

TITLE

Membrane Repair Proteins as Negative Regulators of Cytosolic Delivery Using Attenuated Cationic Lytic Peptide L17E and Cell-Penetrating Peptides: Differences and Similarities

AUTHOR NAMES

Masashi Kuriyama¹, Yoshimasa Kawaguchi¹, Shinji Ito², Junko Satoh², Hisaaki Hirose^{1*}, and Shiroh Futaki^{1,#*}

AUTHOR ADDRESS

¹ Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan

² Medical Research Support Center, Graduate School of Medicine, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan

ABSTRACT

Cytosolic delivery of functional macromolecules is beneficial for intracellular targeting. Although numerous intracellular delivery methods have been developed, the biological factors that govern their efficacy remain poorly understood, thereby limiting the further advancement of existing approaches. L17E is an attenuated cationic amphiphilic lytic (ACAL) peptide developed by our research group that facilitates the cytosolic delivery of macromolecules, including antibodies and functional proteins. The L17E peptide exhibits varying levels of cytosolic delivery even within a single cell line, suggesting heterogeneity in cellular sensitivity to the delivery process. Based on the hypothesis that the specific proteins contribute to this variability, HeLa cells were sorted into L17E-sensitive and L17E-insensitive populations. Comparative proteome analysis of the membrane fractions of these two groups revealed that annexin A2, a membrane repair-related protein, was more abundant in L17E-insensitive cells. Time-lapse imaging and knockdown experiments indicated that annexin A2 negatively regulates L17E-mediated cytosolic delivery by sealing the plasma membrane regions permeabilized by the L17E peptide. To assess whether membrane repair factors also influence cytosolic delivery via conventional cell penetrating peptides (CPPs), we examined the roles of repair-related proteins. We found that the endosomal sorting complex required for transport (ESCRT) complex suppressed the cytosolic translocation of the CPPs, whereas annexin A2 did not. These findings suggest that the L17E peptide and the CPPs utilize distinct membrane repair-associated pathways during delivery, and the expression levels of these repair factors affect delivery efficiency. Modulating such factors may therefore provide a strategy for enhancing peptide-based cytosolic delivery systems.

KEYWORDS

intracellular delivery, attenuated cationic amphiphilic lytic peptide, cell-penetrating peptide, membrane repair, annexin A2, ESCRT complex

INTRODUCTION

Antibodies, owing to their high molecular recognition ability and strong target, have already emerged as highly prominent pharmaceutical modalities.^{1,2} However, due to their large molecular weight and hydrophilicity, antibodies have difficulty permeating the plasma membrane. As a result, most approved antibody therapeutics currently target extracellular molecules.^{3, 4} On the other hand, numerous potential drug targets exist within cells.^{5, 6} Consequently, extensive research has been conducted on intracellular delivery methods for bioactive macromolecules, including proteins such as antibodies, peptides, and therapeutic nucleic acids such as siRNA, miRNA, antisense oligonucleotides, aptamers, and DNA/mRNA vaccines, as well as nanoparticles encapsulating these molecules.⁷⁻¹¹ However, although numerous reports exist on the development of intracellular delivery methods, detailed studies on their delivery mechanisms remain limited.¹²⁻¹⁵ Elucidating these mechanisms is expected to contribute to improving existing delivery techniques and creating novel delivery concepts.

Cell-penetrating peptides (CPPs) have emerged as a promising tool for intracellular delivery. These peptides have been shown to internalize into cells via multiple mechanisms, including direct translocation across the plasma membrane and endocytosis.^{16, 17} The efficiency of CPP-mediated cytosolic delivery is influenced by various factors, including physicochemical properties and interactions with cell surface molecules such as membrane-associated proteoglycans.¹⁸⁻²¹ The plasma membrane has intrinsic repair mechanisms activated upon membrane damage, involving calcium-dependent recruitment of repair proteins such as annexins, ESCRT (endosomal sorting complex required for transport) complex, and dysferlin.²² These repair machineries may restore membrane integrity, preventing the cytosolic translocation of CPPs.²³⁻²⁵

We previously developed an attenuated cationic amphiphilic lytic (ACAL) peptide designated L17E, which enables the delivery of antibodies (IgG) and other functional proteins into the cytosol.²⁶ The L17E peptide transiently increases membrane permeability upon addition to cells, allowing bioactive macromolecules, including antibodies, to enter the cytosol within 5 minutes.²⁷ The L17E peptide is conceptually different from conventional CPPs. Conventional CPPs, such as octa-arginine (R8)^{28, 29} and penetratin (Pen),³⁰ are typically conjugated to payloads and used for intracellular delivery.^{29, 31} In contrast, the L17E and other ACAL peptides more strongly interact with and permeabilize the plasma membrane. This enables efficient intracellular delivery of proteins, including antibodies, simply by mixing them with the L17E peptide without the need for conjugation.^{26, 32-34} By examining the differences in L17E-mediated cytosolic delivery efficiency across 11 different cell lines, we identified KCa3.1, a calcium-activated potassium channel encoded by *KCNN4* gene, as a positive regulator of L17E activity.³⁵ L17E treatment was shown to activate KCa3.1 by inducing extracellular Ca²⁺ influx. The expression level of KCa3.1 was found to determine the cell line selectivity of L17E-mediated delivery.³⁵ However, even in the cell lines with high KCa3.1 expression, delivery efficiency did not reach 100%, suggesting that L17E sensitivity varies among individual cells within a single cell line. This prompted us to explore additional determinants of L17E-mediated delivery efficiency within a single cell line.

To this end, we utilized a cell sorter to isolate cells exhibiting cytosolic signals of cargo delivered by the L17E peptide (L17E-sensitive cells; positive HeLa cells) from those lacking cytosolic cargo signals (L17E-insensitive cells; negative HeLa cells). We then compared these two populations to identify the determinants of L17E susceptibility within a single cell line. In positive cells, L17E-mediated delivery efficiency was found to be 5–10% higher than in negative cells. Proteomic analysis revealed that the abundance of annexin A2 – a membrane repair-associated protein³⁶⁻³⁸ – in the membrane fraction was approximately 1.4 times higher in negative HeLa cells than in positive HeLa cells. Live-cell imaging and knockdown experiments further indicated that annexin A2 negatively regulates L17E-mediated

intracellular delivery via a membrane repair mechanism. Membrane repair proteins, including annexin A2, respond to extracellular Ca^{2+} influx. We investigated the effects of Ca^{2+} influx and other membrane repair proteins on the membrane translocation of cell-penetrating peptides (CPPs), which are representative intracellular delivery peptides. The cytosolic translocation of CPPs was enhanced by treatment of a Ca^{2+} chelator, suggesting that Ca^{2+} -dependent membrane repair responses exert an inhibitory effect on CPP internalization. Consequently, the ESCRT complex, another membrane repair-related factor,^{39, 40} was implicated as a suppressive regulator of cytosolic CPP translocation.

RESULTS And DISCUSSION

The L17E Peptide Exhibits Selectivity within Cell Lines on Its Cytosolic Delivery Activity.

In this study, HeLa cells – one of the most widely used human cell lines with a previously reported delivery efficiency of approximately 40% (i.e. percentage of the cells with cytosol-translocated cargos)^{27, 33} – were employed as a model to evaluate the efficiency of L17E-mediated cytosolic delivery. To investigate whether there are differences in L17E sensitivity within a single cell line (or intra-line variability in L17E sensitivity), HeLa cells were treated with Alexa Fluor 488 (AF488)-labeled dextran (10 kDa; hereafter AF488-dex10) in the presence of the L17E peptide. Cells were subsequently sorted based on AF488 fluorescence into populations with high (AF488-dex10-positive) and low (AF488-dex10-negative) cytosolic signals (Figure 1A, B left). Confocal microscopy observation performed immediately after sorting revealed that the high-signal fraction displayed diffuse cytosolic AF488-dex10 signals, whereas the low-signal fraction lacked the discernible cytosolic AF488-dex10 signals (Figure 1B, right).

The high-sensitivity population was designated positive HeLa cells (pHeLa), and the low-sensitivity population as negative HeLa cells (nHeLa) (Figure 1A). After culturing both fractions for approximately four days, L17E-mediated delivery efficiency – defined as the percentage of cytosolic AF488-positive cells – was assessed in pHeLa, nHeLa, and unsorted HeLa cells using newly added AF488-dex10 in the presence of the L17E peptide (Figure 1C). While no significant difference in L17E-mediated delivery efficiency was observed between nHeLa and unsorted HeLa cells, pHeLa cells consistently showed 5–10% higher delivery efficiency than both comparison groups. To confirm these trend using a different marker, the efficiency in propidium iodide (PI) delivery by the L17E peptide (percentage of the cells with cytosolic PI signal) was evaluated using a flow cytometry-based system previously established by our group³⁵ (Figure 1D). Consisted with the above results, pHeLa cells showed approximately 5% higher delivery efficiency of PI compared to the other two cell types. PI is membrane-impermeable and fluoresces only upon binding intracellular nucleic acids, including RNA.⁴¹ PI can thus be used as a model payload to specifically evaluate the extent of cytosolic translocation while eliminating the fraction of payload trapped in endosomes.³⁵ These results indicate that by isolating and culturing HeLa cells that have undergone L17E-mediated cytosolic delivery, a population of cells with high sensitivity to the L17E peptide can be obtained. These findings suggest that even within a single cell line, the sensitivity to the L17E peptide varies between individual cells, and higher sensitivity to the L17E peptide observed in pHeLa cells is maintained for at least 1-2 weeks.

It is also noteworthy that nHeLa cells – those initially exhibiting low L17E sensitivity – demonstrated comparable delivery efficiency after an additional four days of culture (Figure 1B). This observation suggests that not all cells within the sorted low-efficiency fraction remain consistently insensitive to L17E. One possibility is that variability in L17E-mediated delivery efficiency may be influenced by stochastic gene expression, such as random fluctuations in transcription, translation, and degradation rates – even within a genetically identical cell line⁴² – which could affect sensitivity to L17E-mediated delivery. Therefore, additional genes are likely to contribute to L17E activity. Notably, pHeLa cells consistently exhibit higher sensitivity to L17E, and in this study, we identified annexin A2 as one of the key factors underlying this differential response.

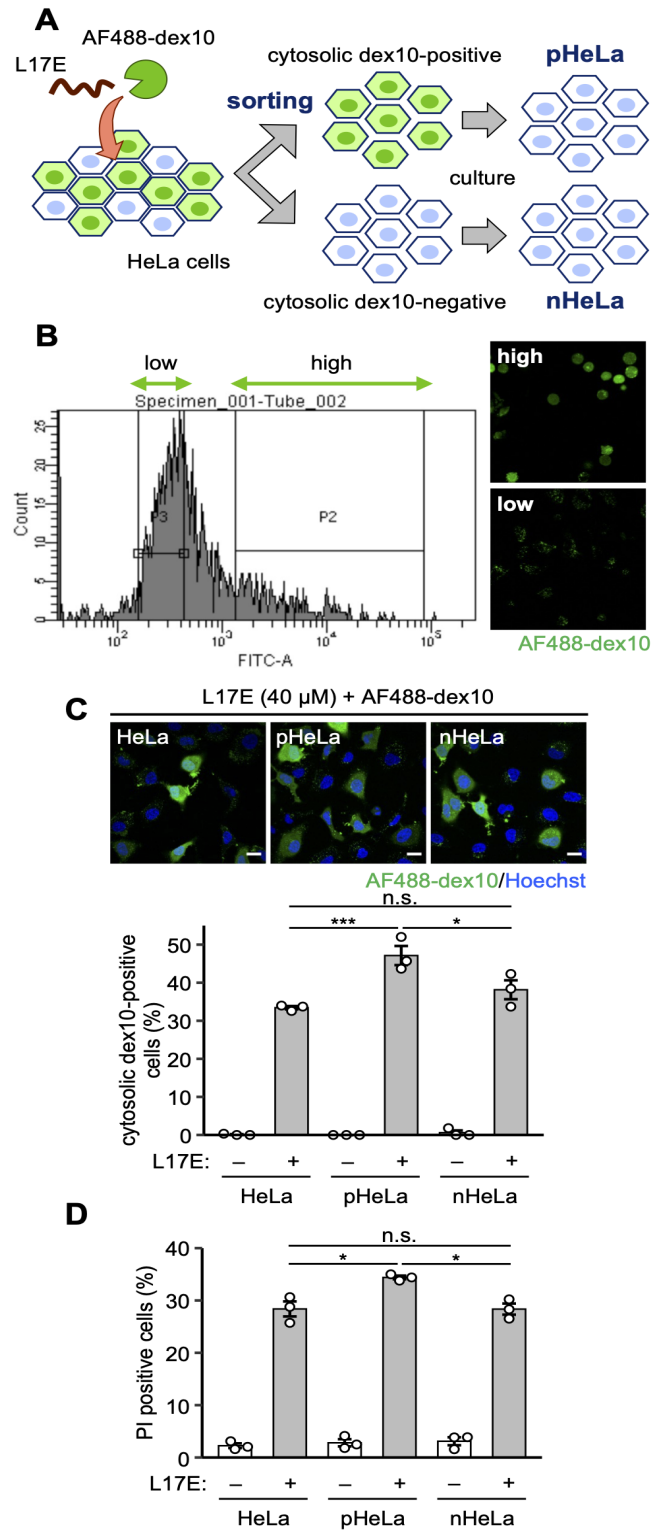


Figure 1. (A) Experimental overview. HeLa cells treated with AF488-labeled dextran 10 kDa (AF488-dex10) in the presence of the L17E peptide were separated into cytosolic AF488-dex10-positive and -negative populations using cell sorter. The collected cells were cultured and named pHeLa (cells derived from cytosolic dex10-positive HeLa cells) and nHeLa (cells derived from cytosolic dex10-negative HeLa cells), respectively. (B) Selection of cells based on AF488-dex10 fluorescence by cell sorter (left). Confocal microscopy observation of cells immediately after sorting (right). (C) Delivery of AF488-dex10 by the L17E peptide in HeLa, pHeLa, and nHeLa cells. Cells were treated with AF488-dex10 and the L17E peptide (40 μ M) for 10 min.²⁷ Representative confocal images (top), and proportion of the cells with cytosolic diffuse

AF488-dex10 signals (bottom). Nuclei were stained with Hoechst 33342 (blue). Scale bars, 20 μm . (D) PI delivery by L17E in HeLa, pHeLa, and nHeLa cells. Cells were treated with PI and the L17E peptide (40 μM) for 5 min. Flow cytometry analysis (bottom) of PI internalized cells. Data represent the mean $\pm$ standard error (SE) of three independent biological replicates (C, D). *, $p < 0.05$; ***, $p < 0.001$; n.s., not significant. One-way ANOVA followed by Tukey–Kramer’s post hoc test (C, D).

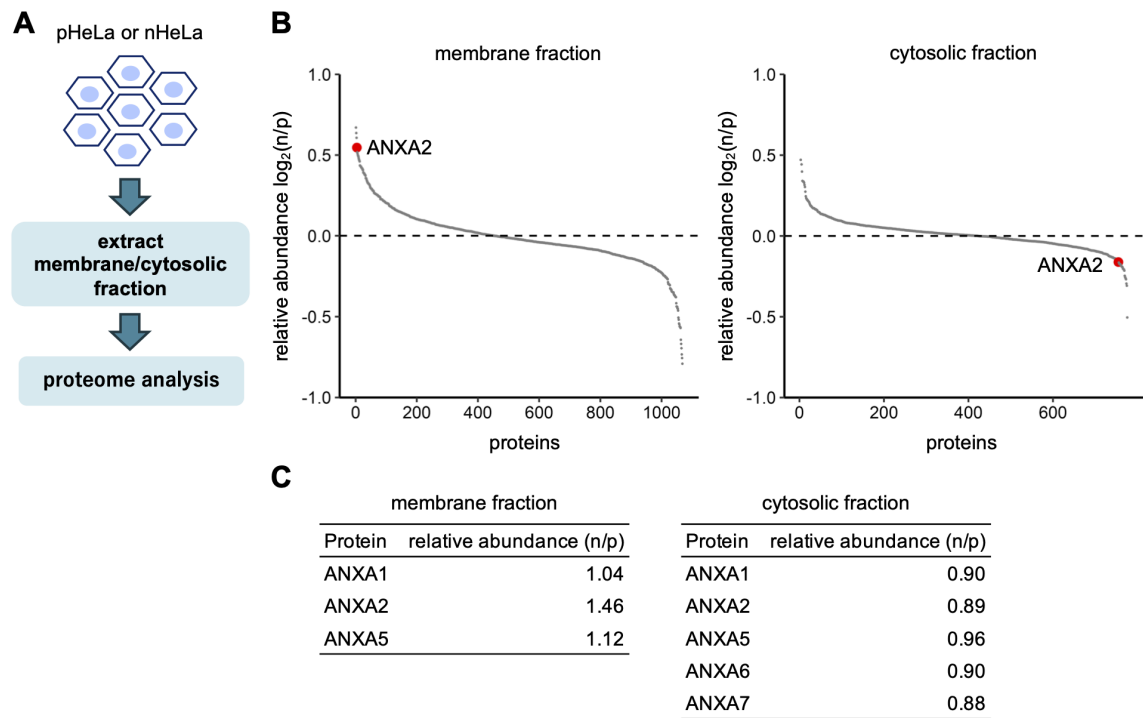


Figure 2. (A) Experimental overview. (B) Proteome comparison between pHeLa and nHeLa in the membrane fraction (left) and cytosolic fraction (right). The abundance of each protein is plotted as the $\log_2$ value of the nHeLa/pHeLa ratio (n/p). (C) nHeLa/pHeLa ratio of annexin family protein level identified in the membrane fraction (left) and cytosolic fraction (right).

Proteome Analysis Identifies Annexin A2 as a Potential Regulator of L17E-Mediated Delivery.

We hypothesized that the difference in delivery efficiency by the L17E peptide between pHeLa and nHeLa cells was due to differences in protein expression. First, we examined the expression level of KCa3.1, which we previously identified as a determinant of the variation in L17E activity among various cell lines.³⁵ The mRNA levels of *KCNN4*, which encodes KCa3.1, in pHeLa and nHeLa cells were evaluated by quantitative PCR (qPCR). However, no marked differences in *KCNN4* mRNA levels were observed between the original HeLa (unsorted), pHeLa, and nHeLa cells (Figure S1A), implying that other cellular factors are also involved in regulating L17E activity in the single cell line. To comprehensively investigate potential cellular factors determining L17E activity, we conducted comparison of protein levels between pHeLa and nHeLa by proteomic analysis (Figure 2A). Proteins from the membrane and cytosolic fractions were extracted from both pHeLa and nHeLa. We confirmed that Na⁺/K⁺-ATPase and Ca²⁺-ATPase4 (PMCA4), both membrane marker proteins, were excluded from the cytosolic fractions, whereas glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β -actin, both cytosol marker proteins, were relatively enriched in cytosolic fractions (Figure S2). Comparison of the protein levels in the membrane and cytosolic fractions revealed differences in the abundance of several proteins between pHeLa and nHeLa (Figure 2B). KCa3.1 was not identified in this analysis; therefore, differences in KCa3.1 expression at protein level between pHeLa and nHeLa could not be evaluated. Among the identified proteins, we focused on annexin A2, a membrane repair-associated protein,³⁶⁻³⁸ because cytosolic delivery by L17E depends on transient permeabilization of plasma membrane.^{27, 35} annexin A2 was approximately 1.4-fold more abundant in the membrane fraction of nHeLa cells compared to pHeLa cells, whereas its abundance in the cytosolic fraction was slightly decreased in nHeLa (Figure 2B, C). qPCR analysis revealed no significant differences in annexin A2 mRNA levels among original HeLa, pHeLa, and nHeLa cells, suggesting that annexin A2 membrane localization differs in pHeLa and nHeLa cells (Figure S1B). These results led us to hypothesize that differences in the abundance of annexin A2 in membrane fraction may affect membrane repair activity between nHeLa and pHeLa cells, potentially leading to variations in intracellular delivery efficiency via the transient membrane damage induced by L17E.

Annexin A2 is Involved in Membrane Repair Response to Membrane Permeabilization by L17E Treatment.

Among the annexin A family members identified through our proteomic analysis, annexin A1,^{36, 43} and annexin 5,^{44, 45} which are also known to be associated with membrane repair, showed no significant differences in protein levels between pHeLa and nHeLa in the membrane fraction. To investigate whether the localization of annexin A1, A2, and A5 changes in response to transient plasma membrane permeabilization induced by L17E treatment, their respective EGFP (enhanced green fluorescent protein) fusion proteins [annexin A1 (ANXA1)-EGFP, annexin A2 (ANXA2)-EGFP, and annexin A5 (ANXA5)-EGFP] were transiently expressed in HeLa cells. The intracellular localization of these fusion proteins upon L17E treatment was monitored by time-lapse imaging using confocal microscopy in conjugation with PI fluorescence. As a control, cells expressing EGFP alone showed no change in intracellular EGFP localization following L17E treatment (Figure 3A, EGFP). In contrast, cells expressing ANXA2-EGFP exhibited significant accumulation of ANXA2-EGFP at the plasma membrane at sites where PI influx into the cytosol occurred following L17E treatment. Notably, PI influx upon L17E treatment was observed in only a limited area at the cell periphery rather than across the entire plasma membrane, as previously observed for the influx of arginine-rich CPPs.^{46, 47} Strong accumulation of ANXA2-EGFP was observed at the PI influx points (Figure 3A, ANXA2-EGFP). In contrast, only marginal accumulation of fluorescence signals was observed

at PI influx points for both ANXA1-EGFP and ANXA5-EGFP after the L17E treatment (Figure 3A, ANXA1-EGFP and ANXA5-EGFP), suggesting a minimal contribution of annexin A1 and A5 to the L17E-mediated delivery. The proportion of cells showing EGFP signal accumulation at the plasma membrane among those with observable PI influx during L17E treatment was then analyzed. For ANXA2-EGFP, more than 90% of cells (31 of 33 cells) showed signal accumulation (Figure 3B, ANXA2-EGFP). In contrast, 30% of cells (9/30 cells) expressing ANXA1-EGFP and approximately 24% of cells (7/29) expressing ANXA5-EGFP exhibited membrane accumulation (Figure 3B, ANXA1-EGFP and ANXA5-EGFP). As a control, EGFP alone showed no accumulation (0 of 21 cells) (Figure 3B, EGFP). These results suggest that among the tested EGFP-tagged annexins, ANXA2-EGFP exhibited the most pronounced L17E-dependent membrane accumulation. In contrast, when ionomycin – a Ca^{2+} ionophore that induces a stronger Ca^{2+} influx than L17E (Figure S3) – was applied to elevate cytosolic Ca^{2+} levels, all three ANXA-EGFP proteins (ANXA1-EGFP, ANXA2-EGFP, and ANXA5-EGFP) were recruited to the plasma membrane in HeLa cells (Figure 3C). These findings indicate that the transiently expressed EGFP-tagged annexins were functional, and that differences in Ca^{2+} sensitivity among these annexins may account for their differential responses to L17E-mediated Ca^{2+} influx. Thus, among the annexin family members identified in the proteomic analysis, annexin A2 appears to play the most significant role in repairing membrane damage induced by L17E (Figure 2C). This may explain why only annexin A2, among annexin A1, A2, and A5, showed differences in protein levels in the membrane fraction between pHeLa and nHeLa (Figure 2C). Notably, although annexin A6 has been reported to play an important role in membrane repair⁴⁸ and was relatively abundant in cytosolic fraction, its presence in the membrane fraction was not prominent (Figure 2C). Given that membrane-localized repair proteins are likely more relevant for repairing the membrane perturbation caused by L17E, we did not further evaluate the contribution of annexin A6.

Annexins are cytosolic proteins that commonly bind to negatively charged phospholipids – particularly phosphatidylserine (PS), phosphatidic acid (PA) and phosphatidylinositol 4,5-bisphosphate [$\text{PI}(4,5)\text{P}_2$] – at elevated Ca^{2+} levels.^{48,49,50} Annexin A1, A2, and A5 share the well-conserved annexin core domains that possess common Ca^{2+} -dependent binding properties to these negatively charged phospholipids. However, these proteins have distinct N-terminal domains with different Ca^{2+} sensitivities. These N-terminal domains are also involved in binding to partner proteins, stabilizing their membrane binding and facilitating membrane repair, including S100 proteins.⁵¹ Therefore, the more pronounced accumulation of annexin A2 at the plasma membrane over annexin A1 and A5 following L17E treatment may be explained by the differences in their Ca^{2+} binding ability. As support for this, Ca^{2+} responsiveness has been reported to vary among the annexin A family,⁵² and annexin A2 has a 4-fold higher interaction with S100A11 protein in the presence of Ca^{2+} compared to annexin A1.⁵³

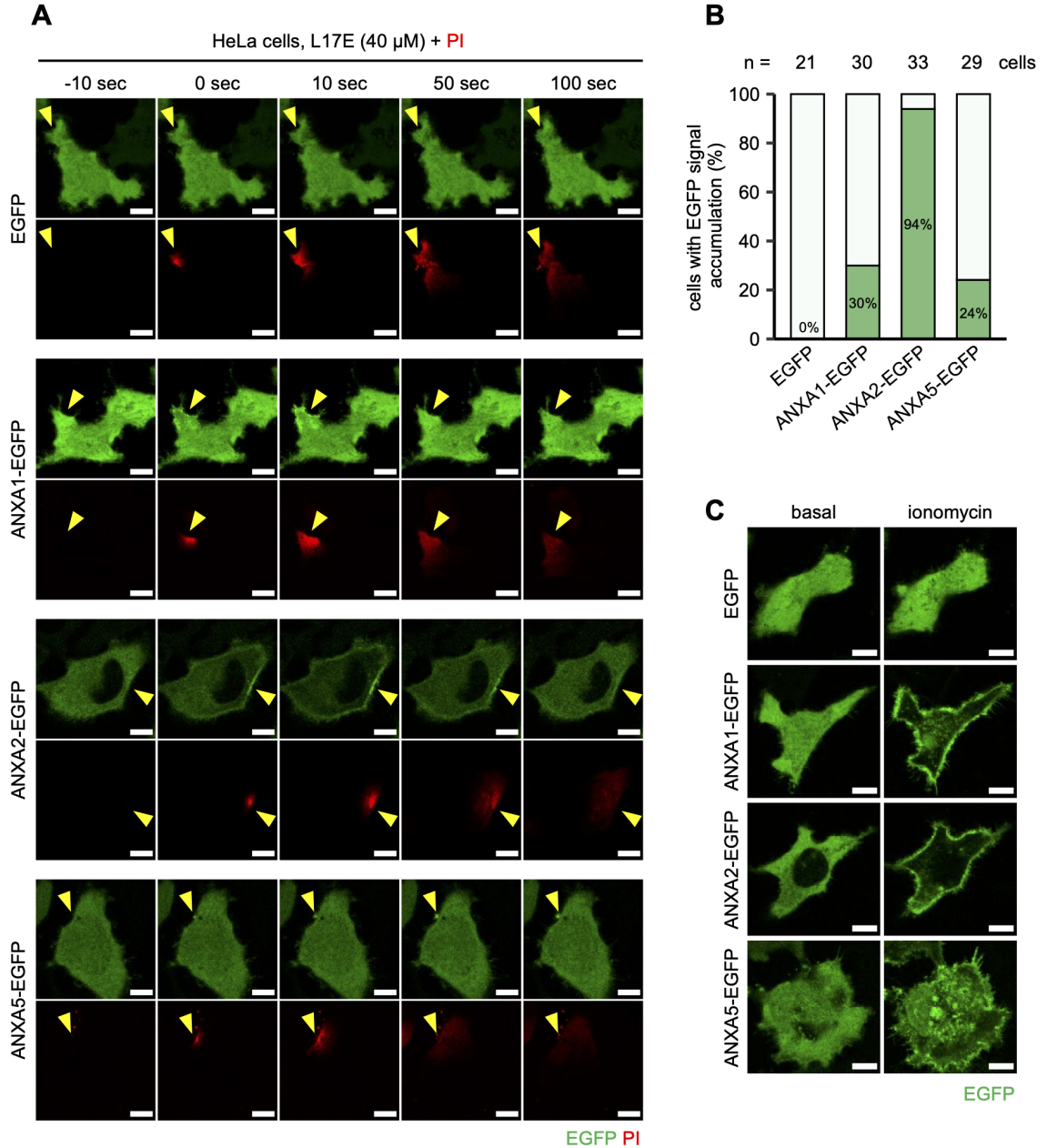


Figure 3. (A) Confocal time-lapse imaging of HeLa cells transiently expressing EGFP, ANXA1-EGFP, ANXA2-EGFP, or ANXA5-EGFP. Cells were treated with PI (50 μ g/mL) and the L17E peptide (40 μ M). Yellow arrowheads indicate PI influx points. The time after the initiation of PI influx is shown above the images. (B) Proportion of cells showing EGFP signal accumulation among those with observed PI influx. Green: cells with EGFP signal accumulation; light green: cells without accumulation of the signal. Cells from three independent biological replicates were analyzed. (C) Confocal time-lapse imaging of HeLa cells transiently expressing EGFP, ANXA1-EGFP, ANXA2-EGFP, or ANXA5-EGFP. Cells were treated with ionomycin (5 μ M). Images show the cells before and after treatment with ionomycin. Note that the lack of significant nuclear staining of ANXA2-EGFP may be due to the presence of a nuclear export sequence (NES).⁵⁴ Scale bars, 10 μ m.

Annexin A2 Negatively Regulates L17E-Mediated Cytosolic Delivery in HeLa and T24 cells.

To investigate whether annexin A2 modulates L17E activity, we performed si-RNA mediated knockdown of annexin A2. The knockdown efficiency in HeLa cells was confirmed by western blotting (Figure 4A). Annexin A2 knockdown did not significantly affect the L17E-induced cytotoxicity (Figure S4). However, annexin A2 knockdown slightly but significantly increased the efficiency of L17E-mediated delivery of PI (Figure 4B). Furthermore, in T24 cells – which exhibits low *KCNN4* expression and minimal intracellular delivery efficiency³⁵ – annexin A2 knockdown significantly increased delivery efficiency (Figure 4C, D). These results suggest a possibility that suppressing annexin A2 may enhance L17E-mediated delivery even in cell lines with low *KCNN4* expression and correspondingly low L17E sensitivity. The results also indicate that annexin A2 functions as a negative regulator of L17E activity in both HeLa and T24 cells. The amount of annexin A2 recruited to the plasma membrane varies among individual cells within a single cell line and appears to determine cellular sensitivity to L17E, resulting in heterogeneity of delivery efficiency (Figure 4E).

Interestingly, overexpression of ANXA family members in HeLa cells and HT1080 cells – which were previously shown to exhibit high-efficiency intracellular delivery by L17E³⁵ – did not markedly enhance L17E-mediated delivery. We examined the effects of annexin A1, A2, and A5 by transiently overexpressing ANXA1-EGFP, ANXA2-EGFP, and ANXA5-EGFP, respectively. Overexpression of ANXA1-EGFP, ANXA2-EGFP, or ANXA5-EGFP did not alter the L17E-mediated delivery efficiency compared to overexpression of EGFP alone as control (Figure S5). These findings indicate that while reducing annexin A2 expression enhances L17E activity, increasing the expression of annexin A2 or other annexin A family members (annexin A1 or A5) does not suppress it. This suggests that endogenous annexin A2 may be functionally sufficient, and that its overexpression does not significantly enhance the membrane repair activity. It should also be noted that overexpression may not substantially affect the amount of functional annexin A2 localized on the plasma membrane.

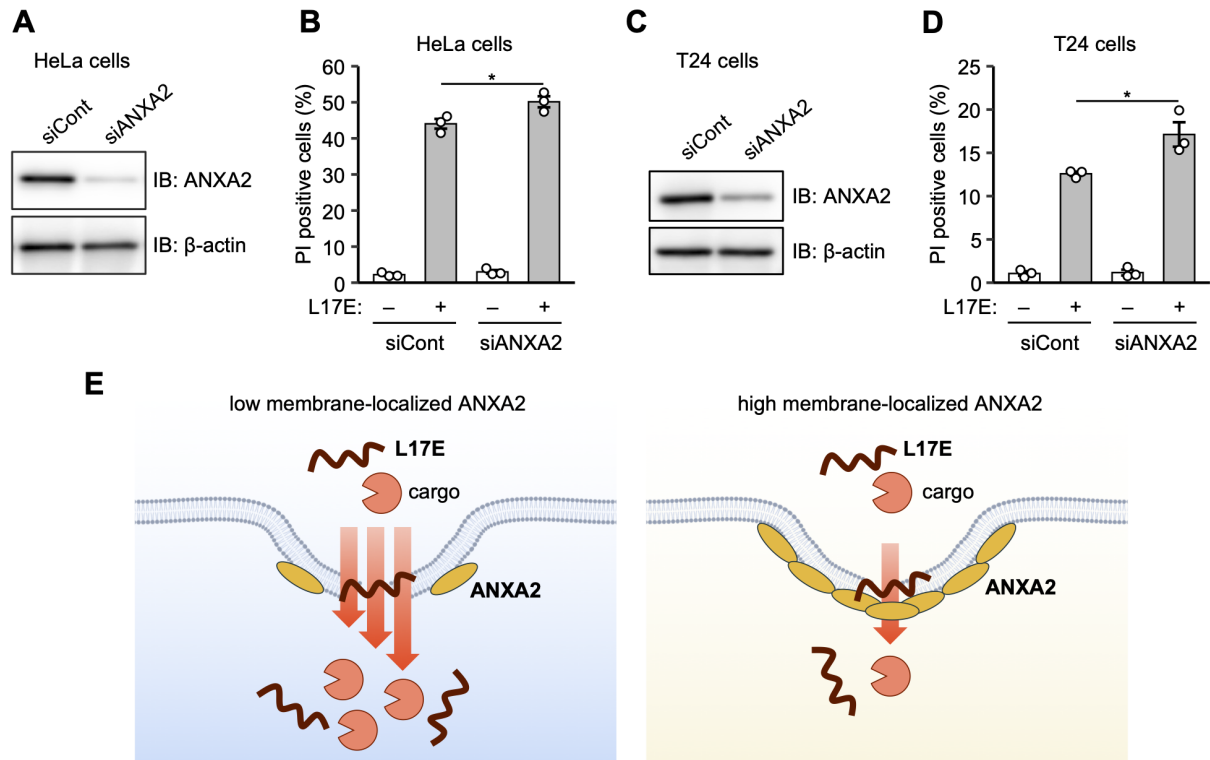


Figure 4. (A) Western blot analysis for annexin A2 in HeLa cells transfected either with control siRNA (siCont) or annexin A2 siRNA (siANXA2). (B) PI delivery by L17E in annexin A2 knockdown HeLa cells. Cells were transfected either with siCont or siANXA2 and treated with PI and L17E (40 μ M) for 5 min. PI delivery efficiency was analyzed by flow cytometry. (C) Western blot analysis for annexin A2 in T24 treated as (A). (D) PI delivery by L17E in annexin A2 knockdown T24 cells treated as (B). (E) Proposed involvement of annexin A2 in L17E activity. Cells with low membrane-localized annexin A2 level are more sensitive to L17E-mediated cytosolic delivery (left). Cells with high membrane-localized annexin A2 level are less sensitive to L17E due to its strong membrane repair activity. Data represent the mean $\pm$ SE of three independent biological replicates; *, $p < 0.05$. One-way ANOVA followed by Tukey–Kramer’s post hoc test (B, D).

Similarities and Differences in the Contribution of KCa3.1 and Annexin A2 to L17E- and CPP-mediated Intracellular Delivery.

We have previously shown that KCa3.1 channel is a positive regulator,³⁵ and now demonstrate that annexin A2 a negative regulator of the L17E activity. It has been reported that the expression of potassium channels, including KCa3.1, and the maintenance of membrane potential are critical for the cytosolic translocation of conventional CPPs.^{20, 55} Additionally, prior to our findings, Widmann and colleagues proposed that potassium channels, including the KCa3.1 channel, contribute to cellular uptake of the Tat-RasGAP317-326 conjugate peptide.²⁰ However, the contributions of membrane repair mechanisms and Ca^{2+} to CPP-mediated cytosolic delivery remain poorly understood.^{23, 24} As described in Introduction, L17E possesses distinct features compare to conventional CPPs. This prompted us to evaluate what extent L17E- and CPP-mediated delivery share similar mechanisms of action.

As previously reported, L17E-mediated cytosolic PI delivery was markedly inhibited by the knockout of *KCNN4* (encoding KCa3.1 channel) or by depolarization in K buffer (HEPES-based buffer containing 140 mM KCl instead of NaCl)³⁵ (Figure 5A). Note that a model cargo, PI, was added to the cell culture in the presence of the L17E peptide as a mixture, rather than as a conjugate – our previous study showed that simply mixing the L17E peptide with cargoes without covalent conjugation provides more efficient delivery than using covalently conjugated forms.⁵⁶ In contrast, intracellular delivery using representative CPPs, such as R8 and Pen, is generally performed with covalently bound CPP-cargo conjugates. A similar tendency was observed in the confocal microscopy analysis of the cytosolic distribution of fluorescein isothiocyanate (FITC)-labeled CPPs, including the R8 and Pen peptides (FITC-R8 and FITC-Pen), in *KCNN4* knockout cells or cells treated with K buffer (Figure 5B(i),(ii)), where FITC is used as a model cargo. We have also recently shown that L17E peptide tagged with tetra-arginine (L17ER4) functions as a more efficient CPP than R8 peptide or unmodified L17E peptide for protein delivery.⁵⁶ Cytosolic translocation of L17ER4 or L17E peptides bearing FITC at the C-termini [L17ER4(FITC) and L17E(FITC), respectively] was similarly diminished under *KCNN4* knockout or K buffer conditions (Figure 5(iii), (iv)). However, the results under the Ca^{2+} depletion condition were markedly different between L17E peptide and the others (Figure 5, BAPTA-AM). Treatment with intracellular Ca^{2+} chelator BAPTA-AM suppressed cytosolic PI delivery by the L17E peptide (Figure 5A), a result that was consistent with the diminished cytosolic translocation of the L17E(FITC) peptide (Figure 5B(iv)). On the other hand, translocation of FITC-R8, FITC-Pen and L17ER4(FITC) peptides was increased when the cells were treated with BAPTA-AM (Figure 5B(i)-(iii)). Notably, none of these CPPs induced cytotoxicity upon BAPTA-AM (Figure S6).

These differential responses to BAPTA-AM suggest distinct role for KCa3.1 and membrane repair mechanisms in L17E- versus conventional CPP-mediated intracellular delivery. We previously demonstrated that KCa3.1 activation via extracellular Ca^{2+} influx triggered by the interaction with the L17E peptide is essential for L17E-mediated cargo delivery into cells.^{35, 57, 58} In this study, we also showed that annexin A2 negatively regulates L17E activity. Membrane repair is known to be triggered by Ca^{2+} influx following plasma membrane damage, and membrane repair proteins such as annexins act in a Ca^{2+} -dependent manner.⁵⁹ Thus, BAPTA-AM treatment may have two opposing effects on L17E-mediated delivery: inhibition of KCa3.1 activation (a negative effect) and inhibition of annexin A2-mediated membrane repair (a positive effect). The fact that PI delivery was greatly diminished by BAPTA-AM treatment suggests that the inhibition of KCa3.1 plays a more dominant role than annexin A2 suppression in L17E delivery. Conversely, while *KCNN4* knockout and K buffer treatment suppressed CPP-mediated FITC delivery, BAPTA-AM treatment enhanced CPP-mediated FITC uptake compared to untreated wild-type (WT) cells. A possible explanation is that, unlike in L17E-mediated delivery, Ca^{2+} -triggered KCa3.1 activation may

not be essential for R8 uptake; rather, the resting membrane potential maintained by KCa3.1 and other potassium channels (e.g., KCNQ5 and KCNK5²⁰) may play a more significant role. Therefore, under BAPTA-AM treatment, a fraction of R8 can still translocate into cells, and membrane repair more critically regulates CPP-mediated delivery. Adjobo-Hermans et al. reported that treatment of HeLa cells with 10 μ M BAPTA-AM decreased cytosolic entry of nona-arginine (R9).⁶⁰ This result contrasts with our observation in Figure 5B. These discrepancies may be attributed to differences in the cell lines used (U2OS versus HeLa) or to the insufficient concentration (10 μ M) of BAPTA-AM to fully inhibit the mediated repair. Further investigation is required to resolve this discrepancy.

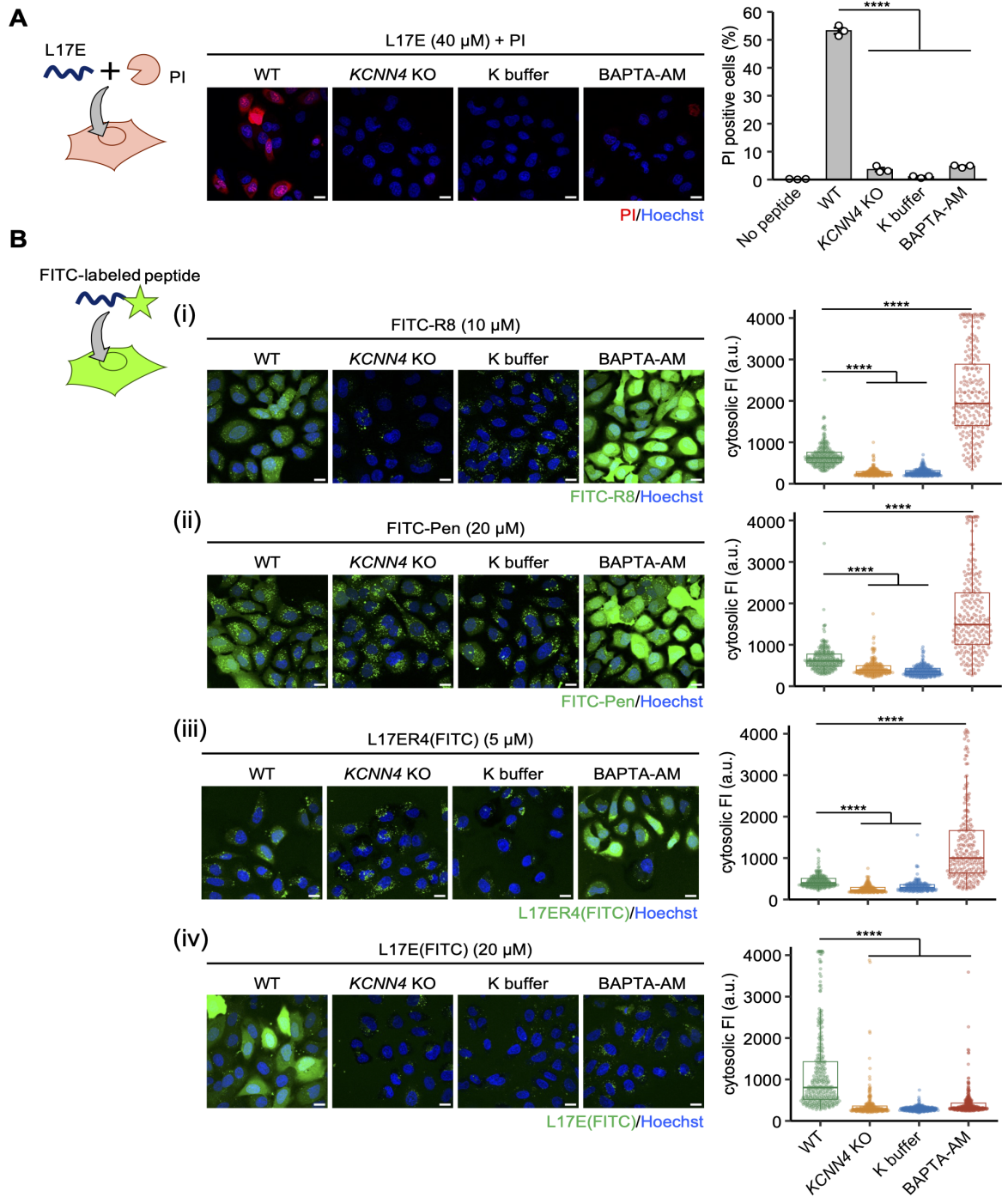


Figure 5. Effects on KCa3.1 channel, membrane potential, and Ca^{2+} in (A) cytosolic PI translocation in the presence of L17E and (B) cytosolic FITC delivery using the conjugates with CPPs (R8, Pen, and L17ER4) and the L17E peptide. In A, cells were treated with PI and the L17E peptide (40 μ M) for 5 min, and the cytosolic PI translocation was analyzed by confocal microscopy observation (left) and flow cytometry (right). Data represent the mean $\pm$ SE of three independent biological replicates. In B, cells were treated with FITC-labeled peptides of given concentration for 5 min [L17E(FITC)] or 30 min [FITC-R8, FITC-Pen, and L17ER4(FITC)], and the cytosolic translocation of these peptides was analyzed by confocal microscopy observation and the fluorescence intensity of FITC was analyzed (right). Data from three independent biological replicates are given in box plots. Shown are median (horizontal

bars), first and third quartiles (boxes), range (vertical bars). WT: wild-type U2OS cells; *KCNN4* KO: *KCNN4* knockout U2OS cells; K buffer: U2OS cells (wild type) incubated in K buffer; BAPTA-AM: U2OS cells (wild type) treated with 40 μ M BAPTA-AM. Scale bars, 20 μ m. ****, $p < 0.0001$. Kruskal-Wallis test followed by Steel-Dwass test. The derivatives labeled with FITC at the N-terminus of R8 and Pen peptides,⁶¹ and at the C-terminus of L17ER4 and L17E peptides,⁵⁶ as used in our previous studies, were employed. The concentration of each FITC-labeled peptide was adjusted to enable evaluation of the effect of each inhibitor while avoiding significant cytotoxicity to cells (see also Figure S6).

Cytosolic Translocation of CPPs Is Regulated Not by Annexin A2 but by ESCRT.

We then tested whether annexin A2 is involved in the cytosolic translocation of these CPPs. Although the possible effect of repair proteins on the membrane translocation of CPPs has been suggested,²³ few studies have explored this in detail. The ESCRT complex is known to participate in the repair of relatively small membrane damages and are recruited to damaged sites in a Ca^{2+} -dependent manner.^{39, 40} ESCRT proteins assemble into complexes that facilitate the removal of damaged membrane regions. Although the protective effect of the ESCRT complex against the membrane damage caused by the Tat-RasGAP317-326 conjugate peptide has been reported,²⁵ its contribution to facilitating membrane translocation of CPPs remains largely uncharacterized.

Interestingly, L17E-mediated cytosolic delivery into HeLa cells was enhanced by annexin A2 knockdown (Figure 4B). However, only a subtle decrease in the cytosolic translocation of conventional CPPs (R8 and Pen), and a subtle increase in that of the L17E-based CPP (L17ER4), were observed – though these changes were statistically significant (Figure 6A). Further study is necessary to explain the slight decrease in cytosolic translocation observed for conventional CPPs after annexin A2 knockdown. One possible explanation is that annexin A2 is only marginally involved in the membrane repair response following conventional CPP treatment, at least in HeLa cells.

To evaluate the involvement of the ESCRT complex in the cytosolic translocation of these CPPs, a dominant-negative VPS4a^{E228Q} mutant protein, which inhibits ESCRT disassembly, was overexpressed – VPS4a is a protein that facilitates the disassembly of the ESCRT complex.^{62, 63} Because prolonged inhibition of ESCRT function leads to cell death,^{64, 65} HeLa cells in which expression of VPS4a (WT or E228Q) can be controlled by doxycycline (Dox)-inducible system (HeLa VPS4a^{WT} and HeLa VPS4a^{E228Q}) were established (Figure S7A), and time-dependent expression of VPS4a after Dox treatment was confirmed (Figure S7B). The intracellular localization of VPS4a^{E228Q} differed from that of VPS4a^{WT}, indicating that VPS4a^{E228Q} impairs disassembly of the ESCRT complex and accumulates at the endosomal membrane (Figure S7C). Additionally, FITC-labeled CPPs did not induce cytotoxicity even after overexpression of VPS4a^{E228Q} (Figure S7D).

We then evaluated the cytosolic translocation of FITC-labeled CPPs under ESCRT-inhibited conditions. In HeLa VPS4a^{WT} cells, the cytosolic translocation of FITC-labeled R8, Pen, and L17ER4 was unchanged regardless of Dox treatment. In contrast, in HeLa VPS4a^{E228Q} cells, a significant increase in cytosolic translocation was observed for all three FITC-labeled CPPs after Dox treatment (Figure 6B). The cytosolic translocation of FITC-labeled L17E (L17E(FITC)) did not significantly increase in HeLa VPS4a^{E228Q} cells after Dox treatment, suggesting that ESCRT is not involved in L17E-mediated cytosolic delivery (Figure S7E).

These results suggest that ESCRT complex negatively regulates the cytosolic translocation of these CPPs and that inhibition of ESCRT can enhance their cytosolic translocation. Thus, the potential role of the ESCRT complex in the promoting the cytosolic translocation of CPPs such as R8, Pen and L17ER4, and the lack of contribution of ANXA2, were thus demonstrated.

While further investigation is needed to clarify why annexin A2 does not strongly influence CPP-mediated delivery, one possible explanation lies in the different modes of membrane interaction and permeabilization between the L17E peptide and CPPs. Unlike CPPs such as R8 and Pen peptides, the L17E peptide facilitates membrane translocation of large macromolecules like IgG antibodies, which is generally challenging for conventional CPPs – the L17E peptide may cause more extensive membrane disruption, thereby making membrane repair a more critical determinant of delivery efficiency.

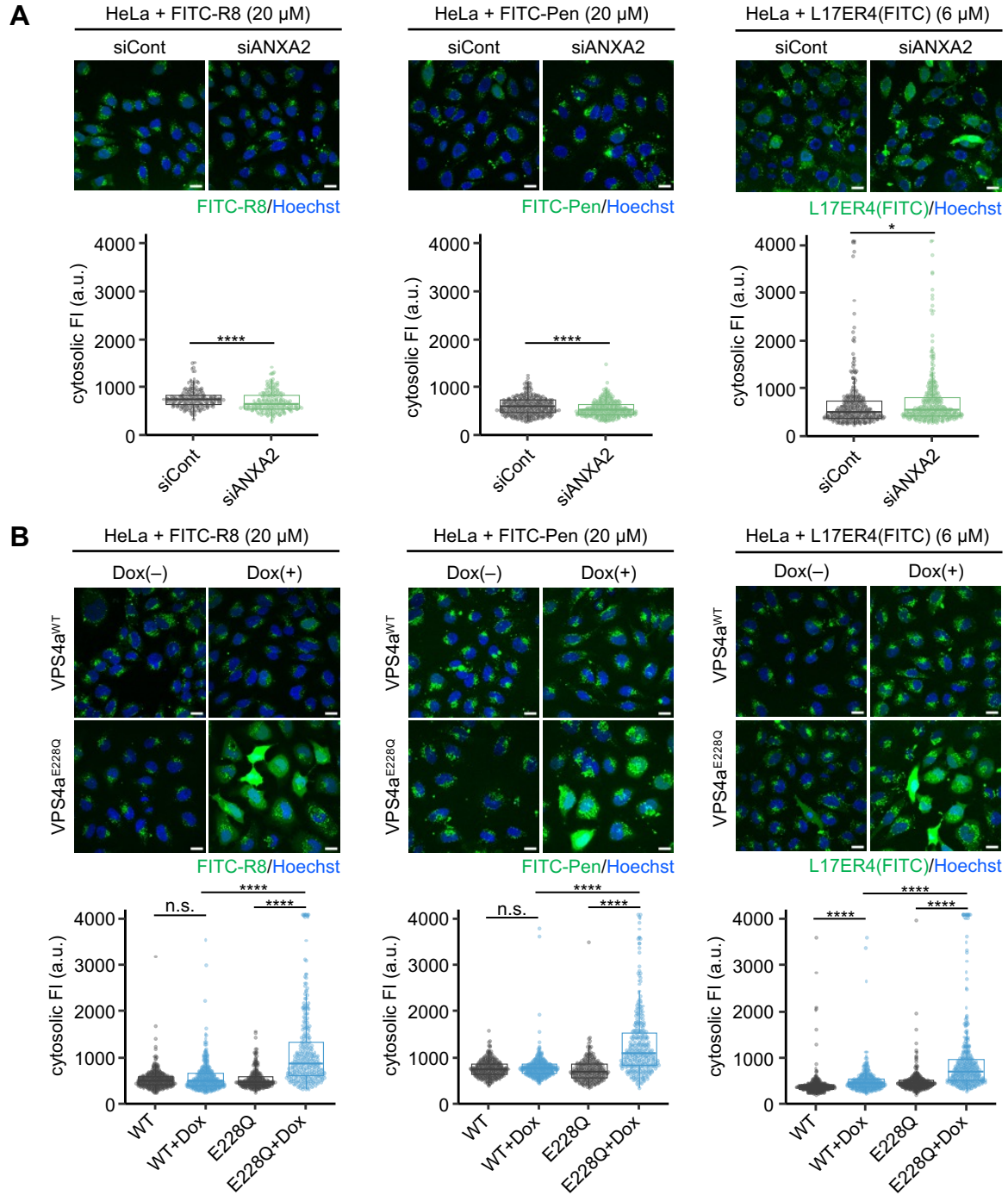


Figure 6. (A) Internalization of FITC-R8, FITC-Pen, and L17ER4(FITC) peptides in ANXA2 KD HeLa cells. HeLa cells were transfected either with control siRNA (siCont) or annexin A2 siRNA (siANXA2) and then treated with FITC-R8 (20 μ M), FITC-Pen (20 μ M), and L17ER4(FITC) (6 μ M) for 30 min, respectively. Confocal microscopy observation of FITC-labeled CPP internalization (top) and quantification of cytosolic fluorescence intensity of FITC-labeled CPPs (bottom). (B) Internalization of FITC-R8, FITC-Pen, and L17ER4(FITC) peptides in HeLa VPS4a^{WT} and HeLa VPS4a^{E228Q} cells. Cells were treated with Dox (1 μ g/mL) for 8 h and then incubated with FITC-R8 (20 μ M), FITC-Pen (20 μ M), and L17ER4(FITC) (6 μ M) for 30 min, respectively. Confocal microscopy observation of FITC-labeled CPP internalization (top) and quantification of cytosolic fluorescence intensity of FITC-labeled CPP (bottom). Scale bars, 20 μ m. Data from three independent biological replicates, except for

FITC-R8 in (A), which was analyzed using two independent replicates, are presented as box and dot plots. *, $p < 0.05$; ****, $p < 0.0001$; n.s., not significant. Wilcoxon rank-sum test (A) or Kruskal-Wallis test followed by Steel-Dwass test (B).

CONCLUSION

In this study, we demonstrated the heterogeneity of delivery efficiency within a single cell line, arising from differences in the activity of membrane repair-related factors. The L17E peptide enables efficient delivery of antibodies into cells when mixed with IgGs, although a relatively high concentration (40 μ M) was required. Further improvement and refinement of the delivery systems are necessary for future *in vivo* applications. Therefore, the aim of this study was to understand the mechanisms of intracellular delivery by L17E, with the ultimate goal of improving peptide-based delivery systems based on this knowledge. Since the mechanisms by which CPPs enter cells are largely unknown, the findings from this study may also have broader implications for other delivery methods. Novel strategies to modulate membrane-repair-related factors could lead to more efficient peptide-based intracellular delivery systems, and deepen our understanding of how cells maintain membrane integrity in response to external membrane-disrupting agents, including peptides, bionanomaterials, and viruses.

To understand the heterogeneity in the cell population that permits high-efficiency cytosolic delivery by the L17E peptide – even within the same cell line – we hypothesized that differences in expression levels of key regulatory proteins might underlie this phenomenon. Proteomic analysis after cell sorting and comparison of protein expression levels between L17E-responsive and non-responsive cells identified annexin A2 as a candidate. Subsequent studies revealed that membrane repair by annexin A2 plays a crucial role in regulating L17E-mediated cytosolic delivery, by preventing membrane permeabilization and thereby suppressing the cytosolic translocation of biomolecules through the plasma membrane. Interestingly, ESCRT was found to play a crucial role in CPP-mediated delivery instead of annexin A2 presumably because of differences in the membrane-interaction modes of these peptides. Collectively, these findings contribute to a deeper mechanistic understanding of peptide-based intracellular delivery and highlight the importance of membrane repair pathways as key modulators of delivery efficiency.

MATERIALS AND METHODS

Materials. α -MEM, D-MEM, doxycycline, and penicillin-streptomycin were purchased from FUJIFILM Wako Pure Chemical Corporation. Fetal bovine serum, bovine serum, and Opti-MEM were purchased from Gibco. Lipofectamine LTX, Lipofectamine RNAiMAX, and Hoechst 33342 were purchased from Invitrogen. Puromycin was purchased from Sigma-Aldrich. Alexa Fluor 488 labeled-dextran 10 kDa and Tet-System Approved FBS were purchased from Thermo Fisher Scientific. Reagents were dissolved in the manufacturer's recommended solvent, if necessary, aliquoted and stored at -30°C .

Peptide synthesis. Peptides were synthesized by Fmoc (9-fluorenylmethyloxycarbonyl)-solid-phase synthesis and the 3-[bis(dimethylamino)methylumyl]-3*H*-benzotriazol-1-oxide hexafluorophosphate (HBTU)/1-hydroxybenzotriazole (HOBt)/diisopropylcarbodiimide (DIEA) coupling system. L17E (IWLTKFLGKHAACKHEAKQQLSKL-amide) was synthesized using Syro I (Biotage) on Rink amide resin (TGS-RAM; Shimadzu). For the synthesis of FITC-R8 (FITC-GABA-RRRRRRRR-amide, GABA: γ -aminobutyric acid), R8 sequence with GABA was synthesized using Syro I (Biotage) on Rink amide resin (TGS-RAM) and then fluorescently labeled using fluorescein isothiocyanate isomer I (FITC-I, Sigma-Aldrich) and DIEA in dimethylformamide (DMF). FITC-penetratin (FITC-Pen, FITC-GABA-RQIKIWFQNRRMKWKK-amide) was similarly prepared using PSSM-8 (Shimadzu). L17E(FITC) (IWLTKFLGKHAACKHEAKQQLSKLGK(FITC)-amide) and L17ER4(FITC) (IWLTKFLGKHAACKHEAKQQLSKLGRRRRGK(FITC)-amide) were prepared as reported previously⁵⁶. Deprotection of protecting groups and cleavage from the resin were conducted using trifluoroacetic acid (TFA)/ethanedithiol (95:5) at room temperature ($\sim 25^{\circ}\text{C}$) for 3 h. Peptides were purified by reversed-phase high-performance liquid chromatography (RP-HPLC). The masses were confirmed by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF-MS). Observed mass (m/z) and calculated mass for $[M+H]^+$ were as followed: L17E (observed: 2859.0, calculated: 2858.7); L17E(FITC) (observed: 3489.9, calculated: 3490.19). L17ER4(FITC) (observed: 4114.1, calculated: 4114.6). FITC-R8 (observed: 1740.8, calculated: 1741.3). FITC-Pen (observed: 2718.7, calculated: 2719.8). All the peptides were dissolved in DMSO, aliquoted, and stored at -30°C . The final concentration of DMSO in the medium was set to no greater than 1% for all cellular treatments with peptides.

Cell culture. HeLa cells were maintained in α -MEM with L-Glutamine and Phenol Red supplemented with 10% (v/v) heat-inactivated bovine serum (BS) [α -MEM(+)]. HeLa VPS4a^{WT} and HeLa VPS4a^{E228Q} cells were maintained in α -MEM with L-Glutamine and Phenol Red supplemented with 10% (v/v) heat-inactivated Tet-System Approved FBS and 1 $\mu\text{g/mL}$ puromycin. Other cell lines were maintained in D-MEM with L-glutamine and Phenol Red supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) [D-MEM(+)]. All cells were cultured in humidified atmosphere containing 5% CO_2 at 37°C . Cells were passaged every 2-4 days and used between passages 1 and 20. Following cell lines were used: HeLa (human cervical cancer, ECACC, 93021013), HT1080 (human fibrosarcoma, JCRB, JCRB9113), T24 (human bladder cancer, JCRB, JCRB0711), and U2OS (human osteosarcoma, ECACC, 92022711). *KCNN4* KO U2OS cells were employed that established previously.³⁵

Plasmids construction. XLone-Puro eGFP was a gift from Xiaoping Bao (Addgene plasmid #140027; <http://n2t.net/addgene:140027>; RRID: Addgene_140027). To construct ANXA1-EGFP, human ANXA1 was amplified with cDNA derived from HeLa cells using the following primer pair: 5-GGACTCGAGATGGCAATGGTATCAGAATTC-3' (forward) and 5'-

GGAGGATCCTTGTTTCCTCCACAAAGAGCC-3' (reverse). The amplified product was introduced into the XhoI/BamHI sites of pEGFP-N1 vector (Clontech). To construct ANXA2-EGFP, human ANXA2 was amplified with cDNA derived from HeLa cells using the following primer pair: 5'-CAAGCTTCGAATTCATGGGCCGCCAGCTAG-3' (forward) and 5'-ACCGGTGGATCCGAGTCATCTCCACCACAC-3' (reverse). The amplified product was introduced into pEGFP-N1 vector using NEBuilder HiFi DNA Assembly (NEB). To construct ANXA5-EGFP human ANXA5 was amplified with cDNA derived from HeLa cells using the following primer pair: 5'-CAAGCTTCGAATTCATGGCACAGGTTCTCA-3' (forward) and 5'-ACCGGTGGATCCGAGTCATCTTCTCCACAG-3' (reverse). The amplified product was introduced into pEGFP-N1 vector using NEBuilder HiFi DNA Assembly (NEB). To construct pTRE3G-HA-VPS4a^{WT}, human VPS4a was amplified with cDNA derived from HeLa cells using the following primer pair: 5'-ATGACAACGTCAACCCTCCAG-3' (forward) and 5'-CTACTAGTTTAACTCTCTTGCCCCAAAGTCC-3' (reverse). The amplified product was introduced into XLone-Puro eGFP vector with HA-tag (Eurofins Scientific SE) using NEBuilder HiFi DNA Assembly. To construct pTRE3G-HA-VPS4a^{E228Q}, the region around E228 of human VPS4a was amplified using the following two pairs of primer: 5'-ATGACAACGTCAACCCTCCAG-3' (forward) and 5'-GGAATCCACCTGATCGATGAAGATG-3' (reverse), 5'-CATCTTCATCGATCAGGTGGATTCC-3' (forward) and 5'-CTACTAGTTTAACTCTCTTGCCCCAAAGTCC-3' (reverse). The amplified products were used to replace the VPS4a^{WT} region in pTRE3G-HA-VPS4a^{WT} using NEBuilder HiFi DNA Assembly.

Transfection. Transfection of plasmids was performed using Lipofectamine LTX (Invitrogen) according to the manufacturer's protocol. The transfection medium was changed 4 h after the transfection and subsequent experiments were performed 24 h after the transfection. Transfection of siRNA (20 nM) was performed using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. The transfection medium was changed 6 h after the transfection, and subsequent experiments were performed 72 h after the transfection. The following siRNAs were used: ON-TARGETplus Non-targeting Pool (Dharmacon, D-001810-10-05), ON-TARGETplus Human ANXA2 siRNA SMARTPool (Dharmacon, L-010741-00-0005).

Isolation of HeLa Cells by Cell Sorting. HeLa cells were seeded on 100-mm dishes (1×10⁶ cells/dish), incubated for 2 days. The cells were washed twice with PBS, followed by incubation with α -MEM(-) [α -MEM with L-Glutamine and Phenol Red supplemented without containing bovine serum (BS)] containing L17E (40 μ M) and AF488-dex10 (100 μ g/mL) at 37°C for 5 min. After washing twice with PBS, the cells were detached from the plate with 0.05% trypsin in PBS for 5 min at 37°C, added α -MEM(+) to prevent further digestion, and collected into centrifuge tubes. The cells were centrifuged (800 × g, 25°C, 5 min), and the resulting cell pellets were resuspended in α -MEM(-). The cells were sorted using a FACSAriaII (BD Biosciences). For each experiment, cells collected from five or six 100-mm dishes were used for sorting. The sorted cells were centrifuged (800 × g, 25°C, 5 min), and the resulting cell pellets were seeded on 100-mm dishes and cultured in α -MEM(+) containing penicillin-streptomycin for at least 3 days. For proteomics sample preparation, cells cultured for 3 or 4 days after sorting were used. For other experiments, cells within 14 days after sorting were used.

Enrichment of Membrane and Cytosolic Protein Fractions. Membrane and cytosolic protein fractions were prepared using the Mem-PER Plus Membrane Protein Extraction Kit

(Thermo Fisher Scientific) according to the recommended protocol. Cells grown to confluence in 100-mm dishes were washed twice with ice-cold PBS, and 1 mL of ice-cold PBS was added to detach the cells using a cell scraper. The detached cells were collected into tubes and centrifuged ($800 \times g$, 4°C , 5 min). The resulting cell pellets were washed with Cell Wash Solution, followed by centrifugation ($800 \times g$, 4°C , 5 min) and another wash with Cell Wash Solution. After a final centrifugation ($800 \times g$, 4°C , 5 min), the pellets were resuspended in Permeabilization Buffer and mixed by inversion at 4°C for 10 min. The samples were then centrifuged ($16,000 \times g$, 4°C , 15 min), and the supernatant was collected as the cytosolic fraction. The remaining pellets were resuspended in Solubilization Buffer and mixed by inversion at 4°C for 30 min. The samples were centrifuged again ($16,000 \times g$, 4°C , 15 min), and the supernatant was collected as the membrane fraction. Protein concentration was measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

Proteome Analysis. Twenty micrograms of protein were precipitated using cold acetone, air-dried, and dissolved in 30 mM ammonium bicarbonate solution containing 8 M urea. The proteins were reduced and alkylated with dithiothreitol and iodoacetamide, respectively, followed by digestion with trypsin. The resulting peptides were purified using the Pierce™ C-18 Spin Column (Thermo Fisher Scientific). An equivalent of 1 μg of purified peptides dissolved in 0.1% formic acid solution was introduced into a nano-flow liquid chromatography system (Nano-LC-Ultra 2D-Plus, Eksigent). After desalting with a trap column, the peptides were separated using an analytical column. The trap column was a $200 \mu\text{m} \times 0.5 \text{ mm}$ ChromXP C18-CL $3 \mu\text{m}$ $120 \text{ \AA}$ (Eksigent), and the analytical column was a $75 \mu\text{m} \times 15 \text{ cm}$ ChromXP C18-CL $3 \mu\text{m}$ $120 \text{ \AA}$ (Eksigent). Separation on the analytical column was performed using a binary gradient of two mobile phases (0.1% formic acid solution and 0.1% formic acid in acetonitrile) at a column temperature of 40°C and a flow rate of 300 nL/min. Peptides eluted from the analytical column were directly introduced into a quadrupole time-of-flight mass spectrometer (TripleTOF 5600+ System, SCIEX) via a flow path connected to the Nanospray III ion source (SCIEX). Peptides were positively charged by the ion source and introduced into the mass spectrometer. Data acquisition was performed using the information-dependent acquisition (IDA) method (SCIEX), comprehensively obtaining MS and MS/MS spectra. Peptides were identified using the ProteinPilot software (version 5.01, SCIEX) with the UniProtKB/Swiss-Prot human protein library (retrieved in July 2024). For comparing proteome compositions across samples, the Progenesis QI for Proteomics software (version 4.2, Nonlinear Dynamics) was employed. The dataset obtained from the measurements was imported into the Progenesis QI for Proteomics platform. After alignment and normalization, peptide peaks were assigned identification results (with confidence above 95%) obtained from ProteinPilot. Relative quantification of individual proteins was calculated using the Hi-N method (N=3, Nonlinear Dynamics).

Real-time qPCR. Total RNA was extracted from cells using NucleoSpin RNA Plus (Takara Bio) according to the manufacturer's protocol, and then 2 μg of total RNA was reverse transcribed to cDNA using PrimeScript RT Master Mix (Takara Bio). Real-time qPCR was performed using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) and QuantStudio 3 Real-Time PCR System (Applied Biosystems). The cycling conditions were 95°C for 3 min, then 40 cycles of denaturation at 95°C for 5 s followed by annealing and extension at 60°C for 30 s. mRNA expression was quantified by the standard curve method. *GAPDH* was used as a reference gene. The following primer pairs were used: 5'-CCTGTTTCGACAGTCAGCCG-3' (*GAPDH* forward) and 5'-CGACCAAATCCGTTGACTCC-3' (*GAPDH* reverse), 5'-CATTCTGACCATCGGCTATGG-3' (*KCNN4* forward) and 5'-

GCCTTGTTAACTCCAGCTTCCG-3' (KCNN4 reverse), 5'-
 TCGGACACATCTGGTGACTTCC-3' (ANXA2 forward) and 5'-
 CCTCTTCACTCCAGCGTCATAG-3' (ANXA2 reverse).

Western blot. Cells were seeded on a 12-well plate (1×10^5 cells/well), cultured for 1 day. For western blot for other than VPS4a, cells were washed twice with ice-cold PBS and lysed with 100 μ L of RIPA buffer (150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate and 50 mM Tris-HCl at pH 7.6) containing protease inhibitor cocktails (Roche), and phosphatase inhibitor cocktails (Roche). Lysates were cleared by centrifugation at $16000 \times g$ for 20 min at 4°C. For western blot for VPS4a, cells were washed twice with PBS and lysed with 100 μ L of SDS hot lysis buffer (1% SDS, 1 mM Na-orthovanadate, and 10 mM Tris-HCl at pH 8.0) preheated to 95°C and then heated at 95°C for 10 min. The protein concentrations were measured by BCA protein assay using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) and then unified to 0.5 μ g/ μ L. Lysates containing 5 (for other than VPS4a) or 10 (for VPS4a) μ g of protein were applied into a polyacrylamide gel (SuperSep Ace, 5–20%, 13 well; FUJIFILM Wako Pure Chemical Corporation) and fractionated by SDS-PAGE. The blots were transferred to a polyvinylidene difluoride membrane using Trans-Blot Turbo Transfer System (Bio-Rad), blocked with 5% BSA (for other than VPS4a) or 5% skim milk (for VPS4a) in Tris-buffered saline (TBS) containing Tween-20 (TBST) for 1 h at 25°C. The blots were then incubated overnight at 4°C with appropriate primary antibodies in blocking buffer. After washing the membrane with TBST for 10 min three times, the blots were further incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies in blocking buffer for 1 h at 25°C. After washing the membrane with TBST for 10 min three times, chemiluminescence was detected using ECL prime (GE Healthcare) and Amersham ImageQuant 800 (Cytiva). Images were analyzed using ImageJ software (NIH). The following antibodies were used: anti-ANXA2 (rabbit monoclonal, 1:1,000, Cell Signaling Technology, 8235), anti-PMCA4 (mouse monoclonal, 1:1,000, Sigma-Aldrich, P1494), anti-Na,K ATPase (rabbit polyclonal, 1:1,000, CST, #3010), anti-GAPDH (rabbit polyclonal, 1:10,000, Sigma, G9545), anti-HA (mouse monoclonal, 1:10,000, Sigma-Aldrich, H9658), anti- β -actin (mouse monoclonal, 1:10,000, Sigma-Aldrich, A2228), anti-rabbit IgG (HRP) (goat polyclonal, 1:2,000, Cell Signaling Technology, 7074), and anti-mouse IgG (HRP) (goat polyclonal, 1:5,000, GeneTex, GTX213111-01).

Dextran (10 kDa) delivery assay. The L17E-mediated cytosolic delivery of Alexa Fluor 488-labeled dextran 10 kDa (AF488-dex10) was evaluated by confocal microscopy observation. Cells were seeded on 35 mm glass-bottomed dishes (2×10^5 cells/dish) and incubated for 1 day. Cells were washed twice with PBS and incubated with AF488-dex10 (200 μ g/mL) and L17E (40 μ M) in α -MEM(–) for 10 min at 37°C. Cells were then washed twice with PBS and stained nuclei with Hoechst 33342 (5 μ g/mL) for 5 min at 37°C. Observation was performed with an FV3000 CLSM system equipped with a 60 $\times$ objective lens (UPlanSApo, oil immersion, NA 1.35). Images were analyzed with ImageJ software.

PI delivery assay. The cytosolic delivery of propidium iodide (PI) mediated by L17E was evaluated by flow cytometry analysis. Cells were seeded on 24-well plate (5×10^4 cells/dish) and incubated for 1 day. Cells were washed twice with PBS and incubated with PI (50 μ g/mL) in the presence of L17E (40 μ M) in α -MEM(–) (for HeLa cells) or D-MEM(–) (for other cell lines) for 5 min at 37°C. After washing twice with PBS, the cells were detached from the plate with 0.05% trypsin in PBS for 5 min at 37°C, added α -MEM(+) (for HeLa cells) or D-MEM(+) (for other cell lines) to prevent further digestion, and collected into centrifuge tubes. The cells were then centrifuged ($800 \times g$, 5 min) and the resulting pellets were washed with PBS. Flow

cytometry analysis was performed with 10,000 gated events using an Attune NxT Flow Cytometer (Thermo Fisher Scientific). For siRNA transfection, cells seeded on 6-well plate were transfected with each siRNA and re-seeded on 24-well plate 48 h after the transfection. Following experiments were conducted 24 h after re-seeding (72 h after the transfection). For plasmid transfection, cells seeded on 24-well plate were transfected with each plasmid and following experiments were conducted 24 h after the transfection. For BAPTA-AM treatment, cells were pretreated with BAPTA-AM (40 μ M) in D-MEM(–) for 30 min at 37°C and then treated with peptides in the absence of BAPTA-AM. For K buffer treatment, cells were treated with peptides in HEPES buffer [140 mM KCl, 5.4 mM NaCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 10 mM D-glucose, 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) at pH 7.4] containing 10% (v/v) heat-inactivated FBS.

Time-lapse Observation of EGFP-Labeled ANXA Localization. HeLa cells were seeded on 35-mm glass-bottomed dishes (1.5×10^5 cells/dish), cultured for 1 day and transfected with 200 ng of pEGFP-N1, pANXA1-EGFP, pANXA2-EGFP, or pANXA5-EGFP. After 1 day incubation, the cells were washed twice with PBS and the culture medium was replaced with α -MEM(–). The cells were placed at 37°C in a microchamber (STXG-IX3WX-SET; Tokai Hit) attached on the FV3000 microscope stage. Time-lapse imaging was started immediately after removal of α -MEM(–) from the glass-bottomed dish. DIC and fluorescence images were acquired every 5 sec for a total of 5 min. L17E (40 μ M) and PI (50 μ g/mL) in α -MEM(–) (200 μ L) were added to the glass part of the dish 30 sec after the start of imaging. Imaging was performed using an FV3000 confocal laser scanning microscope (CLSM) system (Olympus) equipped with a 60 $\times$ objective lens (UPlanSApo, oil immersion, NA 1.35; Olympus). Analysis was performed with ImageJ software (version 2.14.0, NIH).

Ca²⁺ imaging using Fluo-8. HeLa cells were seeded on 35-mm glass-bottomed dishes (2×10^5 cells/dish) and cultured for 1 day. Cells were washed twice with PBS and pre-incubated with Fluo-8 AM (5 μ M, AAT Bioquest) in α -MEM(–) for 40 min. Cells were then washed twice with PBS and the culture medium was replaced with Hanks' balanced salt solution (HBSS; 400 mg/L KCl, 60 mg/L KH₂PO₄, 8,000 mg/L NaCl, 350 mg/L NaHCO₃, 60 mg/L Na₂HPO₄ · H₂O, 1,000 mg/L D-glucose, 1.8 mM CaCl₂, 1 mM MgCl₂, and 20 mM HEPES, pH 7.4). The cells were placed at 37°C in a microchamber (STXG-IX3WX-SET; Tokai Hit) attached on the FV3000 microscope stage. Time-lapse imaging was started immediately after removal of HBSS from the glass-bottomed dish. DIC and fluorescence images were acquired every 10 sec for a total of 5 min 30 sec. DMSO, L17E (40 μ M), or ionomycin (5 μ M) in HBSS (200 μ L) were added to on the glass part of the dish 30 sec after the start of imaging. Imaging was performed using an FV3000 CLSM system equipped with a 60 $\times$ objective lens (UPlanSApo, oil immersion, NA 1.35). Quantification of Fluo-8 fluorescence intensity of each cell was performed using ImageJ software (NIH).

Cytosolic Translocation of FITC-Labeled Peptides. Cells were seeded on 35-mm glass-bottomed dishes (2×10^5 cells/dish) and cultured for 1 day. The cells were incubated in α -MEM(+) (for HeLa cells) or D-MEM(+) (for U2OS cells) containing FITC-labeled peptides at the concentrations indicated in the corresponding figure legends for 30 min at 37°C. After washing the cells with PBS twice, the cells were stained nuclei with Hoechst 33342 (5 μ g/mL) in α -MEM(–) (for HeLa cells) or D-MEM(–) (for U2OS cells) for 5 min at 37°C. Observation was performed with an FV3000 confocal laser scanning microscope (CLSM) system (Olympus) equipped with a 60 $\times$ objective lens (UPlanSApo, oil immersion, NA 1.35; Olympus). Images were analyzed using ImageJ software (NIH). Quantification of the cytosolic translocation of FITC-labeled peptides was performed using ImageJ software (version 2.14.0,

NIH) by measuring the fluorescence intensity within Hoechst 33342-stained region of each cell. Cells from more than five images were analyzed for each sample. siRNA transfection, BAPTA-AM treatment, and K buffer treatment were done described as in PI delivery assay section.

Establishment of Tet-Inducible VPS4a Stable HeLa Cell Lines. HeLa cell lines expressing VPS4a (WT or the E228Q mutant) in a Dox-dependent manner (HeLa VPS4a^{WT} and HeLa VPS4a^{E228Q} cells) were established using the Tet-On system with the piggyBac vector system. HeLa cells were seeded on a 6-well plate (1×10^5 cells/dish) and cultured for 1 day. pTRE3G-HA-VPS4a^{WT} or pTRE3G-HA-VPS4a^{E228Q} was co-transfected with the Transposase Expression Vector (System Biosciences, PB210PA-1) at a ratio of 600 ng:200 ng per well. After transfection, the cells were cultured for 1 day, incubation in the presence of puromycin (1 μ g/mL) for 9 days. After selection, the cells were maintained in α -MEM medium containing 10% (v/v) Tet-System Approved FBS (heat-inactivated) and puromycin (1 μ g/mL). Single-cell cloning was not performed, and the polyclonal population was used for experiments.

Induction of VPS4a Expression by Dox. HeLa VPS4a^{WT} and HeLa VPS4a^{E228Q} cells were seeded for experiments and cultured for 1 day. Before the experiment, the cells were cultured for 8 h in α -MEM medium containing 10% (v/v) heat-inactivated Tet-System Approved FBS, puromycin (1 μ g/mL), and Dox (1 μ g/mL) to induce the expression of VPS4a (WT or E228Q). Subsequent experiments were conducted in medium without puromycin and Dox.

Immunostaining. HeLa VPS4a^{WT} and HeLa VPS4a^{E228Q} cells were seeded on 35-mm glass-bottomed dishes (1×10^5 cells/dish), cultured for 1 day and incubated with Dox (1 μ g/mL) for 16 h. The cells were washed twice with PBS, fixed with 4% *p*-formaldehyde (PFA) (Nacalai Tesque) for 15 min at 25°C, permeabilized with 0.1% Triton X-100 in PBS for 15 min at 25°C, and blocked with 5% BSA in PBS containing Tween-20 (PBST) for 30 min at 25°C. The cells were then incubated with anti-HA antibody (mouse monoclonal IgG, 1:1,000, Sigma, H9658) in PBST containing 5% BSA for 1 h at 25°C. After washing the cells with PBST for 10 min three times, the cells were incubated with anti-mouse IgG (Alexa Fluor 488) (1:2000, Invitrogen, A11001) in in PBST containing 5% BSA for 1 h at 25°C. After washing the cells with PBST for 10 min three times, the cells were stained nuclei with Hoechst 33342 (5 μ g/mL) in PBS for 5 min at 25°C. Observation was performed with an FV3000 confocal laser scanning microscope (CLSM) system (Olympus) equipped with a 60 $\times$ objective lens (UPlanSApo, oil immersion, NA 1.35; Olympus). Images were analyzed using ImageJ software (NIH).

Cytotoxicity Assay (WST-8 Assay). The WST-8 assay was performed using the Cell Counting Kit-8 (Dojindo) according to the manufacturer's protocol. Cells were seeded on a 96-well plate (1.5×10^4 cells/well) and cultured for 1 day. For BAPTA-AM treatment, cells were washed twice with PBS and incubated with BAPTA-AM (40 μ M) in D-MEM(–) for 30 min. The cells were treated with α -MEM(+) or D-MEM(+) containing FITC-labeled peptides at the concentrations indicated in the corresponding figure legends for 30 min at 37°C. After treatment, the cells were washed twice with PBS, and 100 μ L of α -MEM(–) and 10 μ L of CCK-8 solution were added to each well. The cells were incubated at 37°C for 2 h, and the absorbance ($A_{450\text{ nm}} - A_{600\text{ nm}}$) was measured. The results were shown as the mean $\pm$ standard deviation (SD) from triplicate in one of two experiments.

Statistical Analysis. All data are presented as the mean $\pm$ standard error (SE) from three independent biological replicates ($n = 3$), unless otherwise specified. Statistical analyses were performed using JMP Pro 17 (SAS Institute). For comparison of two groups, an unpaired Student's t-test was used. For multiple comparison analyses, one-way analysis of variance

(ANOVA) followed by Tukey-Kramer's post hoc test or Dunnett's post hoc test was used. For non-parametric comparisons between two groups, the Wilcoxon rank-sum test was used. For non-parametric multiple comparisons, the Kruskal-Wallis test followed by the Steel-Dwass test was used. The calculated P-values were considered significant at $p < 0.05$.

ASSOCIATED CONTENT

Supporting Information

Figures S1 – S7 (PDF): Figures S1–S7 (PDF): qPCR analysis for mRNA levels of *KCNN4* or *ANXA2* in HeLa, nHeLa and pHeLa cells; western blot analysis for Na⁺/K⁺-ATPase, PMCA, GAPDH, and β-actin; assessment of intracellular Ca²⁺ concentration using Fluo-8; cytotoxicity assay for HeLa cells transfected either with ANXA2 siRNA; PI delivery efficiency in ANAX2-EGFP expressing HeLa and HT1080 cells; cell viability assay for U2OS cells treated with BAPTA-AM; CPPs-mediated delivery into VPS4a^{E228Q}-expressing HeLa cells.

AUTHOR INFORMATION

Corresponding Authors

Hisaaki Hirose — *Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan*; orcid.org/0000-0001-6301-2760; Email: hisaaki.hirose@gmail.com

Shiroh Futaki — *Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan*; orcid.org/0000-0002-0124-4002; Email: futaki@scl.kyoto-u.ac.jp

Authors

Masashi Kuriyama — *Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan*; orcid.org/0000-0002-4135-0854

Yoshimasa Kawaguchi — *Institute for Chemical Research, Kyoto University, Uji, Kyoto 611-0011, Japan*; orcid.org/0000-0002-4180-7869

Shinji Ito — *Medical Research Support Center, Graduate School of Medicine, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan*

Junko Satoh — *Medical Research Support Center, Graduate School of Medicine, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan*

Author Contributions

Conceptualization, M.K., H.H., and Y.K.; Methodology, M.K., H.H., Y.K., and S.I.; Investigation, M.K., S.I., and J.S.; Resources, M.K.; Writing – Original Draft, M.K., H.H., Y.K., and S.F.; Writing – Review & Editing, M.K., H.H., Y.K., and S.F.; Visualization, M.K. and S.I.; Supervision, H.H. and S.F.; Funding Acquisition, H.H., Y.K., and S.F.

Funding Sources

This work was supported by JST CREST (Grant Number JPMJCR18H5) and JSPS KAKENHI (Grant Numbers JP23K17412 and JP24H00051) to S.F. This work was also supported by JSPS KAKENHI (Grant number JP22K05313) and the Inamori Foundation to H.H, JSPS KAKENHI (Grant number JP22K15250 and JP24H00842) and JST ACT-X (Grant Number 22715691) to Y.K. M.K. is grateful for JST SPRING (Grant Number JPMJSP2110) and the JSPS Research Fellowship for Young Scientists (Grant Number JP23KJ1287).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank Dr. Motonari Uesugi, Dr. Masahiro Abo, and Ms. Asmaa M. A. S. Farrag for their help for cell sorting.

FOOTNOTE

#Present address

Graduate School of Pharmaceutical Sciences, Kyoto University
46-29, Yoshida-Shimoadachi-cho, Sakyo-ku, 606-8501, Japan

REFERENCES

1. Leader, B.; Baca, Q. J.; Golan, D. E., Protein therapeutics: a summary and pharmacological classification. *Nat. Rev. Drug Discov.* **2008**, *7* (1), 21-39.
2. Ebrahimi, S. B.; Samanta, D., Engineering protein-based therapeutics through structural and chemical design. *Nat. Commun.* **2023**, *14* (1), 2411.
3. Lagassé, H. A.; Alexaki, A.; Simhadri, V. L.; Katagiri, N. H.; Jankowski, W.; Sauna, Z. E.; Kimchi-Sarfaty, C., Recent advances in (therapeutic protein) drug development. *Fl000Res.* **2017**, *6*, 113.
4. Shepard, H. M.; Phillips, G. L.; C, D. T.; Feldmann, M., Developments in therapy with monoclonal antibodies and related proteins. *Clin. Med. (Lond)* **2017**, *17* (3), 220-232.
5. Santos, R.; Ursu, O.; Gaulton, A.; Bento, A. P.; Donadi, R. S.; Bologa, C. G.; Karlsson, A.; Al-Lazikani, B.; Hersey, A.; Oprea, T. I.; Overington, J. P., A comprehensive map of molecular drug targets. *Nat. Rev. Drug. Discov.* **2017**, *16* (1), 19-34.
6. Xie, X.; Yu, T.; Li, X.; Zhang, N.; Foster, L. J.; Peng, C.; Huang, W.; He, G., Recent advances in targeting the "undruggable" proteins: from drug discovery to clinical trials. *Signal. Transduct. Target Ther* **2023**, *8* (1), 335.
7. Chou, L. Y.; Ming, K.; Chan, W. C., Strategies for the intracellular delivery of nanoparticles. *Chem. Soc. Rev.* **2011**, *40* (1), 233-245.
8. Fu, A.; Tang, R.; Hardie, J.; Farkas, M. E.; Rotello, V. M., Promises and pitfalls of intracellular delivery of proteins. *Bioconjug. .* **2014**, *25* (9), 1602-1608.
9. Stewart, M. P.; Sharei, A.; Ding, X.; Sahay, G.; Langer, R.; Jensen, K. F., In vitro and ex vivo strategies for intracellular delivery. *Nature* **2016**, *538* (7624), 183-192.
10. Goswami, R.; Jeon, T.; Nagaraj, H.; Zhai, S.; Rotello, V. M., Accessing intracellular targets through nanocarrier-mediated cytosolic protein delivery. *Trends Pharmacol. Sci.* **2020**, *41* (10), 743-754.
11. Chan, A.; Tsourkas, A., Intracellular protein delivery: approaches, challenges, and clinical applications. *BME Front.* **2024**, *5*, 0035.
12. Copolovici, D. M.; Langel, K.; Eriste, E.; Langel, Ü., Cell-penetrating peptides: design, synthesis, and applications. *ACS Nano* **2014**, *8* (3), 1972-1994.
13. Guo, Z.; Peng, H.; Kang, J.; Sun, D., Cell-penetrating peptides: possible transduction mechanisms and therapeutic applications. *Biomed. Rep.* **2016**, *4* (5), 528-534.
14. Durymanov, M.; Reineke, J., Non-viral delivery of nucleic acids: insight into mechanisms of overcoming intracellular barriers. *Front. Pharmacol.* **2018**, *9*, 971.
15. Futaki, S., Functional peptides that target biomembranes: design and modes of action. *Chem. Pharm. Bull. (Tokyo)* **2021**, *69* (7), 601-607.
16. Heitz, F.; Morris, M. C.; Divita, G., Twenty years of cell-penetrating peptides: from molecular mechanisms to therapeutics. *Br. J. Pharmacol.* **2009**, *157* (2), 195-206.
17. Madani, F.; Lindberg, S.; Langel, Ü.; Futaki, S.; Gräslund, A., Mechanisms of cellular uptake of cell-penetrating peptides. *J. Biophys.* **2011**, *2011*, 414729.
18. Nakase, I.; Tadokoro, A.; Kawabata, N.; Takeuchi, T.; Katoh, H.; Hiramoto, K.; Negishi, M.; Nomizu, M.; Sugiura, Y.; Futaki, S., Interaction of arginine-rich peptides with membrane-associated proteoglycans is crucial for induction of actin organization and macropinocytosis. *Biochemistry* **2007**, *46* (2), 492-501.

19. Kawaguchi, Y.; Takeuchi, T.; Kuwata, K.; Chiba, J.; Hatanaka, Y.; Nakase, I.; Futaki, S., Syndecan-4 is a receptor for clathrin-mediated endocytosis of arginine-rich cell-penetrating peptides. *Bioconjug. Chem.* **2016**, *27* (4), 1119-1130.
20. Trofimenko, E.; Grasso, G.; Heulot, M.; Chevalier, N.; Deriu, M. A.; Dubuis, G.; Arribat, Y.; Serulla, M.; Michel, S.; Vantomme, G.; Ory, F.; Dam, L. C.; Puyal, J.; Amati, F.; Luthi, A.; Danani, A.; Widmann, C., Genetic, cellular, and structural characterization of the membrane potential-dependent cell-penetrating peptide translocation pore. *Elife* **2021**, *10*, e69832.
21. Perr, J.; Langen, A.; Almahayni, K.; Nestola, G.; Chai, P.; Lebedenko, C. G.; Volk, R. F.; Detres, D.; Caldwell, R. M.; Spiekermann, M.; Hemberger, H.; Bisaria, N.; Aiba, T.; Sanchez-Rivera, F. J.; Tzelepis, K.; Calo, E.; Mockl, L.; Zaro, B. W.; Flynn, R. A., RNA-binding proteins and glycoRNAs form domains on the cell surface for cell-penetrating peptide entry. *Cell* **2025**, *188* (7), 1878-1895.e25.
22. Cooper, S. T.; McNeil, P. L., Membrane repair: mechanisms and pathophysiology. *Physiol. Rev.* **2015**, *95* (4), 1205-1240.
23. Palm - Apergi, C.; Lorents, A.; Padari, K.; Pooga, M., The membrane repair response masks membrane disturbances caused by cell - penetrating peptide uptake. *FASEB J.* **2008**, *23* (1), 214-223.
24. Lorents, A.; Kodavali, P. K.; Oskolkov, N.; Langel, Ü.; Hallbrink, M.; Pooga, M., Cell-penetrating peptides split into two groups based on modulation of intracellular calcium concentration. *J. Biol. Chem.* **2012**, *287* (20), 16880-16889.
25. Serulla, M.; Ichim, G.; Stojceski, F.; Grasso, G.; Afonin, S.; Heulot, M.; Schober, T.; Roth, R.; Godefroy, C.; Milhiet, P. E.; Das, K.; García-Sáez, A. J.; Danani, A.; Widmann, C., TAT-RasGAP³¹⁷⁻³²⁶ kills cells by targeting inner-leaflet-enriched phospholipids. *Proc. Natl. Acad. Sci. U. S. A.* **2020**, *117* (50), 31871-31881.
26. Akishiba, M.; Takeuchi, T.; Kawaguchi, Y.; Sakamoto, K.; Yu, H. H.; Nakase, I.; Takatani-Nakase, T.; Madani, F.; Gräslund, A.; Futaki, S., Cytosolic antibody delivery by lipid-sensitive endosomolytic peptide. *Nat Chem* **2017**, *9* (8), 751-761.
27. Akishiba, M.; Futaki, S., Inducible membrane permeabilization by attenuated lytic peptides: a new concept for accessing cell interiors through ruffled membranes. *Mol. Pharm.* **2019**, *16* (6), 2540-2548.
28. Wender, P. A.; Mitchell, D. J.; Pattabiraman, K.; Pelkey, E. T.; Steinman, L.; Rothbard, J. B., The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: peptoid molecular transporters. *Proc. Natl. Acad. Sci. U. S. A.* **2000**, *97* (24), 13003-13008.
29. Futaki, S.; Suzuki, T.; Ohashi, W.; Yagami, T.; Tanaka, S.; Ueda, K.; Sugiura, Y., Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. *J. Biol. Chem.* **2001**, *276* (8), 5836-5840.
30. Joliot, A.; Prochiantz, A., Homeoproteins as natural Penetratin cargoes with signaling properties. *Adv. Drug. Deliv. Rev.* **2008**, *60* (4-5), 608-613.
31. Futaki, S.; Nakase, I., Cell-surface interactions on arginine-rich cell-penetrating peptides allow for multiplex modes of internalization. *Acc. Chem. Res.* **2017**, *50* (10), 2449-2456.
32. Yu, H. H.; Sakamoto, K.; Akishiba, M.; Tamemoto, N.; Hirose, H.; Nakase, I.; Imanishi, M.; Madani, F.; Gräslund, A.; Futaki, S., Conversion of cationic amphiphilic lytic peptides to cell - penetration peptides. *Pept. Sci.* **2019**, *112* (1), e24144.

33. Sakamoto, K.; Akishiba, M.; Iwata, T.; Murata, K.; Mizuno, S.; Kawano, K.; Imanishi, M.; Sugiyama, F.; Futaki, S., Optimizing charge switching in membrane lytic peptides for endosomal release of biomacromolecules. *Angew. Chem. Int. Ed. Engl.* **2020**, *59* (45), 19990-19998.
34. Tamemoto, N.; Akishiba, M.; Sakamoto, K.; Kawano, K.; Noguchi, H.; Futaki, S., Rational design principles of attenuated cationic lytic peptides for intracellular delivery of biomacromolecules. *Mol. Pharm.* **2020**, *17* (6), 2175-2185.
35. Kuriyama, M.; Hirose, H.; Kawaguchi, Y.; Michibata, J.; Maekawa, M.; Futaki, S., KCNN4 as a genomic determinant of cytosolic delivery by the attenuated cationic lytic peptide L17E. *Mol. Ther.* **2025**, *33* (2), 595-614.
36. Lennon, N. J.; Kho, A.; Bacsikai, B. J.; Perlmutter, S. L.; Hyman, B. T.; Brown, R. H., Jr., Dysferlin interacts with annexins A1 and A2 and mediates sarcolemmal wound-healing. *J. Biol. Chem.* **2003**, *278* (50), 50466-50473.
37. Koerdt, S. N.; Gerke, V., Annexin A2 is involved in Ca^{2+} -dependent plasma membrane repair in primary human endothelial cells. *Biochim. Biophys. Acta Mol. Cell. Res.* **2017**, *1864* (6), 1046-1053.
38. Bittel, D. C.; Chandra, G.; Tirunagri, L. M. S.; Deora, A. B.; Medikayala, S.; Scheffer, L.; Defour, A.; Jaiswal, J. K., Annexin A2 mediates dysferlin accumulation and muscle cell membrane repair. *Cells* **2020**, *9* (9), 1919.
39. Jimenez, A. J.; Maiuri, P.; Lafaurie-Janvore, J.; Divoux, S.; Piel, M.; Perez, F., ESCRT machinery is required for plasma membrane repair. *Science* **2014**, *343* (6174), 1247136.
40. Scheffer, L. L.; Sreetama, S. C.; Sharma, N.; Medikayala, S.; Brown, K. J.; Defour, A.; Jaiswal, J. K., Mechanism of Ca^{2+} -triggered ESCRT assembly and regulation of cell membrane repair. *Nat. Commun.* **2014**, *5*, 5646.
41. Narcis, J. O.; Tapia, O.; Tarabal, O.; Piedrafita, L.; Caldero, J.; Berciano, M. T.; Lafarga, M., Accumulation of poly(A) RNA in nuclear granules enriched in Sam68 in motor neurons from the SMNDelta7 mouse model of SMA. *Sci. Rep.* **2018**, *8* (1), 9646.
42. Elowitz, M. B.; Levine, A. J.; Siggia, E. D.; Swain, P. S., Stochastic gene expression in a single cell. *Science* **2002**, *297* (5584), 1183-1186.
43. McNeil, A. K.; Rescher, U.; Gerke, V.; McNeil, P. L., Requirement for annexin A1 in plasma membrane repair. *J. Biol. Chem.* **2006**, *281* (46), 35202-35207.
44. Bouter, A.; Gounou, C.; Bérat, R.; Tan, S.; Gallois, B.; Granier, T.; d'Estaintot, B. L.; Pöschl, E.; Brachvogel, B.; Brisson, A. R., Annexin-A5 assembled into two-dimensional arrays promotes cell membrane repair. *Nat. Commun.* **2011**, *2*, 270.
45. Demonbreun, A. R.; Quattrocchi, M.; Barefield, D. Y.; Allen, M. V.; Swanson, K. E.; McNally, E. M., An actin-dependent annexin complex mediates plasma membrane repair in muscle. *J. Cell Biol.* **2016**, *213* (6), 705-18.
46. Hirose, H.; Takeuchi, T.; Osakada, H.; Pujals, S.; Katayama, S.; Nakase, I.; Kobayashi, S.; Haraguchi, T.; Futaki, S., Transient focal membrane deformation induced by arginine-rich peptides leads to their direct penetration into cells. *Mol. Ther.* **2012**, *20* (5), 984-93.
47. Brock, R., The uptake of arginine-rich cell-penetrating peptides: putting the puzzle together. *Bioconjug. Chem.* **2014**, *25* (5), 863-868.
48. Gerke, V.; Creutz, C. E.; Moss, S. E., Annexins: linking Ca^{2+} signalling to membrane dynamics. *Nat. Rev. Mol. Cell Biol.* **2005**, *6* (6), 449-461.
49. Jaiswal, J. K.; Lauritzen, S. P.; Scheffer, L.; Sakaguchi, M.; Bunkenborg, J.; Simon, S. M.; Kallunki, T.; Jäättelä, M.; Nylandsted, J., S100A11 is required for efficient plasma membrane repair and survival of invasive cancer cells. *Nat. Commun.* **2014**, *5*, 3795.

50. Ashraf, A. P. K.; Gerke, V., Plasma membrane wound repair is characterized by extensive membrane lipid and protein rearrangements in vascular endothelial cells. *Biochim. Biophys. Acta Mol. Cell. Res.* **2021**, *1868* (7), 118991.
51. Gerke, V.; Gavins, F. N. E.; Geisow, M.; Grewal, T.; Jaiswal, J. K.; Nylandsted, J.; Rescher, U., Annexins-a family of proteins with distinctive tastes for cell signaling and membrane dynamics. *Nat. Commun.* **2024**, *15* (1), 1574.
52. Potez, S.; Luginbühl, M.; Monastyrskaya, K.; Hostettler, A.; Draeger, A.; Babiychuk, E. B., Tailored protection against plasmalemmal injury by annexins with different Ca²⁺ sensitivities. *J. Biol. Chem.* **2011**, *286* (20), 17982-17991.
53. Rintala-Dempsey, A. C.; Santamaria-Kisiel, L.; Liao, Y.; Lajoie, G.; Shaw, G. S., Insights into S100 target specificity examined by a new interaction between S100A11 and annexin A2. *Biochemistry* **2006**, *45* (49), 14695-705.
54. Madureira, P. A.; Hill, R.; Lee, P. W.; Waisman, D. M., Genotoxic agents promote the nuclear accumulation of annexin A2: role of annexin A2 in mitigating DNA damage. *PLoS One* **2012**, *7* (11), e50591.
55. Rothbard, J. B.; Jessop, T. C.; Lewis, R. S.; Murray, B. A.; Wender, P. A., Role of membrane potential and hydrogen bonding in the mechanism of translocation of guanidinium-rich peptides into cells. *J. Am. Chem. Soc.* **2004**, *126* (31), 9506-9507.
56. Shinga, K.; Iwata, T.; Murata, K.; Daitoku, Y.; Michibata, J.; Arafiles, J. V. V.; Sakamoto, K.; Akishiba, M.; Takatani-Nakase, T.; Mizuno, S.; Sugiyama, F.; Imanishi, M.; Futaki, S., L17ER4: A cell-permeable attenuated cationic amphiphilic lytic peptide. *Bioorg. Med. Chem.* **2022**, *61*, 116728.
57. Joiner, W. J.; Wang, L. Y.; Tang, M. D.; Kaczmarek, L. K., hSK4, a member of a novel subfamily of calcium-activated potassium channels. *Proc. Natl. Acad. Sci. U. S. A.* **1997**, *94* (20), 11013-11018.
58. Wulff, H.; Castle, N. A., Therapeutic potential of KCa3.1 blockers: recent advances and promising trends. *Expert Rev. Clin. Pharmacol.* **2010**, *3* (3), 385-396.
59. Andrews, N. W.; Almeida, P. E.; Corrotte, M., Damage control: cellular mechanisms of plasma membrane repair. *Trends Cell Biol.* **2014**, *24* (12), 734-742.
60. Zuconelli, C. R.; Schmidt, S.; Wallbrecher, R.; van Oostrum, J.; Bartels, Y. L.; Didan, Y.; Berendsen, M. L. T.; Brock, R.; Adjobo-Hermans, M. J. W., Modulation of Orail by cationic peptides triggers their direct cytosolic uptake. *Biochim. Biophys. Acta. Biomembr.* **2020**, *1862* (3), 183155.
61. Nakase, I.; Niwa, M.; Takeuchi, T.; Sonomura, K.; Kawabata, N.; Koike, Y.; Takehashi, M.; Tanaka, S.; Ueda, K.; Simpson, J. C.; Jones, A. T.; Sugiura, Y.; Futaki, S., Cellular uptake of arginine-rich peptides: roles for macropinocytosis and actin rearrangement. *Mol. Ther.* **2004**, *10* (6), 1011-1022.
62. Bishop, N.; Woodman, P., ATPase-defective mammalian VPS4 localizes to aberrant endosomes and impairs cholesterol trafficking. *Mol. Biol. Cell* **2000**, *11* (1), 227-239.
63. Ritter, A. T.; Shtengel, G.; Xu, C. S.; Weigel, A.; Hoffman, D. P.; Freeman, M.; Iyer, N.; Alivodej, N.; Ackerman, D.; Voskoboinik, I.; Trapani, J.; Hess, H. F.; Mellman, I., ESCRT-mediated membrane repair protects tumor-derived cells against T cell attack. *Science* **2022**, *376* (6591), 377-382.
64. Raiborg, C.; Stenmark, H., The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins. *Nature* **2009**, *458* (7237), 445-452.
65. Vietri, M.; Radulovic, M.; Stenmark, H., The many functions of ESCRTs. *Nat. Rev. Mol. Cell Biol.* **2020**, *21* (1), 25-42.

TABLE OF CONTENT

