UBL3 modification influences protein sorting to small extracellular vesicles

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Exosomes, a type of small extracellular vesicles (sEVs), derived from multivesicular bodies (MVBs), mediate cell-to-cell communication by transporting proteins, mRNAs, and miRNAs. However, the molecular mechanism by which proteins are sorted to sEVs is not fully understood. Here, we report that ubiquitin-like 3 (UBL3)/membrane-anchored Ub-fold protein (MUB) acts as a posttranslational modification (PTM) factor that regulates protein sorting to sEVs. We find that UBL3 modification is indispensable for sorting of UBL3 to MVBs and sEVs. We also observe a 60% reduction of total protein levels in sEVs purified from Ubl3 knockout mice compared with those from wild-type mice. By performing proteomics analysis, we find 1241 UBL3-interacting proteins, including Ras. We also show that UBL3 directly modifies Ras and oncogenic RasG12V mutant, and that UBL3 expression enhances sorting of RasG12V to sEVs via UBL3 modification. Collectively, these results indicate that PTM by UBL3 influences the sorting of proteins to sEVs.

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EVs are nanometre-sized vesicles secreted from various cell types. Exosomes, a type of sEVs, derived from multicellular bodies (MVBs), mediate cell-to-cell communication by transporting proteins, mRNAs, and miRNAs. The delivery of proteins between cells by sEVs, including exosomes, is related to tumour progression and neurodegenerative diseases. Furthermore, neurodegenerative disease-related proteins such as amyloid beta, tau, α-synuclein, and prions are also packaged inside sEVs, and spread in the brain. The delivery of proteins between cells by sEVs, including exosomes, is a conserved UBLs, UBL3/MUB protein has been identified in Arabidopsis thaliana and is a membrane protein localised by prenylation. However, the role of UBL3/MUB as a PTM factor remains poorly understood.

In this study, we provide evidence that UBL3/MUB acts as a PTM factor that regulates protein sorting to sEVs.

Results

Analysis of UBL3 as a post-translational modification factor. In the present work, to identify PTM factors, we used a bioinformatics method. We extracted UBL3/MUB (Supplementary Fig. 1), which is known to contain a ubiquitin-like (UBL) domain and is an evolutionarily conserved membrane protein localised by prenylation in animals, filamentous fungi, and plants. However, the role of UBL3 as a PTM factor remains poorly understood. To clarify the role of UBL3 as a PTM factor, we expressed Flag-UBL3 in MDA-MB-231 breast cancer cells and purified UBL3 proteins by immunoprecipitation, followed by western blotting with UBL3 antiserum. The estimated molecular weight of Flag-UBL3 is 16 kDa. Interestingly, in the Flag-UBL3 expressing cells, the UBL3 signal was observed as a smear band up to a high molecular weight only under non-reducing conditions. The smear signal disappeared after the addition of 2-mercaptoethanol to the samples, before loading onto an SDS-polyacrylamide gel. To verify whether UBL3 modification occurs in vivo, we established Ubl3 knockout (KO) mice and analysed PTM in brain lysates, as the expression of UBL3 in the brain is relatively high. We observed endogenous UBL3-related PTM in lysates from the cerebral cortex, and showed that the degree of PTM was reduced in Ubl3 KO mice. We next investigated UBL3 modification using UBL3 mutants; we focused on all cysteine residues as UBL3 modification is dependent on non-reducing conditions. UBL3 has only two cysteine residues at its C-terminus (C113 and C114); therefore we constructed three UBL3 mutants—C113A, C114A, and C113/114A (Fig. 1c)—and studied these along with the wild-type UBL3. In the UBL3C113/114A mutant, UBL3 modification was completely abolished not only in MDA-MB-231 cells but also in every cell line examined (Fig. 1d).

UBL3 modification is indispensable for sorting of UBL3 to MVBs and sEVs. We next examined subcellular localisation of UBL3 by immunocytochemistry, as the degree of UBL3 modification was found to be reduced in the cytoplasmic fraction. Following the introduction of EGFP-UBL3, colocalisation with various organelles was studied. More than 40% of the total EGFP-UBL3 signal was found to be colocalised with endogenous CD63, a marker for MVBs, supporting that UBL3 is enriched in MVBs. UBL3 did not show colocalisation with the markers for recycling endosome (Rab11), mitochondria (COXIV), endoplasmic reticulum (Calnexin), Golgi (GM130), peroxisome (PMP70), or nuclear membrane (Lamin B1). To clarify the localisation of UBL3 in MVBs and the plasma membrane, we performed immunoelectron microscopic analysis. Unlike wild-type UBL3, the mutants did not show colocalisation with CD63 (median values: UBL3, UBL3C113A, UBL3C114A, UBL3C113/114A, and UBL3Δ1 with endogenous CD63). To clarify the localisation of UBL3 in MVBs and the plasma membrane, we performed immunoelectron microscopic analysis (Fig. 2d, e). When Flag-UBL3 was introduced to MDA-MB-231 cells, the UBL3 signal was clearly detected in the plasma membrane, but not in mitochondria or the nuclear membrane. UBL3Δ1 was localised in the plasma membrane but not in MVBs.

These results indicate that membrane localisation is not sufficient and that UBL3 modification is required for MVB localisation. One fate of MVBs is to fuse with the plasma membrane for release as exosomes. The other fate is to fuse with lysosomes, which degrade the MVB contents. In the present study, we focused on the extracellular secretion of UBL3, as this protein colocalises with MVBs to a degree greater than with lysosomes. Cells release extracellular vesicles of various sizes. These vesicles, some of which are derived from the plasma membrane, include large- and medium-sized EVs purified at 2000 x g and 10,000 x g, respectively. sEVs are purified at 100,000 x g and are derived in part from the plasma membrane (non-exosomal sEVs) or are derived from MVBs (exosomes). We have purified EVs from 2K-, 10K-, and 100K-cenrifuged pellets from MDA-MB-231 cells and found that...
UBL3 was more concentrated in the 100K fraction than in the 2K and 10K fractions (Fig. 3a). We verified that sEVs purified from the 100K pellet contained nanometre-sized vesicles ranging from 50 to 100 nm in diameter by negative EM staining (Supplementary Fig. 5a). We found that the majority of UBL3 was present in sEVs containing CD63 (Supplementary Fig. 5b). Additionally, we found that UBL3 was packaged within the sEVs (Supplementary Fig. 5c). In order to determine whether endogenous UBL3 was released in sEVs, we prepared primary cultured cells from wild-type and Ubl3 KO mice, purified sEVs from the

Fig. 1 Analysis of UBL3 as a post-translational modification factor. a UBL3-dependent posttranslational modification was detected by immunoprecipitation (IP) with anti-Flag antibodies from MDA-MB-231 cells transfected with Flag-UBL3, followed by western blotting with UBL3 antiserum (right panel). b The tissue extracts from the cerebral cortex of WT and Ubl3 KO mice were immunoprecipitated with anti-UBL3 antibodies. c, f Schematic structures of Flag-tagged wild-type or mutant UBL3. d, g Detection of UBL3 modification in these cells. IP products were boiled without 2-mercaptoethanol (βME−). A portion of the samples was treated with 2-mercaptoethanol (βME+). e, h Subcellular localisation of UBL3 in MDA-MB-231 cells transfected with Flag-UBL3 (wild-type and mutants). Twenty μg per lane
culture supernatant, and blotted them using anti-UBL3 antibodies. Endogenous UBL3 was only found in sEVs from wild-type mice (Supplementary Fig. 5d). Finally, UBL3 modification was observed not only in cell lysates but also in sEVs (Supplementary Fig. 5e). In order to clarify that UBL3 modification is indispensable for sorting to sEVs, the presence of UBL3 and its mutants in 100K sEV was studied. Among these, UBL3 cysteine mutants were not sorted to sEV fractions. On the other hand, UBL3Δ1 was detected at much reduced levels compared with those of the wild-type UBL3 (Fig. 3b). Purified 100K pellets
Fig. 2 The localisation of UBL3 to MVBs depends on UBL3 modification. a Representative projected images of MDA-MB-231 cells transfected with EGFP-UBL3 and co-stained with markers for MVB (CD63, n = 10), early endosome (EEA1, n = 10), lysosome (LysoTracker, n = 10), or recycling endosome (Rab11, n = 10). The regions in the dotted box are shown as a single confocal image in the inset. Scale bars, 10 and 1 μm. b Quantitative analysis of EGFP-UBL3 fluorescence intensity in a. *p < 0.05; **p < 0.001 by Kruskal-Wallis/Dunn’s multiple-comparisons test. c Representative images of MDA-MB-231 cells transfected with EGFP-UBL3 (wild-type, n = 5; mutants, n = 5) and co-stained with CD63 values shown as % of total UBL3. Scale bars, 10 and 1 μm. d Immune-EM images of wild-type UBL3 and UBL3Δ1 in MDA-MB-231 cells. Scale bars, 500 nm (left and right panels) and 200 nm (middle panel). e Quantification of the numbers of gold colloids per area in d. MVB, n = 10; Plasma membrane, n = 10. *, p < 0.05; ***, p < 0.001 by two-tailed Student’s and Welch’s t-tests.

Fig. 3 The level of total proteins in the sEVs is reduced in Ubl3 knockout mice. a The cell lysate (CL) and pellets from the conditioned medium of MDA-MB-231 cells transfected with 3xFlag-UBL3 vectors were blotted with various antibodies. b The presence of UBL3 and its mutants in sEVs. c Electron microscopic analyses of purified sEVs by negative staining. Scale bars, 100 nm. d Upper left panel, protein staining for the sEVs from the serum in WT and Ubl3 KO mice. Lower panels, purified serum sEVs were blotted with anti-tubulin and CD9 antibodies with βME. Right panel, relative intensity of total proteins in serum sEVs. n = 9 pairs. e Total RNA levels in the serum sEVs. WT, n = 5; KO, n = 5. n.s., p > 0.05 by Mann-Whitney test. a, b, βME + condition: Flag, Flotillin-1, GP96, Actinin-4, Calreticulin, GAPDH, and Alix antibodies. βME- condition: CD63, and CD9 antibodies.
contained both exosomes and non-exosomal sEVs. The latter include vesicles directly secreted from the plasma membrane\(^1\), this explains why UBL3A1, which shows plasma membrane but not MVB localisation, was detected in the 100K pellets in small amounts (Figs. 1h, 2d, e, 3b). This finding indicates that the UBL3 modification is important for sorting of UBL3 to sEVs. Furthermore, when exosome release was inhibited by Rab27a siRNA\(^2\), the levels of UBL3 modification products were increased (Supplementary Fig. 5f). The above results indicate that UBL3 modification is required for localisation of UBL3 in MVBs, and that the majority of UBL3 is secreted in sEVs originating from the fusion of MVBs released as exosomes. As UBL3 was localised in the plasma membrane, UBL3 modification may also influence non-exosomal sEVs.

**The level of total proteins in the sEVs is reduced in UBL3 knockout mice.** To elucidate the functional role of UBL3 in the sorting of proteins to sEVs, we quantified the protein contents of purified sEVs derived from Ubl3-KO mouse sera. The genotype had no effect on the concentration of sEV particles or the average vesicular diameter (Fig. 3c, Supplementary Fig. 6a, b). Intriguingly, the total level of sEV proteins in the sera from the Ubl3 KO mice was 60% lower than that from the wild-type mice, although the total protein content in the serum and plasma were not different (Fig. 3d, Supplementary Fig. 6c). We did not find any significant difference in the RNA profiles and RNA levels (Fig. 3e and Supplementary Fig. 6d, e). Specific sEV-enriched miRNAs levels were not statistically different (Supplementary Fig. 6f). These results indicate that the Ubl3 KO mice produce sEVs with reduced proteins, but with normal levels of miRNAs in the serum. In other words, the sorting of proteins in the serum sEVs is influenced by UBL3.

**UBL3 modification influences the sorting of proteins to the sEV.** For a better understanding of the physiological function of UBL3 modification, we performed comprehensive proteomics analysis to identify proteins that interacted with UBL3 in a manner dependent on the two C-terminal cysteine residues. Lysates prepared from 3xFlag-UBL3 expressing in MDA-MB-231 cells were purified with Flag antibody-beads, subjected to on-bead digestion, and analysed by nanoflow liquid chromatography tandem mass spectrometry (LC-MS/MS) gradients on a Q Exactive HF mass spectrometer (Fig. 4a). The dataset covered 3882 proteins in total at a false discovery rate (FDR) of 1%. The average of HF mass spectrometer (Fig.4a). The dataset covered 3882 proteins in total at a false discovery rate (FDR) of 1%. The average of Pearson correlation coefficients was 0.988 within triplicate pull-downs. Label-free quantification (LFQ)-based unsupervised hierarchical clustering of significantly regulated proteins (ANOVA, FDR = 0.05 & S0 = 1) revealed 1447 possible UBL3-interacting proteins that were upregulated in Flag-UBL3 compared to Flag-UBL3C113/114A or the Flag empty vector (Fig. 4a and Supplementary Table 1). Next, we grouped the UBL3-interacting proteins based on their subcellular localisation as defined by the Gene Ontology Cellular Components (GOCC). We found that 31% of proteins (454 out of 1447 proteins; \(p\)-value of 0.00405, Fisher exact test) were categorised as the GOCC term ‘extracellular vesicular exosome’ (Fig. 4b, and Supplementary Data file). In addition, the pairwise comparison of UBL3 with UBL3C113/114A revealed that 1241 proteins significantly interact with UBL3, emphasising the role of cysteine residues in UBL3 modification (two-sample Student’s \(t\)-test, FDR = 0.05 & S0 = 1). Of these proteins, 29% (369) of them were also annotated as extracellular vesicular exosome. Moreover, Fisher exact test for the statistically significant proteins showed an enrichment for the annotation of extracellular vesicular exosome (\(p\)-value of 0.00828). Among these, tubulins (TUBA1A, 1B, 1C, and 4A; and TUBB, B2A, B3, B4A, B4B, B6, and B8) were found in 3xFlag-UBL3 samples (Fig. 4c, Supplementary Fig. 7a and Supplementary Data file). Tubulins are reported to be present in sEVs and other EVs\(^{21,24}\). Endogenous tubulin alpha can be detected in sEVs with the commonly used specific antibody (DM1A); therefore we chose tubulin alpha as a model case to study sorting of endogenous proteins to sEVs via UBL3 modification. When EGFP-UBL3 introduced to MDA-MB-231 cells was immunopurified from anti-GFP-beads, tubulin was found to be modified by UBL3 and observed as shifted bands only under non-reducing conditions (Supplementary Fig. 7b). We then studied the sorting of tubulin by UBL3 in secreted vesicles prepared by differential centrifugation. Intriguingly, the tubulin signal was specifically augmented by UBL3 expression in the 100K, but not in the 2K or 10K fractions (Supplementary Fig. 7c). Under non-reducing conditions, the majority of tubulin in the 100K sEV pellet was found to have a higher molecular weight than under reducing conditions. We used UBL3 mutants to investigate the relationship between UBL3 modification of tubulin and the sorting to sEVs. The increase in tubulin in the sEVs was specific to wild-type UBL3; the UBL3C113/114A and UBL3A1 mutants exhibited no such effects (Supplementary Fig. 7d). We also observed that the protein levels of tubulin in the serum sEVs were reduced in Ubl3 KO mice (Fig. 3d). We therefore conclude that UBL3 modifies endogenous tubulin posttranslationally and regulates its sorting to sEVs.

We also found that at least 22 disease-related molecules were included as UBL3-interacting proteins (Fig. 4c and Supplementary Fig. 8a). Intriguingly, some of these molecules are related to oncogenesis and tumour progression/metastasis, namely, HRAS, KRAS, TGFBR1, TGFBR2, RB1, ITGA6, ITGB4, mTOR, TSC2 and APLP2. In addition, immune-responsive molecules (IRF3 and IKBKG), mTOR signalling molecules (mTOR, RPTOR and TSC2), Notch signalling molecules (NOTCH1, NOTCH2, NOTCH3), BMP signalling molecules (BMPR1A and BMPR2), and even molecules involved in neurodegenerative/neuronal diseases (PSEN1, ATXN10, HIP1R, APLP2 and NPC1) are identified as UBL-interacting proteins. Among these, Ras family members are proto-oncogenes\(^25\) and have been reported to be enriched in sEVs\(^26\). Therefore, we chose H-Ras as a model protein to study the sorting to sEVs via UBL3 modification. We found that exogenous wild-type Ras and constitutively active oncogenic RasG12V mutant\(^25\) interacted with UBL3, and were observed as shifted bands only under non-reducing conditions (Supplementary Fig. 8b). Moreover, exogenous wild-type Ras proteins were more sorted to sEV by UBL3 (Supplementary Fig. 8c). We also found that wild-type UBL3 but not UBL3C113/114A enhanced sorting of RasG12V to sEVs (Fig. 4d).

To examine whether sEVs encapsulated with UBL3 and oncogenic RasG12V caused the activation of Ras signalling in the recipient cells, we added PKH67-labelled sEV purified from MDA-MB-231 cells that were transfected with RasG12V and either mock, 3xFlag-UBL3 or 3xFlag-UBL3C113/114A to recipient MDA-MB-231 cells. As an indicator of Ras activation in the recipient cells, we measured the phosphorylation level of ERK, which is a downstream signalling molecule\(^25\). Phosphorylated ERK (pERK) activity in PKH67-labelled sEV-incorporated cells was examined by antibody staining. We found that purified sEVs from MDA-MB-231 cells transfected with wild-type UBL3 and RasG12V significantly enhanced the level of pERK in the recipient cells compared to sEVs from UBL3C113/114A and RasG12V transfected cells (Fig. 4e, f). These results indicated that increased sorting of RasG12V to sEVs by UBL3 modification enhanced activation of Ras signalling in the recipient cells.

We also performed a comparative analysis using other UBLs. Unlike UBL3, other UBLs, including ubiquitin, SUMO1, and SUMO2, were not enriched either in MVBS or sEVs (Fig. 5a-c).

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Finally, we attempted to determine whether UBL3 serves as a useful tag for protein delivery to sEVs. When EGFP or biotinylated-protein tagged with UBL3 was introduced to MDA-MB-231 cells, EGFP-UBL3 and biotinylated-UBL3 were found in sEVs (Fig. 6). These observations confirmed that UBL3 serves as a tag for the delivery of proteins to sEVs.

Discussion

In the current study, we characterised UBL3 as a PTM factor. The glycine residues at the C-termini of ubiquitin, SUMO, and Nedd8 are covalently attached to lysine residues in target proteins in general. In contrast, our data suggest that UBL3 modifies target proteins by disulfide bonding through cysteine residues at its C-terminus; therefore although UBL3 has a ubiquitin-like domain, UBL3 modification is found to be completely different from conventional ubiquitin and ubiquitin-like modifications. From the result of UBL3Δ1 mutant (Fig. 1g), the membrane localisation of UBL3 alone is not sufficient for the UBL3 modification. Thus, in addition to CAAX motif for the membrane localisation in the C-terminal of UBL3, it is possible that...
**Fig. 4** UBL3 modification influences the sorting of proteins to the sEV. **a, b** Heat map of z-scored LFQ intensities of the significantly regulated proteins (ANOVA, FDR = 0.05 & S0 = 1) in all three conditions (3xFlag-UBL3, 3xFlag-UBL3C113/114A or 3xFlag empty vector) revealed the UBL3 interacting proteins (Cluster 1). The colour key denotes normalised protein abundances (z-score). Profiles of all proteins (1447) found in Cluster 1 are shown and 454 of those (31%) with ‘extracellular vesicular exosome’ annotations are highlighted in dark blue. **c** Volcano plots showing the p-values vs. the log2 protein abundance differences in Flag-UBL3 compared with either Flag-UBL3C113/114A or Flag empty vector. The significance cut-off is based on an FDR = 0.05 and S0 = 1. Disease-related molecules, black colour. HRAS and KRAS, red colour. TUBA1A, 1B, 1C, and 4A, orange colour. TUBB, B2A, B3, B4A, B4B, B6, and B8, blue colour. d sEV pellets were blotted with anti-Ras antibodies. e Images of phosphorylated ERK (pERK) in PKH67-labelled sEV-incorporated MDA-MB-231 cells. Purified sEVs from the conditioned medium of MDA-MB-231 cells transfected with RasG12V and either mock, 3xFlag-UBL3, or 3xFlag-UBL3C113/114A or with mock and 3xFlag-UBL3 were labelled with PKH67 dye (green) and added to MDA-MB-231 cells. Scale bars, 10 μm. f Each plot shows pERK fluorescence in PKH67-labelled sEV-incorporated cells normalised to the average pERK values in the two neighbouring PKH67-labelled sEV-unincorporated cells from each image in e. RasG12V-mock, n = 18; RasG12V-UBL3, n = 19; RasG12V-UBL3C113/114A, n = 19; Mock-UBL3, n = 21. ***, p < 0.001 by Kruskal-Wallis/Dunn’s multiple-comparisons test.

**Fig. 5** Subcellular localisation and sorting to sEV of UBLs. **a** Representative images of MDA-MB-231 cells transfected either with EGFP-ubiquitin, -SUMO1, -SUMO2 or -UBL3 and co-stained with markers for MVBs (CD63). Scale bars, 10 and 1 μm. **b** Quantitative analysis of EGFP-ubiquitin, -SUMO1, -SUMO2, and -UBL3 fluorescence intensity in a. EGFP-ubiquitin, n = 10; EGFP-SUMO1, n = 10; EGFP-SUMO2, n = 10; EGFP-UBL3, n = 10; EGFP, n = 10. *, p < 0.05; ***, p < 0.0001 by Kruskal-Wallis/Dunn’s multiple-comparisons test. **c** EGFP-UBL3, but not EGFP, EGFP-ubiquitin, EGFP-SUMO1, or EGFP-SUMO2, was preferentially enriched in the sEVs of the cell culture media. Before sample loading, the samples were boiled with βME.
important amino acid sequences for the UBL3 modification in the C-terminal region exist. In the future UBL3 study, it is important to analyse molecules that recognise the C-terminal sequence for UBL3 modification.

Additionally, we report that UBL3 modification is essential for the sorting of UBL3 to MVs and sEVs. We identify 1214 UBL3-modified proteins in the sEVs of the cell culture media. After the western blot analysis, gels were stained with SYPRO Ruby (lower panel). Before loading, the samples were boiled with βME. The same amounts of protein were loaded on the gels (Cell Lysate, 20 μg per lane; sEVs, 1 μg per lane).

**Methods**

**Cell culture.** MDA-MB-231-luc-D3H2LN breast cancer cells (Xenogen Co, CA), HEK293T cells (RIKEN Cell Bank, Japan), and HeLa cells (RIKEN Cell Bank, Japan) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (GIBCO, 11875-093) or Dulbecco’s modified Eagle’s medium (DMEM, GIBCO, 11965-092) with PS (100 units of penicillin G per mL, 10 μg of streptomycin sulphate per mL) and 10% foetal bovine serum (FBS). Cultures were incubated in a 5% CO2 at 37 °C. All the cell lines were routinely tested for mycoplasma contamination (Biological Industries, EZ-PCR Mycoplasma Test kit).

Following previous papers, we prepared primary cells from the adipose tissue-derived stromal vascular fraction (SVF) and myoblasts from wild-type and Ubl3 KO mice. Briefly, to prepare primary SVF cells, inguinal and visceral adipose tissues were excised from mice and digested with 3.33 mg mL−1 type II collagenase (Nitta-gelatin, type L) with gentle rocking in Hank’s balanced salt solution for 60 min at 37 °C. After sequential filtration through 40- and 100-μm filters (BD), the isolated SVF cells were suspended in FBS and cultured in DMEM (GIBCO, 11965-092) with 10% FBS and PS. Myoblasts were cultured using the following growth medium in a culture dish coated with collagen type I (IWAKI, 44020-10) for 3–5 days; Ham’s F-10 Nutrient Mix (GIBCO, 11350-043) containing 20% FBS, 2.5 ng mL−1 bgFGF (Katayama Chemical Industries, 161-010-5), β-glutamine, penicillin, and streptomycin (Thermo Fisher Scientific, PS-glutamine). For myotube differentiation, the medium was changed to a differentiation medium (5% horse serum in DMEM [GIBCO, 11995-065]) with PS-glutamine for 5 days.

**Plasmid construction.** Various expression vectors were subcloned either to pcDNA3 (Invitrogen), pEGFP (Clontech), or pcDNA6/BioErase (Invitrogen) using conventional molecular biology techniques and PCR. The sequence of Rab27a shRNA (5'-GCTGCCAATGGGACAAACATA-3') was used and inserted into pcDNA 6.2-GW/miR vector (Invitrogen).

In the Ras experiments, we used H-Ras expression vector set (Clontech, pCMV-Ras vector, and pCMV-RasV12 vectors). GFP-ubiquitin was a gift from Nico Dantuma (Addgene plasmid # 11928).

**Establishment of Ubl3−/− (knockout; KO) mice.** The targeting strategy is illustrated in Supplementary Fig. 2a. The targeting construct was linearised by SpeI digestion. CCE (129/SvEv) (a gift from Dr. E. Robertson) embryonic stem (ES) cells were electroporated using a SpeI-linearised targeting vector and selected in G418. Heterozygous ES cells were injected into the blastocysts of C57BL/6 strain mice to generate germ-line chimeras. Chimeric mice were backcrossed with C57BL/6 mice for 10 generations. Genotypes were determined by Southern blotting or by PCR using the following three primers: Ubl3-KO-3', 5’-ACCA GGTCTCTATGCTAGTCTAGA-3’; Ubl3-KO-5', 5’-CCACCTACGTCCCTTC CAGAACC-3’. Ubl3-KO-Neo, 5’-GCTGAAGGCGTCTGCGTGTTG-3’. All procedures related to the care and treatments of animals were in accordance with institutional and National Institutes of Health guidelines and were approved by the Animal Care and Use Committee at Fujita Health University.

**Antibodies.** To obtain the rabbit anti-UBL3 antibody, 1.5 mg of glutathione S-transferase (GST)-fused mouse UBL3 protein expressed in bacteria was injected four times each into nine rabbits. Recombinant, exogenous, and endogenous UBL3 proteins were specifically recognised in only one rabbit’s serum. The serum was passed through the GST column three times to remove non-specific antibodies. We called the resulting flow-through (FT) the UBL3 antisera. To obtain a more specific antibody for the UBL3 protein, a polyclonal UBL3 antisera was passed through the GST column. The resultant FT was further passed through a column conjugated with a brain lysate from Ubl3 KO mice to exclude the recognition of other UBL-UBL3 column. We called this eluate the anti-UBL3 antibody. The antibodies used in the experiments were anti-GAPDH (Cell Signalling, 14C10, 1:1000), Flag-M2 (Sigma, F3165, 1:1000), Flag-T7 (BD, Clone 18, 1:250), C6D6 (Invitrogen, Ts63, 1:1000 for WB, 1:5000 for IP), EAA1 (BD, 610862, 1:5000), Rab11 (BD, 610555, 1:150), COXIV (Cell Signalling, 4850, 1:1000), Calnexin (Enzo, ADI-SPA-860, 1:500), GM130 (BD, 610822, 1:100), PMP70 (Thermo, PAI-650, 1:5000), Lamin B1 (Abcam, ab16048, 1:800), GP96 (Enzo, 9G10, 1:500), alpha Actinin-4 (Genetex, C2C3, 1:250).
Calreticulin (Cell Signalling, 2891, 1:500), Aβx (Abnova, H00010015-B02, 1:300), CD9 (Affymetrix, 14-0891, 1:500; Invitrogen, Ti9, 1:500), alpha-Tubulin (Sigma, DMA1A, 1:2000; anti-ubiquitin, anti-ERK (MBL, 598, 1:1000; Thermo, A1122, 1:1000); pan-Ras (Cell Signalling, 3339, 1:1000; Millipore, MABS195, 1:1000), Phospho-ERK (Cell Signalling, 4370, 1:200), horse radish peroxidase (HRP)-conjugated streptavidin (Invitrogen, 19534-050, 1:5000), Alexa 488- or 555-conjugated antibodies (Thermo), and HRP-conjugated secondary antibody (eliScience, Cell Signalling, and Abcam)). In a differential centrifugation experiment, we used the antibody combinations against the following markers: sEV-markers, CD36 and CD9; multiple EV marker, CD63; and secondary antibodies for 1 h; and mounted with Aqua-Poly/Mount (Polysciences). Lysosomes were labelled with 50 μM LysoTracker Red (Thermo) for 1 h at 37°C.

Confocal images were acquired with a 63X objective lens (NA 1.4) on a confocal laser microscope (Carl Zeiss, LSM780-DUO-NLO, ZEISS 2012 SP1 Ver. 8. 1. 0. 0). Representative images of each sample were presented in Supplementary Fig 9. Images were analysed by using Fiji (Image J); the values of pERK signal in PKH67-labelled sEV-incorporated cells were presented as the mean signal intensity measured over an area of 2.5–5 μm² in a region of the cytoplasm adjacent to the nucleus, and then normalised to the signal to the average values of pERK in the two neighbouring PKH67-labelled sEV-unincorporated cells, in each image.

Immunoelectron microscopy. 3xFlag-UBL3 (wild-type or UBL3ΔΔ) was mock-transfected MDA-MB-231 cells were fixed with 4% PFA/PBS for 30 min at 4°C, and subsequently fixed with 4% PFA and 0.1% glutaraldehyde (GA)/PBS for 30 min at 4°C. After being washed with PBS, the cells were fixed with 1% osmium tetroxide/PBS for 30 min, dehydrated through a graded ethanol series, transferred to n-butyl glycidyl ether (QY-1), and embedded in epoxy resin (TAAB, EPON812). Ultrathin sections were cut on an ultramicrotome (Reichert-Nissei, Ultracut N) at 100-nm thickness, placed on a nickel grid (VECO, 2352), etched with 5% sodium metaperiodate for 10 min, and washed with PBS. The sections were incubated with anti-Flag antibodies (1:100) for 2 h. After washing with PBS, the sections were incubated with 15 nm gold-conjugated goat-antimouse IgG antibodies (Cyto-diagnostics, 1:50) for 1 h. After being washed with PBS, the sections were fixed in 2.5% GA/PBS for 10 min and washed with Milli-Q water to stabilise the antigen-antibody binding. The immunostained sections were further stained with uranyl acetate for 30 min and lead citrate for 1 min, and then observed under a transmission electron microscope (Hitachi, H-7650, H-7650 control Ver. 01. 10, EMIP Ver. 05. 04). The number of gold particles per unit area in the MVBS, plasma membrane, mitochondrion, and nuclear membrane were counted.

Electron microscope analysis for sEVs. Ultracomic analysis was performed following the conventional protocol with slight modification. Briefly, the sEVs isolated by the differential ultracentrifugation protocol from the conditioned medium with or without 3xFlag-UBL3 were fixed with 2% paraformaldehyde and subsequently deposited on electron microscopy (EM) grids (Nissin EM, Excel Support Film). The sEVs on the EM grids were washed with PBS, further fixed for 5 min with 1% GA/0.1M phosphate buffer, and washed eight times every 2 min with ultrapure water. The EM grid was negatively stained with 10% EM stain (Nissin EM) and analysed using a transmission electron microscope.

Isolation of EVs and RNA analyses. sEVs were isolated from a conditioned medium according to the conventional protocol with slight modification. Briefly, exosome-depleted PBS (centrifuged for 16 h at 100,000 × g) was used to prepare the conditioned medium. After incubation for 24 h, the culture medium (approximately 90 mL from 3 × 10⁶ cells) was collected and centrifuged at 300 g × 10 min at 4°C. The supernatant was centrifuged at 300,000 × g for 30 min at 4°C. The supernatant was centrifuged again at 100,000 × g for 30 min at 4°C. The resulting supernatant was centrifuged again at 100,000 × g for 30 min at 4°C. The resulting pellet was washed with 1 mL of PBS and collected using ultracentrifugation at 100,000 × g for 30 min at 4°C. The supernatant was centrifuged again at 100,000 × g for 30 min at 4°C. The resulting pellet was washed with 1 mL of PBS and collected by ultracentrifugation at 100,000 × g for 60 min at 4°C, then resuspended in PBS. For the purification of the 2K, 10K and 100K pellets, a differential ultracentrifugation protocol was used. Briefly, exosome-depleted PBS was used to prepare the conditioned medium. After incubation for 24 h, the culture medium (approximately 90 mL from 3 × 10⁶ cells) was collected and centrifuged at 300 g × 10 min at 4°C. The supernatant was centrifuged again at 300,000 × g for 30 min at 4°C. The supernatant was centrifuged again at 1,000,000 × g for 30 min at 4°C. The supernatant was centrifuged again at 100,000 × g for 30 min at 4°C. After washing the supernatant, the pellets were incubated in 150 mM NaCl. CDs were then dewashed four times with 1% Triton-X-100, 1% NP-40, 1% SDS. For pre-clearing, the cell lysates were incubated with 40 μL of Protein G-sepharose beads for 1 h at 4°C with rotation. The resulting supernatants were incubated with 40 μL of Protein G-sepharose beads for 1 h at 4°C with rotation. The method for the immune-isolation of sEVs using anti-CD9 or CD63 antibodies was referred to from Kowal J et al.22. Briefly, before performing the immune-isolation, anti-CD9 (Millipore, MM2/57, 1:10), anti-CD63 (BD, H5C6, 1:50) and control mouse polyclonal IgG (Millipore, D21-271, 1:100) incubated with 100 μL of protein A magnetic beads (Thermo, R88845) for 16 h at 4°C with rotation. The resulting protein A magnetic beads were washed three times with ice-cold 0.1% Tween/PBS, resuspended in 500 μL of ice-cold 0.01% Tween/PBS. Two μL purified sEVs were added to the magnetic beads and incubated for 1 h at 4°C with rotation. The bead-bound sEVs were collected and washed three time in 500 μL of ice-cold 0.01% Tween/PBS. For the detection of UBL3 inside the sEVs, sEVs were purified from the media of biotinylated-tagged UBL3-transfected MDA-MB-231 cells, washed with PBS or SDS buffer (1% SDS, 20% glycerol, and 0.01% bromophenol blue, without 2-mercaptoethanol) was added to the beads and the beads were boiled for 3 min (βME –). A portion of the samples was treated with 2-mercaptoethanol and boiled for 3 min (βME+). These samples were botted with UBL3 antisense (1:100 dilution). For the detection of endogenous UBL3 modification, 50 μg of frozen tissue was pulsed (BMS, ShakeMan2) and homogenised with RIPA buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS). For pre-clearing, the cell lysates were incubated with 40 μL of Protein G-sepharose beads for 1 h at 4°C with rotation. The resulting supernatants were incubated with biotinylated-ubiquitin antibody (1:1000 dilution) in 20 μL of Protein G-sepharose beads for 1 h at 4°C with rotation.
100,000 × g (Beckman, TLA-110 rotor) for 60 min at 4 °C. The 100K pellet was then resuspended in PBS, centrifuged at 100,000 × g for 10 min at 4 °C, and then resuspended in PBS. The resulting centrifugation was performed before the purification of the 100K pellet. For the mouse serum, after collection, whole blood was allowed to clot by leaving it undisturbed for 30 min at RT. To remove the clot, the whole blood was centrifuged twice at 8,000 × g for 10 min. For the preparation of mouse plasma, after collection, the whole blood was added to a heparin solution (Mochida Pharmaceutical, heparin Na 5000 units per 5 mL), rotated slowly, centrifuged at 9100 × g for 5 min, and the supernatant was used as the plasma.

In each experiment, the volume of serum from the U373 KO mice was matched to that of its wild-type littermates (approximately 350–500 μL). The harvested serum was immediately diluted with an equal volume of PBS and centrifuged at 2000 × g for 30 min at 4 °C. The supernatant was centrifuged at 12,000 × g for 45 min at 4 °C. The supernatant was diluted fivefold with PBS and then filtered through a 0.22-μm filter. The sample was then centrifuged at 110,000 × g (Beckman, SW55Ti rotor) for 70 min at 4 °C. The nEV pellet was washed with 1 mL of PBS and ultra-centrifuged at 100,000 × g (Beckman, TLA-110 rotor) for 60 min at 4 °C, and resuspended in PBS. To quantify the total RNA amounts of serum sEVs, RNA was purified from the whole serum sEVs from each mouse using the mirNeasy Kit (QIAGEN). The purified RNA solution was measured by a Bioanalyzer 2100 system (Agilent, 2100 Expert Ver. B 02. 08. 91648, RNA 6000 Pico kit). For real-time qPCR analysis, the purified RNA solution (40%) was reverse-transcribed into complementary DNA (cDNA), and 10% of the resulting cDNA solution for each miRNA was used in real-time qPCR analysis.

Protein concentrations of the sera, plasma, and cell lysates were measured by BCA (Thermo). To quantify the protein concentration of the 2K, 10K, and 100K pellets, each pellicle was diluted with 2% SDS and measured by Micro-BCA (Thermo).

Measuring total protein amount in the serum sEVs. Ten percent of the purified serum sEVs from each experiment was boiled with βME containing an SDS sample buffer and separated by SDS-polyacrylamide gel electrophoresis. The resulting gel was fixed in 50% methanol and 10% acetic acid for 30 min and washed in 10% methanol and 7% acetic acid for 30 min. The gel was stained in SYPRO Ruby Gel Stain (Life Technologies) in the dark. The gel was washed in 10% methanol and 8% acetic acid for 30 min, and then washed in ultrapure water for 10 min. The gel was then scanned using a fluorescence scanner (GE Healthcare, Typhoon 9400, Typhoon Scanner Control Ver. 5.0) set at 532 nm. The total protein intensity was analysed using the Image software.

Proteomics analyses. MDA-MB-231 cells were transfected with plasmids using Lipofectamine 2000. After 24 h, cells were washed and collected by scraping into ice-cold 1% Triton buffer for 5 min at 4 °C, and lysed with 1% Triton buffer for 20 min at 4 °C. Crude nuclei and unbroken cells were excluded by centrifugation at 20,000 × g for 20 min at 4 °C. The supernatant was then scanned using a Gel Stain (Thermo) for 3 h in the dark. The gel was washed in 10% methanol and fixed in 50% methanol and 10% acetic acid for 30 min and washed in ultrapure water for 10 min. The gel was then scanned using a fluorescence scanner (GE Healthcare, Typhoon 9400, Typhoon Scanner Control Ver. 5.0) set at 532 nm. The total protein intensity was analysed using the Image software.

Measurement of sEVs by nanoparticle tracking analysis. Nanoparticle tracking analysis (NTA) was carried out using NanoSight LM100HS with a blue laser system (NanoSight, Amesbury) on isolated serum sEV particles diluted 25-fold with PBS37. The system focuses a laser beam through a suspension of the particles of interest. They were visualised by light scattering on a conventional optical microscope aligned perpendicularly to the beam axis, which collects light scattered from every particle in the field of view. A 60-s video records all events for further analysis by NTA software. The Brownian motion of each particle was tracked between frames to calculate its size using the Stokes–Einstein equation. Each sample was measured three times, and we used an averaged value for the statistical analysis.

Statistical analysis. Statistical analyses were carried out using Prism 6.03 (GraphPad Software), Microsoft Excel, and PERSEUS (Ver. 1.5.4.1). The Mann-Whitney test or Wilcoxon signed-rank test and Kruskal-Wallis/Dunn’s multiple-comparisons test were, respectively, applied for comparisons between 2 groups and 3 or more groups except proteomics analyses. We used more than two independent samples for each experiment. Horizontal bars indicate median values for each group.

Data availability. Proteomics raw datasets are deposited in jPOST (under accession codes JPS000315, PXDD007167).

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**Author Contributions**

H.A., T.O., M.M., M.S., and K.T. contributed to the study design. H.A., N.A.I., K. Hitachi, O.K., T.O., H.Y., T.K., K. Hatanaka., K.I., Y.Y., N.K., M.N., A.U., T.I., T.Y., H.S., and M.K. participated in data collection and interpretation. H.A., K. Hitachi, T. K., H.S., and K. Hatanaka cloned constructs. K.N. contributed new reagents/analytic tools. K. Hatanaka generated the anti-UBL3 antibody. H.A., M.S., and K.T. wrote the paper. All authors discussed and commented on the manuscript.

**Additional information**

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**Competing interests:** The authors declare no competing interests.

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