti-miR-133 and anti-miR-92a can influence multiple pathways, including cholesterol efflux and vascular inflammation [22]. Therefore, if such agents are to be used clinically, the precision of their delivery must be ensured to avoid the potential risks of off-target delivery to normal tissues and cells. In summary, although current therapeutic strategies have shown certain efficacy in specific diseases, the application is quite limited and their safety issues cannot be ignored.

2.3. Therapeutic implications

At present, some researchers have already made progress in analyzing the mechanisms of exosomal miRNA sorting. Through studying the mechanisms, we may understand under what conditions and by

what means exosomal miRNAs are sorted and thus influence disease progression. These findings can guide the development of more upstream therapeutic strategies, thereby avoiding the risks associated with current treatments, overcoming their limitations, and achieving a transition from symptomatic treatment to etiological treatment.

3. Molecular mechanisms of exosomal miRNA sorting

In 2010, Zhang *et al.* compared the miRNA profiles of THP1 cells and their exosomes under different inflammatory stimuli during the process of exosomal miRNA packaging, and found that the sorting of miRNAs into exosomes was not directly correlated with the intracellular miRNA levels, but instead displayed an active nature [11]. Subsequently, Liu *et al.* further demonstrated that exosomes exhibit clear features of active sorting with respect to the miRNAs they carry [24], namely, that the degree of enrichment of a given exosomal miRNA is independent of its abundance in the cytoplasm, and that this sorting can dynamically respond to various physiological and pathological stimuli [11]. This provides the basis for exosome-mediated specific intercellular communication. However, through what molecular mechanisms do cells accomplish such precise selection and packaging?

Garcia-Martin *et al.* and O'Brien *et al.* found that this process is not governed by a single pathway. To date, two molecular mechanisms have been identified that are independent yet interconnected. One is mediated by RNA-binding protein (RBP) sequence-specific recognition [25]; the other is ceramide-dependent membrane remodeling [26]. These two mechanisms may function in a complementary manner, though direct evidence for their coordinated action within the same cell remains to be established. It should be noted that, to date, only two molecular mechanisms of exosomal miRNA sorting have been identified at the mechanistic level. This limited mechanistic foundation reflects the early stage of the field and underscores the importance of a classification framework that can guide future mechanistic investigation.

3.1. RBP-mediated sequence-specific recognition mechanism

Considerable progress has been made in elucidating the mechanisms underlying the specific sorting of exosomal RNAs. The principal mechanism is that cells can recognize specific motifs contained in RNA molecules, especially miRNAs [24,25], and accomplish sorting with the assistance of RBPs capable of recognizing these motifs (Figure 2).

Characteristics and functions of sorting motifs: The key information determining the fate of a miRNA is directly encoded in its nucleotide sequence [25]. Therefore, to compare intracellular and exosomal miRNA profiles derived from different cell types by inserting or deleting specific sequence motifs, Garcia-Martin *et al.* successfully identified two classes of short functional sequence motifs with opposite functions. One class is the exosomal export motif (EXOmotif), which is enriched in guanine (G) and cytosine (C), such as 'CGGGAG' and 'UGUGU'; miRNAs carrying such motifs are generally secreted extracellularly. The other class is the cellular retention motif (CELLmotif), which is enriched in adenine (A) and uracil (U), such as 'AGAAC' and 'UUAAA'; this motif causes miRNAs to be retained intracellularly, thereby inhibiting their secretion (Figure 2C) [25]. Through genetic experiments, they found that insertion of the potent EXOmotif 'CGGGAG' into the sequence of miR-34c, which is normally retained predominantly within cells, increased the efficiency of its exosomal secretion by 20-fold.

Conversely, deletion of the CELLmotif from endogenous miRNAs led to their substantial export into EVs. These findings demonstrate that such motifs are key elements regulating the direction of miRNA sorting.

RBPs recognize specific motifs and execute sorting: RNA sequences are recognized by their corresponding specific RBPs, which use their RNA-binding domains to pair with the relevant motifs and thereby initiate the downstream sorting program. Available evidence indicates that the RBPs involved in this process are diverse, and that the underlying sorting mechanism is highly conserved. Alyref and Fus proteins can specifically recognize and bind the EXOmotif 'CGGGAG' [25]; hnRNPA2B1 can recognize the 'GGAG' motif [27]; and Syncrip (hnRNPQ) preferentially recognizes the 'GGCU/A' sequence [28] (Figure 2A). Among these, Y-box binding protein 1 (YBX1) not only binds specific miRNAs such as miR-223, but its C-terminal domain can also directly induce Liquid-Liquid Phase Separation (LLPS) (Figure 2B) [24]. The resulting YBX1-miRNA condensates are proposed to concentrate target miRNAs in the vicinity of the MVB membrane, potentially increasing the efficiency of their packaging into ILVs. This finding suggests that phase separation may play an important role in the spatially specific recruitment of RNA cargoes, at least for a subset of sorting events.

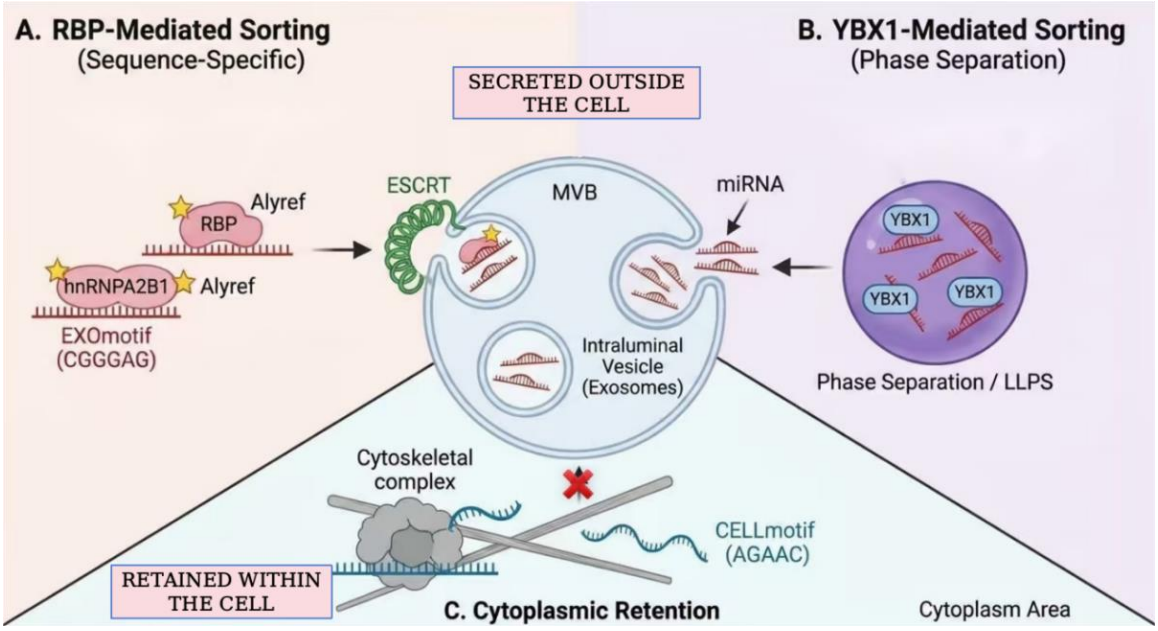

Figure 2. RBP-mediated sequence-specific active sorting of exosomal miRNAs. **(A)** Schematic of RBP recognition of EXOmotifs: hnRNPA2B1 recognizes the GGAG motif; Alyref protein recognizes CGGGAG. RNAs are transported to MVBs via the ESCRT complex; **(B)** YBX1-mediated LLPS sorting pathway: The C-terminal domain of YBX1 drives LLPS to form condensates that concentrate miRNAs, which are then delivered to MVBs and encapsulated into exosomes;**(C)** Cytoplasmic retention regulatory mechanism: miRNAs carrying the CELLmotif (AGAAC) sequence bind to cytoskeletal complexes and are retained in the cytoplasm, preventing their entry into MVBs for exosomal secretion. Diagram created with Deepsider.

Fine regulation of sorting activity and specificity by post-translational modifications: In order to adapt to changes in the internal and external environment, the activity and substrate specificity of sorting mechanisms are subject to precise dynamic regulation by post-translational modifications. Such modifications alter the functions of RBPs. Yu *et al.* found that hnRNPA2B1 must undergo SUMOylation,

which may alter its protein conformation or interaction interfaces, in order to effectively recognize the ‘GGAG’ motif and execute subsequent sorting steps [8]. In addition, such modifications can confer disease specificity on the sorting machinery. Zhang *et al.* found that in lung cancer cells, the methyltransferase SETD3 methylates a specific lysine residue on YBX1, significantly enhancing the binding of YBX1 to an oncogenic ncRNA fragment derived from hY4 RNA (hY4F). As a result, in cancer cells with high SETD3 expression, methylated YBX1 preferentially loads hY4F into exosomes, thereby promoting tumor growth [29]. This clearly illustrates the pathological reprogramming of a sorting pathway through modification of an RBP. At the same time, modifications of miRNAs themselves, such as 3′ uridylation, can also influence their sorting direction, making them more likely to be incorporated into exosomes [27].

3.2. Ceramide-dependent membrane remodeling mechanism

Unlike the highly sequence-specific recognition mechanism described above, the second mechanism primarily acts by regulating membrane lipid composition and membrane morphodynamics during exosome biogenesis. In doing so, it globally affects the efficiency of exosome production and the RNA-loading environment, thereby altering the direction of RNA sorting.

Ceramide-dependent membrane dynamics pathway: The fundamental driving force is the lipid second messenger ceramide, which is mainly generated through hydrolysis of membrane sphingomyelin catalyzed by neutral sphingomyelinase 2 (nSMase2). Ceramide molecules possess a typical cone-shaped geometry; thus, when they accumulate locally on the membrane of MVBs, they induce negative curvature of the lipid bilayer, directly promoting inward budding of the MVB membrane and thereby formation of ILVs [30]. Therefore, the activity of nSMase2 directly determines the rate and quantity of exosome generation. More importantly, Leidal *et al.* found that inhibition of nSMase2 activity globally decreases exosome secretion and is accompanied by specific changes in the abundance of certain miRNAs, such as miR-210 [30]. However, this effect is not achieved through sequence recognition. Rather, it is more likely due either to the reduction in exosome production, which decreases the opportunities for all RNA cargoes to be loaded, or to ceramide-mediated alterations in the membrane lipid microenvironment, such as membrane curvature and lipid raft properties, which non-specifically affect the physicochemical tendency of different RNA-protein complexes to associate with membranes and their aggregation states on the membrane surface. It should be noted that the ceramide-dependent pathway described here governs membrane curvature and ILV budding, and is mechanistically distinct from macroautophagy. A separate but related finding by Leidal *et al.* (2020) showed that the LC3-conjugation machinery, which is also involved in autophagy, participates in loading RBPs into EVs; however, this represents a distinct molecular event from the ceramide-driven membrane remodeling described above.

Potential auxiliary integrative role of the ESCRT machinery: ESCRT is the core molecular machinery that executes membrane neck scission and completes vesicle release. Its canonical function is to recognize and sort ubiquitinated membrane proteins. However, its accessory protein Alix can directly undergo protein-protein interactions with the RBP (SYNCRIP), which participates in RNA sorting [31]. This indicates that the ESCRT machinery does not directly participate in the early recognition and recruitment of RNAs, but instead functions at the terminal stage of sorting. This interaction suggests that ESCRT accessory proteins may assist in the final integration of RNA-RBP complexes during vesicle scission, though the precise mechanistic details remain to be determined.

In summary, the selective sorting of exosomal RNAs is a complex, multilayered, and coordinated process. Based on current studies, the molecular framework can be summarized into two distinct sorting processes.

The first is a sequence-based, highly specific recognition pathway. In this pathway, sorting motifs within RNA sequences are recognized by specific RBPs, such as hnRNPs and Y-box proteins, and are efficiently enriched spatially through processes like LLPS. The sorting of RNAs is further subject to dynamic and fine regulation by post-translational modifications, thereby determining the species of miRNAs to be loaded.

The second is a membrane remodeling and production-capacity regulatory pathway. In this pathway, ceramide regulates membrane curvature to promote budding, and the ESCRT machinery may assist in scission and integration. This pathway primarily regulates exosome output and provides the necessary membrane environment for cargo loading, thereby determining loading capacity and loading conditions.

4. Exosomal miRNA sorting phenomenon

Before describing the sorting phenomena in detail, it is necessary to clarify the conceptual framework. A defining property shared across all documented cases of active exosomal miRNA sorting is abundance-independence: the degree to which a miRNA is enriched in exosomes does not correlate with its intracellular expression level. This decoupling is not itself a classification criterion, but rather the fundamental property distinguishing active sorting from passive encapsulation. Building on this, we classify actively sorted exosomal miRNAs into two categories based on whether sorting operates as a stable cellular default or is dynamically reconfigured by stimuli. The first category, constitutive sorting, reflects the intrinsic, stimulus-independent miRNA packaging preferences of a given cell type. This is a default state maintained under homeostatic conditions. The second category, adaptive sorting, describes the dynamic reprogramming of exosomal miRNA cargo that occurs in response to physiological or pathological stimuli. These two categories are mutually exclusive at the level of individual sorting events: constitutive sorting describes the default state of a given miRNA in the absence of perturbation, while adaptive sorting describes the stimulus-driven deviation from that baseline. It should be noted that mutual exclusivity applies at the level of individual sorting events, not at the level of cellular sorting behavior as a whole. Under stimulated conditions, constitutive and adaptive sorting operate in parallel: some miRNAs continue to follow their default packaging preferences while others are selectively reprogrammed in response to the stimulus. The classification is therefore a tool for analyzing discrete sorting events, rather than a claim about the exclusive nature of global cellular sorting programs. Importantly, adaptive sorting is itself abundance-independent. Stimulus-driven changes in exosomal miRNA content frequently occur without corresponding changes in intracellular abundance. This, in turn, underscores that abundance-independence is a shared property of both categories.

4.1. Constitutive sorting of exosomal miRNAs

Constitutive sorting refers to the default, stimulus-independent miRNA packaging preferences that a given cell type maintains under homeostatic conditions. A hallmark of constitutive sorting is its abundance-independence: the exosomal enrichment of a given miRNA under basal conditions bears no direct relationship to its intracellular expression level, indicating active selective recruitment rather than passive encapsulation of cytoplasmic contents during ILV formation. Constitutive sorting manifests in

two directions: miRNAs present at low intracellular abundance yet selectively enriched in exosomes, and miRNAs highly abundant intracellularly yet actively excluded from exosomes (Figure 3A) (Table 1).

Table 1. Summary of reported exosomal miRNA sorting events.

Category	Stimulus/Condition	Cell/Tissue	miRNA(s)	Direction	Known Mechanism
Constitutive sorting	Homeostasis	Multiple cell types	miRNAs with EXOmotif (GGAG)	↑ in exosomes	hnRNPA2B1-mediated basal sorting
	Homeostasis	Multiple cell types	miRNAs with EXOmotif (CGGGAG)	↑ in exosomes	Alyref/Fus protein-mediated basal sorting
	Homeostasis	Multiple cell types	miRNAs with EXOmotif (GGCU/A)	↑ in exosomes	Syncrip (hnRNPQ)-mediated basal sorting
	Homeostasis	Multiple cell types	miR-223	↑ in exosomes	YBX1 binding via C-terminal LLPS
	Homeostasis	Multiple cell types	miRNAs with CELLmotif (AGAAC/UUAAA)	↑ in cells	CELLmotif-mediated intrinsic intracellular retention
	Homeostasis	HEK293T cells; mesenchymal stem cells	miR-451	↑ in exosomes	Cell-type-specific constitutive enrichment
	Homeostasis	Multiple cell types (high-throughput sequencing)	sEV-enriched miRNAs (panel)	↑ in exosomes	Cell-type-specific constitutive sorting (Garcia-Martin *et al.*)
Adaptive sorting	Hyperglycemia (diabetes)	Pancreatic cells	miR-29a, miR-375	↑ in exosomes	Unknown
	Aging	Human dermal fibroblasts	miR-21-3p, miR-17-3p	↑ in cells	Unknown
	Aging	Human dermal fibroblasts	miR-15b-5p, miR-30a-3p	↑ in exosomes	Unknown
	Colorectal cancer	Colorectal cancer cells	miR-19b-3p	Shifts from constitutive to adaptive sorting under cancer conditions	Unknown
	Cancer (gastric, lung, colorectal)	AZ-521; SBC-3, DMS-35, NCI-H69; SW480, SW620	let-7 family members	↓ in exosomes	Unknown
	Cancer (breast, lung)	Breast cancer cells; A549 cells	miR-210	↑ in exosomes	Unknown
	Hypoxia		miR-135b, miR-210	↑ in exosomes	Unknown
	Hypoxia	Lung cancer cells	miR-23a	↑ in exosomes	Unknown

Table 1. *Cont.*

Category	Stimulus/Condition	Cell/Tissue	miRNA(s)	Direction	Known Mechanism
Adaptive sorting	90 min Downhill running exercise	Exosomes in Sprague-Dawley rats	miR-1, miR-133a, miR-133b, miR-206, miR-208a, miR-499	↑ in exosomes	Unknown
	OA/PA stimulation	THP-1	miR-150, miR-30d, miR-26b, miR-222	↑ in exosomes	Unknown
	H_2O_2 stimulation	THP-1	miR-150, miR-23a	↑ in exosomes	Unknown
	Liver injury/inflammation	Plasma	miR-155	↑ in exosomes	Unknown
	LPS-induced inflammation	Human blood cells	miR-155	↑ in exosomes	Unknown
	TNF-α-induced inflammation	Adipocytes	miR-155	↑ in exosomes	Unknown

↑, increased; ↓, decreased abundance in exosomes relative to baseline or unstimulated control. OA, oleic acid; PA, palmitic acid; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-alpha; LLPS, liquid-liquid phase separation; HTS, high-throughput sequencing; IDR, intrinsically disordered region;

* Abundance-independence has been directly verified for all constitutive sorting cases listed and for selected adaptive sorting cases. For remaining adaptive sorting cases, systematic simultaneous quantification of intracellular and exosomal miRNA levels has not yet been reported.

Low intracellular abundance but high exosomal abundance: Using deep sequencing, Guduric-Fuchs *et al.* found that miR-451 shows the strongest tendency toward export in HEK293T cells [32]. miR-451 also exhibits high export levels in mesenchymal stem cells [33], resulting in higher levels of miR-451 in exosomes than within the cells themselves. In addition, miR-223, which is of low intracellular abundance, is regulated by YBX1 and is selectively recruited into exosomes, where it becomes highly enriched [24]. These studies demonstrate that miRNAs present at low levels inside cells can be enriched in exosomes.

High intracellular abundance but low exosomal abundance: Kosaka *et al.* measured the ratios of exosomal to donor-cell levels for endogenous miR-16, endogenous miR-21, exogenous miR-143, exogenous miR-146a, and exogenous miR-155 secreted into conditioned medium by HEK293 and COS-7 cells. The results showed that all five miRNAs were present at significantly lower levels in exosomes than in the donor cells. Except for exogenous miR-146a, whose exosomal level was approximately 15% of that in donor cells, the levels of the other four miRNAs in exosomes were all no more than 4% of their intracellular levels [34]. Similarly, miR-218 exhibited an obvious retention tendency in HEK293T cells [32]. Moreover, they further observed that the proportions of retention or export for 12 miRNAs in human microvascular endothelial cells (HMEC-1) and primary outgrowth endothelial progenitor cells (OECs) were similar to those observed in HEK293T cells [32]. Since gene expression differs among different cell types, the abundance of the same miRNA can vary across cells. Based on this, the observation that the same miRNA exhibits similar retention or export features in different cell types further demonstrates that exosomal miRNA sorting is abundance-independent, indicating the existence of specific cellular mechanisms that selectively retain certain miRNAs in cells or enrich them in exosomes.

In particular, Garcia-Martin *et al.* also systematically demonstrated this phenomenon across multiple cell types by high-throughput sequencing of miRNA expression levels in cells and EVs, and respectively

designated the two categories of miRNAs as cell-enriched miRNAs and sEV-enriched miRNAs [25]. This stable, cell-type-specific sorting preference is maintained under homeostatic conditions and independent of external perturbation. And this is precisely what we designate here as constitutive sorting.

4.2. Adaptive sorting of exosomal miRNAs

Adaptive sorting describes the dynamic reprogramming of exosomal miRNA cargo when cells are exposed to endogenous or exogenous stimuli. It includes physiological cues and pathological conditions such as inflammation, metabolic stress, and cancer. Upon stimulation, cells alter both the identity and abundance of miRNAs packaged into exosomes; these changes can in turn modify the state of recipient cells, triggering downstream physiological or pathological effects. Critically, as shown below, adaptive sorting frequently occurs independently of changes in intracellular miRNA abundance, confirming that it represents active cargo reprogramming rather than a passive reflection of altered gene expression (Figure 3B).

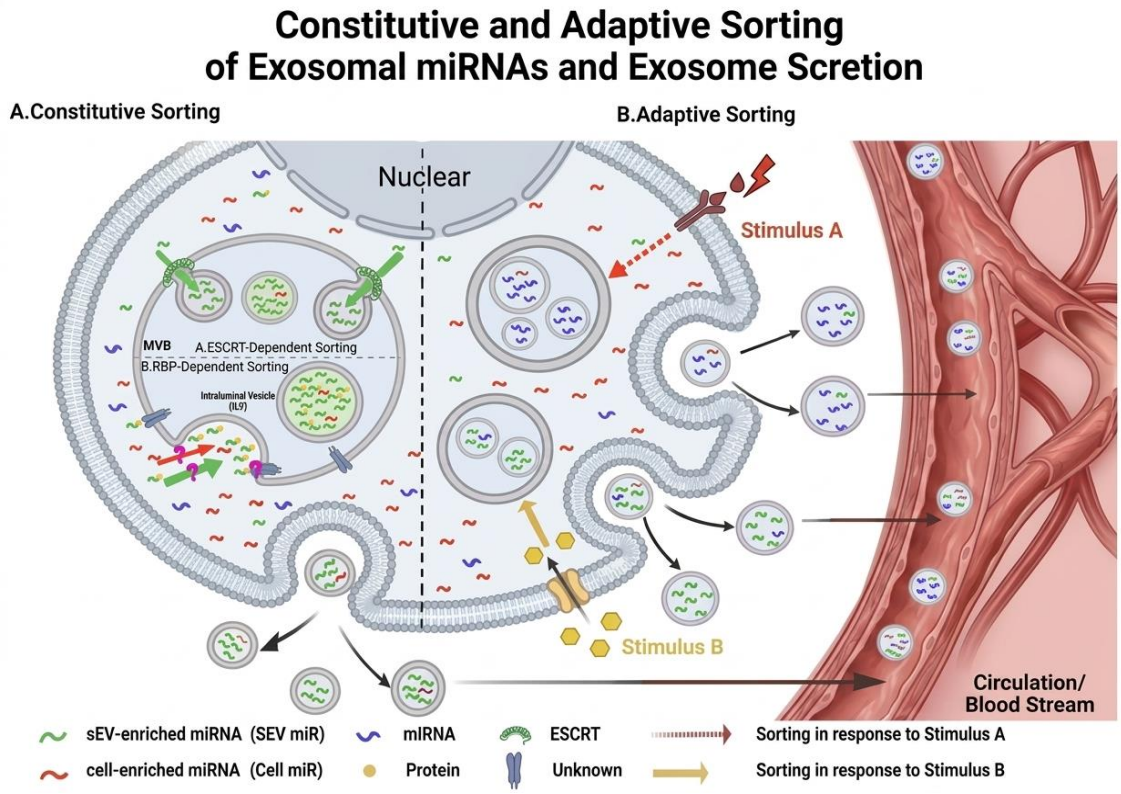

Figure 3. Constitutive and adaptive sorting of exosomal miRNAs and exosome secretion. **(A)** Constitutive sorting: under homeostatic conditions, miRNAs present at low intracellular abundance can be selectively enriched in exosomes (upper left), while miRNAs highly abundant intracellularly may be actively excluded from exosomes (bottom left). This default sorting preference is cell-type-specific and stimulus-independent; **(B)** Adaptive sorting (right): upon exposure to physiological or pathological stimuli (e.g., hyperglycemia, hypoxia, inflammation, cancer), cells dynamically reprogram their exosomal miRNA cargo independently of changes in intracellular miRNA abundance. Diagram created with Deepsider.

The multiple features of cellular responses to stimuli also indicate the presence of active selection in exosomal miRNA sorting.

Different exosomal miRNA sorting profiles in a specific tissue/cell type before and after stimuli: Under stimuli, a given cell or tissue displays differential selectivity toward different miRNAs. After 1h of stimuli with 25 mmol/L glucose, the levels of some of miRNAs in islet-cell-derived exosomes, such as miR-29a and miR-375, increased to 2- to 3-fold those before stimuli, whereas certain miRNAs remained essentially unchanged [6]. Similar findings were observed in MIN6 cells [6]. Likewise, during the development of insulin resistance, different exosomal miRNAs exhibit different trends in abundance. For example, the exosomal levels of miR-122-5p and miR-26b-3p increase, whereas those of miR-4461 and miR-20a-5p decrease [15]. Terlecki-Zaniewicz *et al.* also found that during aging, miR-21-3p and miR-17-3p are retained in senescent human dermal fibroblasts while miR-15b-5p and miR-30a-3p are selectively packaged into sEVs [4]. Moreover, as aging progresses, the proportional composition of different miRNAs in exosomes changes accordingly [4]. Teng *et al.* measured exosomal miRNA levels in primary colon tissue and liver-metastatic colon tumors, and found that the sorting of oncogenic miRNAs such as miR-196a/b, miR-181d-5p, and miR-155-5p were suppressed, whereas the sorting of tumor-suppressive miRNAs such as miR-10a-5p, miR-193a-3p, miR-200b-5p, and miR-222-3p were enhanced [35]. To some extent, the study also indicates that miRNAs with similar functions may be sorted into exosomes in similar patterns under stimuli. Based on previous research, it is not difficult to conclude that under stimuli such as hyperglycemia, aging, and cancer, exosomes exhibit differential sorting toward different miRNAs, and notably, miRNAs with similar functions may be selected in similar ways. To some extent, this phenomenon demonstrates that exosomes can actively sort miRNAs in response to stimuli.

Different exosomal miRNA sorting profiles in the same tissue in response to different stimuli: Research has shown that a given tissue or cell type exhibits stimulus-specific selectivity toward exosomal miRNAs under different stimuli. Zhang *et al.* stimulated THP-1 cells with oleic acid (OA)/palmitic acid (PA), advanced glycation end products (AGEs), and H_2O_2, and found that the types and levels of miRNAs in THP-1-derived exosomes changed [11]. Among these, except that miR-150 expression was increased under all three stimuli, the expression level of miR-30d, miR-26b, and miR-222 was significantly increased only under AGEs and OA/PA stimulation and remained essentially unchanged under H_2O_2 stimulation; in contrast, miR-23a expression was significantly increased only under H_2O_2 stimulation and remained largely unchanged under AGEs and OA/PA stimulation [11]. Similarly, in exosomes derived from the pancreatic β-cell line MIN6, the expression level of miRNAs such as miR-29a, miR-29b, and miR-223 was significantly increased under high glucose, potassium chloride, and arginine stimulation, but remained nearly unchanged under stimulation with high levels of free fatty acids (FFAs); miR-29c and miR-375 were significantly increased under all four stimulation conditions; whereas miR-143 was significantly increased under high potassium chloride and arginine stimulation, but significantly decreased under high FFA stimulation [6]. In addition to chemical stimulation, different types of exercise can also induce stimulus-specific sorting of miRNAs into exosomes. For example, 90 min of downhill running significantly increased the circulating exosomal levels of six miRNAs—miR-1, miR-133a, miR-133b, miR-206, miR-208a, and miR-499—in Sprague-Dawley rats. In contrast, 90 min of uphill running caused no significant changes regarding all selected miRNA levels [36]. Pathological stimuli such as cancer have similar effects. Tumor hypoxia is a complex pathophysiological phenomenon in which oxygen consumption increases due to rapid tumor proliferation and abnormal angiogenesis. Oxygen supply becomes insufficient and cannot meet the demands of tumor growth [37]. Under

conditions of insufficient oxygen supply, the levels of miRNAs in exosomes change markedly, thereby producing a series of biological effects. Hypoxia induces increased levels of miR-135b and miR-210 in exosomes derived from A549 cells, while miR-23a is upregulated in hypoxic exosomes from lung cancer cells; both effectively promote angiogenesis [3], thereby facilitating oxygen acquisition and further promoting tumor cell proliferation.

Different exosomal miRNA sorting profiles in different tissue in response to the same stimulus (tissue specificity of adaptive sorting): In addition, different tissues or cell types may show similar selectivity toward certain miRNAs under the same stimulus. Inflammation induces increased levels of miR-155, miR-122, and miR-146a in plasma during liver injury [19]. Scoditti *et al.* induced adipose tissue inflammation with TNF-α and found that under this inflammatory condition, the expression levels of miR-155 and miR-34a were both increased in adipocyte-derived exosomes [38]. Lipopolysaccharide (LPS) is a macromolecule with strong pro-inflammatory property. After systemic in vivo injection of LPS into mice to induce systemic inflammation, Balusu *et al.* measured the levels of four inflammation-related miRNAs—miR-155, miR-1a, miR-9, and miR-146a—in exosomes secreted by choroid plexus epithelial cells into the cerebrospinal fluid, and found that all were upregulated [39]. Likewise, under LPS stimulus, the level of miR-155 in exosomes derived from human blood cells was also elevated compared with the unstimulated condition [11]. Under the inflammatory stimuli used in the above studies, adipose tissue, choroid plexus epithelial cells, and human blood cells all showed increased exosomal miR-155 expression, thereby amplifying inflammatory signaling. This indicates that under inflammatory conditions, miR-155 is commonly highly expressed in exosomes derived from multiple cell types. Therefore, by detecting exosomal miR-155 levels, it may be possible to roughly assess whether inflammatory responses are present in the body. Cancer-related stimuli can likewise induce tissue-specific sorting of exosomal miRNAs. Through comparative analysis, the expression levels of let-7 family members were lower in cancer-cell-derived exosomes from the gastric cancer cell line AZ-521, the lung cancer cell lines SBC-3/DMS-35/NCI-H69, and the colorectal cancer cell lines SW480/SW620 [40]. Meanwhile, miR-210 is selectively enriched in glioma cells [41], breast cancer cells [42], and A549 cells under lung cancer conditions [43], and promotes tumor angiogenesis in both breast cancer and lung cancer.

Stimulus-responsive miRNA sorting is independent of changes in intracellular abundance and can even alter the intrinsic sorting pattern of exosomal miRNAs: More importantly, exosomal miRNA sorting after stimuli may also occur independently of changes in intracellular miRNA expression levels. Teng *et al.* classified the miRNAs examined in their study into two groups according to their oncogenic or tumor-suppressive properties, and measured both intracellular and exosomal miRNA levels in primary colon tissue and liver-metastatic colon tumors [35]. The data showed that oncogenic miRNAs were upregulated in tumor cells, whereas tumor-suppressive miRNAs were downregulated. However, in exosomes, the sorting of oncogenic miRNAs was suppressed, whereas the sorting of tumor-suppressive miRNAs was enhanced [35]. The opposite trends in intracellular and exosomal miRNA levels are consistent with an abundance-independent sorting pattern. In some cases, stimuli can even alter the original sorting pattern of a certain miRNA, converting a cell-enriched miRNA into an sEV-enriched miRNA, or vice versa. miR-19b-3p has oncogenic property and is present at very low levels in normal plasma [44]. Under normal physiological conditions, miRNAs such as miR-19b-3p are highly expressed in brown adipose tissue (BAT), C2C12, 3T3-L1, AML12, and SVEC cells, but are expressed at very low or nearly undetectable levels in exosomes derived from these five cell types [25]. However,

circulating miR-19b-3p levels are elevated in patients with colorectal cancer and this phenomenon is strongly associated with advanced disease stage and poor prognosis [45]. Under cancer-related stimuli, miR-19b-3p shifts from being cell-enriched to sEV-enriched, thereby playing an important role in colorectal cancer metastasis and promoting cancer progression. In a mouse model of acute kidney injury, renal tubular epithelial cells release exosomal miR-19b-3p, which is subsequently taken up by macrophages and stimulates M1 polarization, thereby further aggravating inflammation [46]. These studies more fully demonstrate that orderly sorting is of great importance in maintaining normal physiological function. Once the sorting pattern of a specific miRNA changes under stimulatory conditions, disease progression may be profoundly affected. It should be noted that the evidence for abundance-independence in adaptive sorting, while supported by available studies, is less systematically established than for constitutive sorting. In many reported adaptive sorting cases, only exosomal miRNA levels were measured without simultaneous quantification of intracellular abundance. The rigorous verification of abundance-independence across a broader range of adaptive sorting contexts therefore represents an important methodological priority for future studies.

In summary, the sorting of exosomal miRNAs is adaptive—dynamically reconfigured in response to stimuli. Under conditions such as exogenous chemicals, exercise, aging, hyperglycemia, hypoxia, and cancer, cells exhibit differential sorting toward specific miRNAs (Table 1), and notably, miRNAs with similar functions may be selected in similar ways across different cell types. To some extent, this phenomenon is consistent with the hypothesis that exosomes can actively reprogram their miRNA cargo in response to stimuli, at least in part independently of changes in intracellular abundance.

5. Outstanding scientific questions to be addressed

At present, the widespread nature of exosomal miRNA sorting phenomena and the important roles of this process in disease initiation and progression have been widely recognized. Exosomal miRNA-based therapy, such as injection of antisense miRNA oligonucleotides, has become a new direction in disease treatment research and has already made some progress. However, this therapeutic approach still carries certain risks and limitations. First, because antisense miRNA oligonucleotides can regulate multiple signaling pathways, the precision of RNA drug delivery is reduced, creating the risk of off-target effects and impacting the physiological functions of normal cells. Second, this approach exerts therapeutic effects by inhibiting the function of miRNAs already secreted; it cannot treat diseases caused by reduced exosomal miRNA levels, nor address the upstream problem of aberrant sorting. These limitations underscore a fundamental unmet need: therapeutic strategies that intervene at the level of sorting itself, before pathogenic miRNAs are packaged and released.

When viewed through the classification framework proposed here, the mechanistic landscape is strikingly uneven. The two known mechanisms are RBP-mediated sequence-specific recognition and ceramide-dependent membrane remodeling. Both operate under basal conditions and account for only a subset of constitutive sorting events. No molecular mechanism has yet been identified that governs adaptive sorting: we do not know how a cell senses a stimulus and converts that signal into selective reprogramming of its exosomal miRNA cargo. This is arguably the most critical gap in the field, because adaptive sorting is the form most directly implicated in disease progression—the mechanism by which tumor cells, inflamed tissues, and metabolically stressed organs alter intercellular communication. Beyond this overarching gap, even constitutive sorting mechanisms remain incomplete: miRNAs from

the same family may share sequence motifs yet sort in opposite directions; some miRNAs carry EXOmotif sequences yet are constitutively retained in cells. These anomalies suggest additional regulatory layers. These layers potentially involve miRNA secondary structure, RNA modifications, or subcellular compartmentalization, and they remain to be discovered. Resolving both the adaptive sorting gap and these constitutive sorting anomalies represents the most pressing agenda for the field.

5.1. Prospective mechanistic directions for adaptive sorting research

Based on existing evidence, we propose four mechanistic directions that may govern adaptive sorting and warrant prioritized investigation.

(1) Stimulus-dependent post-translational regulation of sorting RBPs. Stimulus-activated kinase cascades (e.g., MAPK, PI3K/AKT) may phosphorylate sorting RBPs such as hnRNPA2B1, Syncrip (hnRNPQ), and YBX1, altering their miRNA-binding affinity or subcellular localization and thereby reprogramming sorting preferences. Precedent for this concept exists: stimulus-activated kinases are known to phosphorylate RBPs and alter their RNA-binding specificity. Future studies systematically mapping the PTM landscape of sorting RBPs under stimulated versus basal conditions would directly test this hypothesis.

(2) Stimulus-driven remodeling of MVB membrane lipid composition. Stimuli known to activate sphingomyelinase—including inflammatory cytokines, hypoxia, and oxidative stress—could dynamically alter ceramide content and broader MVB lipid composition, creating distinct membrane microdomains that redirect miRNA sorting during ILV formation. This direction is particularly attractive because it would explain how diverse stimuli converge on a common biophysical mechanism to produce adaptive sorting outcomes.

(3) Dynamic regulation of liquid–liquid phase separation (LLPS) as a sorting switch. YBX1-mediated constitutive sorting depends on LLPS driven by its C-terminal intrinsically disordered region (IDR). Stimulus-induced dissolution or reorganization of such condensates could release or redirect previously sequestered miRNAs, generating adaptive sorting responses. This hypothesis connects the adaptive sorting gap directly to the rapidly advancing field of biomolecular condensate biology.

(4) Epitranscriptomic regulation via RNA modifications. Stimulus-induced changes in N6-methyladenosine (m6A) modification of miRNAs—mediated by dynamic regulation of writers (METTL3/METTL14) or erasers (FTO, ALKBH5)—could alter RBP recognition of EXOmotif-containing miRNAs, providing an epitranscriptomic layer of adaptive sorting regulation.

6. Summary and prospects

The sorting of exosomal miRNAs plays a crucial role in various diseases and has become a key research focus in the development of new therapeutics. In this review, we propose a classification framework that distinguishes two categories of active exosomal miRNA sorting: constitutive sorting, which reflects the default, stimulus-independent packaging preferences of a given cell type, and adaptive sorting, which represents the dynamic reprogramming of exosomal cargo in response to physiological or pathological stimuli. Both categories are characterized by abundance independence—the decoupling of exosomal miRNA content from intracellular expression levels—which we identify as a shared property rather than a classification criterion. Applying this framework to existing mechanistic knowledge reveals a critical

asymmetry: currently known molecular mechanisms account for only a subset of constitutive sorting events, while the mechanisms governing adaptive sorting—the category most directly relevant to disease—remain almost entirely unknown. We hope this classification will more precisely direct future mechanistic research, particularly toward adaptive sorting, ultimately advancing our understanding of disease pathogenesis and enabling more targeted therapeutic strategies.

A deeper understanding of the mechanisms underlying exosomal miRNA sorting, particularly those governing adaptive sorting in disease contexts, may make it possible to intervene directly at the sorting step. We could then either block the packaging of pathogenic miRNAs or enhance the sorting of protective ones. Such upstream interventions would offer a fundamentally different and potentially more precise therapeutic strategy than current approaches that target miRNAs after secretion. We therefore call on the field to prioritize the mechanistic dissection of adaptive sorting as the next major frontier in exosomal miRNA biology.

Declaration of generative AI and AI-assisted technologies

During the preparation of this manuscript, the authors used generative AI tools only to improve language and readability. Specifically, the authors used Deepseek for language polishing only in limited sections. The authors also used Deepsider for figure material generation only. The authors take full responsibility for the content of the manuscript.

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Authors' contribution

Conceptualization, Y.Z.; writing—original draft preparation, Z.H. and Y.Z.; writing—review and editing, Y.Z., Z.H. and Y.Z.; visualization, Z.H. and Y.Z.; supervision, Y.Z., Z.H. and Y.Z.; project administration, Y.Z.; funding acquisition, Y.Z. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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