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Export of RNA-derived modified nucleosides by equilibrative nucleoside transporters defines the magnitude of autophagy response and Zika virus replication

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ABSTRACT

RNA contains a wide variety of posttranscriptional modifications covalently attached to its base or sugar group. These modified nucleosides are liberated from RNA molecules as the consequence of RNA catabolism and released into extracellular space, but the molecular mechanism of extracellular transport and its pathophysiological implications have been unclear. In the present study, we discovered that RNA-derived modified nucleosides are exported to extracellular space through equilibrative nucleoside transporters 1 and 2 (ENT1 and ENT2), with ENT1 showing higher preference for modified nucleosides than ENT2. Pharmacological inhibition or genetic deletion of ENT1 and ENT2 significantly attenuated export of modified nucleosides thereby resulting in their accumulation in cytosol. Using mutagenesis strategy, we identified an amino acid residue in ENT1 that is involved in the discrimination of unmodified and modified nucleosides. In ENTs-deficient cells, the elevated levels of intracellular modified nucleosides were closely associated with an induction of autophagy response as evidenced by increased LC3-II level. Importantly, we performed a screening of modified nucleosides capable of inducing autophagy and found that 1-methylguanosine (m^1G) was sufficient to induce LC3-II levels. Pathophysiologically, defective export of modified nucleosides drastically induced Zika virus replication in an autophagy-dependent manner. In addition, we also found that pharmacological inhibition of ENTs by dilazep significantly induced Zika virus replication. Collectively, our findings highlight RNA-derived modified nucleosides as important signaling modulators that activate autophagy response and indicate that defective export of these modified nucleoside can have profound consequences for pathophysiology.

Introduction

RNA contains a wide variety of modifications that are post-transcriptionally and enzymatically added to the base or sugar of nucleotides. To date, over 130 different types of modifications have been identified in various RNA species across all domains of life [1]. These modifications are important for maintaining fundamental RNA functions such as structural integrity, intracellular localization, and decoding efficiency [2,3]. Deficits in RNA modification have been implicated in diverse diseases including type 2 diabetes, myopathy, liver failure, and cancer [4–7]. Thus, RNA modifications are indispensable for homoeostatic regulation of human physiology as well as pathogenesis of various diseases via regulation of post-transcriptional gene expression and translation.

Like protein, RNAs are subject to catabolism as the consequence of RNA turnover or quality control processes [8–10]. RNA is broken down into single nucleosides via various degradation machinery including the exosome and lysosome [11–14]. Degradation of RNA generates a pool of unmodified adenosine, guanosine, uridine, and cytidine in the cells. Subsequently, these unmodified nucleosides are subject to several downstream pathways: they can be salvaged for the usage in RNA transcription as building blocks, metabolized to end products such as uric acid, or transported to the extracellular space [14]. Notably, extracellular adenosine can act as an active signaling molecule that binds to adenosine receptors and has a wide range of pathophysiological roles [15,16].

Because of the presence of abundant post-transcriptional modifications in RNA, intracellular RNA catabolism can yield various modified nucleosides [17]. Of note, most of these modified nucleosides are excluded from salvage pathways because of lack of specific kinases for the formation of 5′-nucleotide [18,19]. The modified nucleosides are also resistant to further breakdown. Consequently, these modified nucleosides are released to the extracellular space and are abundantly present in serum and urine of animals [20–22]. Extracellular modified nucleosides have been utilized as potential

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biomarkers for certain diseases such as cancer and AIDS [23,24]. Intriguingly, a recent study has also shown that extracellular modified adenosine can activate adenosine receptor with higher potency than unmodified adenosine [17]. However, how the RNA-derived modified nucleosides are transported to the extracellular space and why these modified nucleosides need to be transported remain unanswered in the field of RNA biology.

A family of specialized solute carrier nucleoside transporter proteins, also known as equilibrative nucleoside transporters (ENTs), are the transporters for unmodified nucleosides [25,26]. The ENT family contain four members in human, namely ENT1, ENT2, ENT3, ENT4. ENT1, ENT2, ENT3 are the transporters for purine and pyrimidine nucleosides and are ubiquitously distributed in mammalian tissues [25,26]. While ENT1 and ENT2 are localized to the plasma membrane [27], ENT3 is localized to the membrane of lysosome [28,29]. ENT4 is the last identified ENT family member and shows a low sequence identity to other ENTs [30]. Interestingly, ENT4 has been identified as a plasma membrane monoamine transporter because of its ability to uptake monoamine [31,32], indicating that the molecular function of ENT4 is distinct from the other three ENTs. Transport of nucleosides by ENT4 is selective to adenosine with its maximum efficiency being in an acidic environment [33]. The abilities of ENTs in the transport of nucleosides have contributed to fundamental cellular processes including DNA and RNA biosynthesis as well as intracellular signaling pathway related to cyclic nucleotide [34,35]. Consequently, ENTs have been implicated in many physiological functions such as regulation of anxiety-like behaviour, alcohol preference, inflammation, and bone formation [36–39].

Because modified nucleosides are the major product of RNA catabolism, we aimed to investigate whether ENTs are capable of transporting modified nucleosides. We employed pharmacological inhibition of ENTs activities and genetic deletion of ENTs in cells, followed by mass spectrometry-based quantification of modified nucleosides in the extracellular and intracellular milieus. We also investigated the intracellular signaling pathways potentially modulated by ENT-mediated nucleoside transport. We found that RNA-derived modified nucleosides were actively exported through ENT1 and ENT2, and that ENTs-deficiency progressively induced accumulation of intracellular modified nucleosides, which activated autophagy response through suppression of AKT-Beclin-1 signaling, leading to hyper-replication of Zika virus (ZIKV).

Results

Profiling of intracellular- and extracellular-modified nucleosides by mass spectrometry

To investigate the transport of nucleosides, we measured the intracellular and extracellular levels of modified nucleosides that are abundantly present in various cell lines derived from oral cancer (HOC313 and SAS), breast cancer (MDA231 and MCF7), and gastric cancer (NUGC3, HGC27, MKN45). The levels of unmodified nucleosides, adenosine (A), guanosine (G),

uridine (U), were abundant in cells when compared to the extracellular medium (Fig. 1A). In contrast, the levels of modified nucleosides (18 out of 20 modified nucleosides) were abundantly accumulated in the extracellular medium of all cell lines, except 7-methylguanosine (m^7G) and inosine (I). Inosine is abundantly present in mRNA and tRNA but can also be generated from deamination of free adenosine [1]. The intracellular accumulation of inosine thus reflects the complex metabolism of adenosine rather than RNA catabolism. The extracellular level of m^7G, which is a common post-transcriptional modification present in mRNA, tRNA, and rRNA, was detected at a very low level when compared to the intracellular level (Fig. 1A).

A principal component analysis of intracellular and extracellular modified nucleosides across different cell lines shows that the two first principal components (PC1 and PC2) accounted for a total of 90.1% of the variance of the intracellular and extracellular nucleosides, with PC1 accounting for 76.2% of the variance (Fig. 1B). Notably, the PC1 was the principal component responsible for the separation of intracellular nucleosides and extracellular nucleosides. Furthermore, the biplot shows that most of the modified nucleosides are associated with the extracellular nucleosides and the intracellular nucleosides are represented by unmodified nucleosides including A, G, U, and two modified nucleosides, m^7G and I (Fig. 1C).

It should be noted that cytosolic nucleosides exhibited a clear difference among different cell lines, which can be separated by PC2 (13.9%, see y-axis of Fig. 1B). To gain further insight into this difference, we analysed cytosolic levels of modified nucleosides by principle component analysis (PCA) and heatmaps. Interestingly, each cell line showed a unique pattern of cytosolic nucleosides (Figure S1A-B), with PC1 accounting for 68.3% of the variance (Figure S1B). PC1 includes most of modified nucleosides, which were highly accumulated in the cytosol of gastric cancer cell line MKN45 and oral cell line SAS cells compared to other cell lines (Figure S1A-B). PC2 accounts for 14.1% of the variance, in which unmodified nucleosides and several modified nucleosides (m^7G, m^3C, m^6A) can be separated from the rest of nucleosides as shown by the biplot (Figure S1C). Thus, intracellular modified nucleosides are differently regulated in each cell line, which might reflect cell-type-specific RNA epitranscriptome and catabolism.

In addition to cancer lines, we investigated MCF-10-2A cell line, a non-tumorigenic epithelial cell line (Figure S2). We performed separate heatmap and PCA analysis because the medium and supplements used for MCF-10-2A cells were different from that for cancer cell lines. Nonetheless, similar to cancer cell lines, a majority of modified nucleosides, except t^6A and ac^4C, were abundantly found in the supernatant, and unmodified nucleosides as well as m^7G and I were accumulated in cytosol (Figure S2). Thus, a majority of modified nucleosides are released into extracellular medium regardless of the origin of cells.

Association between extracellular modified nucleosides and modification levels in RNA

Since free modified nucleosides are derived from RNA catabolism, we compared the levels of free nucleosides in the
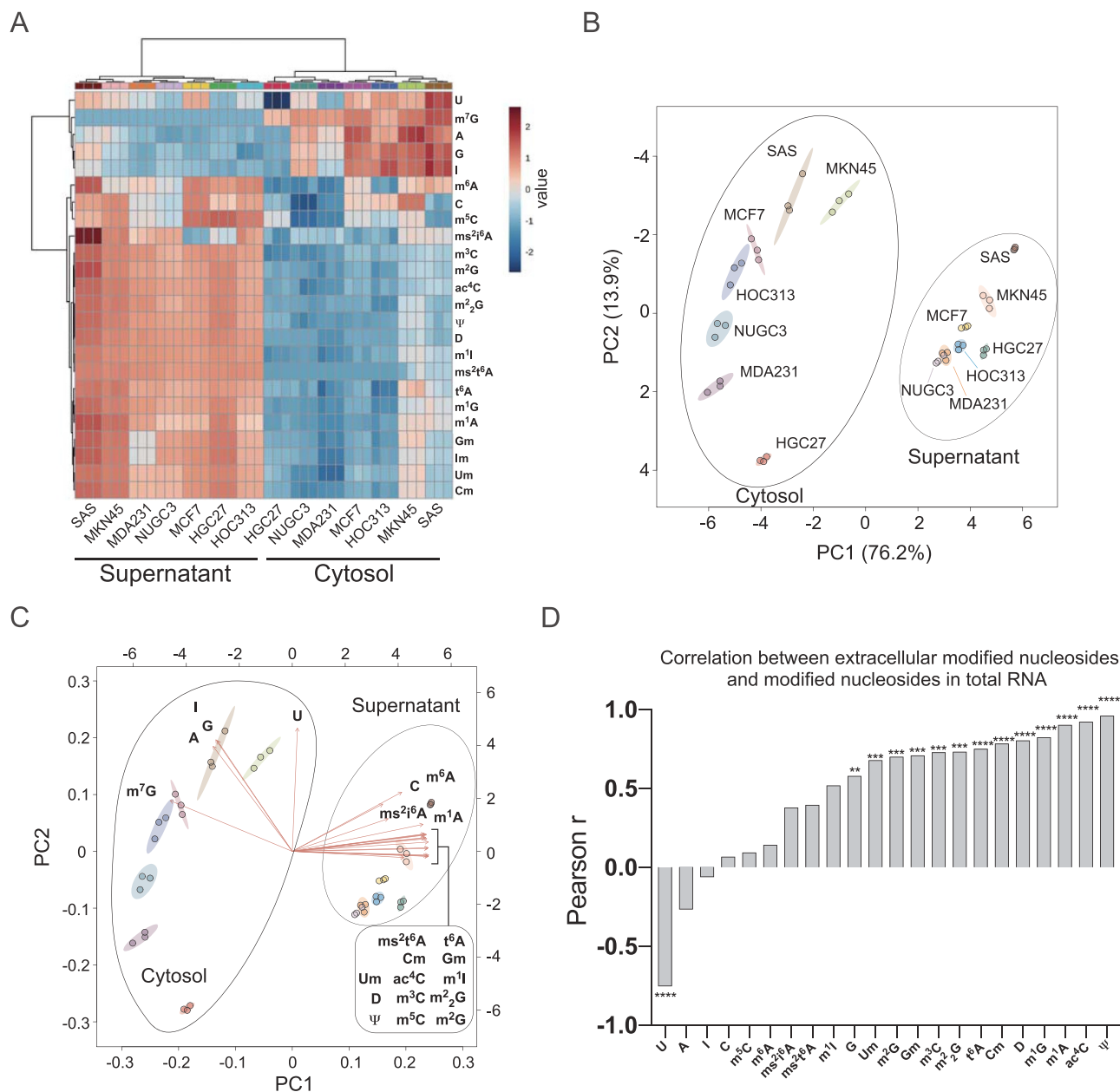
Figure 1. Profiling of extracellular and intracellular modified nucleosides across seven cell lines.

(A) Hierarchical clustering and heatmap analysis of modified nucleoside levels in supernatant and cytosol of the indicated cell lines. n = 3 biological replicates for each cell line. Colour scale represents the auto-scaled value. m^{7}G: 7-methylguanosine; m^{6}A: N^6-methyladenosine; m^{5}C: 5-methylcytidine; ms^{2}i^{6}A: 2-methylthio-N^6-isopentenyladenosine; ms^{2}t^{6}A: 2-methylthio-N^6-threonylcarbamoyladenosine; m^{3}C: 3-methylcytidine; m^{2}G: N^2-methylguanosine; ac^{4}C: N^4-acetylcytidine; m$^2_2$G: N^2,N^2-dimethylguanosine; Ψ: pseudouridine; D: dihydrouridine; m^{1}I: 1-methylinosine; t^{6}A: N^6-threonylcarbamoyladenosine; m^{1}A: N1-methyladenosine; Gm: 2′-O-methylguanosine; Im: 2′-O-methylinosine; Um: 2′-O-methyluridine; Cm: 2′-O-methylcytidine. (B) Principal component analysis (PCA) of all modified nucleosides in supernatant and cytosol of the indicated cell lines. Samples are indicated by colours corresponding to each cell line. The shaded area represents a 95% confidence interval. Note that samples can be clearly separated by the first dimensional components, indicating that the levels of these modified nucleosides in the supernatant are markedly different from the levels in cytosol. (C) PCA biplot of all nucleosides in supernatant and cytosol of the indicated cell lines. Note that most modified nucleosides are associated with supernatant samples (arrows pointed to the right) and unmodified nucleosides and 7-methylguanosine (m^{7}G) and Inosine (I) are associated with cytosol samples (arrows pointed to the left and above). (D) Total RNAs were extracted from the seven cell lines followed by Nuclease P1 digestion and mass spectrometry analysis. The levels of modified nucleosides in total RNA of these cell lines were compared to the corresponding levels of modified nucleosides in supernatant. For each modified nucleoside indicated in (D), Pearson correlation coefficient was calculated to examine correlation. **$P < 0.01$, ****$P < 0.0001$. n = 21 (7 cell lines x 3 biological replicates).

extracellular medium with their levels in the total RNAs. A majority of modified nucleosides in the extracellular medium showed significant and positive correlation with their modification levels in RNAs (Fig. 1D). Interestingly, unmodified U showed a negative correlation between extracellular level and intra-RNA level.

To further show that extracellular modified nucleosides are derived from intracellular RNA modifications, we performed a pulse-chase experiment using stable isotope methionine (Figure S3), taking advantage of the fact that a majority of modified nucleosides are methylated nucleosides. NUGC3 cells were labelled with ^{13}C-methionine for 12 h, followed by

additional incubation in culture medium containing normal methionine up to 96 h (Figure S3A). Because extracellular ^{13}C-methylated nucleosides are solely derived from intracellular ^{13}C-methylated RNA, detection of ^{13}C-methylated nucleosides in the culture medium will provide a direct proof that extracellular modified nucleosides are derived from intracellular modified nucleosides, at least for methylated nucleosides. As expected, ^{13}C-methylated nucleosides, which were not present in the fresh medium immediately after labelling, were rapidly released into extracellular medium 12 h after labelling and slowly increased during chasing period (Figure S3B). Collectively, these results suggest that in cell culture, a majority of modified nucleosides are exported from cytosol to the extracellular medium and the abundance of these extracellular modified nucleosides are correlated with their levels in RNAs.

The effect of pharmacological inhibition of ENTs on accumulation of intracellular modified nucleosides

ENTs can import and export unmodified nucleosides to maintain their homoeostatic levels [25,26]. To examine whether ENTs are also responsible for transport of modified nucleosides, we treated cells with various ENT inhibitors and examined extracellular and intracellular modified nucleosides. Dilazep and dipyridamole are FDA-approved vasodilators that can inhibit nucleoside reuptake by blocking both ENT1 and ENT2 with distinct affinities (Fig. 2A) [40]. TC-T 6000 is a potent inhibitor with IC$_{50}$ of 74.4 nM for ENT4 but can also inhibit ENT1 and ENT2 at high doses (>1.4 μM) [41]. We found that application of Dilazep and Dipyridamole up to 50 μM dose-dependently increased the levels of intracellular modified nucleosides and concomitantly suppressed the levels of extracellular modified nucleosides in two distinct cell lines, SAS cells and MKN45 cells, with dipyridamole showing a higher potency than dilazep (Fig. 2A). In contrast, TC-T 6000 showed less effect on the levels of intracellular and extracellular modified nucleosides when compared to dilazep and dipyridamole, despite its ability to affect transport of unmodified nucleosides (Fig. 2A). Moreover, a mitochondrial tRNA-derived modified nucleoside, ms^{2}i^{6}A [5], was suppressed both intracellularly and extracellularly by all three inhibitors (Fig. 2A). Collectively, these results suggest that ENT1 and ENT2 inhibition effectively attenuated export of a majority of modified nucleosides, leading to their intracellular accumulation.

Accumulation of intracellular modified nucleosides by genetic deletion of ENTs

In addition to the blockage of nucleoside transporter, ENT inhibitors can inhibit phosphodiesterase and potentially exert secondary effects to nucleoside metabolism [26]. To provide direct evidence, we generated knockout cells with deficiency of ENT1 (ENT1 KO) or ENT2 (ENT2 KO) by CRISPR-Cas9 and examined the levels of intracellular modified nucleosides (Figure S4A-C). Deficiency of ENT1 alone induced moderate accumulation of certain methylated nucleosides (m^{1}A, m^{6}A, m^{1}I, Gm, m^{5}C, Cm, Um), while deficiency of

ENT2 alone only induced accumulation of m^{5}C, but not other nucleosides in cells (Fig. 2B). To examine whether deficiency of both ENT1 and ENT2 has synergistic effect on transport inhibition, we deleted *ENT2* alleles using the ENT1 KO cells to obtain double knockout (DKO) cells (Figure S5A-D). As expected, deficiency of both ENT1 and ENT2 strongly induced accumulation of intracellular modified nucleosides; the degree of upregulation ranged from 1.6-fold to 63.7-fold change when compared to parental control cells (Fig. 2B). Interestingly, several adenosine derivatives (t^{6}A, ms^{2}t^{6}A, ms^{2}i^{6}A), which contain bulky modification at the N^6 or C^2 position of the purine base, showed no change even in the DKO cells. These results suggest that ENT1/2 cannot export these nucleosides because they are too large to pass through the transport domains and that other transporters or mechanisms may be involved in the extracellular transport of these bulky, modified nucleosides. Collectively, these results suggest that both ENT1 and ENT2 are involved in export of modified nucleosides, with ENT1 being preferentially utilized for certain modified nucleosides.

Reversal of defective export in ENT1/2 knockout cells by re-introduction of ENTs

Our successful generation of ENT1/2 double knockout cells provides a unique opportunity to evaluate the role of each ENT in export of modified nucleosides. To this end, we transduced KO cells with lentivirus carrying *ENT1* or *ENT2* or both and examined the levels of intracellular modified nucleosides (Figure S5D and Fig. 3A). For all modified nucleosides examined in this study, re-introduction of ENT1 in DKO cells was sufficient to reduce their intracellular levels to nearly basal levels (Fig. 3A). In contrast, re-introduction of ENT2 in DKO cells only moderately reduced intracellular modified nucleoside levels (Fig. 3). Importantly, introduction of both ENT1 and ENT2 in the DKO cells completely restored the levels of intracellular modified nucleosides to the levels in control cells (Fig. 3A). Taken together, our results demonstrate that in addition to the conventional transport activity towards unmodified nucleosides, both ENT1 and ENT2 are capable of exporting modified nucleosides, with ENT1 showing higher efficacy than ENT2.

The dysregulation of intracellular modified nucleosides in ENTs-deficient cells may be caused by the decrease of cell viability upon ENT-deficiency. To investigate this possibility, we examined the cell viability of control cells, DKO cells, and DKO cells with reintroduction of ENT1 or ENT1/2 (Fig. 3(B, C)). When cells were cultured in normal culture medium supplemented with 10% FBS and 4500 mg/L glucose, cell viability did not largely differ among cell lines (Fig. 3B). Interestingly, when cells were cultured in a low nutrient condition (medium supplemented with 0.1% FBS and 1000 mg/L glucose), all cell lines showed a sharp decrease in cell viability after two days in culture (Fig. 3C). Importantly, cell viability of DKO cells was significantly lower than control cell, which can be partially rescued by reintroduction of ENT1 and fully rescued by ENT1/2-reintroduction (Fig. 3C). Thus, during normal assay conditions, dysregulation of intracellular modified nucleosides was not due to the decrease of cell viability
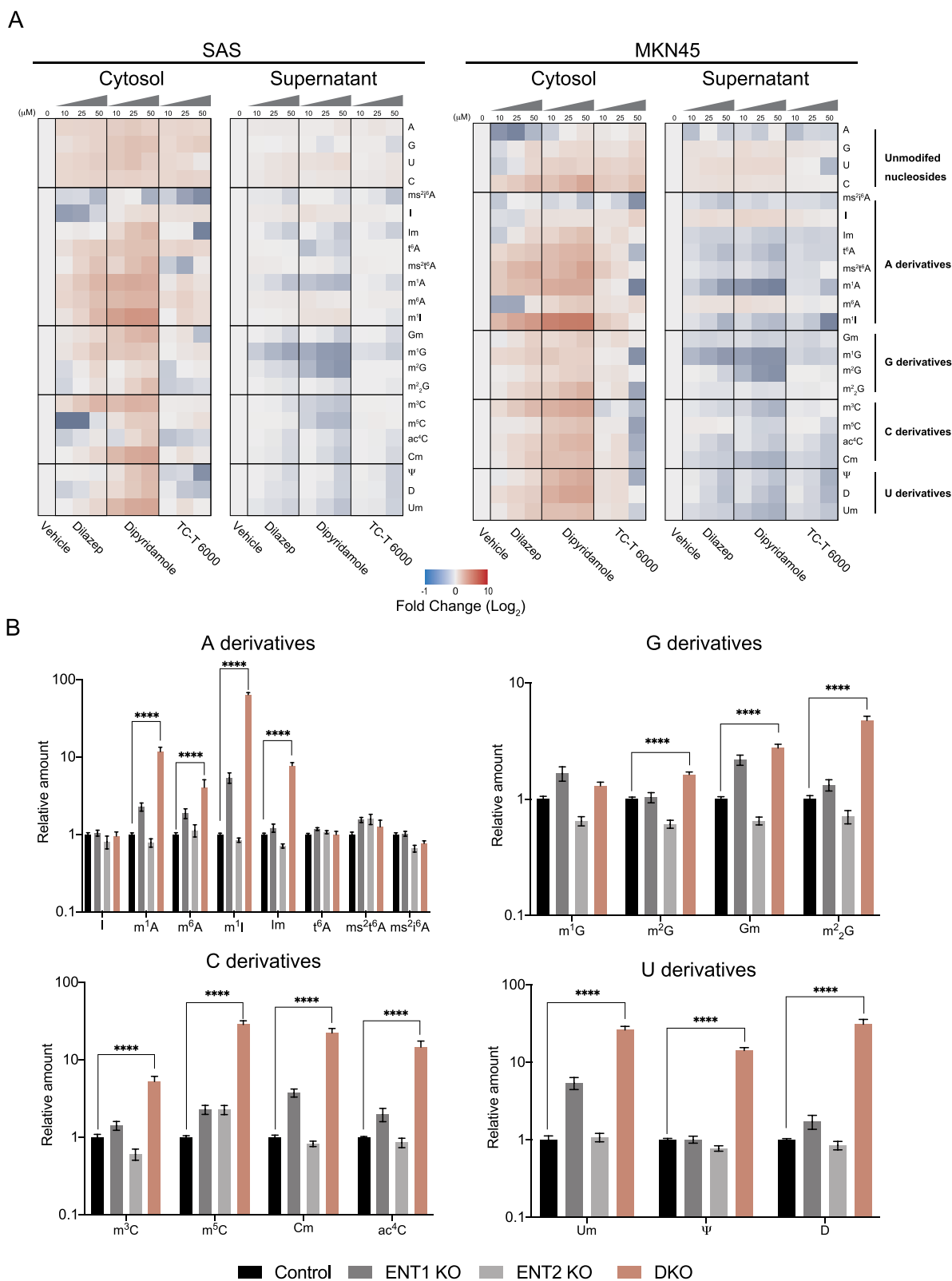
Figure 2. Impairment of modified nucleosides transport by blockage or deletion of ENTs.

(A) The oral cancer line SAS and gastric cancer line MKN45 were treated with vehicle or dilazep, dipyridamole or TC-T 6000 at 10 μM, 25 μM, 50 μM for 24 h. The level of each nucleoside in cytosol and supernatant of inhibitor-treated cells was normalized to the level in cells treated with vehicle. Nucleosides were categorized as unmodified nucleosides and modified nucleosides, and modified nucleosides were further classified to four subclasses based on their origin. Each data point represents the average of two biological replicates. (B) Intracellular modified nucleosides of control NUGC3 cells, NUGC3 cells with deficiency of ENT1 (ENT1 KO), ENT2 (ENT2 KO), and both ENT1 and ENT2 (DKO) were examined by mass spectrometry. The amount of each modified nucleoside was normalized to the amount in control cells. For each modified nucleoside, the difference within the four cell lines was analysed using One-way ANOVA followed by post-hoc Tukey test. n = 10 ~ 11 biological replicates. ****$P < 0.0001$. Note that most modified nucleosides except I, ms^{2}t^{6}A, and ms^{2}i^{6}A was significantly and abundantly accumulated in DKO cells.
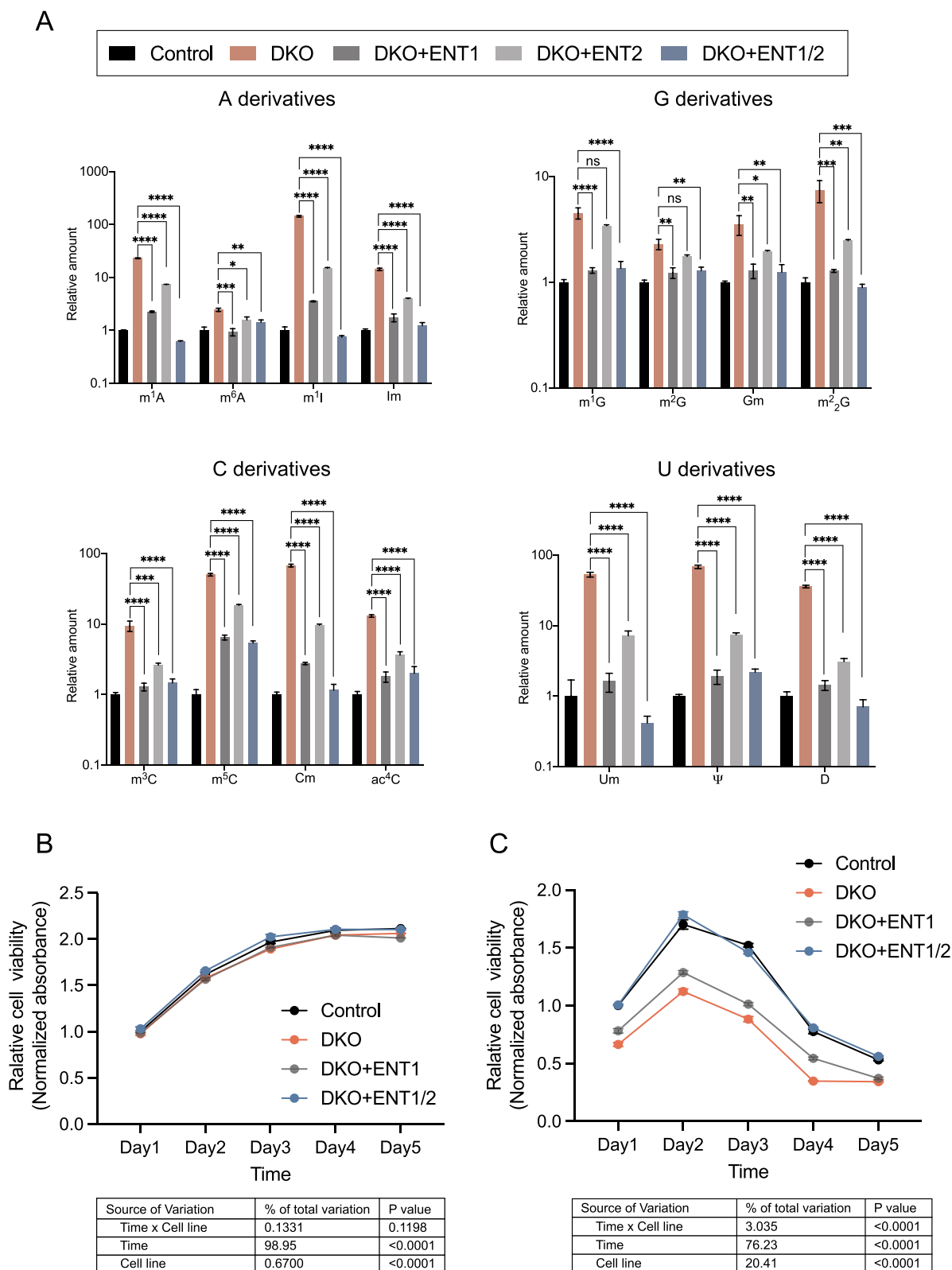
Figure 3. Rescue of modified nucleosides transport by re-introduction of ENTs in DKO cells.

(A) Intracellular modified nucleosides in control NUGC3 cells, DKO cells, and DKO cells stably expressing ENT1 (DKO+ENT1), ENT2 (DKO+ENT2), ENT1 and ENT2 (DKO +ENT1/2) were examined by mass spectrometry. The amount of each modified nucleoside was normalized to that in control cells. For each modified nucleoside, the difference within the five cell lines was analysed using One-way ANOVA followed by post-hoc Tukey test. n = 3 biological replicates. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. (B-C) Control NUGC3 cells, DKO cells, and DKO cells stably expressing ENT1 (DKO+ENT1), ENT1 and ENT2 (DKO+ENT1/2) were cultured in (B) normal medium supplemented with 4500 mg/L glucose and 10% FBS or (C) low nutrient medium supplemented with 1000 mg/L glucose and 0.1% FBS for 5 days. Viability of each cell line was assessed by WST-8 assay every day. Statistical analysis was performed using repeated measure of Two-way ANOVA followed by post-hoc Tukey test. n = 3 biological replicates. The statistical values were shown below each graph.

but solely dependent upon ENT1/2 levels. However, our results unexpectedly suggest that ENT1/2-deficiency has a marked impact on cell viability under low nutrient condition.

Generation of ENT1 mutants with distinct effects on the transport of modified and unmodified nucleosides

Previous structural study [26,42] and mutagenesis studies [25] have identified key amino acids in human ENTs that are responsible for the efficacy of unmodified nucleoside transport or modulation of inhibitor sensitivity. To investigate whether these amino acids are also important for modified nucleosides, we introduced mutant ENT1 to DKO cells (Figure S5D) and measured the amount of intracellular modified nucleosides (Fig. 4). Glycine 24 (G24) is localized to the first transmembrane domain and is conserved in eukaryotes (Fig. 4A, Figure S6A-C). A previous study has shown that G24 is required for nucleoside recognition and uptake [43]. As expected, the ENT1-G24R mutant failed to reduce the accumulation of unmodified adenosine. Importantly, ENT1-G24R mutant also had no effect on the accumulation of modified nucleosides in the infected DKO cells (Fig. 4(B,C)). Thus, G24 is absolutely required for transport of both unmodified and modified nucleosides.

Leucine 92 of human ENT1 is conserved in vertebrates (Fig. 4A, Figure S6A) and is localized to the second transmembrane domain (Figure S6D). Random mutagenesis has shown that when mutated to Glutamine (Q), this amino acid residue can impair intracellular uptake of purine nucleosides [44]. In agreement with previous findings, we found that introduction of the ENT1-L92Q mutant in DKO cells failed to suppress intracellular adenosine level (Fig. 4B). Interestingly, despite the inhibitory effect on adenosine, the ENT1-L92Q mutant significantly and markedly reduced intracellular modified nucleosides in DKO cells (Fig. 4C). These results suggest that L92 is involved in export of unmodified adenosine but not modified nucleosides.

In addition to these two mutants, we generated an Isoleucine 216 to Threonine (I216T) mutant, which is a natural variant identified in humans [45]. I216 shows moderate conservation in eukaryotes (Figure S6A) and is localized to the sixth transmembrane domain (Fig. 4A, Figure S6E). Similar to WT ENT1, expression of ENT1-I216T mutant effectively reduced intracellular accumulation of modified nucleosides in DKO cells (Fig. 4C). These results are in agreement with previous finding and demonstrate that the I216T mutation of ENT1 does not affect nucleoside transport.

Induction of autophagy through deficiency of ENTs

Dysregulation of intracellular and extracellular adenosine level has been associated with autophagy, but the mechanism is as yet unknown [46,47]. The aberrant accumulation of intracellular nucleosides, both modified and unmodified, prompted us to examine the status of autophagy in ENT1/2-deficient cells. The conversion of the soluble form of microtubule-associated protein 1A/1B-light chain 3 (LC3-I) to the lipid-bound form (LC3-II) is required for the formation of the autophagosome and is one of the hallmarks of autophagy induction [46,47]. We examined LC3 levels in control cells, DKO cells, and DKO cells expressing ENT mutants by Western blotting (Fig. 5(A,B)). Remarkably, DKO cells exhibited abundant LC3-II levels compared to control cells even when cells were cultured in normal medium containing 10% foetal bovine serum (Fig. 5A). Importantly, re-introduction of ENT1 alone or both ENT1 and ENT2 successfully reduced LC3-II levels in the DKO cells (Fig. 5(B,C)). Furthermore, re-introduction of ENT1-G24R mutant, a non-functional ENT1, failed to reduce LC3-II accumulation in the DKO cells (Fig. 5 (B,C)). Next, we examined the spatial distribution of LC3 proteins in these cells by immunofluorescence staining. Similar to the results of Western blotting, DKO cells as well as DKO cells expressing ENT1-G24R showed an intense and aggregated LC3 staining, whereas the strong LC3 staining in DKO cells was diminished in DKO cells expressing ENT1 or ENT1/2 (Fig. 5D). These results suggest that defective ENT1/2-mediated nucleoside export can induce autophagy response.

Effect of ENTs deficiency on AKT-Beclin-1 signaling

Cellular signaling of autophagy induction involves multiple pathways that are regulated by phosphorylation [46]. We found that among these pathways, phosphorylation of AKT at Ser473 was markedly decreased in DKO cells compared to control cells, suggesting that AKT activity was suppressed in DKO cells (Fig. 5E). Beclin-1 is an AKT substrate. Its phosphorylation at Ser295 by AKT is implicated in the inhibition of autophagy induction [48]. In accordance with the decrease in AKT phosphorylation in DKO cells, phosphorylation of Beclin-1 at Ser295 was also markedly decreased in DKO cells (Fig. 5E). Importantly, expression of ENT1 or ENT1/2 in DKO cells effectively restored AKT phosphorylation as well as Beclin-1 phosphorylation (Fig. 5E).

Loss of ENT1/2 resulted in the accumulation of unmodified and modified nucleosides, both of which can potentially contribute to autophagy induction. To clarify the role of modified nucleosides in the induction of autophagy, we examined LC3 levels in DKO cells expressing the ENT1-L92Q mutant, which impairs export of unmodified adenosine but permits export of modified nucleosides (Fig. 4). The upregulated LC3-II levels in DKO cells were significantly reduced by expression of ENT1-L92Q in DKO cells (Fig. 5(F,G)). Furthermore, expression of ENT1-L92Q mutant effectively restored phosphorylation of AKT and Beclin-1 (Fig. 5(F,G)). Taken together, these results suggest that accumulation of intracellular modified nucleosides can trigger autophagy response through suppression of AKT-Beclin-1 signaling.

Identification of autophagy-related modified nucleosides

The potential role of modified nucleosides in autophagy induction prompted us to investigate which modified nucleoside is capable of inducing an autophagy response. To this end, we treated control NUGC3 cells cultured in normal medium with individual modified nucleosides for 24 h and examined LC3-II levels by western blotting (Fig. 6A). Among the tested nucleosides, exogenous addition of Im
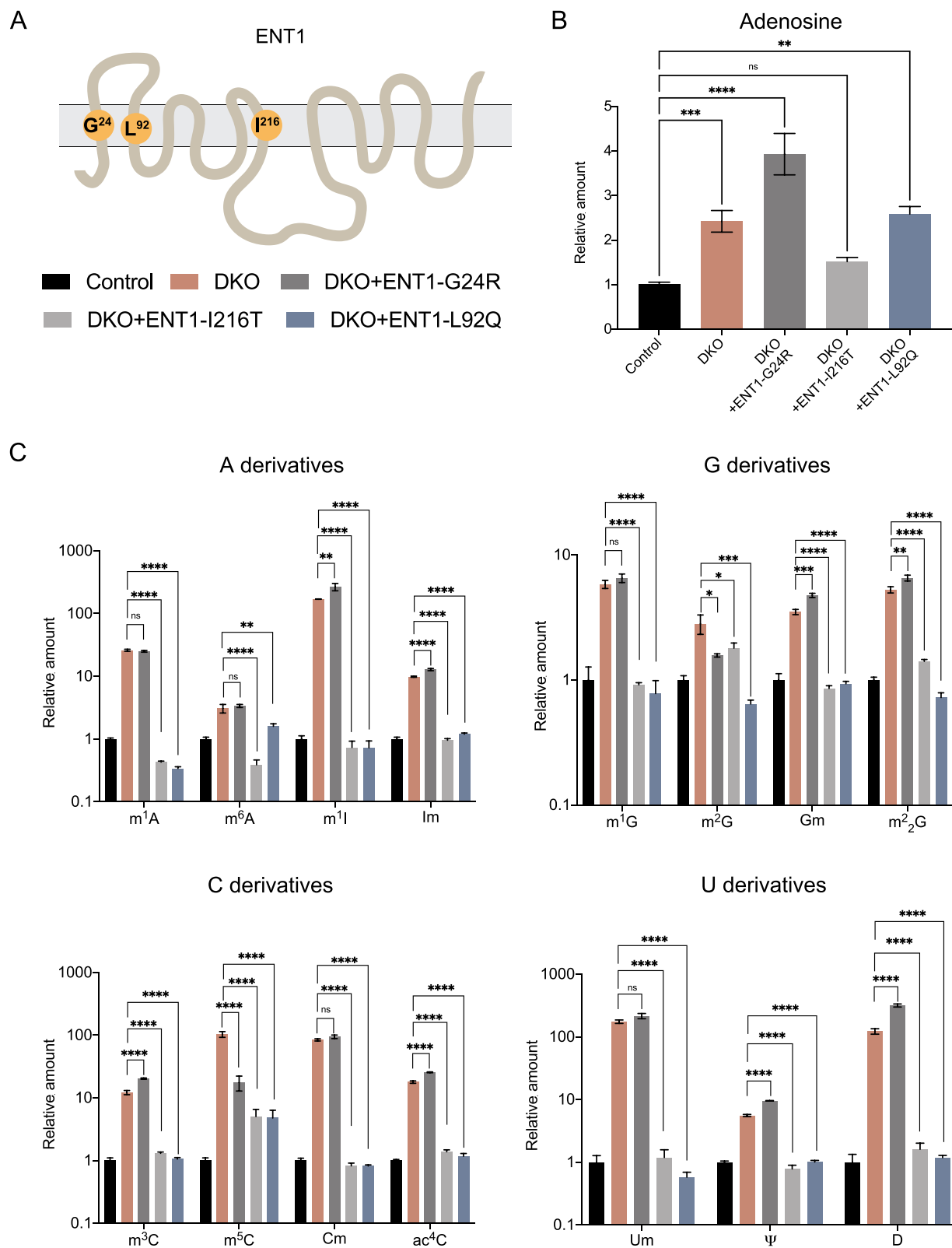
Figure 4. Amino acids involved in nucleosides transport.

(A) Schematic illustration of human ENT1. Glycine 24 (G24), Leucine 92 (L92) and Isoleucine 216 (I216) located in the first, second, sixth transmembrane domain are shown. Mutant ENT1s with G24 mutated to Arginine (G24R), L92 mutated to Glutamine (L92Q), I216 mutated to Threonine (I216T) were used to transduce DKO cells. (B) Relative amount intracellular adenosine in control cells, DKO cells, and DKO cells transfected with various mutant *ENTs*. n = 3 biological replicates for each. **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ by One-way ANOVA followed by post-hoc Tukey test. (C) Relative amount of intracellular modified nucleosides in indicated cell lines were compared. Note that intracellular modified nucleosides were significantly accumulated in G24R mutant cells but were reduced in I216T and L92Q mutant cells. n = 3 biological replicates for each. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ by One-way ANOVA followed by post-hoc Tukey test.
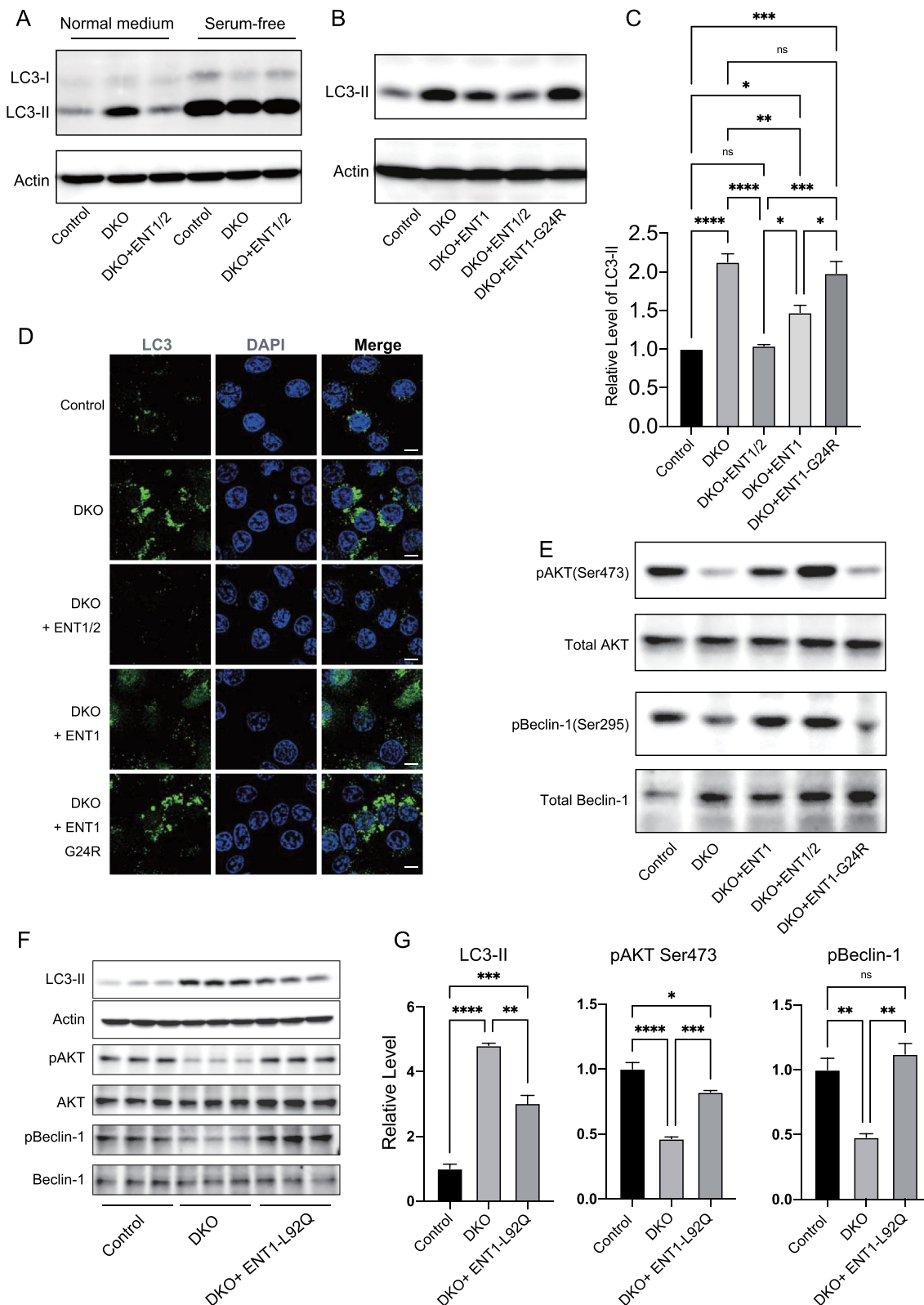
Figure 5. Induction of autophagy in cells having defective nucleoside transport.

(A) Representative images of LC3-I, LC3-II, and Actin levels in control cells, DKO cells, and DKO cells expressing ENT1/2 cultured in DMEM containing 10% FBS (normal medium) or no FBS (serum-free). (B) Representative images of LC3-II and Actin levels in control cells, DKO cells, and DKO cells expressing indicated ENT1 mutants under serum-containing culture condition. (C) Quantification of LC3-II levels relative to Actin in indicated cell lines. Note that DKO cells showed an increased LC3-II level, which was suppressed by expressing ENT1, ENT1/2, but not by G24R mutant ENT1. n = 3 biological replicates for each. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ by One-way ANOVA followed by post-hoc Tukey test. (D) Representative immunocytochemistry images of LC3 in indicated cell lines. Nucleus was stained by DAPI. Note that the large puncta of LC3 were observed in DKO cells and DKO cells expressing G24R mutant. Bars = 10 μm. (E) Representative images of phosphorylated AKT (pAKT (Ser473)), total AKT, phosphorylated Beclin-1 (pBeclin-1 (Ser295)) in indicated cell lines. Note that phosphorylation of AKT and Beclin-1 was suppressed in DKO cells and the decrease of phosphorylation was rescued by expression of ENT1 or ENT1/2 in DKO cells. (F) Representative images of LC3-II, pAKT (Ser473), pBeclin-1 (Ser295), total AKT, total Beclin-1, Actin, in control cells, DKO cells, and DKO cells expressing L92Q mutant ENT1. (G) Quantification of western blot results (F). Note that L92Q ENT1 significantly suppressed LC3-II induction and concomitantly increased AKT and Beclin-1 phosphorylation in DKO cells. n = 3 biological replicates for each. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ by One-way ANOVA followed by post-hoc Tukey test.

and m^{1}G significantly induced LC3-II induction, with m^{1}G showing the highest effect (P < 0.01, versus control, Fig. 6B). Next, we examined whether exogenous Im and m^{1}G can be imported into cells (Fig. 6C). Mass spectrometry analysis revealed that m^{1}G treatment significantly upregulated intracellular m^{1}G to a level that was comparable to the level in DKO cells 24 h after treatment. By contrast, intracellular Im did not show upregulation 24 h after
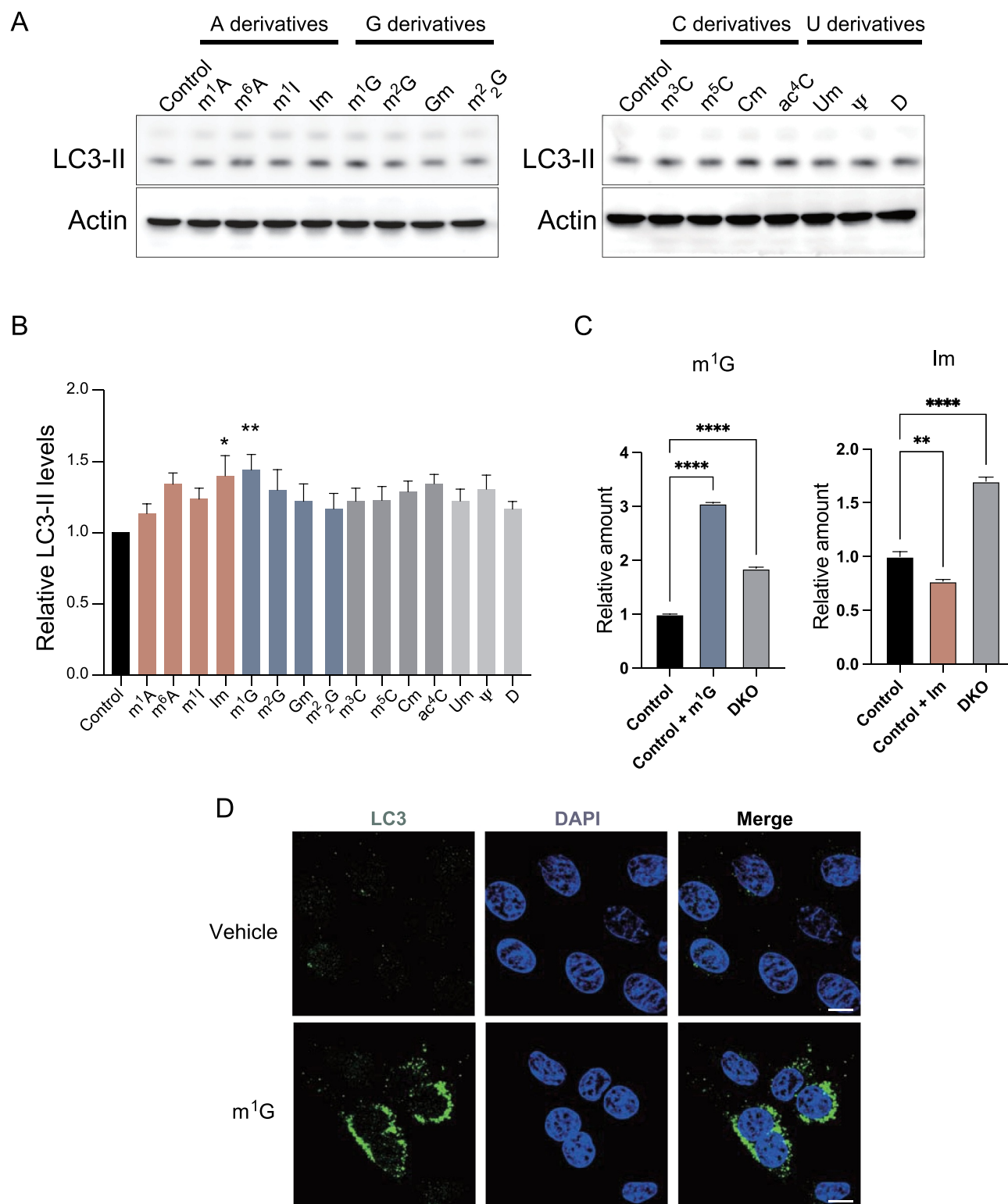

Figure 6. Induction of autophagy response by modified nucleosides.

(A) Representative western blot images of LC3-II and Actin in control NUGC3 cells treated with indicated modified nucleosides at a final concentration of 1 μM for 24 h. (B) Relative LC3-II levels in cells treated with each modified nucleoside. n = 3 biological replicates for each. *P < 0.05, **P < 0.01, by One-way ANOVA followed by post-hoc Tukey test. (C) Relative intracellular m^{1}G and Im levels in control cells, control cells treated with 1 μM m^{1}G and Im for 24 h, DKO cells. (D) Representative immunocytochemistry images of LC3 in control cells and control cells treated with 1 μM m^{1}G for 24 h. Nucleus was stained by DAPI. Bars = 10 μm.

treatment. It is conceivable that the import and export rate of Im may be faster than m^1G. Finally, we stained m^1G-treated cells with anti-LC3 antibody and found the m^1G-treatment indeed induced LC3-positive aggregation, an indicative of formation of autophagosome (Fig. 6D). These results suggest that excessive m^1G was capable of inducing an autophagy response. It should be noted that our results do not exclude the possibility that autophagy response can be triggered by other modified nucleosides besides Im and m^1G. In fact, m^6A and ac^4C showed tendency to induce LC3-II levels when added to cells (m^6A: P = 0.06 vs. Control; ac^4C: P = 0.055 vs. Control). Moreover, the level of m^1G-mediated LC3-II induction was not as prominent as ENT1/2-deficiency-mediated LC3-II indication, which also suggests that other modified nucleosides are involved in autophagy response. Thus, it is most likely that various modified nucleosides can synergistically induce autophagy response when they are excessively accumulated in the cells.

Potentiation of Zika virus (ZIKV) replication as a result of defective nucleoside transport

Finally, we sought to investigate the pathological role of ENTs-mediated nucleoside transport. ENTs have been implicated in viral infection owing to their ability to import anti-viral drugs as nucleoside analogues [49–51]. Interestingly, some RNA viruses, including coronaviruses and flaviviruses, can utilize autophagy machinery in host cells for viral replication [52]. For example, ZIKV infection can induce autophagy via the Akt signaling pathway, which in turn facilitates ZIKV replication [53].

Given the accumulation of nucleosides and increase in the basal autophagy response in cells with deficiency of ENT1 and ENT2, we infected the DKO cells with ZIKV and examined how defective nucleoside transport would impact ZIKV replication. In agreement with a previous report [53], we found that ZIKV infection significantly induced LC3-II levels in control cells (Fig. 7(A,B)). Puncta of LC3 were clearly observed in these cells after ZIKV infection at a Multiplicity of infection (MOI) value of 1 (Fig. 7C). Interestingly, examination of intracellular nucleosides revealed that many modified nucleosides were accumulated in cells infected with ZIKV at a MOI of 1 (Fig. 7D). However, ZIKV infection upregulated the expression level of ENT1 (Figure S7), suggesting that the accumulation of modified nucleosides in these cells was not attributed to ENT1-deficiency.

To investigate the impact of ENT-deficiency on viral replication, we extracted total RNA from ENT1/2-deficient or ENT1-mutant cells infected with ZIKV at a MOI of 0.01 for 3 days, followed by quantitative RT-PCR of viral genome RNA. Remarkably, DKO cells showed >20-fold increase of ZIKV genome RNA compared to control cells (Fig. 7E). Notably, re-introduction of ENT1 alone or ENT1/2, but not the non-functional ENT1-G24R mutant, effectively suppressed the hyper-replication of ZIKV genome RNA in DKO cells (Fig. 7E). In addition, we collected the supernatants and measured the titre of ZIKV, which represents the number of infectious ZIKV particles released from host cells (Fig. 7F, Figure S8A). Similar to the viral genome results, the relative titre of ZIKV in DKO cells was >10-fold higher than that in control cells. Furthermore, re-introduction of ENT1 or ENT1/2, but not the non-functional ENT1-G24R, significantly suppressed ZIKV released from DKO cells. Importantly, when DKO cells were re-introduced with the ENT1-L92Q mutant, which can selectively restore export of modified nucleosides, the level of ZIKV genomic RNA as well as the titre of ZIKV in the supernatant was significantly decreased upon ZIKV infection (Fig. 7(G,H), Figure S8B). These results clearly suggest that accumulation of intracellular modified nucleosides contributed to the hyper-replication of ZIKV.

To understand the impact of ENT-inhibition of virus replication and its potential clinical relevance, we examined virus replication in control cells in the presence of dilazep, the ENT1/2 inhibitor and an FDA-approved drug for treatment of cardiopathy and renal disorders (Figure S9). Similar to ENT1/2-deficient cells, ZIKV replication was significantly upregulated in the presence of dilazep as evidenced by the increase in ZIKV genome copy number and virus particle number (Figure S9). While these results are preliminary, it would be of interest to investigate their clinical relevance for patients receiving dilazep who have ZIKV infection.

Finally, we investigated the role of autophagy in ZIKV replication by shRNA-mediated knockdown of ATG5, one of the master regulators of autophagy initiation. The shRNA against ATG5 (shATG5) achieved 75%~95% suppression of ATG5 protein levels despite the moderate effect on ATG5 mRNA levels (Figure S10). In addition, shATG5 also significantly reduced LC3-II levels in DKO cells (Figure S10). Importantly, ATG5 knockdown drastically reduced ZIKV genome RNA replication in DKO cells (Fig. 7I), suggesting that the upregulation of autophagy response in DKO cells is responsible for the hyper-replication of ZIKV in these cells.

Discussion

Homoeostatic regulation of intracellular and extracellular nucleosides is vital to various biological processes such as RNA transcription, DNA replication, energy production, immune response, and learning and memory [54]. Extensive studies have been focused on the unmodified nucleosides, particularly adenosine [16]. It has been revealed that ENT-mediated bidirectional transport is critical to maintain the basal levels of unmodified nucleosides in both extracellular and intracellular environments [25]. In addition to unmodified nucleosides, diverse post-transcriptional modifications have been identified in RNA [1]. As a consequence of RNA catabolism, RNA-derived modified nucleosides are constantly generated in the cells and released into biological fluids [20,22]. In fact, mammalian serum and urine contain abundant modified nucleosides with some species having even higher concentrations than unmodified nucleosides [17]. Importantly, some modified nucleosides can even act as bioactive molecules that can activate G-protein coupled receptors to induce allergic reaction and inflammation in vivo [17].

In agreement with the in vivo results, the present study revealed that abundant modified nucleosides were released into culture medium of various cell lines independent of their origins. Importantly, for many modified nucleosides,
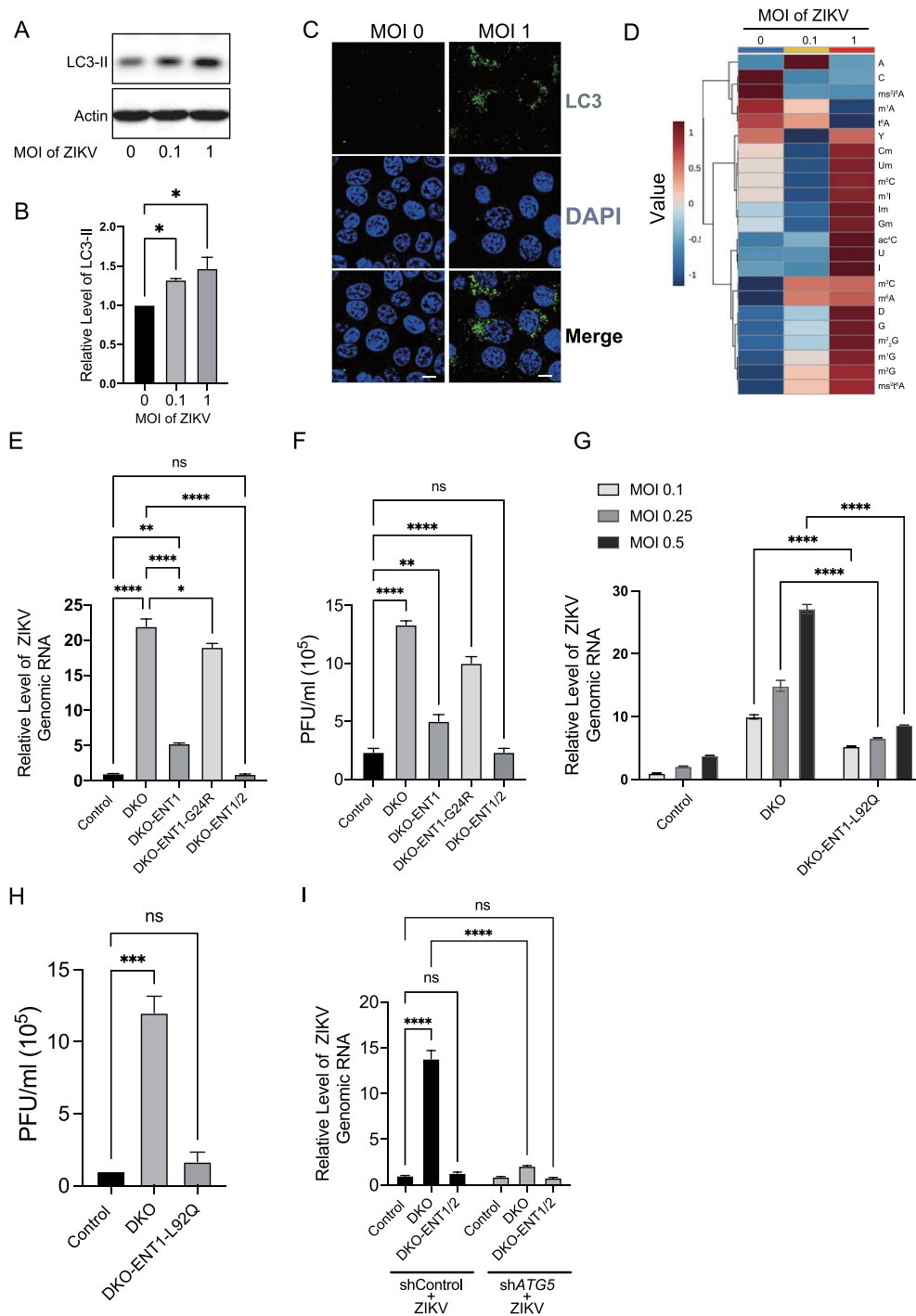
Figure 7. Facilitation of Zika virus (ZIKV) replication in cells having defective nucleoside transport and its association with autophagy.

(A) Representative images of LC3-II and Actin in NUGC3 cells infected with ZIKV at indicated Multiplicity of infection (MOI) for 3 days. (B) Quantification of LC3-II level (A) shows ZIKV infection induced accumulation of LC3-II. n = 3, *$P < 0.05$ by One-way ANOVA followed by post-hoc Tukey test. (C) Immunostaining of LC3 in NUGC3 cells with mock-infection and ZIKV infection at MOI = 1. Note that puncta of LC3 was observed in ZIKV-infected cells. (D) Heatmap analysis of intracellular nucleosides in NUGC3 cells infected with ZIKV at indicated MOI. (E) Quantitative PCR examination of the relative levels of genomic ZIKV in control cells, DKO cells, and mutant ENTs-expressing DKO cells infected with ZIKV at MOI = 0.01. Note that ZIKV genome RNA was abundantly detected in DKO cells and DKO cells expressing the non-functional G24R mutant ENT1. n = 3 biological replicates for each. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$ by One-way ANOVA followed by post-hoc Tukey test. ns: not significant. (F) Plaque-forming unit (PFU) of ZIKV-containing supernatant collected from each cell line was calculated according to plaque assay (Figure S6A). n = 3 for each, **$P < 0.01$, ***$P < 0.0001$ by One-way ANOVA followed by post-hoc Tukey test. ns: not significant. (G) Control cells, DKO cells, and DKO cells expressing ENT1-L92Q mutant were infected with ZIKV at the indicated MOI for 3 days followed by quantitative RT-PCR examination of viral genomic NS5. n = 3 for each, ***$P < 0.0001$ by One-way ANOVA followed by post-hoc Tukey test. (H) PFU of ZIKV-containing supernatant collected from control cells, DKO cells, DKO cell expression ENT1-L92Q was calculated according to plaque assay (Figure S6B). n = 3 for each, ***$P < 0.0001$ by One-way ANOVA followed by post-hoc Tukey test. ns: not significant. (I) Control cells, DKO cells, and mutant ENTs-expressing DKO cells were infected with lentivirus containing ATG5-targeting shRNA or control shRNA, followed by ZIKV infection at MOI = 0.01. n = 3, ***$P < 0.0001$ by One-way ANOVA followed by post-hoc Tukey test. ns: not significant.

their amounts in the culture medium are correlated with their abundance in RNA. These results demonstrate that extracellular transport of modified nucleosides is a fundamental cellular function, and the amount of extracellular modified nucleosides can be used to estimate the corresponding modification status in RNA.

In addition to the extracellular modified nucleosides, the levels of intracellular modified nucleosides also showed unique patterns in the cell lines with diverse origins. Notably, m^7G, m^3C and m^6A were relatively high in the cytosol of the oral cancer line SAS, gastric cancer cell line MKN45, and breast cancer line MCF7 when compared to other cell lines. Notably, a previous study has shown that a low level of m^6A modification in mRNA is associated with the breast cancer prognosis. In line with the previous report, our results show that intracellular m^6A is relatively low in a high metastatic breast cancer cell line MDA-MB-231 (MDA) cells, when compared to MCF7 cells, which is less metastatic (Figure S1A). Collectively, our results suggest that modified nucleosides are differentially regulated in each cancer cell line, and that intracellular and extracellular modified nucleosides can be used as potential diagnostic markers for cancer.

Molecular mechanism of modified nucleosides export

In the present study, we provide compelling evidence that ENTs, which have long been known as passive transporters for unmodified nucleosides, can also transport the majority of modified nucleosides from the cytosol to extracellular medium. Using ENT1/2 double knockout cells in combination with a rescue strategy, we found that both ENT1 and ENT2 can transport a wide variety of modified nucleosides. Interestingly, while expression of ENT2 in DKO cells only moderately reduced intracellular modified nucleosides, expression of ENT1 alone effectively reduced intracellular modified nucleosides to nearly basal level. Thus, modified nucleosides, at least those examined in this study, are preferentially transported through ENT1 as compared to ENT2. Notably, our mutagenesis data indicate that while the G24 amino acid residue of ENT1 is absolutely required for transport of both unmodified and modified nucleosides, L92 of ENT1 is only required for transport of unmodified adenosine. Human ENT1 shows 46% identity to ENT2 (Figure S11) at the amino acid level. Interestingly, G24 and L92 of ENT1 are also conserved in ENT2 (Figure S11), suggesting other unidentified residues of ENT1 are involved in the recognition and transport of modified nucleosides.

Despite the biological importance of ENTs, the understanding their mechanisms is limited due to the lack of structural information. In 2019, Wright and Lee reported the first experimental structure of hENT1 bound to NBMPR and dilazep, the ENT1 inhibitors [42]. It is proposed that ENT1 undergoes a dynamic conformational transition from outward-open state to inward-facing state during transport cycle. Notably, the first transmembrane domain (TM1) is particularly important for the conformational transition. The TM1 of ENT1 contains two alpha-helix structures, which are connected by two amino acids and is facing the central cavity

of ENT1 (Figure S6B). Of note, Gly 24 is the last amino acid residue of the first helix of TM1 (Figure S6C). It is conceivable that the mutation of Gly 24 might impair proper folding of TM1, which perturbs conformational transition, ultimately leading to the blockage of the opening of central cavity.

The structure of ENT1 in combination with NBMPR demonstrates that the L92 residue in TM2 is involved in purine binding, possibly through hydrophobic contact (Figure S6D). Because modification at either ribose or nucleobase adds extra bulkiness to the nucleosides, it is conceivable that unlike adenosine, modified nucleosides do not structurally interact with L92, which might explain why L92Q mutant is only inhibitory to unmodified adenosine but not modified nucleosides. I261 is localized to TM6, which constitutes the outside wall of ENT1 and is not directly involved in substrate recognition (Figure S6E). From the structural point of view, the effect of I261T to the structural dynamics of ENT1 is very limited, which possibly explains why I261T mutation had no effect on nucleoside transport.

A limitation of our study is that although we directly measured the intracellular and extracellular modified nucleosides using mass spectrometry, the detailed kinetics of export remained unexplored. Future study using radioisotope-labelled nucleosides in combination with synthetic liposome as well as recombinant ENTs will provide precise kinetic insights. While the current study revealed that ENT1/2 can export modified nucleosides, we have not examined the potential role of ENT4 in this type of transport. ENT4 is also localized to the plasma membrane and has been shown to be involved in nucleoside transport in a pH-dependent manner [33]. Although the expression level of ENT4 is relatively lower than that of ENT1 and ENT2 (Figure S12), we found that application of an ENT4 inhibitor to SAS and MKN45 cells resulted in a slight increase of certain modified nucleosides in the cytosol and a moderate decrease in the cell medium, suggesting that ENT4 can potentially transport modified nucleosides. In the future, generation of triple knockout cells lacking ENT1/2/4 will help elucidate the role of ENT4 in nucleoside transport. In addition, it should be noted that our results do not exclude the possibility that modified nucleosides can be exported in a membrane-bound manner, such as through an exosome. Future studies will be needed to elucidate the molecular basis and the diverse export mechanisms underlying the transport of modified nucleosides.

Modified nucleosides as inducers of autophagy response

An important finding of this study is that ENT1/2-deficiency resulted in a strong autophagy response, even in the absence of nutrient deprivation. Notably, expression of the ENT1-L92Q mutant, which permits export of modified nucleoside but interferes with adenosine transport, completely restored phosphorylation AKT and Beclin-1 in ENT1/2 knockout cells. Furthermore, the ENT1-L92Q mutant significantly suppressed LC3-II induction. These results demonstrate that in addition to unmodified nucleoside, homoeostatic regulation of intracellular modified nucleoside levels via ENTs is actively involved in signalling transduction and aberrant accumulation

of intracellular modified nucleosides can trigger strong cellular responses such as autophagy. However, how cells sense the levels of modified nucleosides thereby leading to the suppression of AKT phosphorylation and induction of autophagy remain to be addressed in the future study. Interestingly, in line with our findings, a recent study reported that dipyridamole, potent inhibitor of ENTs, was capable to induce accumulation of autophagy marker such as LC3-II and suppress proliferation of a prostate cancer cell line [55]. Although the effect of dipyridamole was attributed to its ability to potentiate protein kinase A signaling as a phosphodiesterase inhibitor, our results suggest that the effect of dipyridamole could also be attributed, in part, to the accumulation of intracellular modified nucleosides.

By screening the potency of individual modified nucleosides towards autophagy induction, we found that exogenous m^1G was capable of upregulating intracellular m^1G levels and effectively inducing autophagy response, as evidenced by increase in LC3-II levels. It should be noted that the increase of intracellular m^1G level after m^1G addition was within a physiological range because the level of m^1G level was comparable to the level of ENT1/2-deficient cells. We also found that Im could induce autophagy response, but the intracellular Im did not increase 24 h after treatment. It is conceivable that Im may be exported earlier than m^1G. Our results suggest that increase of intracellular m^1G can trigger autophagy response. However, the exact threshold of m^1G to initiate autophagy response and the molecular mechanism by which m^1G induces autophagy response need to be elucidated in future studies. It is worthwhile to indicate that our results do not necessarily suggest that other modified nucleosides are not responsible for autophagy induction. In fact, other modified nucleosides, such as m^6A and ac^4C, also showed tendency to induce autophagy response (Fig. 6A). Moreover, because most extracellularly added modified nucleosides could not be efficiently imported to cells, it was technically difficult to properly evaluate the effect of these modified nucleosides on autophagy response in the current experimental condition (Fig. 6). Furthermore, given the fact that the level of m^1G-mediated LC3-II induction was not as prominent as ENT1/2-deficiency-induced LC3-II induction, it is most likely that other modified nucleosides are also involved in autophagy response. Taken together, these results suggest that various intracellular modified nucleosides synergistically trigger autophagy response when their intracellular levels are excessive. Nevertheless, the finding that m^1G can induce autophagy provides a mechanistic insight into the modified nucleosides-induced autophagy.

Modified nucleosides export and infectious disease

This study revealed that ENT1/2-deficient cells are potent hosts for massive replication of ZIKV in an autophagy-dependent manner. In addition to genetic deletion of ENT1/2, our study revealed that the ENT1/2 inhibitor dilazep was also capable of potentiating ZIKV replication in NUGC3 cells. ZIKV infection in pregnant woman results in the development of microcephaly in the foetus by affecting the growth

and differentiation of neuronal stem cells. Interestingly, ZIKV infection has been shown to induce an autophagy response which supports viral replication by providing specific membrane structure that is required for the formation of viral particle [56]. Notably, human neural progenitors infected with ZIKV show downregulation of *ENT1 and ENT2* mRNA [57,58]. Thus, downregulation of ENTs may induce aberrant nucleoside transport which may in turn trigger autophagy and accelerate virus replication. Unlike neuronal progenitor cells, ZIKV infection did not reduce the *ENTs* mRNA levels in NUGC3 cells despite the induction of autophagy and accumulation of modified nucleosides. Future studies using ENT1/2-deficient neural stem cells will be needed to elucidate the potential role of ENT-mediated nucleoside transport in ZIKV infection. A previous report has shown that chronic infection of hepatitis C virus, which belongs to the same *Flaviviridae* virus family as ZIKV, causes endocytosis of ENT1, thereby impairing drug delivery for the treatment of hepatitis C [49]. It is possible that ZIKV infection may also induce internalization of ENTs independent of transcriptional regulation, thereby supporting viral replication. Collectively, our results illuminate an important link between ENT1/2-mediated modified nucleoside transport and viral disease.

In conclusion, our study has revealed that RNA-derived modified nucleosides are actively exported by ENT1 and ENT2 (Fig. 8). Moreover, defective export resulted in accumulation of intracellular modified nucleosides, such as m^1G, leading to a strong autophagy response and autophagy-dependent hyper-replication of ZIKV. Thus, export of modified nucleosides is a fundamental biological process that is closely associated with cell physiology and disease development.

Material and methods

Reagents

All reagents and their origins are listed in Table S1. DNA oligos used for cloning and quantitative PCR are listed in Table S2 with sequence information.

Cell culture

Gastric cancer cell lines (HGC27, MKN45, NUGC3), oral cancer cell lines (HOC313 and SAS), breast cancer cell lines (MCF7 and MDA-MB-231), and hepatocellular carcinoma cell line HuH-7 were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4,500 mg/L D-glucose, 2 mM L-glutamine, and 110 mg/L sodium pyruvate, with 10% heat-inactivated foetal bovine serum, at 37°C and 5% CO_2. Vero cells were maintained in DMEM containing 1,000 mg/L D-glucose, 2 mM L-glutamine, and 110 mg/L sodium pyruvate, with 10% heat-inactivated foetal bovine serum, at 37°C and 5% CO_2. MCF-10-2A cells were maintained in DMEM/F-12 medium supplemented with 5% heat-inactivated horse serum, 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 0.01 mg/ml bovine insulin, 200 ng/ml hydrocortisone.
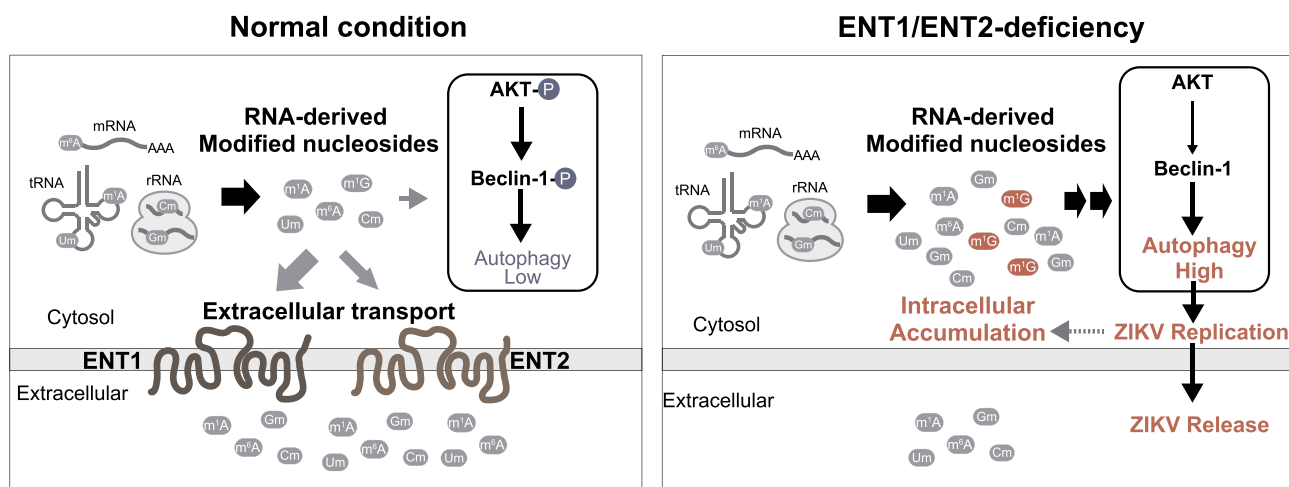
Figure 8. Schematic illustration of ENTs-mediated extracellular transport of modified nucleosides and its pathophysiological implications.

Under normal condition, various modified nucleosides are generated as the consequence of RNA catabolism and these modified nucleosides are exported by ENT1 and ENT2. The resulting low level of intracellular modified nucleosides is associated with activation of AKT and Beclin-1 signaling, leading to the suppression of the basal autophagy response. On the other hand, ENT1/2-deficiency can induce accumulation of intracellular modified nucleosides, which impairs AKT-Beclin-1 signaling, thereby leading to activation of autophagy response and autophagy-dependent ZIKV replication. In addition, excessive intracellular m^1G can also induce autophagy response.

Cell viability assay

Cells were seeded in 96-well plates at a density of 3,000 cells/well and cultured upto 5 days. On each culture day, 10 µL of WST-8 solution was added into each well and incubated for 2 h at 37°C. The absorbance of WST-8 was measured 450 nm using a microplate reader.

Zika virus preparation

Zika virus (ZIKV) strain PRVABC59 was obtained from National Institute of Infectious Diseases of Japan and the use of the virus was approved by the regulatory committee of Kumamoto University (Approval number: 0002). ZIKV stocks were prepared in Vero cells as described previously [59]. Briefly, 2 million Vero cells were cultured in 10-cm dish until confluent. Next, 50µL ZIKV was added to the cells and incubated at 37°C and 5% CO_2 for 3 days. The culture medium was collected and centrifuged at 600 × g for 10 min to precipitate cell debris and stored as viral stock at −80°C until use. For viral infection of gastric-cancer-derived NUGC3 cells, cells were infected for 3 days and examined for viral RNA levels by quantitative RT-PCR as described below.

Plaque assay of ZIKV

The titre of ZIKV was measured by plaque assay as described elsewhere [59]. Briefly, 10-fold serial dilutions of the viral stock were prepared and applied to Vero and HuH-7 cells for 60 min at 37°C. The medium was removed and covered with 1% Agar in DMEM supplemented with 10% FBS. After incubation for 5 days, the Vero and HuH-7 cells were fixed with 4% formaldehyde and stained with 1% crystal violet.

Nucleosides extraction and mass spectrometry

All cells except MCF-10-2A cells were cultured in DMEM supplemented with 10% FBS until confluent. Culture medium was replaced with fresh medium and incubated for 24 h. Nucleosides in cytosol and culture medium were extracted as follows. To extract nucleosides from the supernatants, 500 µL supernatants were mixed with 500 µL 100% methanol. Precipitants were cleared by centrifugation at 15,000 rpm for 10 min. Next, 500 µl of centrifugal supernatant was transferred to Nanosep 3 K Omega ultrafiltration columns and centrifuged at 12, 000 × g for 2 h. The eluate (300 µl) was dried using a Savant SpeedVac SPD1010 (Thermo Fisher Scientific) and the pellet resuspended in ultrapure water. To extract intracellular nucleosides, cells were washed three times with phosphate-buffered saline (PBS) and nucleosides were extracted with 1 mL 80% methanol. The extracts were cleared by centrifugation at 12,000 rpm for 10 min. The supernatant was dried by evaporation and the pellet was resuspended in ultrapure water.

Samples were analysed with a triple-quadruple mass spectrometer (LCMS-8050, Shimazu, Kyoto, Japan) equipped with an electrospray ionization (ESI) source and a liquid chromatography system. Nucleosides were separated using a C18 column as described previously [17]. Modified and unmodified nucleosides were detected by the multiple reaction monitoring (MRM) method in positive ion mode. MRM parameters for nucleosides examined in this study can be found in previous study [17].

Pulse-chase metabolic labelling with ^{13}C-methionine

Pulse-chase of methylated nucleosides was performed as described previously [17]. A final concentration of 1 mM stable isotope labelled methionine (^{13}C-methionine) was added to NUGC3 cells in methionine-/cysteine-free DMEM

medium supplemented with 10% dialysed FBS for 12 h. Subsequently, cells were washed with PBS for 3 times and replaced with normal culture medium. Culture medium was collected 0 h, 12 h, 24 h, 48 h, 72 h, 96 h after changing to normal medium, followed by mass spectrometry analysis.

Generation of knockout cells by CRISPR-Cas9

To generate ENTs-knockout cells by CRISPR-Cas9, DNA oligos targeting each ENT were purchased from Hokkaido System Science and cloned into the LentiCRISPR v2 vector (Addgene #52,961). Ten µg of LentiCRISPR v2 vector was mixed with 7.5 µg psPAX2 (Addgene #12,260) and 2.5 µg pMD2.G (Addgene #12,259) and transfected into 293 FT cells using Lipofectamine 3000 reagent in Opti-MEM™. After overnight incubation, the medium was replaced with fresh medium and cultured for 48 h. Supernatant containing viral particles was collected and filtered through a 0.45 µm sterile filter unit containing a Durapore® PVDF membrane (Millipore). One mL of viral supernatant was used to transduce NUGC3 cells cultured in a 6-well plate. At 4 days after transduction, cells were dissociated and seeded into a 10-cm dish at a density of 40 cells/dish in the presence of 2 µg/ml puromycin. To generate ENT1 and ENT2 double knockout (DKO) cells, lentivirus was generated using lentiGuide-Hygro -dTomato vector (Addgene #99,376) carrying oligo DNA targeting ENT2. The lentivirus was added to ENT1-knockout cells and DKO cells were selected in the presence of 2 µg/ml puromycin and 400 µg/ml hygromycin B.

Re-expression of ENT1 and ENT2 in DKO cells

Total RNA of HEK293FT cells was subjected to reverse transcription using PrimeScript RT Master mix with oligo-dT primers. Human *ENT1* and *ENT2* were amplified using regular PCR and cloned into pENTR/D-TOPO vector. PCR products encoding *ENT1* and *ENT2* were further cloned into pLenti7.3/V5-DEST Gateway vector and pLX303 vector (Addgene #25,897), respectively. pLenti7.3/V5-DEST carries the *GFP* gene, which enabled FACS sorting of DKO cells infected with Lentivirus carrying *ENT1*. Briefly, infected cells were treated with 0.05% trypsin and resuspended in PBS. Cells expressing GFP were sorted into 15 mL tubes using an SH800 cell sorter (SONY) and centrifuged at 1,000 × g for 5 min. The resulting cells were plated in 10 cm dish for recovery and GFP-positive colonies were cloned using a cloning disk (Sigma). After ENT1-expressing DKO cells were established, lentivirus generated from the pLX303 vector carrying *ENT2* was infected into the DKO-ENT1 cells. The infected cells were treated with blasticidin to select clones expressing both ENT1 and ENT2 in the DKO cells.

Genotyping

Knockout cells generated using CRISPR-Cas9 was confirmed by genomic sequencing. Briefly, genomic DNA was extracted using QIAamp DNA Mini and Blood Mini kit according to the manufacturer's instruction. Ten ng DNA was subjected to PCR using KOD DNA polymerase, followed by electrophoresis in 1% agarose gel. PCR products were isolated from agarose gel and extracted using QIAquick Gel Extraction Kit according to the manufacturer's instruction. BigDye terminator v3.1 was used for sequence analysis of PCR product. Sequence of specific primers is listed in Supplementary Table S1.

Immunocytochemistry and confocal imaging

Cells were fixed with 4% paraformaldehyde for 15 min and treated with 0.1% triton-PBS for 15 min. Samples were incubated with blocking solution (3% bovine serum albumin in PBS) for 1 h at room temperature, followed by incubation with anti-LC3 antibody diluted in blocking solution at 4°C overnight. Cells were washed with PBS and incubated with secondary antibody for 1 h at room temperature. Cell nuclei were stained with 0.1 µg/ml 4,6-diamidino-2-phenylindole (DAPI) for 15 min at room temperature. A FV3000 confocal microscope (Olympus) was used to observe cellular fluorescence using FluoView software (Olympus).

RNA isolation and quantitative RT-PCR

Total RNA from cells and tissues were isolated using TRI Reagent and processed according to the manufacturer's instructions. The isolated RNA was diluted to 50 ng/µl. Recombinant DNase I was added to RNA samples and incubated for 20 min at 37°C to reduce genomic DNA background. The RNA was then reverse-transcribed into cDNA using PrimeScript™ RT Master Mix. Quantitative RT-PCR was performed using gene-specific primers and SYBR Premix ExTaq™. The sequences of gene-specific primers (18S as a reference) are shown in the Supplemental Table S1. For each condition, we prepared three technical replicates and performed three independent experiments unless otherwise noted.

Western blotting

Protein from each cell line was extracted using lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 1% NP-40) with protease inhibitor cocktail. Cell lysates were sonicated and centrifuged at 10,000 × g for 5 min at 4°C. Protein concentrations were measured using a Pierce BCA protein assay kit. Protein samples were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane. Detection was performed using an ECLPlus chemiluminescence kit (GE Healthcare). All primary antibodies were used at a 1:1,000 dilution in 5% non-fat milk (skim milk), and secondary antibodies were used at a 1:5,000 dilution in 5% skim milk.

Screening of modified nucleosides

For screening of modified nucleosides that can induce an autophagy response, commercially purchased modified nucleosides (m^1A, m^6A, m^1I, Im, m^1G, m^2G, Gm, m^2_2G, m^3C, m^5C, Cm, ac^4C, Um, ψ, D) were added to

NUGC3 cells at a final concentration of 1 μM for 24 h. Cells were washed with PBS for 3 times and collected for examining intracellular modified nucleosides by mass spectrometry, western blot, and immunocytochemistry, as described above.

Quantification and statistical analysis

All data were analysed using GraphPad Prism 9 software. Unpaired Student's t-tests was used to assess differences between two groups, and one-way ANOVA tests followed by Tukey's multiple comparisons were used to examine differences among multiple groups. Heatmap and principal component analysis were performed using MetaboAnalyst 5.0 website [60]. A two-tailed p-value of 0.05 was considered significant. Data are presented as means ± standard error of mean (SEM).

Disclosure statement

No potential conflict of interest was reported by the authors.

Author Contributions

S.S., H.F., T.K. performed experiments and analysed data. T.C. and H. O. provided methodology. S.S. and F.Y.W. wrote the manuscript. K. T. and F.Y.W. supervised the project. F.Y.W. conceptualized the project.

Data availability

All data and cell resources presented in this study are available upon request.

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