Extracellular mRNA transported to the nucleus exerts translation-independent function

Takeshi Tomita1,2, Masayoshi Kato1,2, Taishi Mishima3, Yuta Matsunaga3, Hideki Sanjo4, Ken-ichi Ito5, Kentaro Minagawa6, Toshimitsu Matsui7, Hiroyuki Oikawa8, Satoshi Takahashi8, Toshifumi Takao9, Noriki Iwai10, Takashi Mino10, Osamu Takeuchi10, Yoshiro Maru3✉, & Sachie Hiratsuka1,2✉

RNA in extracellular vesicles (EVs) are uptaken by cells, where they regulate fundamental cellular functions. EV-derived mRNA in recipient cells can be translated. However, it is still elusive whether “naked nonvesicular extracellular mRNA” (nex-mRNA) that are not packed in EVs can be uptaken by cells and, if so, whether they have any functions in recipient cells. Here, we show the entrance of nex-mRNA in the nucleus, where they exert a translation-independent function. Human nex-interleukin-1β (IL1β)-mRNA outside cells proved to be captured by RNA-binding zinc finger CCCH domain containing protein 12D (ZC3H12D)-expressing human natural killer (NK) cells. ZC3H12D recruited to the cell membrane binds to the 3′-untranslated region of nex-IL1β-mRNA and transports it to the nucleus. The nex-IL1β-mRNA in the NK cell nucleus upregulates antiapoptotic gene expression, migration activity, and interferon-γ production, leading to the killing of cancer cells and antimetastasis in mice. These results implicate the diverse actions of mRNA.
Endogenous mRNA in cells is recognized as a key molecule in the central dogma of biology. Extensive research has been conducted to understand the structure and function of mRNA, and the consensus is that mRNA is translated into protein in the cytoplasm. In addition to endogenous mRNA, the presence of extracellular mRNA in extracellular vesicles (EVs), such as microvesicles and exosomes, has been reported. EV-derived mRNA can be transferred to other cells, where these are translated into corresponding proteins in vitro and in vivo. Remarkably, EV-containing mRNA from human endothelial progenitor cells is transferred to quiescent endothelial cells (ECs) and activates an angiogenic program. The observation suggests a functional significance of mRNA in EVs for angiogenesis. The potential significance of mRNA in EVs has also been suggested in the field of tumor biology because tumor-derived EVs contain mRNA for growth factors that could be transferred into recipient cells in a translation-dependent fashion.

Tumor metastasis is one of the most important issues in which intercellular communications are involved. Many valuable studies have revealed that metastasis involves complex systemic events between host cells and tumor cells. In particular, the host resident tissue and immune cells create a premetastatic phase, with a tumor-friendly environment, before tumor cells physically appear in the distant organ. Potential antimetastatic immune cells in the premetastatic soil were recently discovered. Specifically, B220+/CD11c+/NK1.1+ natural killer (NK) cells were found relocated from the liver to the lungs of tumor-bearing mice. These cells accumulate in fibrinogen- and hyperpermeability regions and demonstrate two antimetastatic activities: they eliminate enriched fibrinogen and possess tumoricidal abilities with interferon-γ (IFN-γ) production.

In this study, a comparison of gene expression profiling between B220+/CD11c+/NK1.1+ NK cells in the liver and lungs is carried out to elucidate the molecular mechanisms that underlie the relocation of B220+/CD11c+/NK1.1+ NK cells from the liver to the lungs and their antimetastatic abilities. The profiling indicates the highest fold change of mRNA for zinc finger (ZF) CCCH domain containing protein 12D (ZC3H12D), a putative tumor suppressor in lymphoma and lung cancer patients. In lung cells (Supplementary Table S1, GSE76235), ZC3H12D belongs to a nonvesicular exRNA (nex-RNA) associated with microvesicles, exosomes, and ribonucleoproteins (RNPs). Based on data that TCM induced the relocation of B220+/CD11c+/NK1.1+ NK cells from the liver to the lungs of tumor-bearing mice, these cells accumulate in fibrinogen- and hyperpermeability regions and demonstrate two antimetastatic activities: they eliminate enriched fibrinogen and possess tumoricidal abilities with interferon-γ (IFN-γ) production.

In this study, a comparison of gene expression profiling between B220+/CD11c+/NK1.1+ NK cells in the liver and lungs is carried out to elucidate the molecular mechanisms that underlie the relocation of B220+/CD11c+/NK1.1+ NK cells from the liver to the lungs and their antimetastatic abilities. The profiling indicates the highest fold change of mRNA for zinc finger (ZF) CCCH domain containing protein 12D (ZC3H12D), a putative tumor suppressor in lymphoma and lung cancer patients. In lung cells (Supplementary Table S1, GSE76235), ZC3H12D belongs to a nonvesicular exRNA (nex-RNA) associated with microvesicles, exosomes, and ribonucleoproteins (RNPs). Based on data that TCM induced the relocation of B220+/CD11c+/NK1.1+ NK cells from the liver to the lungs of tumor-bearing mice, these cells accumulate in fibrinogen- and hyperpermeability regions and demonstrate two antimetastatic activities: they eliminate enriched fibrinogen and possess tumoricidal abilities with interferon-γ (IFN-γ) production.

Results
An RNA-binding protein located on the cell surface. To determine whether ZC3H12D+ leukocyte mobilization was induced by primary tumors, immunohistochemical (IHC) examination of the lungs of tumor-bearing mice was conducted. Their tumors originated from E0771 breast cancer cells or Lewis lung carcinoma (LLC) cells. The number of ZC3H12D+CD45+ cells in the lungs increased with primary tumor growth (Supplementary Fig. S1a: E0771 data). Zc3h12d expression in leukocytes derived from tumor-bearing mice was enhanced by tumor-bearing lungs in a coculture system in vitro (Supplementary Fig. S1b). Because ZC3H12D was recognized as a tumor suppressor gene in follicular lymphoma and lung cancer patients, the role of ZC3H12D in metastasis was further examined. The primary tumor formed by E0771 implantation in Zc3h12d−/− mice exhibited a similar growth rate to wild-type mice (Supplementary Fig. S1c), indicating that ZC3H12D in host cells may be an insufficient tumor suppressor of rapid primary tumor growth. However, lung metastasis was more severe in Zc3h12d−/− than in wild-type mice (Supplementary Fig. S1c), implying that ZC3H12D acted as a tumor suppressor molecule in metastasis.

To clarify the ZC3H12D localization pattern in immune cells stimulated by a tumor, ZC3H12D protein was observed in peripheral blood mononuclear cells (PBMCs) derived from mice without tumors and tumor-bearing mice using a flow cytometer. ZC3H12D was detected on the cell surface of PBMCs from mice without tumors (Fig. 1b, top). Note that the specificity of this ZC3H12D antibody was validated using a knockout sample. In contrast, in PBMCs from tumor-bearing mice, ZC3H12D was detected in the intracellular sphere but not on the cell surface (Fig. 1b, bottom). This tumor-mediated change in the ZC3H12D localization pattern was further confirmed using two other anti-ZC3H12D antibodies (Supplementary Fig. S1d). Tumor-conditioned medium (TCM), used as a tumor cell culture, as it is expected to contain elements secreted from tumor cells, upregulated ZC3H12D 30 min after stimulation on splenic leukocytes (Fig. 1c). Three hours later, ZC3H12D protein completely moved inside the cell (Fig. 1c). Because ZC3H12D is considered an RNA-binding protein, it was suspected that nucleic acid was the explanatory factor in this phenomenon. Strikingly, pretreatment of TCM with RNase (TCM + RNase) did not affect the localization pattern of ZC3H12D (Fig. 1d, wt panel). The addition of RNA isolated from TCM resulted in the same phenomenon (Fig. 1d, wt panel, RNA-TCM column). These data suggested that RNA drives the translocation of ZC3H12D. In contrast, this was not observed when splenic leukocytes from Zc3h12d−/− mice were used (Fig. 1d).

Nonvesicular interleukin-1β (IL1β)-mRNA was detected in lung TCM. The tumor-related environment contains extracellular RNA (exRNA) associated with microvesicles, exosomes, and ribonucleoproteins (RNPs). Based on data that TCM induced the RNA-dependent response of the ZC3H12D location (Fig. 1d), it was hypothesized that some specific exRNA binds to ZC3H12D. Because exRNA should be exposed to ZC3H12D on leukocytes, the authors tried to examine which nonvesicular exRNA (nex-RNA) was increased in the lung microenvironment stimulated by a tumor. Microarray analysis was carried out on the lung EC TCM. The top 20 ranked genes are listed in Supplementary Table S2. The authors then searched for mRNA differences in expression between wild-type and Zc3h12d−/− mouse spleens. Spleens were chosen because they had the highest ZC3H12D expression compared to other organs tested in this study. The rationale was that ZC3H12D depletion effects might be seen by comparing wild-type and Zc3h12d−/− spleen data. This comparison revealed that immunoglobulin κ variable (Igkv), resistin like-γ (Retnlg), IL1β, and matrix metalloproteinase 8 (Mmp8) were upregulated in the Zc3h12d−/− sample (Supplementary Table S3). Among upregulated genes, this study focused on IL1β mRNA because this gene exhibited relatively high expression of nex-RNA derived from TCM-stimulated lung cells (Supplementary Table S2). Also, β-actin was chosen as a negative control in this study because it was abundantly found in TCM-stimulated lungs (Supplementary Table S2), and its expression levels did not change between wild-type and Zc3h12d−/− mice (Supplementary Table S3). First, this study tested whether IL1β mRNA was detected in lung TCM prepared by the coculture of lung specimens with various TCMs using LLC, E0771, or B16 melanoma cells. To determine the relative
expression levels of IL1β-mRNA, a gene-specific primer (GSP) was used for the first-strand synthesis in quantitative reverse transcription-polymerase chain reaction (RT-qPCR; Fig. 1e, top). In Fig. 1e, lung TCMs contained larger amounts of IL1β-mRNA than nonstimulating lung CM, whereas liver TCMs had lower amounts than lung TCMs. To quantitate extracellular human (h) IL1β-mRNA, CMs were collected from human breast cancer cells, including MCF7, MDAMB231, T47D, and HCC1954 (Fig. 1f, left). Along with these samples, hIL1β-mRNA was also analyzed in CM from human lung ECs stimulated by the aforementioned TCMs (Fig. 1f, right) because mouse lung culture medium stimulated with TCM contained mouse (m)IL1β-mRNA (Fig. 1e).
Next, this study investigated whether there is nonvesicular IL1β-mRNA in human breast cancer TCM because it was reported that human glioma stem cell culture contained mRNA in EVs and nonvesicular RNPs. The isolation conditions for nonvesicular (called extraexosome) and exosome fractions (Fig. 1g) were first set up, and the results were validated by RPLP2, RPS8, and RPS27 TaqMan probe-based qPCR (Fig. 1h). MDAMB231-TCM included more hIL1β-mRNA in the extraexosome fraction than in the exosome fraction (Fig. 1h). Extraexosomal mIL1β-mRNA in mouse lung TCM was increased (Fig. 1i) and considered to be PolyA(+), as they were reverse transcribed using oligo(dT) primer (Supplementary Fig. S1e). GSP-RT-qPCR also confirmed an increase of mIL1β-mRNA in the lung TCM (Supplementary Fig. S1e). Also, nonvesicular mIL1β-mRNA in TCM-stimulated mice was directly captured by ZC3H12D protein beads (Fig. 1j). In contrast, mRNA of β-actin, gapdh, retnlg, mmp8, and Igkv were not enriched by bead pull-down (Fig. 1j; mmp8 and Igkv were not detected).

**ZC3H12D binds to nex-IL1β-mRNA.** Whether nex-mRNA could bind to ZC3H12D on the cell surface was examined next. ZC3H12D was used because it is expressed in murine RAW 264.7 macrophage cells, and human THP1 cells. To visualize the interaction between IL1β-mRNA and ZC3H12D protein, an mZC3H12D-overexpressing RAW cell line (ZC3H12D RAW) was set up, and fluorescein isothiocyanate (FITC)-labeled IL1β-mRNA was applied. Thirty minutes after TCM stimulation, the colocalization signals in ZC3H12D RAW cells were more prominent (Pearson's $R = 0.47 \pm 0.04$; $n = 3$) than cells stimulated by control NoCM (Fig. 2a, arrow and Supplementary Fig. S2a). In ZC3H12D +THP1 cells isolated using a cell sorter and an anti-ZC3H12D antibody, similar colocalization signals were observed after applying IL1β-mRNA-FITC (Supplementary Fig. S2b; Pearson's $R = 0.62 \pm 0.08$; $n = 3$). Whether there was a direct interaction between IL1β-mRNA in lung TCM and ZC3H12D protein was also investigated. After application of lung TCM to ZC3H12D RAW cells, cells were treated with ultraviolet (UV) irradiation to fix RNA on the proteins. Then, ZC3H12D protein with FLAG tag was isolated using anti-FLAG magnetic beads, and the amount of IL1β-mRNA bound to ZC3H12D-FLAG-beads was quantified by RT-qPCR. The data showed that ZC3H12D protein enriched IL1β-mRNA in ZC3H12D RAW cells (Fig. 2b). In contrast, the gapdh- and β-actin-mRNA control showed little enrichment in the condition (Fig. 2b). Direct interactions between ZC3H12D protein and IL1β-mRNA were investigated using fluorescence correlation spectroscopy (FCS). This technique has been used to demonstrate a direct protein-RNA interaction in vitro. The correlation curves of fluorescence-labeled ZC3H12D were generated in the presence of β-actin- or IL1β-mRNA (Fig. 2c). The curve fitting revealed that FCS data are a mixture of three elements: ZC3H12D protein, IL1β-mRNA complex, and fast-decay component. The protein-RNA complex ratio was evaluated based on the ratio of the elements (Fig. 2d). FCS data indicate that the dissociation constant for ZC3H12D-β-actin- and IL1β-mRNA were in the order of 1 nM. It was also confirmed that the 3'UTR of IL1β-mRNA had direct interaction with ZC3H12D protein (Fig. 2e, f). Because the 5'UTR of IL1β-mRNA had a different sequence from β-actin-mRNA, the binding might include nonspecific and sequence-specific interactions in vitro.

**ZC3H12D transports nex-IL1β-mRNA into the nucleus.** To determine the intracellular kinetics of IL1β-mRNA captured by ZC3H12D, three-dimensional (3D) cell analyses were conducted using a series of z-stack confocal microscopy images. The 5'-Cap and 3'-Poly(A) are hallmark structures of eukaryotic mRNA, therefore, the FITC-labeled naked IL1β-mRNA and its 5'-Cap and 3'-Poly(A) adduct were tested in this assay. Surprisingly, both were observed in the nucleus of ZC3H12D RAW cells 3 h after application. IL1β-mRNA-Cap(+)PolyA(+)FITC was more clearly observed than IL1β-mRNA-FITC in the nucleus, whereas the control β-actin-mRNA-Cap(+)PolyA(+)FITC gave almost no signal (Fig. 3a left). To evaluate whether exogenous IL1β-mRNA was translated to functionally modify the cell, IL1β-mRNA with nonsense mutations (IL1β-stop-mRNA) was prepared. This mRNA has three stop codon mutations in the protein coding sequence (CDS) to not generate the IL1β protein. Interestingly, IL1β-stop-mRNA is clearly transported to the nucleus (Fig. 3a, right). ZC3H12D seemed to move into speckles based on the immunostaining data that ZC3H12D showed relatively dominant coexisting with SC35, a speckle marker, rather than other nuclear body markers, such as promyelocytic leukemia (PML), fibrillarin, and SFPQ for PML body, nucleolus, and paraspeckle, respectively (Supplementary Fig. S2c, d). Also, the flow cytometric analysis of THP1 cells revealed that ZC3H12D cell surface...
expression was observed in 0.3% of the cells with CM stimulation. In contrast, the population decreased to 0.1% in the presence of TCM stimulation. This decrease indicates that TCM stimulated the intracellular internalization of ZC3H12D from the cell surface. ZC3H12D-THP1 cells incorporated IL1β-mRNA and IL1β-stop-mRNA into the nucleus, although the signals were weaker than in NK cells (Supplementary Fig. S3a). To quantify the amount of external RNA transported into the nucleus, a chimeric IL1β-mRNA was synthesized by combining 5′-hIL1β-mRNA and 3′-mIL1β-mRNA (Fig. 3b). Note that the qPCR probe was designed to amplify hIL1β-mRNA located in the 5′-region but not endogenous mouse transcripts (top, Fig. 3b; see Supplementary Fig. S3b for more detail). These data indicate that chimeric RNA was transported into the nucleus regardless of the Cap or Poly(A)
modification (Fig. 3b, bottom left), although small amounts were detected in the cytoplasm (Fig. 3b, bottom right). To confirm the involvement of ZC3H12D in this phenomenon, this assay was repeated using B220+CD11c+NK1.1+ cells16,31 derived from TCM-stimulated wild-type and Zc3h12d−/− mice. The localization of IL1β-mRNA-FITC and IL1β-stop-mRNA-FITC in the nucleus of wild-type mouse cells was prominent 3 h after the application (Fig. 3c). In contrast, there were minimal signals in the nuclei of those cells from Zc3h12d−/− mice (Fig. 3c).

ZC3H12D recognizes the 3′-UTR of IL1β-mRNA. A sequence-specific uptake via ZC3H12D protein was investigated using IL1β-mRNA. For this purpose, two partial IL1β-mRNA, a CDS and a 3′-UTR, were first prepared. The former consists of a short 5′-UTR and a CDS to give 897 nt; the latter is 451 nt long. These RNAs were tested in a cell uptake assay (Fig. 3d). The uptake of FITC-labeled IL1β-(3′-UTR)-mRNA in ZC3H12D RAW was greater than that of IL1β-(CDS)-mRNA (Fig. 3d). Moreover, pre-incubation with IL1β-(3′-UTR)-mRNA blocked the uptake of FITC-labeled full-length IL1β-mRNA in the nucleus, but neither IL1β-(CDS)-mRNA nor βactin-mRNA had this effect (Supplementary Fig. S3c). Taken together, the 3′-UTR in IL1β-mRNA is recognized by ZC3H12D RAW cells in a region-specific manner.

Some RNA-binding proteins recognize AU-rich elements (AREs) and/or stem-loop structures in 3′-UTR for controlling the stability of target mRNA32. In this study, an electrophoretic mobility shift assay (EMSA) revealed that ZC3H12D bound to specific sites of single-stranded RNA (ssRNA) with 50 nt in the 3′-UTR of IL1β-mRNA (Fig. 4a, No. 5 probe in Fig. 4b and Supplementary Fig. S4a). This binding was confirmed by inhibition assay using cold RNA oligonucleotide (Fig. 4a, c). These data indicate that ZC3H12D interacts with relatively large elements around AREs in IL1β-mRNA with no typical stem-loop sequence. Subsequent competition assays revealed that the binding site of ZC3H12D contains a typical AU-rich sequence (UAUUUAU) but that its short fragments (20 nt) exhibited much lower affinities than the 50 nt oligonucleotide (Fig. 4a, d and Supplementary Fig. S4b). In the nucleus, binding is expected to be lost under conditions with other ARE-binding proteins, such as HuR33.

RNA uptake may not be supported by Regnase-1. Many ZF proteins are involved in intracellular RNA metabolism in innate immunity34,35. Among the ZC3H12 family, ZC3H12A (also known as Regnase-1) regulates IL6-mRNA with binding to the stem-loop in the 3′-UTR34-36. Also, ZC3H12D potentially interacts with ZC3H12A31. Whether ZC3H12A had a cooperative function in the ZC3H12D-dependent uptake of IL1β-mRNA was therefore investigated. In the microarray data (Supplementary Table S3), Zc3h12a expression levels in the Zc3h12d−/− spleen were close to those in the wild-type spleen. The gene expression profiles of B220+CD11c+NK1.1+NK cells in tumor-bearing lungs and livers were also examined. The data showed that Zc3h12d was markedly upregulated during the relocation of cells from the liver to the lungs, whereas Zc3h12a expression was constant (Supplementary Table S1); this suggested that there is no direct correlation between the expression of Zc3h12a and Zc3h12d. The signal of ZC3H12D revealed a similar pattern in B220+CD11c+NK1.1+ NK cells derived from Zc3h12a−/− (Regnase-1−/−) mice and wild-type mice (Supplementary Fig. S5a). Concordantly, NK cells derived from both genotypes exhibited IL1β-mRNA uptake in the nucleus (Fig. 5a, right; Supplementary Fig. S5b) when those cells derived from Zc3h12d−/− mice lost the uptake again (Fig. 5a, left; Supplementary Fig. S5b). These data indicated that ZC3H12A might not be involved in the ZC3H12D-dependent uptake system in B220+CD11c+NK1.1+ NK cells.

Entrained IL1β-mRNA in the nucleus may have functions. When IL1β-mRNA with or without Cap(+)PolyA(+) was applied to B220+CD11c+NK1.1+ cells, IL1β-mRNA-Cap(+) PolyA(+)–FITC was more clearly detected than IL1β-mRNA-FITC in the nucleus of B220+CD11c+NK1.1+ cells from wild-type mice. In contrast, IL1β-mRNA-FITC was pooled outside of the nuclei of Zc3h12d−/− mice. Interestingly, some wild-type cells showed an irregular nucleus 6 h after application of 20–100 ng/mL Cap(+)PolyA(+) (Supplementary Fig. S5c, 100 ng/mL). To remove the possibility that this irregular nucleus shape was due to the addition of FITC-modified substances, the same phenomenon was observed using unlabeled IL1β-mRNA. In this case, an increase in histone H2AX phosphorylation, a marker for DNA stress/damage37, was observed in the nucleus of B220+CD11c+NK1.1+ cells from wild-type mice but not observed in Zc3h12d−/− mice (Fig. 5b). B220+CD11c+NK1.1+ cells derived from Regnase-1−/− mouse exhibited H2AX phosphorylation upon incorporation with IL1β-mRNA· Cap(+)PolyA(+) (Supplementary Fig. S5d). Note that H2AX performs both structural and functional roles in chromatin regulation beyond a specific DNA double-strand break marker37. Next, this study tried to determine whether entrainment of IL1β-mRNA in the nucleus induces cell death because of the increase of cellular stress (Fig. 5c). The time course of cell death after treatment with βactin-mRNA, IL1β-mRNA and IL1β-stop-mRNA was assessed. In this assay, B220+CD11c+NK1.1+ NK cells derived from TCM-stimulated wild-type and Zc3h12d−/− mice showed a slight increase of necrosis signals (Fig. 5c). When 20 ng/mL IL1β-mRNA and IL1β-stop-mRNA were applied to B220+CD11c+NK1.1+ NK cells from wild-type, signal intensities were suppressed compared to those with βactin-mRNA (Fig. 5c). B220+CD11c+NK1.1+ NK cells from Zc3h12d−/− mice showed almost no response for any RNA (Fig. 5c). This indicates that the incorporation of IL1β-mRNA in the nucleus may contribute to NK cell survival.

Whether incorporation of IL1β-mRNA into the nucleus affects intranuclear activities, such as transcription, was then examined.
This study first examined whether nuclear-transported \( IL1\beta \)-mRNA was associated with RNA polymerase II (Pol II), regulating the initiation and elongation of transcription of mRNA. To distinguish the applied \( IL1\beta \)-mRNA from endogenous \( IL1\beta \)-mRNA, a qPCR probe that anneals human-specific sequences only was designed. After the application of chimera \( IL1\beta \)-mRNA to ZC\(^+\)RAW, the transported chimera \( IL1\beta \)-mRNA was fixed with UV irradiation and coimmunoprecipitated by an anti-Pol II antibody (Fig. 5d). This study then searched for the gene expression changes affected by \( IL1\beta \)-mRNA uptake in the same microarray data (Supplementary Table S3). Seven genes were picked based on the following criteria that would suggest
that their behavior is similar to that of \( \text{IL1}\beta \): they were classified as nucleus-related by Gene Ontology and showed a relatively higher expression level in \( \text{Zc3h12d}^-/- \) than in wild-type mice in the microarray data set (Supplementary Table S4). The gene expression levels of these seven genes were compared between RAW and \( \text{Zc3h12d}^-/- \) after treatment with IL1\( \beta \)-mRNA. qPCR revealed that \( \text{Dusp1} \) and \( \text{Il1rn} \) were expressed at higher levels in \( \text{Zc3h12d}^-/- \) than in control RAW (Supplementary Fig. S6), and the content of these transcripts in nuclear RNA increased in conjunction with the uptake of IL1\( \beta \)-mRNA (Fig. 5e). Furthermore, \( \text{Dusp1} \) and \( \text{Il1rn} \) expression was upregulated in B220\(^{+}\)CD11c\(^{+}\)NK1.1\(^{+}\)NK cells after application of IL1\( \beta \)-mRNA (Fig. 5f).

**ZC3H12D entrapment of IL1\( \beta \)-mRNA induces antimetastatic activity.** Whether IL1\( \beta \)-mRNA elicits migration activity in ZC3H12D\(^{-/-}\)NK cells because those cells migrated from the liver to the lungs in tumor-bearing mice was investigated. B220\(^{+}\)CD11c\(^{+}\)NK1.1\(^{+}\)NK cells derived from wild-type mice exhibited migration activity with IL1\( \beta \)-mRNA, but not \( \text{actin}\)-mRNA, and this migration activity was absent in ZC3H12D\(^{-/-}\) (Fig. 6a). To demonstrate that ZC3H12D protein can capture naturally produced IL1\( \beta \)-mRNA, migration assays were conducted using nX-RNA isolated from TCM. To confirm the effect of ZC3H12D protein, migration assays were repeated using TCM passed through ZC3H12D-FLAG tag bound on anti-FLAG beads. In this experiment, TCM passed through ZC3H12D protein-bound beads [designated as ZC3H12D(+) column] and passed through anti-FLAG beads [designated as ZC3H12D(-) column, used as a control] were prepared. nX-RNA were isolated from both TCMs and used for the migration assay. This migration assay revealed that nX-RNA treated with the ZC3H12D(+) column had reduced chemotactic activity compared to that of nX-RNA treated with the ZC3H12D(-) column in B220\(^{+}\)CD11c\(^{+}\)NK1.1\(^{+}\)NK cells (Supplementary Fig. S7a), suggesting that IL1\( \beta \)-mRNA-ZC3H12D interactions play a vital role in cell migration activity.

This study tried to evaluate the effect of IL1\( \beta \)-mRNA on IFN-\( \gamma \) production in B220\(^{+}\)CD11c\(^{+}\)NK1.1\(^{+}\)NK cells. Cells derived from wild-type mice were clearly stimulated by IL1\( \beta \)-mRNA, but only a faint induction was observed in cells from ZC3H12D\(^{-/-}\) mice (Fig. 6b). Furthermore, to determine the tumoricidal effects of B220\(^{+}\)CD11c\(^{+}\)NK1.1\(^{+}\)NK cells, rhodamine-labeled E0771 cells were cocultured with IL1\( \beta \)-mRNA-primed NK cells to count dead tumor cells stained with Zombie, a green fluorescent dye. In this assay system, to account for the effects of RNA priming on the tumor cells, NK cells were washed before being applied to tumor cells. These data revealed that IL1\( \beta \)-mRNA priming increased the tumoricidal activity of NK cells originating from wild-type mice but not from ZC3H12D\(^{-/-}\) mice (Fig. 6c). Again, B220\(^{+}\)CD11c\(^{+}\)NK1.1\(^{+}\)NK cells from ZC3H12D\(^{-/-}\) mice had similar tumoricidal activity (Supplementary Fig. S7b). To check if IL1\( \beta \)-mRNA-primed macrophages enhance this activity, NK cells were cocultured with bone marrow-derived macrophages (BMDMs) stimulated by IL1\( \beta \)-mRNA (Fig. 6d, top). Both IL1\( \beta \)-mRNA-primed BMDMs derived from wild-type and ZC3H12D\(^{-/-}\) mice did not have additive effects on tumoricidal activity in vitro (Fig. 6d, lower).

As ZC3H12D recognized the 3'-UTR of IL1\( \beta \)-mRNA, the functional region in the 3'-UTR was further examined. To define the functional element in the 3'-UTR of IL1\( \beta \)-mRNA, it was further divided into four fragments containing 150 nt, where each fragment had at least one 50 nt overlap with its neighboring fragments. The third fragment showed higher activity than the others in the migration assay of B220\(^{+}\)CD11c\(^{+}\)NK1.1\(^{+}\)NK cells derived from wild-type mice but remained unnoticed from ZC3H12D\(^{-/-}\) mice (Supplementary Fig. S7c). Next, NK activation via ZC3H12A\(^{32,34,35}\), a subfamily of ZC3H12 proteins, was examined. In a migration assay, full-length and 3'-UTR IL1\( \beta \)-mRNA induced chemotactic activity in NK cells from wild-type mice and ZC3H12a\(^{-/-}\) (\( \text{Regnase-1} \)\(^{-/-}\) mice\(^{36}\)), whereas CDS IL1\( \beta \)-mRNA produced almost the same activity levels as those from control RNA (Supplementary Fig. S7d). In addition, IFN-\( \gamma \) induction by IL1\( \beta \)-mRNA was similar in cells derived from both genotypes (Supplementary Fig. S5e). Thus, these data indicated that a central region of the 3'-UTR in IL1\( \beta \)-mRNA is recognized in a ZC3H12D protein-dependent manner.

In cancer, IL1\( \beta \) protein is prevalent in tumor promotion in association with proinflammatory immune cells\(^{41}\). Whether IL1\( \beta \)-mRNA-primed B220\(^{+}\)CD11c\(^{+}\)NK1.1\(^{+}\)NK cells have an antitumortastic ability in vivo was investigated. In this assay system, NK cells were obtained from TCM-stimulated mouse spleens and primed with synthetic IL1\( \beta \)-mRNA. These NK cells were then applied to another TCM-stimulated mouse before injection of rhodamine-labeled tumor cells via the tail vein. To evaluate lung metastasis, lungs were isolated after injection, and the cell numbers in the lungs were counted (Fig. 6e, model). IL1\( \beta \)-mRNA-primed wild-type B220\(^{+}\)CD11c\(^{+}\)NK1.1\(^{+}\) cells reduced the number of metastatic tumor cells homing into the lungs (Fig. 6f). In contrast, IL1\( \beta \)-mRNA-primed NK cells from ZC3H12D\(^{-/-}\) mice have only a slight antitumor effect (Fig. 6f, graph).
Human NK cell has ZC3H12D-IL1β-mRNA axis. Whether hZC3H12D plays a significant role in the mRNA uptake system was investigated. hZC3H12D has two alternatively spliced gene products: one is a long form (58 kDa: hZC58), the primary structure of which is very similar to that of mouse protein, and the other is a short form (36 kDa: hZC36), which lacks a C-terminal domain. These two isoforms share the RNA-binding and ZF domains, but the proline-rich domain, which is considered an interaction site for ZC3H12A protein, is unique to hZC58 (Fig. 7a, top). For a mRNA uptake assay, 786-O cells, human renal carcinoma cells stably expressing hZC58 or hZC36, were first established. These IHC analyses revealed that ZC3H12D was widely distributed in the cell membrane and cytoplasm of hZC58 cells, whereas it was found in a relatively limited area in hZC36 cells (Fig. 7a, bottom). These cells were incubated with FITC-labeled hIL1β (hIL1β-mRNA), and the resulting images indicated that hIL1β-mRNA-FITC was detected more often in the nucleus of hZC58 cells than in hZC36 cells (Fig. 7b), indicating that the C-terminal domain of hZC58 containing the proline-rich domain plays a vital role in the transportation of mRNA into the nucleus. The uptake of hIL1β-mRNA in human CD56⁺CD3⁻ NK⁴²,⁴³ cells isolated from human PBMCs (hPBMCs) was next examined.
In flow cytometric analysis, ZC3H12D was detected on the cell surface of 46% of CD56^+CD3^-NK cells. FITC-labeled hIL1β-mRNA, but not control hβactin-mRNA, was incorporated into the nucleus of CD56^+CD3^-NK cells (Fig. 7c). Small interfering RNA (siRNA)-mediated knockdown of ZC3H12D in CD56^+CD3^-NK cells was carried out to elucidate whether ZC3H12D protein was involved in the NK cell uptake process. The suppression of ZC3H12D protein levels by siRNA electroporation (Supplementary Fig. S8a) clearly reduced hIL1β-mRNA uptake (Fig. 7d) in comparison to control siRNA- or ZC3H12A-siRNA-transfected cells.
NK activation after uptake of hIL1β-mRNA was examined. IFN-γ immunostaining revealed that CD56+CD3 NK cells increased in IFN-γ positivity after hIL1β-mRNA stimulation (Fig. 7e), and their IFN-γ expression levels were as high as those induced by recombinant IL12 44 as an IFN-γ inducer for NK cells 45. Human NK cells are divided into two distinct subsets, CD56bright and CD56dim, and the tumor-related microenvironment is differently affected in those NK cells on their tumoricidal and proliferative functions 47. As ZC3H12D was expressed in both subsets (Supplementary Fig. S8b), CD56bright and CD56dim NK cell fractions from hPBMCs were purified, and hIL1β-mRNA uptake was tested separately to find that it was in 38% of CD56bright and 50% of CD56dim NK cells. To mimic the tumor microenvironment, NK cells were first stimulated with TCM derived from human breast cancer MDAMB231 cells, and IFN-γ production response was then observed upon application of IL-2 protein and/or hIL1β-mRNA (Fig. 7f, top). Three days after TCM stimulation, hIL1β-mRNA but not IL2 protein increased IFN-γ production in CD56dim NK cells (Fig. 7f, bottom left). The second stimulation by hIL1β-mRNA induced IFN-γ production at the same level as the first stimulation (Fig. 7f, bottom right; Supplementary Fig. S8c). Conversely, CD56bright NK cells did not show induction (Supplementary Fig. S8d). The NK cell activation marker, NKG2D, in CD56bright NK cells was slightly increased after the application of hIL1β-mRNA (Supplementary Fig. S8e).

In summary, ZC3H12D in NK cells selectively captures nEX-IL1β-mRNA enriched in the tumor-stimulated lung microenvironment and transports it into the nucleus. To localize ZC3H12D protein at the cell surface, its proline-rich C-terminal domain is necessary, although it is apart from the RNA-binding site. IL1β-mRNA transport into the nucleus elicited three functions: (1) enhancing tumoricidal activity with cell migration ability and IFN-γ production, (2) regulation of antiapoptotic gene expression by associating with RNA Pol II, and (3) controlling the NK cell survival and prolonged IFN-γ production in a tumor microenvironment (Fig. 8).

Discussion

Human serum and plasma contain various classes of RNA molecules, such as mRNA, microRNA, Piwi-interacting RNA, transfer RNA, and other miscellaneous ncRNA molecules 48–51. Recently, nonvesicular RNP complexes, including UTR and the CDS of mRNA, were reported in human glioma cultured medium, indicating the existence of long types of mRNA 24. This revealed that primary tumor-stimulated lung but not liver contained nonvesicular full-length IL1β-mRNA. Human full-length IL1β-mRNA with Poly(A) was more detected as an unpacked vesicular form than packing form in EVs in breast cancer-derived TCM. Furthermore, the specific interaction of IL1β-mRNA was mediated by ZC3H12D protein, which recognizes its 3′-UTR; this is not surprising, as many mRNA-binding proteins bind to secondary structures, including stem loops and short consensus sequences, such as AREs, which are situated in the 3′-UTR of many transcripts in the cytoplasm 32. Also, extracellular double-stranded RNA is nonspecifically recruited into the cytoplasm by the sID family, resulting in the usage of cellular RNA interference silencing 32–54. So far, several types of RNA uptake inside cells have been reported, and some of them are utilized in gene therapies 55. Most importantly, this study revealed that ZC3H12D captures nEX-mRNA at the cell surface; then, the mRNA-bound complex is transported into the nucleus across the cytoplasm. ZC3H12D belongs to the ZC1H2 family and shares an N-terminal domain (NTD), PiIT N-terminus-like (PIN) domain, and ZF domain with other members. Among them, ZC3H12A (Regnase-1) is the most deeply investigated. Its biochemical studies revealed that recombinant Regnase-1 (NTD-PIN-ZF) recognized the stem-loop element in the 3′-UTR of IL-6 mRNA and degraded it in an Mg2+-dependent manner 36–38. Regnase-1 and ZC3H12D regulate mRNA decay by recognizing the 3′-UTR of IL-2, IL-6, IL-10, and TNFα 20. There is no surprise that both proteins regulated the same RNA. Regarding the PIN-ZF domain sequence, Regnase-1 and ZC3H12D are similar to each other, and their aspartate residues requiring a cleavage reaction are entirely conserved. In contrast, IL-17a mRNA degradation was regulated by ZC3H12D but not Regnase-1 20. This minor difference in the enzymatic specificity is attributed to the difference in their amino acid sequences. The homology between these two proteins in the NTD is relatively low 45%. Thus, it is assumed that the NTD modified the biochemical functions of ZC3H12D. It is also assumed that ARE containing RNA bound to ZC3H12D was apart from Mg2+ sitting at the catalytic center so that it was not degraded as other stem-loop substrates. Furthermore, ZC3H12D binding to long synthetic RNA with ARE (50 nt) is much stronger than short synthetic RNA (20 nt), implying that the binding affinity of ZC3H12D is susceptible to various structural factors. The FCS measurements unveiled that ZC3H12D has a non-specific binding site for long (>1000 nt) RNA. Taken together, it is speculated that long RNA with ARE triggers a structural change in the ZC3H12D–RNA complex to give it biological functions. Phylogenetic relationship analysis revealed that the ZC3H12 family ancestor, nematode protein REGE-1, targets ETS-4–mRNA different from target genes by mammalian ZC3H12 and controls proinflammatory mRNA 57. It is suggested that a unique substrate
recognition ability was adopted in this protein to regulate RNA based on their structures\textsuperscript{57} during branching between ZC3H12D and other Regnase family genes.

The premetastatic and metastatic microenvironments contain abundant proinflammatory proteins and exRNA\textsuperscript{7}. In tumor growth and metastasis, tumor-associated RNA packaged in EVs are nonspecifically incorporated into the tumor and host cells, usually resulting in greater malignancy in cases with suppressive phenotypes\textsuperscript{58,59}. The mechanism of this cytoplasmic recognition of RNA has been reported to involve EVs entering the cytoplasm of cells and the RNA they contain being recognized by sensor molecules, such as TLR3 and RIG-1\textsuperscript{60,61}. This study revealed that
Interestingly, some of the ZC3H12D genes may be regulated by transported nex-mRNA in the nucleus. The ratio of rhodamine-labeled E0771 cells that became Zombie$^+$ dead cells in the assay. The dead E0771 cells increased after coculture with IL1β-mRNA- and IL1β-stop-mRNA-primed B220+CD11c$^+$NK1.1$^+$ cells from TCN-stimulated wild-type mice rather than from cells from Zc3h12d$^-/$ mice (n = 3 wells per group). a Tumoricidal assay system. Tumoricidal assay in vitro after cocultivation of tumor cells with B220+CD11c$^+$NK1.1$^+$ cells after coculture with BMDMs primed with IL1β-mRNA (top). To exclude the direct effects of IL1β-mRNA on NK cells, BMDMs were washed with basal medium after priming. The ratio of rhodamine-labeled E0771 cells that became Zombie$^+$ dead cells in the assay (bottom, n = 4 wells per group).

**Methods**

**Reagents.** The following primary antibodies and factors were used in this study: anti-ZC3H12D antibodies for mouse (Abcam, ab1000862, dilution 1:200), E14 (Santa Cruz Biotechnology, sc-169840, dilution 1:100) for fluorescence-activated cell sorting (FACS) analysis, and anti-ZC3H12D (Proteintech, 24991-1-AP, dilution 1:100) for western blotting. Anti-ZC3H12D antibodies for human (Abcam, ab1000862, dilution 1:100), anti-ZC3H12A antibody for human (N3C, GeneTex, GTX110807, dilution 1:100), and histone H2AX139ph (phospho-Ser139) antibody (GT2311, GeneTex, GTX628789, dilution 1:200) were used in IHC analyses. For mouse cell sorting and FACS analyses, the following were used: for mouse CD11c$^+$ anti-mouse CD11c (BioLegend, 117308, dilution 1:100-200); isotype control, PE mouse IgG1, Amersham IgG (eBioscience, 12-4888-81, dilution 1:100); Alexa Fluor 647 anti-mouse anti-CD11c (BioLegend, 11732, dilution 1:100); and isotype control, Alexa Fluor 647 Amersham hamster IgG (BioLegend, 400526, dilution 1:100). For mouse IL1β and CD56, D12/V421 anti-CD56 (BioLegend, 11732, dilution 1:100); isotype control, Brilliant Violet 421 mouse IgG2a, (BioLegend, 400260, dilution 1:100); APC anti-mouse NK1.1 antibody (BioLegend, 108710, dilution 1:100); and isotype control, APC mouse IgG2a, (BD Pharmingen, 550882, dilution 1:100). For mouse CD103, PE/Cy7 anti-mouse/human CD45R/B220 antibody (BioLegend, 103212, dilution 1:200); APC anti-mouse/human CD45R/B220 antibody (BioLegend, 103212, dilution 1:200); and isotype control, APC Rat IgG2a, (BD Pharmingen, 554960, dilution 1:200). For CD45: PE/Cy7 anti-mouse CD45 antibody (BioLegend, 304014, dilution 1:200) and isotype control, PE/Cy7 rat IgG2b (BioLegend, 400127, dilution 1:200). Cell sorting for human CD56$^+$ and NKG2D$^+$ cells derived from murine IL1β-mRNA, IL1β$^-$mRNA showed the irregular shape of the nucleus, although cell death was not increased as a whole. In addition, IFN-γ production levels in NK cells were not IL1β-mRNA dose dependent. These data imply that a relatively small amount of IL1β-mRNA is enough to elicit antitumor activity in NK cells. Therefore, it is speculated that the nex-mRNA-ZC3H12D-axis in B220+CD11c$^+$NK1.1$^+$ NK cells suppressed lung metastasis in an adoptive immunotherapy mouse model.

CD56bright NK cells are assumed to be the human counterpart of murine B220+CD11c$^+$NK1.1$^+$ cells. Both are in lymphoid organs and effectively produce IFN-γ. The ZC3H12D expression levels in CD56$^+$ and CD56bright NK cells were alike. However, IL1β-mRNA-mediated IFN-γ production was stronger in CD56$^+$ than CD56bright NK cells. In contrast, IL1β-mRNA induced NKG2D in CD56bright NK cells, implying that NKG2D-dependent antitumor responsiveness was enhanced in cells$^{49}$. Together, IL1β-mRNA may induce tumoricidal activity on CD56$^+$ and CD56bright NK cells in vivo.
were tested negative for mycoplasma contamination by using MycoAlert Mycoplasma Detection Kit (Lonza, LT-07-218). Cells were not authenticated. TCM was obtained by incubating cells overnight in a serum-free medium without cell death. Gathered TCMs were centrifuged at 215 × g for 5 min, and the supernatant was further centrifuged at 21,500 × g for 10 min. TCMs were then stored at −80 °C before experiments.

**TCM and CD45^+ cell culture system using organ tissues.** In the organ culture experiments, 2 mm^2 lung or liver tissue specimens were serum-free cultured overnight in high glucose DMEM with gentamicin. After collection of the culture media, samples were first centrifuged at 215 × g for 10 min, and the supernatant was further centrifuged at 21,500 × g for 30 min at 4 °C. For the coculture system of tissue and CD45 cells, lungs were incubated in DMEM with FBS (1%) in an upper
The positive control was incubated with 20 ng/mL IL12 protein in an IL2 culture medium (IFN-γTCM-pretreated CD56dimCD3-NK cells). The assay system is shown (top). Two donor NK cells were independently used. No control basal culture medium cells treated with siRNA. Control (con) is a nontargeting siRNA (bottom). Each cell image is composed of 15-stacked 3D images. Experiments were repeated twice with similar results.

forms encode 58 and 36 kDa proteins, respectively. Both have a common N-terminal sequence, including a ZF domain (orange; top). IHC analysis of ZC3H12D in the long form (hZC58)- and short form (hZC36)-overexpressing 786-O cells (bottom).

The tumor-associated nex-IL1β-mRNA with function. a Scheme for two splicing isoforms of hZC3H12D. The long and short forms encode 58 and 36 kDa proteins, respectively. Both have a common N-terminal sequence, including a ZF domain (green), and the long form has a proline-rich domain (orange; top). b Representative confocal microscopy images of hIL1β-mRNA-FITC uptake in the hZC58 or hZC36 cell nucleus (top). Quantitative signals of hIL1β-mRNA-FITC in the hZC58 or hZC36 cell nucleus (bottom). 3D images per group: n = 38 for hZC58 and n = 50 for hZC36. c Uptake of hIL1β-mRNA-FITC into CD56+CD3NK cell nucleus 3 h after applying 50 ng/mL RNA with 200 U/mL IL-2 protein. Confoocal microscopy imaging showing the DAPI-stained nucleus in single images (top) and 3D stacks combining 15 images from top to bottom (middle). 3D stack images show a comparison of nuclear uptake between hIL1β-mRNA and hβactin-mRNA (bottom). d Nuclear signals of hIL1β-mRNA-FITC in CD56+CD3NK cells treated with siRNA. Control (con) is a nontargeting siRNA (n = 13, 10, and 11 cells for control, ZC3H12D-siRNA, and ZC3H12A-siRNA, respectively. Each cell image is composed of 15-stacked 3D images). e IHC analysis of IFN-γ induction 48 h after RNA application with 200 U/mL IL2 to CD56+CD3NK cells. The positive control was incubated with 20 ng/mL IL2 protein in an IL2 culture medium (IFN-γ signals from n = 16, 21, and 20 ZC3H12D+ cells for no, IL1β-RNA, and IL2 protein, respectively). f IHC analysis of IFN-γ induction 3 and 6 days after stimulation of hIL1β-mRNA and IL2 protein for MDAMB231-TCM-pretreated CD56dimCD3NK cells. The assay system is shown (top). Two donor NK cells were independently used. No control basal culture medium (bottom left, n = 42, 45, 51, and 51 cells for no, hIL1β-RNA, and IL2 protein, and hIL1β-RNA plus IL2 protein, respectively). Bottom right, n = 34, 44, 37, and 60 cells for no, hIL1β-RNA, and IL2 protein, and hIL1β-RNA plus IL2 protein, respectively). Bars, 5 µm. In the graphs, the averages ± SEM, and the results of Student’s t-tests (two-sided) or one-way ANOVA with Bonferroni correction are shown. The P values are shown in the figure. Source data are provided as a Source Data file.

Fig. 8 Model of the translation-independent function of nex-mRNA. The tumor-associated nex-IL1β-mRNA 3’-UTR was trapped by ZC3H12D protein on the NK cell surface and introduced to the nucleus via its C-terminal containing a proline-rich domain. This may regulate antiapoptotic gene expression associated with RNA Pol II and control NK cell migration, survival, and sustainability of IFN-γ production.

well culture insert (400 nm pores). CD45+ cells isolated from the lungs were cultured in the lower wells, which were stimulated by organ tissues in the upper wells for 24–48 h.

Primary culture of human cells. Primary human lung microvascular cells (Applied Cell Biology Research Institute, ACBRI 468) were cultured in EBM2–2 Microvascular Endothelial Cell Growth Medium-2 SingleQuots’ Supplements and Growth Factors (Lonza). After becoming subconfluent in a 10 cm dish, human TCMs were added at 15–20% and incubated for 24 h. The culture medium was then gathered as lung EC-CMs. Primary hPBMCs (Lonza CC-2702) were cultured in LGM-3 medium (Lonza) with 200 IU/mL IL2. Cells were received from full consent donors with IRB-approved protocols.

Experimental metastatic model. B220+CD11c+NK1.1+ cells derived from TCM-stimulated mouse spleens were primed by IL1β-RNA, and IL2 protein for CD11c+NK1.1+ cells derived from wild-type and Z3H12d+/- and Z3H12d-/- mouse spleens, cells were purified with a cell sorter (MoFlo Astrios® (Beckman Coulter) and FACSAria® III (BD Biosciences)). B220+CD11c+NK1.1+ cells derived from wild-type and Z3H12d+/- mice were not prestimulated by TCM. Human CD56+CD3 NK cells were obtained by FACSAria® III. Mouse livers and lungs were digested with 0.5 mg/mL collagenase, 1 mg/mL dispase, and DNase at 37 °C for 45 min to obtain B220+CD11c+NK1.1+ cells. Cell suspensions were incubated with mouse CD45-microbeads (MACS; Miltenyi Biotec, Auburn, CA, USA), and the cell counts were normalized to the total tissue surface area. Age- and sex-matched littermates were used for the experiments.

For the spontaneous metastatic assay, E0771 cells were macroscopically detected in the lungs of tumor-bearing mice after primary tumor resection. Syngeneic tumor grafts were generated via subcutaneous or mammary fat pad implantation of 5 × 106 tumor cells into 7- to 8-week-old mice.
captured cells were used for in vitro culture in Supplementary Fig. S1. For the detection of ZCH12D in mouse PBMCs in Fig. 1b, staining was carried out as described below, and cells were analyzed using a flow cytometer (CytoFico, Beckman Coulter). For the staining of the cell surface, PBMCs were gathered using Ficol-Paque (Histopaque*-1083; Sigma-Aldrich) and/or hemolysis and stained with 0.5 μg antibodies/10^6 cells in 100 μL volume. For intracellular staining, cell fixation and permeabilization (Fixation/Permeabilization Kit; BD Pharmingen) treatments were administered before the addition of antibodies. BD FACSDiva (BD Bioscience) was also used for flow cytometry, and data analyses were done by using FACSDiva v8 (BD Bioscience) and FlowJo v7.6 (FlowJo LLC).

Immunoprecipitation. To crosslink protein-RNA complexes, cells cultured in a 10 cm dish were washed with PBS twice and received 254 nm UV irradiation (1500 J/m²) before protein extraction. Cells were dispersed in 10 mM HEPS (pH 7.6), 150 mM NaCl, 0.1 mM EDTA, and 0.5% sodium cholate with protease inhibitors (G6521; Promega) and RNase Out (Thermo). An antibody preincubated with protein G magnetic beads (Thermo) was added to the extract in the presence of RQ1 RNase-Free DNase (Promega) and incubated for 1 h at 4°C. The magnetic beads were washed with Tris-buffered saline + Tween-2 (TBST) four times and further washed with TBST + 1 M NaCl. Then, the beads were incubated with protease K (1 h at 37°C). Then, phenol-chloroform extraction and ethanol precipitation were performed.

Uptake of RNA. Before RNA application, primary cells were incubated in 1% FBS culture medium for 1 h, and the cell lines were preincubated with or without 1% FBS for 3 h. Human NK cells were not starved. To quench the RNA uptake, 4% FBS for 3 h. Human NK cells were not starved. To quench the RNA uptake, 4% FBS for 3 h. To crosslink protein-RNA complexes, cells cultured in a 10 cm dish were washed with PBS twice and received 254 nm UV irradiation (1500 J/m²) before protein extraction. Cells were dispersed in 10 mM HEPS (pH 7.6), 150 mM NaCl, 0.1 mM EDTA, and 0.5% sodium cholate with protease inhibitors (G6521; Promega) and RNase Out (Thermo). An antibody preincubated with protein G magnetic beads (Thermo) was added to the extract in the presence of RQ1 RNase-Free DNase (Promega) and incubated for 1 h at 4°C. Then, phenol-chloroform extraction and ethanol precipitation were performed.

IFN-γ induction and tumoricidal assay. B220⁺CD11c⁻NK1.1⁺ cells were stained using an anti-mouse IFN-γ antibody 16 to 24 h after incubation with 10 ng/mL mlIL1β (Thermo). Human NK cells were stained with an antibody against IFN-γ antibody 48 h after priming with 50 ng/mL mlIL1β-RNA in a 1LM-3 medium (Lonza) supplemented with 200 μIU/mL IL2. For the positive control of IFN-γ induction, cells were incubated with 40 ng/mL mlIL12-RNA. The Zombie Green Fixable Viability Kit (BioLegend) was used to detect the dead PHK26-stained tumor cells 24 h after incubation with B220⁺CD11c⁻NK1.1⁺ cells that were primed with 10 ng/mL mlIL1β-RNA for 16–18 h.

Real-time apoptosis and necrosis assay. ZC⁺ RAW, B220⁺CD11c⁻NK1.1⁺ NK, and hZC58 cells were traced after the application of IL1β-RNA or factin-RNA (Promega). To detect cell death, RealTime-Glo Annexin V Apoptosis and Necrosis Assay (Promega) are used. In this assay, Annexin V binding is detected with a luciferase reporter. In this assay, Annexin V binding is detected with a luciferase reporter.

Vector construct and establishment of ZCH12D stably expressing cells. hZCH12D expression vectors (hZC58 and hZC56), in which the hZCH12D CDS is cloned into a PCMV6-entry vector (C-terminal myc-FLAG tag), were purchased from OriGene Technologies, Inc. (Rockville, MD, USA). mZCH12D was PCR-amplified using the primer set 5′-GGTACATGATGACGCATTAGTT-3′ and 5′-CTGGAGTTTACATACAGACAGCAGCC-3′ and then cloned into a pcR Blunt II vector (Thermo). The cloned fragment was double-digested by NotI and XhoI and subcloned into pcDNA3 attached with a C-terminal FLAG tag. To establish mZCH12D or hZCH12D stably expressing cell lines, one of the aforementioned constructs was transfected into a cell line, and cell lines were cultured for more than 2 weeks in the presence of 400 μg G418. After G418 selection, cell lysates were tested by western blotting with DDDK antibody (MBL Co., Ltd, Japan, PM020, dilution 1:1000) probing to confirm the expression of ZCH12D-FLAG proteins.

Vector constructs for RNA synthesis. mlIL1β ps were PCR-amplified (primer set for full length: 5′-AACAAAACCTGCACTGTTGCAAG-3′ and 5′-GTGGTTGGTACACTTTATTTATTTTTACACCAATT-3′; for CDS: 5′-AACAAACCATGCTTGAAGTGGA-3′ and 5′-TTAGGAAGACACGTTTACAGTGAAAG-3′; for UTR: 5′-ATAGATGCGGTCAGCTTCTAATG-3′ and 5′-GTGGTTGTGGTTTGAATTTTCATGTATTTTATTTTACCAATGAAG-3′) and cloned into a pcR Blunt II vector. To prepare mlIL1β nonsense mutant (IL1β-stop mutation), human IL1β gene (Promega) was further amplified and introduced into a pcRF Blunt II vector. To prepare the human-mouse chimeric IL1β construct, the NotI-Ndel fragment in the mlIL1β-pcR Blunt II vector was replaced with a double-degenerate fragment excised from the hIL1β plasmid. As a result, this chimera encoded hIL1β (5′-UTR + first half of CDS; 502 bp) combined with mlIL1β (second fragment of CDS + 3′-UTR; 843 bp) at the Ndel site. mlIL1β-3′ UTR was further divided into four fragments. The following primer sets were used to amplify the fragments (first fragment: 5′-AGTATGGTGACAAGTTTTGTTTTG-3′ and 5′-AACAGAATGTGGCATGTGTTGG-3′; second fragment: 5′-CGCGCAAAACGACTGCTC-3′ and 5′-CTTGGATTTTACATACACACTATGATTTTACCAATGAAG-3′). The ampli- cons were then cloned into a pcR Blunt II vector. There fragments were 148–153 bp, and each had one at least 1 bp overlap with its neighboring fragments. These constructs were linearized by SpeI digestion and used for the in vitro translation system (RiboMAX Large-Scale RNA Preparation System-17; Promega, WI, USA). To prepare fluorescence-labeled RNA, Fluorescein RNA Labeling Mix (Sigma-Aldrich) was mixed in the RNA synthesis reaction. To introduce Cap modification, synthesized RNA was mixed with Vaccinia virus Capping enzyme (M2080; New England Biolabs (NEB), MA, USA) and Cap 2′-O-Methyltransferase (M2004; NEB) after the reaction. Purified RNA was phenol-chloroform extraction and ethanol precipitation. Subsequently, part of the resulting RNA was mixed with Poly(A) polymerase (M0276; NEB) to make a Poly(A) adduct. Before use, all RNAs were purified using a spin column-based purification system (PureLink RNA Mini Kit; Thermo).

EMSA. ssRNA oligonucleotides were purchased from Eurofins Genomics. A Biotin Labeling Kit (89818; Thermo) was used to introduce biotin labeling at the 3′ end of the oligonucleotides. To study the interaction between RNA oligonucleotides and ZCH12D protein, a LightShift Chemiluminescent RNA EMSA Kit (20158; Thermo) was used. Purified ZCH12D protein was mixed with biotin-labeled oligonucleotides in the presence of glycerol, reaction buffer components [final concentration: 100 mM HEPS (pH 7.3), 200 mM KAc, 10 mM MgCl₂, and 10 mM dithiothreitol], and tRNA supplied with the kit; the mixture was then run in a 5% to 20% gradient gel (Fujiwara; NEB) to make a Poly(A) adduct. Before use, all RNAs were purified using a spin column-based purification system (PureLink RNA Mini Kit; Thermo).

Immunoprecipitation for western blot or qPCR. To crosslink protein-RNA complexes, cells cultured in a 10 cm dish were washed with PBS twice and received 254 nm UV irradiation (1500 J/m²) before protein extraction. Cellular proteins were extracted with RIPA buffer [50 mM HEPS (pH 7.6), 150 mM NaCl, 0.5% Triton X-100, and 0.5% sodium cholate] with protease inhibitors (G6521; Promega) and RNase Out (Thermo). The cloned fragment was double-digested by KpnI-XhoI and subcloned into pcDNA3 attached with a C-terminal FLAG tag. To establish mZCH12D or hZCH12D stably expressing cell lines, one of the aforementioned constructs was transfected into a cell line, and cell lines were cultured for more than 2 weeks in the presence of 400 μg/mL G418. After G418 selection, cell lysates were tested by western blotting with DDDK antibody (MBL Co., Ltd, Japan, PM020, dilution 1:1000) probing to confirm the expression of ZCH12D-FLAG proteins.

RNA preparation from cytoplasmic and nuclear fractions. Cells were dispersed in 10 mM HEPS (pH 7.6), 150 mM KCl, 2 mM MgCl₂, and 0.1 mM EDTA with protease inhibitors (G6521; Promega), and NP-40 was added to yield a final concentration of 0.2%. After centrifugation (600 g for 1 min), the supernatant was further centrifuged (21,500 × g for 15 min), and an RNA-containing pellet was obtained by phenol-chloroform extraction and ethanol precipitation. After 600 g centrifugation, the precipitating nuclear fraction was mixed with Trizol (Thermo) to isolate RNA.

Extraxosome and exosome fractions from TCM and lung TCM. TCMs and lung TCM that serum-free cocultured with or without TCMs (lung TCM/CM) were centrifuged 300 g for 5 min, and the supernatants were further centrifuged 3000 g x 10 min and filtered. Exosomes can be fractionated by size-exclusion...
chromatography, ultracentrifugation, magnetic bead pull-down assay, and polymer precipitation. Among them, ExoQuick-TC (System Biosciences), a polymer used to precipitate exosomes, is the best method to eliminate exosomes from CM. The samples were treated by ExoQuick-TC (System Biosciences) overnight and then centrifuged 1500 × g for 30 min. The supernatants were further centrifuged 20,000 × g. The resulting supernatants were treated by RNAiso Blood (Takara Bio) to obtain RNA as extracellular RNA. The exosome pellets were washed by PBS and treated by Trizol Reagent (Thermo Fisher Scientific) to isolate exosome RNA. All procedures were carried out at 4 °C.

RNA pull-down assay by ZC36 protein beads from mouse serum. Mouse blood was taken from the heart, and serum was separated using Microtainer tubes (Gold; Becton Dickinson). Then, serum was mixed with ZC36-protein-anti-DYKDDDDK magnetic beads for 1 h at 4 °C. Beads were washed with PBS three times, and RNA attaching to the beads was isolated by Trizol reagent.

Protein purification and protein column. The hZC63 expression vector (hZC63/pCMV6-Entry) was delivered into HEK293T cells. Forty-eight hours after transfection, cells were harvested and stored at −80 °C. RIPA buffer with protease inhibitors was added to the frozen cells, and the lysate was centrifuged at 21,500 × g for 15 min. The supernatant was 0.2 μm filtered and loaded on a column holding anti-DYKDDDDK tag beads (FujiFilm Wako Pure Chemical). Then, the column was washed with PBS (10 bed volume), PBS + 1 mM NaCl (10 bed volume), and PBS (10 bed volume). This column was used as a ZC3H12D protein column to absorb substances capable of binding ZC3H12D protein. ZC3H12D protein on the column was eluted by PBS, PBS + 100 mM NaCl, and 100 μg/mg DYKDDDDK peptide (FujiFilm Wako Pure Chemical), and the eluent was concentrated with an Amicon Ultra centrifugal filter unit (Merck, NJ, USA). The affinity column-purified ZC3H12D protein was further purified using a Q-Sepharose HP column attached to a chromatography system (GE Healthcare, NJ, USA). ZC protein diluted in 50 mM HEPES (pH 7.4) was loaded on a Q-Sepharose HP column eluted with a NaCl gradient (0–1 M NaCl). Q-Sepharose-purified ZC3H12D protein was used for FCS. First, equal molar Alexa Fluor 488 NHS ester (Thermo) was mixed with ZC3H12D protein and incubated for 2 h at room temperature. After the labeling reaction, ZC3H12D protein was purified using the Q-Sepharose HP column again. The purified sample was concentrated for storage and diluted in 50 mM HEPES (pH 7.4) and 100 mM NaCl buffer before FCS measurements.

FCS. FCS measurements were performed using a home-built confocal microscope setup. The excitation light was obtained from a diode laser (OPO-1000P-488; Tama Electric, Hamamatsu, Japan) with a wavelength of 480 nm and output power of 100 μW. The laser light was coupled to a single-mode optical fiber to shape the spatial intensity profile. The output from the fiber was introduced to a collimator, expanded by two lenses, and introduced to a water immersion objective (Plan Apo pd 100×; NA 1.20; Nikon, Tokyo, Japan) by a dichroic mirror (MD499; Thorlabs, Berlin, Germany). The autocorrelation functions were accumulated for an acquisition time of 0.1 to 1 s, analyzed using Igor Pro v7 (WaveMetrics, OR, USA). Initially, the autocorrelations obtained for Alexa Fluor 488-labeled ZC3H12D protein were fitted using a 2D diffusion model with one diffusing component and the amplitude caused by the triplet state. The autocorrelations obtained for the other samples were globally fitted using a 2D diffusion model with two diffusing components and the triplet state amplitude based on the assumption that the diffusion time of each component was constant across different conditions. The hydrodynamic radii of the samples were calculated by referring to the measured diffusion time for rhodamine 110, for which the diffusion coefficient in water was reported.

ICHC and cell counts. Anti-ZC3H12D (ab1000862; Abcam) antibody was used to stain spleen cells, B220+CD11c+CD8a- cells, ZC3H12D+CD11c- cells, ZC3H12D+CD8a- cells, ZC3H12D+CD3- cells, CD56+CD3-NK cells, and frozen tissue sections. In Supplementary Fig. S1, the immunostained cell area values are shown as the number of pixels normalized to the DAPI signal. Labeled tumor cells were detected by confocal or fluorescence microscopy and normalized by the total surface area.

Knockdown using siRNA. For knockdown of ZC3H12D and ZC3H12A, CD56+CD3-NK cells were treated with 300–1000 nM siRNA (ON-TARGET plus hZC3H12D (540152) and hZC3H12A (801499) siRNA (SMARTpool)) using electroporation with an Amaxa Nucleofector system (Lonza). The control siRNA was ON-TARGET plus Nontargeting pool (Dharmacon Horizon Discovery). The efficiency of knockdown at the protein level was examined with IHC staining. An RNA uptake assay was carried out 24 h after the application of 300 nM siRNA.

Microarray analysis. Microarray screening of spleen tissues from TCM-stimulated wild-type and Zc312d−/− mice was performed using a GeneChip® Clarion D Assay, Mouse Transcriptome Array 2.0 (Affymetrix). The microarray data of B220+CD11c+CD8a- cells from liver and lung tissue obtained using a GeneChip Mouse Genome 430 2.0 Array (Affymetrix) were previously reported58. Microarray data were deposited at the National Center for Biotechnology Information: Supplementary Table S1, GSE76235; Supplementary Table S2, GSE161219; Supplementary Tables S3 and S4, GSE140042.

qPCR. exRNA samples were isolated from 5 to 10 mL TCM, lung TCM, liver TCM, and human lung EC-CMs using RNAiso Blood (Takara) with Ethachinmate (Nippon Gene). Total RNA samples were isolated from cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and used to generate cDNA with reverse transcriptase (SuperScript VILO, Invitrogen). For the reverse transcription with GSP, SuperScript III reverse transcriptase (Invitrogen) was used. cDNA from exRNA was amplified using TaqMan PreAmp Master Mix (Applied Biosystems, Foster City, CA, USA) before qPCR analysis. qPCR was performed using SYBR Green Master Mix or TaqMan Fast Advanced Master Mix (Applied Biosystems) in a 7900HT Fast Real-Time PCR System (Applied Biosystems). The gene expression levels were calculated from Ct values, and the relationship between the Ct value and the logarithm of the copy number of the target gene was confirmed to be linear using serial dilutions of the corresponding isolated DNA as a standard. In addition, gene expression levels of nuclear and cytoplasmic samples were normalized to that of H3 and Actin, respectively. The primer sequences and probes are listed in Supplementary Table S5.

Colocalization and statistical analyses. For colocalization analysis, Image J v1.52 (National Institute of Health) with coloco, a plugin for colocalization analysis (https://imagej.net/Coloc_2), was used. Data are expressed as mean ± standard error of the mean. Statistical evaluation was conducted as indicated. *P < 0.05 was considered statistically significant. All Student’s t-tests were unpaired and two-sided. Analysis of variance was always employed with Bonferroni’s correction. P value calculations were carried out by using Prism v8 software (GraphPad Software, San Diego, CA, USA). The experiments were repeated more than twice for representative images.

Reporting summary. Further information on research design in Supplementary Table S5 linked to this article.

Data availability. No restrictions on data availability. Microarray data [Supplementary Table S1, GSE76235; Supplementary Table S2, GSE161219; Supplementary Tables S3 and S4, GSE140042] are deposited in the Gene Expression Omnibus (GEO) database. Other data are available from the corresponding author upon request. Source data are provided with this paper.

Received: 26 May 2020; Accepted: 27 May 2021; Published online: 16 June 2021

References

1. Kowalski, P. S., Rudra, A., Miao, L. & Anderson, D. G. Delivering the messenger: advances in therapies for therapeutic mRNA delivery. Mol. Ther. 27, 710–728 (2019).

2. Baj-Krzyworzeka, M. et al. Tumour-derived microvesicles carry several surface determinants and mRNA of tumour cells and transfer some of these determinants to monocytes. Cancer Immunol. Immunother. 55, 808–818 (2006).

3. Valadi, H. et al. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat. Cell Biol. 9, 654–659 (2007).

4. Deregbus, M. C. et al. Endothelial progenitor cell derived microvesicles activate an angiogenic program in endothelial cells by a horizontal transfer of mRNA. Blood 110, 2440–2448 (2007).

5. Bruno, S. et al. Mesenchymal stem cell-derived microvesicles protect against acute tubular injury. J. Am. Soc. Nephrol. 20, 1053–1067 (2009).
6. Herrera, M. B. et al. Human liver stem cell-derived microvesicles accelerate hepatic regeneration in hepatocyte-depleted rats. J. Cell Mol. Med. 14, 1605–1618 (2010).
7. Peinado, H. et al. Pre-metastatic niches: organ-specific homes for metastases. Nat. Rev. Cancer 17, 302–317 (2017).
8. Lambert, A. W., Pattnabiraman, D. R. & Weinberg, R. A. Emerging biological principles of metastasis. Cell 168, 670–691 (2017).
9. McAllister, S. S. & Weinberg, R. A. The tumor-induced systemic environment as a critical regulator of cancer progression and metastasis. Nat. Cell Biol. 16, 717–727 (2014).
10. Fidler, I. J. Timeline—the pathogenesis of cancer metastasis: the ‘seed and soil’ hypothesis revisited. Nat. Rev. Cancer 3, 453–458 (2003).
11. Hiraitsuka, S. et al. MMIP induction by vascular endothelial growth factor receptor-1 is involved in lung-specific metastasis. Cancer Cell 2, 289–300 (2002).
12. Erler, J. T. et al. Hypoxia-induced lysyl oxidase is a critical mediator of bone marrow cell recruitment to form the premetastatic niche. Cancer Cell 15, 35–44 (2009).
13. Hiraitsuka, S., Watanabe, A., Aburatani, H. & Maru, Y. Tumour-mediated upregulation of chemokreceptors and recruitment of myeloid cells predetermines lung metastasis. Nat. Cell Biol. 8, 1369–1375 (2006).
14. Hiraitsuka, S. et al. The S100A8-serum amyloid A3-TLR4 paracrine cascade establishes a pre-metastatic phase. Nat. Cell Biol. 10, 1349–1359 (2008).
15. Kaplan, R. N. et al. VEGFRI-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. Nature 438, 820–827 (2005).
16. Hiraitsuka, S. et al. Hepato-entrained B20po. EMBO Mol. Med. 10, e643 (2018).
17. Hiraitsuka, S. et al. Primary tumours modulate innate immune signalling to create pre-metastatic vascular hyperpermeability. J. Nat. Commun. 4, 1853 (2013).
18. Wang, M. et al. Identification of a novel tumor suppressor gene p34 on human chromosome 6q25.1. Cancer Res. 67, 93–99 (2007).
19. Minagawa, K. et al. Deregulation of a possible tumour suppressor gene, ZC3H12D, by translocation of IGK@ in transformed follicular lymphoma cells. J. Immunol. 192, 1512–1524 (2014).
20. Huang, S. et al. Monocyte chemotactic protein-induced protein 1 and 4 form a complex but act independently in regulation of interleukin-6 mRNA degradation. J. Biol. Chem. 290, 20782–20792 (2015).
21. Wawro, M., Kochan, J., Krzanić, S., Jura, J. & Kassa, A. Intact NMYC/PIN-like domain is crucial for the degradation of inflammation-related transcripts by ZC3H12D. J. Cell Biochem. 118, 487–498 (2017).
22. Li, K. et al. Advances, challenges, and opportunities in extracellular RNA biology: insights from the NIH exRNA Strategic Workshop. J. Circ. Biomark. 20, 2944 (2019).
23. Nabet, B. Y. et al. Exosome RNA unshielding couples stromal activation to create pre-metastatic vascular hyperpermeability. J. Cell Biol. 209, 242–250 (2018).
24. Kim, K. M., Abdelmohsen, K., Mustapic, M., Kapogiannis, D. & Gorospe, M. RNA in extracellular vesicles. Wiley Interdiscip. Rev. RNA 8 (2017).
25. Mathieu, M., Martin-Jaular, L., Lavieu, G. & Théry, C. Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. Nat. Cell Biol. 21, 9–17 (2019).
26. McKelvey, K. J., Powell, K. L., Ashton, A. W., Morris, J. M. & McCracken, S. A. Exosomes: mechanisms of Uptake. J. Circ. Biomark. 4, 7 (2015).
27. McCowan, D. L., Weisman, A. S. & Hunter, C. P. Uptake of extracellular double-stranded RNA by SID-2. Mol. Cell 47, 746–754 (2012).
28. Nguyen, T. A. et al. SIDT2 transports extracellular dsRNA into the cytoplasm for innate immune recognition. Immunity 47, 498–509.e496 (2017).
29. Nguyen, T. A. et al. SIDT2 RNA transporter promotes lung and gastrointestinal tumor. Dev. Sciance 20, 14–24 (2019).
30. Crooke, S. T., Wang, S., Vickers, T. A., Shen, W. & Liang, X. Cellular uptake and trafficking of antisense oligonucleotides. Nat. Biotechnol. 35, 230–237 (2017).
31. Xu, J. et al. Structural study of MCHP1/P-terminal conserved domain reveals a PIN-like RNA. Nucleic Acids Res 40, 6957–6965 (2012).
32. Habacher, C. & Gisquet, R. ZC3H12A/MCHP1/Regnase-1-related endonucleases: an evolutionary perspective on molecular mechanisms and biological functions. Bioessays 39 (2017).
33. Plebanek, M. P. et al. Pre-metastatic cancer exosomes induce immune surveillance by patrolling monocytes at the metastatic niche. Nat. Commun. 8, 1319 (2017).
34. Matei, I., Kim, H. S. & Lyden, D. Unshielding exosomal RNA unleashes tumor growth and metastasis. Cell 170, 223–225 (2017).
35. Liu, Y. et al. Tumour exosomal RNAs promote lung pre-metastatic niche formation by activating alveolar epithelial TLR3 to recruit neutrophils. Cancer Cell 30, 243–256 (2016).
36. Nabet, B. Y. et al. Exosome RNA unshielding couples stromal activation to pattern recognition receptor signaling in cancer. Cell 170, 352–366.e313 (2017).
37. Grimner, A. et al. High-dimensional single-cell analysis identifies organ-specific signatures and conserved NK cell subsets in humans and mice. Immunity 49, 971–986.e975 (2018).
38. Liao, Y. et al. Tumor exosomal RNAs promote lung pre-metastatic niche formation by activating alveolar epithelial TLR3 to recruit neutrophils. Cancer Cell 30, 243–256 (2016).
39. Wagner, W. et al. CD56bright NK cells exhibit potent antitumor responses following IL-15 priming. J. Clin. Invest. 127, 4042–4058 (2017).
40. Evens, A., Mihich, E. & Ehrke, M. J. Distant metastasis from subcutaneously grown E0771 medullary breast adenocarcinoma. Anticancer Res. 25, 3905–3915 (2005).
41. Taylor, D. D., Zacharias, W. & Gercel-Taylor, C. Exosome isolation for proteome analyses and RNA profiling. Methods Mol. Biol. 728, 235–246 (2011).
Acknowledgements
We thank Drs Kensuke Miyake (The University of Tokyo) and Yasuhiro Furuichi (GeneCare Research Institute) for helpful discussions. This study was supported by Japan Society for the Promotion of Science (JSPS) KAKENHI Grant-in-Aid for Scientific Research (B) 19H03500 (S. H.) and (C)20K06554 (T. Tomita) as well as The Yasuda Medical Foundation (S. H.). This work was performed in part under the Collaborative Research Program of Institute for Protein Research, Osaka University, CR-18-05 (T. Tomita).

Author contributions
T. Tomita and S. H. designed the study and analysis. Experiments were performed by T. Tomita, M. K., T. Mishima, Y. M., H. S., K. I., K. M., T. Matsui, H. O., S. T., T. Takao, N. I., T. Mino, O. T., Y. M., and S. H. Data analysis was performed by S. H. The study was supervised by Y. M. and S. H. This manuscript was prepared by T. Tomita and S. H. with input from all authors.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-23969-1.

Correspondence and requests for materials should be addressed to Y.M. or S.H.