Long-range function of secreted small nucleolar RNAs that direct 2′-O-methylation

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Microarray and deep sequencing approaches have led to the discovery of small noncoding RNAs in the extracellular space. The most well studied of these species are miRNAs, which are found in plasma in small membrane-bound vesicles or exosomes and/or bound to lipoproteins (12, 13). The abundance of different miRNAs in plasma, serum, and urine tracks with a spectrum of neurological, cardiovascular, oncologic, metabolic, and hematological disorders, suggesting that miRNAs in the blood can serve as biomarkers for disease processes (14). The observations that miRNAs are transferred from donor to recipient cells in which they impact gene expression and biological functions indicate that miRNAs can play roles in cell–cell communication (13, 15, 16).
RNA-Seq has also revealed the presence of snoRNAs in the medium of cultured cells (17–19) and in human plasma exosomes (20, 21). However, it is unclear whether secretion of this class of noncoding RNAs is regulated, and in contrast to miRNAs, their potential to function in non-cell-autonomous roles is unknown. The present study was undertaken to further characterize the extracellular trafficking of the four intronic box C/D snoRNAs produced from the Rpl13a locus.

**Results**

*Rpl13a* snoRNAs accumulate in the culture medium of lipopolysaccharide (LPS)-stimulated macrophages

The *Rpl13a* snoRNAs U32a, U33, U34, and U35a accumulate outside the nucleus of fibroblasts and cardiomyoblasts in the setting of oxidative stress (9, 10, 22). To extend these findings to another cell type in which oxidative stress plays a central role in pathophysiologically important responses, we treated bone marrow–derived macrophages with LPS, a stimulus known to induce reactive oxygen species and inflammatory gene expression (23). Following exposure of macrophages to LPS, intracellular reactive oxygen species are increased, as evidenced by enhanced 2',7'-dichlorodihydrofluorescein diacetate (DCF) staining (Fig. 1A) and expression of the well-characterized inflammatory response genes, cyclooxygenase 2 and tumor necrosis factor-α (COX2 and TNFα; Fig. 1B). Stimulation with LPS did not cause loss of cellular integrity, as assessed by LDH release (Fig. 1C), or apoptosis, as assessed by caspase 3/7 activity (Fig. 1D). Using snoRNA-specific stem loop primers for reverse transcription with qPCR amplification for detection, we found that the *Rpl13a* snoRNAs rapidly increased in the

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**Figure 1. Macrophages secrete Rpl13a snoRNAs in response to lipopolysaccharide.** Murine macrophages were treated with 50 ng/ml LPS. A, ROS quantified after 8 h by DCF staining. B, qRT-PCR quantification of COX2 and TNFα gene expression relative (rel) to Rplp0 at the indicated times. C, LDH release following treatment with 50 ng/ml LPS for the indicated time. LDH-positive control and freeze-thaw lysis (MAX) are shown as controls. D, apoptosis quantified by a caspase 3/7 activity assay. Cells were treated with 1.5 μM staurosporine (STS) for 4 h as a positive control. E, qRT-PCR of U32a, U33, U34, and U35a snoRNAs in medium relative to *C. elegans* miR39 spike-in. F and G, qRT-PCR quantification of Rpl13a snoRNAs U32a, U33, U34, and U35a relative to Rplp0 in cytosolic (F) and nuclear (G) fractions. Results are mean ± S.E. (error bars) for n = 3 per condition. p < 0.05, using Student’s t test (#) or ANOVA with Dunnett’s multiple-comparison test (*).
medium following 1 h of stimulation and were cleared over the subsequent 3 h (Fig. 1E). Increases in snoRNAs in the medium were not accompanied by increase in cytosolic or nuclear snoRNA abundance (Fig. 1, F and G). Absolute quantification of snoRNAs using standard curves for each species demonstrated that LPS-induced increases in snoRNAs in the medium are significant and specific, but small relative to nuclear and total cellular snoRNA abundance, pools that do not significantly change with treatment (Fig. 2, A–D). Control PCRs indicated that the target-specific stem-loop primed reverse transcription qPCR method detects mature snoRNAs, but not snoRNA sequences in DNA or pre-mRNA templates (Fig. 2E). These findings indicate that LPS induces macrophage snoRNA secretion. Rapid clearance of the snoRNAs from the medium suggests they may be degraded, and decreases in cytosolic snoRNAs at 4 h (significant for U33 and U34 only) may reflect the cumulative export over several hours. In addition to the Rpl13a snoRNAs, LPS also stimulates the secretion of SNORD82, SNORD92, and SNORA73b (Fig. 3), suggesting that LPS broadly stimulates secretion of box C/D and box H/ACA snoRNAs.

**Rpl13a snoRNAs are released with extracellular vesicles**

A growing body of literature provides evidence that RNAs are released from cells in membrane-bound structures, generally referred to as extracellular vesicles (EVs) (24). Release of Rpl13a snoRNAs from LPS-stimulated macrophages was unaf-
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Secreted RNAs can impact the biology of target cells by engaging cell surface receptors or by uptake through endocytosis or macropinocytosis. To determine whether RNA from macrophage-derived EVs is incorporated into recipient cells, EVs were enriched from the medium of donor cells that had been metabolically labeled with ethynyl uridine and incubated for 45 min with unlabelled recipient cells. Following washing to remove surface-bound material, recipient cells were probed with an Alexa Fluor 594–linked click reagent. RNA from donor cell EVs was detected in the nucleus, where it co-localized with fibrillarin (Fig. 7A). The dye-only control indicates that any small amount of free dye that might remain after labeling and cleanup does not cause detectable nucleolar staining. In an alternate approach, RNA and lipids of isolated EVs were stained with Syto RNAselect and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiD), respectively, and incubated with recipient cells. In the recipient cells, labeled RNA was distributed throughout the cytoplasm and nucleus and concentrated in nuclear structures suggestive of nucleoli (arrowheads), a pattern that was distinct from background autofluorescence in the dye-only control (Fig. 7B). DiD was also transferred to recipient cells and was distributed in cytosolic punctae. Together, these findings indicate that both RNA and lipid constituents of EVs are transferred.

Approaches in which RNA is followed by a metabolic label or dye cannot formally distinguish between uptake of exogenous EV components and transfer of free label or dye that has partitioned into the EVs. To specifically assess whether secreted snoRNAs are taken up into recipient cells, we isolated cells from WT mice and from mice with germ line knockout of all four Rpl13a snoRNAs (KO) (28). KO mice are viable and fertile with no overt pathophysiological phenotype under standard housing and feeding conditions. However, pancreatic islets from these mice have decreased ROS tone, resistance to oxidative stress stimuli, and enhanced insulin secretion and mitochondrial proton leak.

WT and KO macrophages were co-cultured in a transwell apparatus that maintains the cells in separate compartments separated by a membrane with 1-μm pores. In this setting, medium and associated EVs were freely shared between the cells of different genotypes, but PCR analysis of DNA confirmed that cellular material was not transferred (Fig. 8A). In situ hybridization to detect snoRNA U33 revealed scattered nuclear puncta and larger areas of nuclear accumulation consistent with nucleoli in singly cultured WT cells (Fig. 8, B and C). Compared with KO macrophages that had been co-cultured with KO macrophages, KO macrophages co-cultured with WT

gradient. Both Rpl13a snoRNAs and EV membrane protein markers were enriched at a density between 1.14 and 1.20 g/cm³, the characteristic density for EVs (Fig. 6A). Rpl13a snoRNAs in these EVs were resistant to RNase treatment but degraded when treated with RNase in the presence of detergent (Fig. 6B), suggesting that the snoRNAs are protected by a membrane. Together, our findings support a model in which macrophages release EVs containing snoRNAs.

EV-associated Rpl13a snoRNAs are internalized by recipient cells

To distinguish between co-fractionation and true association of Rpl13a snoRNAs with EVs, we separated EVs isolated by precipitation or ultracentrifugation from potentially nonvesicular co-sedimented components by floating the EVs into a continuous sucrose density gradient. Both Rpl13a snoRNAs and EV membrane protein markers were enriched at a density between 1.14 and 1.20 g/cm³, the characteristic density for EVs (Fig. 6A). Rpl13a snoRNAs in these EVs were resistant to RNase treatment but degraded when treated with RNase in the presence of detergent (Fig. 6B), suggesting that the snoRNAs are protected by a membrane. Together, our findings support a model in which macrophages release EVs containing snoRNAs.

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macrophages demonstrated significantly increased nuclear punctae, consistent with transfer of U33. Whereas the signal for this single snoRNA species is small compared with the signal for total labeled RNA (Fig. 7), the co-culture approach (Fig. 8) provides further evidence of snoRNA transfer under more physiological conditions.

LPS increases Rpl13a snoRNAs in the serum of murine model and human volunteers

Because LPS triggers EV release of snoRNAs from cultured macrophages, we asked whether this inflammatory stimulus precipitated secretion of snoRNAs in vivo by treating mice with LPS. Compared with PBS-treated mice, animals that received LPS demonstrated a rapid increase in EV-associated Rpl13a snoRNAs in the serum over 4 h, concomitant with an increase in hepatic inflammatory gene expression (Fig. 9, A and B). We extended these findings by quantifying serum snoRNAs in healthy human volunteers undergoing intrabronchial instillation of LPS from tail blood of the parabionts revealed the presence of both the WT and KO alleles in the circulation of each parabiont by 1 week following surgery (Fig. 10B), consistent with reports of rapid development of shared circulation in this model (30). To test for the function of the Rpl13a snoRNAs, we utilized a qPCR-based method for detection of 2′-O-methylation of RNA, the modification that these snoRNAs direct on their canonical rRNA targets (31, 32). This approach revealed the expected increases in amplification across Rpl13a snoRNA–directed 2′-O-methylation sites on the 18S and 28S rRNAs in KO compared with WT macrophages (Fig. 11B).

We applied this method for analysis of Rpl13a snoRNA–directed rRNA modifications to tissues harvested 3 weeks following surgery from KO and WT parabionts. Because new rRNA transcription correlates with cell replication (33) and 2′-O-methylation occurs on nascent rRNAs (34), we chose for study the intestinal epithelium, where cells turn over every 2–3 days (35). Liver tissue served as a negative control, because...
hepatocytes divide approximately once in 150 days in the adult mouse (36), and therefore new rRNA synthesis and 2′-O-methylation are expected to occur at much slower rates. Compared with KO mice that had not undergone parabiosis, enterocytes of KO parabionts showed a significant decrease in amplification (relative quantification; RQ), indicating an increase in modification of all four Rpl13a snoRNA target sites (lower RQ) and phenocopying the WT parabiont and WT animal control (Fig. 12A). By contrast, in liver tissue, we observed no significant difference in modification (RQ) at target sites for U32a and U33 between KO controls and KO parabionts, despite a clear difference between KO controls and either WT controls or WT parabionts (Fig. 12B). Unexpectedly, there was no difference between WT and KO controls in apparent methylation in liver tissue at the U34 or U35a snoRNA target sites, raising the possibility that these target sites are unmodified in WT livers or that other snoRNAs could be up-regulated specifically in the KO livers to compensate for the loss of the Rpl13a snoRNAs. Importantly, GFP expression remained undetectable in the KO parabiont (Fig. 12, C and D), indicating the lack of transfer of cellular material. Taken together, our data provide evidence that Rpl13a snoRNAs travel through the circulation and are taken up by distant tissues in which they function to modify their established RNA targets.

Discussion

Herein, we demonstrate that cultured macrophages secrete Rpl13a snoRNAs in response to the inflammatory stimulus LPS, most likely as cargo inside of EVs. Furthermore, we show that LPS stimulates secretion of these snoRNAs in both mouse models and in human subjects. By leveraging a recently generated mouse model with selective loss of the Rpl13a snoRNAs, we show that these extracellular noncoding RNAs are taken up by cultured macrophages, in which they traffic to the nucleus. In parabiosis experiments, we demonstrate that these snoRNAs are not simply inert passengers in EVs, but are also capable of functioning in distant tissues. Together, our findings provide a new understanding of the biology of snoRNAs and the repertoire of EVs.

EVs secreted from macrophages and other cell types can carry not only membrane and protein components from their cells of origin, but also RNAs that can be detected by sensitive PCR or RNA-Seq–based methods from concentrated preparations of EVs. Microarray and RNA-Seq analyses have provided...
Figure 6. Rpl13a snORs are contained within secreted EVs. A, qRT-PCR for snORs (relative to *C. elegans* miR39 spike-in) and Western blotting of fractions collected from a continuous sucrose gradient (0.25–2 M) after floating EVs enriched by differential centrifugation from overnight macrophage culture (1.6 × 10^7 cells). B, qRT-PCR of snORs from EV-enriched fraction treated with RNase, Triton X-100, and RNase inhibitor (Inh) as indicated. Results are mean ± S.E. (error bars) for *n* = 3. *, *p* < 0.05 using ANOVA with Dunnett’s multiple-comparison test.

Figure 7. EV tRNA. A, WT cells cultured with EVs from ethynyl uridine–labeled WT cells (EU RNA), fixed and stained for labeled RNA with Alexa Fluor 594 azide and α-fibrillarin (FIB) antibody. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Dye control used EVs from cells not labeled with ethynyl uridine. Scale bar, 20 μm. B, representative images of WT cells cultured with DiD– and Syto RNAselect–labeled WT EVs and fixed and stained with 4′,6-diamidino-2-phenylindole. Scale bar, 20 μm. Images are representative of three independent experiments.
evidence that in addition to mRNAs, long noncoding RNAs, and microRNAs, a range of snoRNA species are present in EVs secreted from cultured cells and in EVs isolated from human blood (17–19, 21, 37). Our work extends these observations by demonstrating that macrophage secretion of EV-associated Rpl13a snoRNAs is stimulated by LPS, which is also known to regulate secretion of microRNAs from macrophages (15). Furthermore, we show that the Rpl13a snoRNAs are also secreted in response to this inflammatory stimulus in mice and in humans. For our in vivo analyses, we enriched for circulating EVs by precipitation from serum samples. Whereas it is possible that serum EVs containing Rpl13a snoRNAs originate from macrophages, these snoRNAs are widely expressed, and snoRNA-containing EVs probably originate from additional cell types in vivo.

Secretion of snoRNAs in vivo raises the possibility that these RNAs traffic in a paracrine or endocrine fashion between cells to impact the biology of recipient cells and to regulate organismal physiology, as has been demonstrated for microRNAs (16). In vitro, we used RNA labeling to show that concentrated EVs from donor cells are taken up by recipient cells. We also leveraged primary macrophages from mice with loss of function of the Rpl13a snoRNAs and from WT mice to demonstrate that EV-associated snoRNAs are taken up by recipient cells and traffic to the nucleus. Although we detected the presence of transferred EV RNA and transferred U33 snoRNA, we were unable to consistently detect new 2′-O-methylation in cultured recipient cells treated with concentrated EVs or in co-cultured macrophages. We hypothesize that this is because snoRNAs direct 2′-O-methyl modifications of newly transcribed pre-rRNAs in the nucleolus, but not mature rRNAs (34). Transcription of new rRNA is linked to progression through the cell cycle, which is extremely slow in these nonreplicating cells treated with concentrated EVs or in co-cultured macrophages. We hypothesize that this is because snoRNAs direct 2′-O-methyl modifications of newly transcribed pre-rRNAs in the nucleolus, but not mature rRNAs (34). Transcription of new rRNA is linked to progression through the cell cycle, which is extremely slow in these nonreplicating primary macrophages (33).

By contrast, in the parabiosis model using WT and KO mice, we find that Rpl13a snoRNAs are transferred through the bloodstream and can function in distant tissues, in a setting that does not involve concentration of large quantities of biological fluids. Two features of this model probably facilitated detection of functional snoRNA transfer. First, although the mice were not treated with LPS, sterile inflammation secondary to the surgical procedure may have contributed to EV secretion. Second, our data indicate that shared circulation is established early. Because synthesis of new rRNAs is low in post-mitotic cells and tissues, chronic exposure to snoRNA-containing EVs over several weeks may have enabled detection of snoRNA
signals that regulate snoRNA secretion, the mechanisms for expression or cellular growth. Broader processes for cellular homeostasis, such as protein communication with the extracellular environment. Although it has long been appreciated that the secreted protein cytokines are key mediators of communication between macrophages and distant tissues, evidence is emerging that macrophage-secreted EVs play important roles in pathophysiologic responses to inflammation. In the setting of infections with the intracellular pathogens Mycobacteria and Listeria, macrophages secrete EVs that promote inflammatory signaling, cell migration, and protective T cell–mediated immune responses in vivo (38–40). Macrophage-derived EVs have also been shown to play a critical role in the regenerative response of intestinal stem cells following radiation injury (41). In some instances, these functions have been associated with EV transfer of protein antigens (39) or signaling molecules (41). Thus, macrophage EVs have been implicated to serve as immunoregulators. Our data show that macrophage-derived EVs contain snoRNAs that are known to target rRNAs for 2′-O-methylation, suggesting that the functional repertoire of macrophage EVs may include broader processes for cellular homeostasis, such as protein expression or cellular growth.

Future studies will be required to elucidate the spectrum of signals that regulate snoRNA secretion, the mechanisms for function in rapidly replicating enterocytes, which must synthesize and 2′-O-methylate new rRNAs. Although our studies demonstrate acquisition of new snoRNA-directed modifications, we were unable to consistently detect Rpl13a snoRNAs by qRT-PCR in KO parabiont tissues (not shown). This suggests that snoRNAs are short-lived in recipient tissues relative to the RNA marks they generate and/or that a relatively small number of transferred snoRNA molecules catalyze a substantially larger number of methylation events.

Macrophages play key roles in the response to infection or tissue damage through their activation, polarization, and communication with the extracellular environment. Although it has long been appreciated that the secreted protein cytokines are key mediators of communication between macrophages and distant tissues, evidence is emerging that macrophage-secreted EVs play important roles in pathophysiologic responses to inflammation. In the setting of infections with the intracellular pathogens Mycobacteria and Listeria, macrophages secrete EVs that promote inflammatory signaling, cell migration, and protective T cell–mediated immune responses in vivo (38–40).

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Future studies will be required to elucidate the spectrum of signals that regulate snoRNA secretion, the mechanisms for selection of snoRNAs as EV cargo, and whether specific subclasses of EVs carry snoRNAs. The rapidity of release of EVs containing snoRNAs following treatment with ionomycin or LPS suggests that these stimuli promote release of preformed snoRNA–containing EVs. Although secreted snoRNAs that direct rRNA modifications in recipient cells may mediate cell–cell communication, the physiological role of snoRNA secretion remains to be determined. The finding of augmented mitochondrial proton leak in tissues of the Rpl13a-snoRNA KO mouse suggests that a noncanonical function of the snoRNAs is to promote tightly coupled respiration (28). Based on this, we hypothesize that EV-mediated snoRNA transfer could enhance metabolic efficiency in recipient cells, thereby improving cell survival in the face of stress and/or infection. Characterization of the physiologic contexts that precipitate secretion of snoRNAs could reveal opportunities in which the Rpl13a snoRNAs or other snoRNAs might serve as biomarkers for detection or management of disease. Given that snoRNAs are sensitive to antisense knockdown, these noncoding RNAs have the potential to serve as targets for new therapeutic approaches.
Experimental procedures

Reagents

LPS, BFA, IO, CD, and staurosporine were from Sigma-Aldrich. α-Fibrillarin (ab4566), α-nucleophosmin (ab15440), α-flotillin 1 (ab50673), and α-calnexin (ab22595) antibodies were from Abcam (Cambridge, MA). α-Histone H3 (catalog no. 9717) antibody was from Cell Signaling (Danvers, MA). α-CD9 (catalog no. 564236) was from BD Biosciences.

Mice

FVB and FVB.Cg-Tg(CAG-EGFP)B5Nagy/J were obtained from the Jackson Laboratory (Bar Harbor, ME), and Rpl13a-snoless (KO) mice were maintained in facilities under specific pathogen-free conditions at Washington University (St. Louis, MO). Bone marrow was isolated as described below. Blood samples were obtained from male and female mice 4 h following intraperitoneal injection of 10 mg/kg LPS. All experimental procedures were approved by the Washington University Animal Studies Committee and were conducted in accordance with the United States Department of Agriculture Animal Welfare Act and the Public Health Service Policy for the Humane Care and Use of Laboratory Animals.

Bone marrow–derived macrophages

Bone marrow was harvested from the tibia and femur of male and female mice. Cells were seeded and differentiated for 6 days in 10% CMG14.12 conditioned medium (42). CMG14.12 cells were a gift from the laboratory of Deborah Veis (Washington University). Cells were treated with 50 ng/ml LPS for the indicated times, 10 μg/ml BFA for 90 min, 0.5 μM IO for 15 min, or 2% CD for 60 min. Cell death was measured using the CytoTox96 nonradioactive cytotoxicity assay (Promega, Madison, WI). LDH+ controls contained LDH from the kit (1:5,000 dilution of 1,600 units/ml solution), and the maximum signal was determined by measuring LDH activity in the medium of cells cultured under identical conditions and lysed by quickly freezing and thawing the plate. Apoptosis was quantified by a caspase 3/7 Glo assay (Promega, Madison, WI). ROS was...
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detected by measuring fluorescence on a TECAN Infinite 200 PRO multimode reader (Tecan, Männedorf, Switzerland) after cells were loaded with 10 μM DCF (Thermo Fisher Scientific, Carlsbad, CA). Sequential detergent extraction was performed as described previously to generate nuclear and cytosolic fractions (10).

**EV enrichment, labeling, and transfer**

EVs were isolated from cell culture medium using Total Exosome isolation reagent (Thermo Fisher Scientific) (43), or EVs were isolated by differential centrifugation (44). Serum EVs were isolated with Exoquick (System Biosciences, Mountain View, CA) according to the manufacturer’s instructions (38). Culture medium used for EV labeling/transfer and floatation/RNase treatment experiments was depleted of EVs before use by centrifugation at 120,000 g for 10 min. Conditioned medium was cleared by centrifugation for 10 min at 1,000 g, followed by centrifugation for 20 min at 2,000 × g, and then concentrated using an Amicon Ultra-15 centrifugal filter unit (Millipore, Billerica, MA). EVs were floated into a continuous sucrose gradient for 90 min. Conditioned medium was cleared by centrifugation for 10 min at 1,000 × g, followed by centrifugation for 20 min at 2,000 × g, and then concentrated using an Amicon Ultra-15 centrifugal filter unit with Ultracel-10 membrane (3,500 × g, 20 min; Millipore, Billerica, MA). EVs were floated into a continuous sucrose gradient as described previously (44). A small sample was taken from each fraction, and density was measured at room temperature using an Atago (Bellevue, WA) three-range sugar hand refractometer (0–90%).

For EV transfer experiments, EVs were labeled using Syto RNA select and DiD (Thermo Fisher Scientific) according to the manufacturer’s instructions, and unincorporated dye was removed either by washing in Amicon filters or with Exosome spin columns (Thermo Fisher Scientific) with similar results. Dye controls were treated in an identical manner. Ethynyl uridine–labeled EVs were collected from cells cultured overnight with 0.5 mM ethynyl uridine (45). Following incubation of recipient cells with EVs for 45 min, cells were washed twice with 3% BSA (Sigma-Aldrich) in PBS, fixed with 3.7% paraformaldehyde (Electron Microscopy Science, Hatfield, PA), and permeabilized with 0.5% Triton X-100 (Sigma-Aldrich). Ethynyl uridine was detected using the Click iT RNA imaging kit (Alexa Fluor 594, Thermo Fisher Scientific). For transwell transfer, 106 KO and WT bone marrow cells were grown for 7 days or until confluent in conditioned medium in chambers separated by Thincert 1-μm pore membranes (Greiner Bio-one) in 6-well plates and stained using the Quantigene ViewRNA Cell Plus assay with Alexa Fluor 546 detection, modified to include an additional fixation with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and protease treatment with reagents from the miRNA assay kit. Control wells were assayed for transfer of genomic material using genotyping primers for WT and KO Rpl13a loci (28).

**qRT-PCR**

RNA was purified using TRIzol. Relative quantification of RNA abundance was calculated using the ΔΔCt method on an ABI 7500 fast real-time PCR system. Primer sequences for COX2 were TGA GCA ACT ATT CCA AAC CAG C (forward) and GCA CGT AGT CTT CGA TCA CTA TC (reverse); for TNFα, they were CAT CTT CTC AAA ATT CGA GTG ACA A (forward) and TGG GAG TAG ACA AGG TAG ACC CC (reverse); and for Rplp0, they were ATC CCT GAC GCA CCG CCG TGA (forward) and TGC ATC TGC TTG GAG CCC AGC TT (reverse). The method for qRT-PCR for Rpl13a snoRNAs used target-specific stem loop RT primers, a target-specific forward PCR primer, and a universal reverse PCR primer (46). This method can detect as few as 103 copies of target RNAs, is able to distinguish between small RNA species that differ by only 1 nucleotide, is not affected by genomic contamination, distinguishes mature snoRNAs and miRNAs from their precursor small RNA species, and has been validated against RNA-Seq for snoRNAs (10, 47, 48). mRNA quantification was calculated using Rplp0 as an endogenous control. For quantification of EV RNA, Caenorhabditis elegans mir39 (500 fmol) or luciferase mRNA (0.3 fmol; Promega) was added to EV samples before RNA isolation and used as a reference control. Amplification of reference genes varied by <1 cycle across samples. A plate of 2 × 106 cells was used for cellular fractionation by sequential detergent extraction, and medium was pooled from three plates.

**Microscopy**

Freeze fracture/deep etch EM was performed according to published protocols with minor modifications (49). A 4-μl droplet of sample was placed on top of a 4 × 4-mm acid-cleaned and air-dried coverslip and then topped with a 3-mm round sapphire disc. Samples were immediately frozen by abrupt application of the sample against a liquid helium–cooled copper block with a Cryopress freezing machine. Frozen samples were transferred to a liquid nitrogen–cooled Balzers 400 vacuum evaporator, fractured at −100 ºC by removal of the sapphire disc with the instrument’s nitrogen cooled knife. Samples were etched for 2 min at −100 ºC and rotary replicated with ~2-nm platinum deposited from a 20º angle above the horizontal, followed by an immediate ~4-nm stabilization film of pure carbon deposited from an 85º angle. Replicas were floated onto a dish of concentrated hydrofluoric acid for coverslip removal and then transferred through several rinses of distilled H2O with a loopful of Photoflo, picked up on Formvar-coated grids, and photographed on a JEOL 1400 microscope with attached AMT digital camera. Confocal images were taken on a Zeiss LSM 880 Airyscan confocal microscope with a 0.8-μm pinhole through a focal plane in the center of the nuclei and processed using Zen software (Zeiss, Oberkochen, Germany).

**RNase protection**

EVs enriched by ultracentrifugation were suspended in 250 μl of PBS and treated with 0.3% Triton X-100, 1 unit of RNase A, 40 units of RNase T1 (Ambion RNase mixture, Thermo Fisher Scientific), and 100 units of SUPERase-In RNase inhibitor (Thermo Fisher Scientific) for 30 min at 20 ºC, and then RNase inhibitor was added to all samples. RNA was isolated using TRIzol LS.

**Human subjects**

Blood samples were collected from human volunteers for a study of lung inflammation before and 6 h following endobronchial instillation of 4 ng/kg LPS (29). This study was approved by the Washington University Human Research Protection
Office and complies with the Declaration of Helsinki principles. Informed consent was obtained from all subjects.

**Parabiosis**

The Washington University Mouse Cardiovascular Phenotyping Core performed parabiosis surgery using female WT mice expressing GFP and Rpi13a snoRNA KO mice as described (30). Tail vein blood was sampled serially from the parabionts for PCR analysis of DNA to detect the WT and KO Rpi13a loci with primers GAC AGG TTG CTG CTG AGG AAG TAA ATGG (forward) and CCA GAC CTG CTG TCA GAC TTT AGC CTG (reverse). Three weeks following surgery, parabionts were perfused with PBS to clear blood from tissues and eutonized. Enterocytes and liver were recovered and flash-frozen in liquid nitrogen. For PCR analysis, tissues were homogenized in TRIzol.

**Primer extension qRT-PCR**

Primer extension PCR to detect rRNA methylation was adapted from Dong et al. (31). For each target, 20 ng of total RNA was used in cDNA synthesis using the Super Script III kit (Thermo Fisher Scientific) with 0.3 μl of SSIII enzyme per reaction and dNTP at either the normal stock concentration (50 μM) or diluted 1:1,000 (50 nM). The following primers were used for each RT reaction: m18S RT (U32a, G1328), CAA CTA AGA ACG GCC ATG CA; m18S RT (U33, U1326), AGA ACG GCC ATG CAC C; m28S RT (U34, U2590), GAC TTC CCT TAC CTA C; and m28S RT (U35a, C4188), AAC CTG TCT CAC GAC G. qPCR was done on a 7500 fast real-time PCR system with SYBR Green real-time PCR master mix (Thermo Fisher Scientific). PCR primers used were as follows: m18S F2 (U32a/33), GGC CTG CGG CCT AAT TTG AC; m18S R2 (U33/32), ATG CAC CAC CAC CCA CG; m28S F1 (U34), TCT CGC TGG CCC TTG AAA AT; m28S R1 (U34), ACC TAC ATT GTT CCA ACA AGC TC; m28S F1 (U35a), CTT CGA TGT CGG CTC TCT CT; m28S R1 (U35a), TCA CGA CGG TCT AAA CCC AG. RT efficiency was calculated by normalizing the Ct value for the low dNTP concentration to the high as an endogenous control (RQ). For each modification site within a specific tissue, values are reported relative to the mean WT value.

**Author contributions**—J. M. R. and J. E. S. designed the research with input from P. I. H. and D. S. O.; J. M. R. and R. J. C. performed experiments; J. L. and C. L. H. contributed critical reagents and method development; D. L. C. provided human samples; J. M. R. and J. E. S. wrote the manuscript with input from all other authors.

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