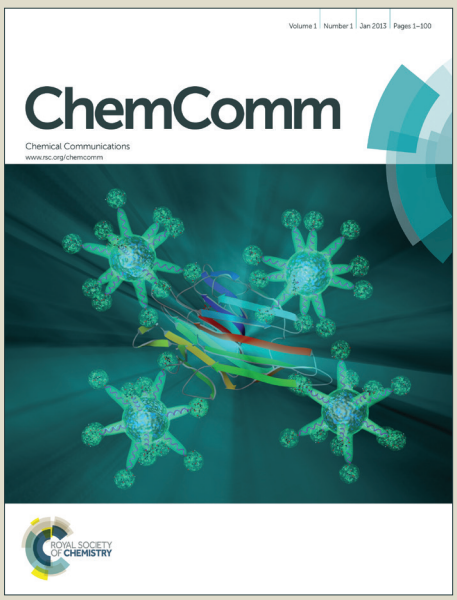
ChemComm

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

Journal Name

RSCPublishing

COMMUNICATION

Novel leucine zipper motif-based hybrid peptide delivers a functional peptide cargo inside cells†

Cite this: DOI: 10.1039/x0xx00000x

Y. Hakata,*[a] S. Tsuchiya,[b] H. Michiue,[c] T. Ohtsuki,[d] H. Matsui,[c] M. Miyazawa[a] and M. Kitamatsu*[b]

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

A hybrid comprising an autophagy-inducing peptide (AIP) and a cell-penetrating peptide (CPP) connected *via* heterodimeric leucine zippers was generated and delivered into cells. The hybrid successfully induced autophagy without significant cell death, while the same AIP directly connected to a CPP caused both autophagy and significant cell death.

Intracellular delivery of functional peptides (artificial bioactive peptides) is important for the development of peptide drugs that target various proteins inside cells. Cell-penetrating peptides (CPPs)[1] such as the Tat protein transduction domain (Tat PTD)[2] and oligo(arginine)s such as R11[3], have been developed as powerful agents for macromolecule delivery systems. In general, these CPPs contain several positively charged amino acids and are directly conjugated to the termini of macromolecules. Although there is no doubt about the usefulness of these types of CPPs in peptide delivery systems, they also have some negative effects on the systems. It has been reported that these positively charged CPPs may interfere with the biological function of the cargo peptide owing to nonspecific interactions with the negatively charged nucleic acids and membrane proteins inside the cells.[4] To eliminate this disadvantage, several systems with cargo peptides that are detachable from the CPP have been developed.[5] These methods, however, have their own problems and complexities. Hence, a new methodology to deliver biomolecules into cells is required.

Leucine zipper (Lz) motifs are seen in many cellular proteins and form an α-helix structure. The Lz motif can be made to carry a positive or negative charge by introducing charged amino acids.[6] A basic lysine residue (K) may be used for the generation of a positively charged Lz (Lz(K)), and an acidic glutamic acid residue (E) may be used for producing a negatively charged Lz (Lz(E)). Furthermore, Lz(K) and Lz(E) are able to form a 1:1 hybrid through electrostatic interactions. Here, a novel method using the heterodimeric Lz-based hybrid peptides for functional peptide transduction into cells was evaluated. In this study, a bioactive peptide was conjugated to Lz(K), while R11 was conjugated to Lz(E). When mixed *in vitro*, these two components spontaneously associated with each other *via* Lz motifs and formed a hybrid peptide that can be transduced to cells. After delivery of the hybrid peptides into cells, the bioactive peptide conjugated to Lz(K) is expected to dissociate from Lz(E)-R11 because the linkages between the Lz motifs are non-covalent.

A

Fam-Lz(K):	Fam-EYQALKKKVAQLKAKNQALKKKVAQLKHK-bA
Tmr-Lz(E):	Tmr-EYQALEKEVAQLEAENQALEKEVAQLEHE-bA
Tmr-Lz(E)-R11:	Tmr-EYQALEKEVAQLEAENQALEKEVAQLEHE-RRRRRRRRRR-bA
Tmr-Lz(K)-R11:	Tmr-EYQALKKKVAQLKAKNQALKKKVAQLKHK-RRRRRRRRRR-bA

B

Beclin 1-Lz(K):	TNVFNATFEIWHDGEFGT-EYQALKKKVAQLKAKNQALKKKVAQLKHK
Beclin(scr)-Lz(K):	VGNDFFINHETTGFATEW-EYQALKKKVAQLKAKNQALKKKVAQLKHK
Lz(E)-R11:	EYQALEKEVAQLEAENQALEKEVAQLEHE-RRRRRRRRRR
Beclin 1-R11:	TNVFNATFEIWHDGEFGT-RRRRRRRRRR
Beclin(scr)-R11:	VGNDFFINHETTGFATEW-RRRRRRRRRR

Fig. 1 (A) Sequences of fluorescently labeled leucine zipper peptides with or without R11 CPP. The red letters indicate the charged amino acids critical for forming a hybrid between Lz(K) and Lz(E). bA represents β-alanine. (B) Sequences of Beclin 1-Lz(K), Lz(E)-R11 and their control peptides.

To assess the hybrid formation between Lz(K) and Lz(E) peptides, four fluorescently-labeled Lz motif peptides with or without an R11 type CPP (Fam-Lz(K), Tmr-Lz(E), Tmr-Lz(E)-R11, and Tmr-Lz(K)-R11; Fam and Tmr are fluorescein and tetramethylrhodamine, respectively; Fig. 1A and Fig. S1, ESI†) were prepared by a conventional solid-phase peptide synthesis (Fig. S2, ESI†). They were used in fluorescent titration experiments, in which gradually increasing amounts of the Tmr-labeled peptides were titrated against a constant amount of Fam-Lz(K) in aqueous buffer at pH 7.0 (Fig. 2A). The fluorescence intensity of Fam-Lz(K) was decreased by the

addition of Tmr-Lz(E), which was due to Förster resonance energy transfer from Fam to Tmr (open circles). The result implies that Fam-Lz(K) interacts closely with Tmr-Lz(E). When a greater than equimolar amount of Tmr-Lz(E) was added to Fam-Lz(K) (Tmr-Lz(E)/Fam-Lz(K) > 1), the rate of decrease in fluorescence intensity of Fam-Lz(K) was significantly reduced. These results indicate that Tmr-Lz(E) formed a 1:1 hybrid with Fam-Lz(K). The titration curve of Tmr-Lz(E)-R11 against Fam-Lz(K) showed a similar behavior (closed circles). In contrast, the fluorescence intensity of Fam-Lz(K) showed no apparent decrease following addition of Tmr-Lz(K)-R11 (closed squares). These results indicate that R11 does not significantly obstruct the specific hybrid formation of Lz(E) and Lz(K).

Next, the hybrid of Fam-Lz(K) and Tmr-Lz(E)-R11 was used to evaluate whether it is taken up by cells. U-251 MG cells were treated with an equimolar mixture of Fam-Lz(K) and Tmr-Lz(E)-R11 (Fig. 2B). Fluorescence signals from both Fam-Lz(K) and Tmr-Lz(E)-R11 were detected in the cells. By contrast, images of U-251 MG cells incubated with Fam-Lz(K) alone showed no Fam signal, indicating that internalization did not occur (Fig. S3, ESI†). These results suggest that Fam-Lz(K) is successfully delivered into cells by forming the hybrid with Tmr-Lz(E)-R11.

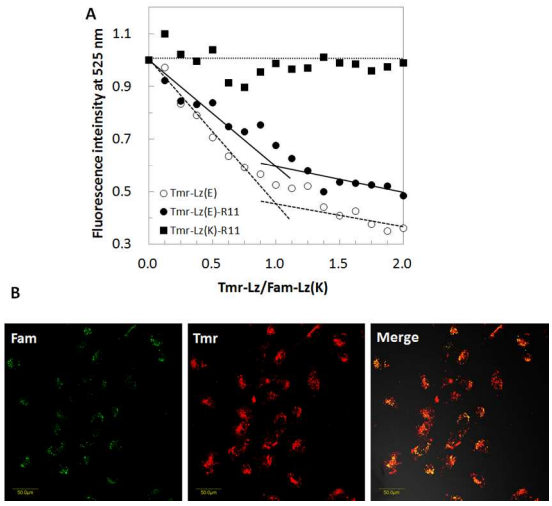


Fig. 2 (A) Fluorescence titration curves of Tmr-labeled peptides against Fam-Lz(K) in aqueous buffer. The concentration of Fam-Lz(K) is constant at 15 nM and a gradually increasing amount of Tmr-labeled peptide was used for titration. The excitation wavelength was set at 500 nm to measure Fam fluorescence intensity. (B) Confocal microscopy images of U-251 MG cells treated with hybrid peptides. The cells were treated with 10 μM of the Fam-Lz(K)/Tmr-Lz(E)-R11 hybrid for 4 h at 37°C. Fam and Tmr fluorescence signals were separately detected. The merged image is also included in the right panel.

To evaluate whether the functional peptide transduced by the Lz-based hybrid had the expected bioactivity in cells, a Lz(K) that was conjugated to a functional autophagy-inducing peptide, Beclin 1 peptide[7], (Beclin 1-Lz(K)) and a negative control peptide that consisted of a scrambled sequence of Beclin 1 peptide (Beclin(scr)-Lz(K)) (Fig. 1B and Fig. S4, ESI†) was synthesized. An R11-bearing Lz(E) (Lz(E)-R11) was also prepared to make hybrid peptides with the above Lz(K)-bearing peptides. Beclin 1-R11 and Beclin(scr)-R11 are Beclin 1 and Beclin 1(scr) peptides directly conjugated to R11, which are the same peptides used in the previous report[7] except for the usage of R11 instead of Tat PTD as the CPP, and are known to induce autophagy after transduction into cells. We used R11 peptide as the CPP since it shows satisfactory permeability to wider variety types of cell lines when compared to Tat PTD[8].

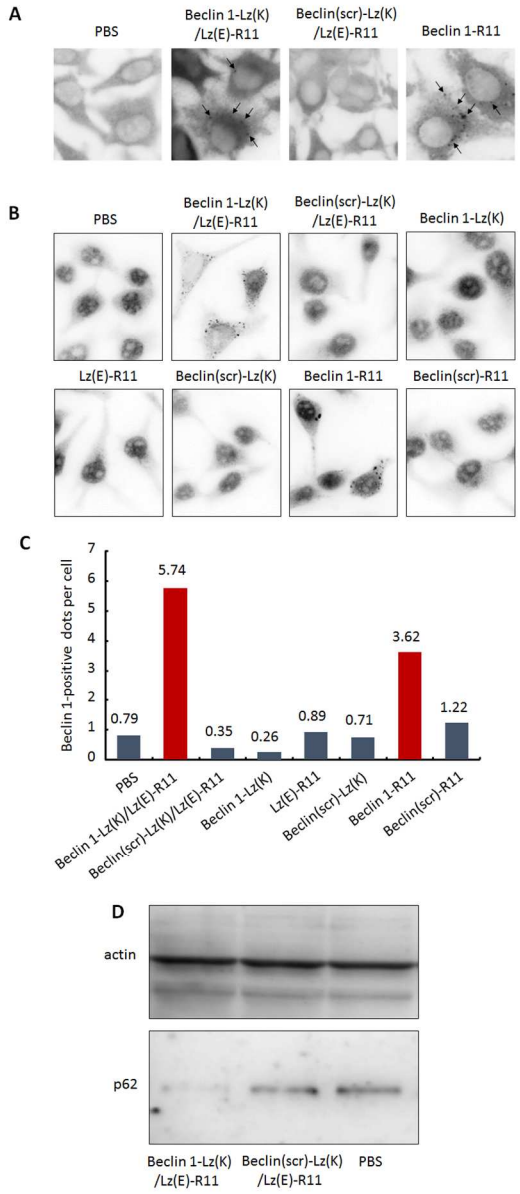


Fig. 3 (A) Autophagy induction in cells treated with the Lz-based hybrid peptides. HeLa cells were treated with 10 μM peptides for 1 h. The cells were fixed and stained with anti-LC3 antibody followed by fluorescently labeled secondary antibody. Representative fluorescence images are shown. Arrows indicate LC3-positive dots, a marker of autophagy. (B) Representative fluorescence images of endogenous Beclin 1 proteins. HeLa cells were treated with the indicated peptides. The cells were fixed and stained with anti-Beclin 1 antibody followed by fluorescently labeled secondary antibody. (C) The number of Beclin 1-positive dots per cell cytoplasm. Beclin 1-positive dots observed in (B) were counted. Approximately 30 cells of each sample were evaluated and the mean of Beclin 1 dots per cell cytoplasm was calculated. (D) The Lz-based hybrid peptides induced p62/SQSTM degradation. HeLa cells were treated with 10 μM peptides for 3 h. The cells were lysed and the cell extract was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). An autophagy marker p62/SQSTM1 was detected on an immunoblot.

Beclin 1-Lz(K) or Beclin(scr)-Lz(K) was mixed with Lz(E)-R11 peptides to prepare the desired hybrid peptides, Beclin 1-Lz(K)/Lz(E)-R11 and the negative control Beclin(scr)-

Lz(K)/Lz(E)-R11. HeLa cells were treated with these hybrids or Beclin 1-R11 (Fig. 3A). As expected, treatment of cells with Beclin 1-R11 resulted in augmented number of LC3-II dots in the cytoplasm, a marker for autophagy induction, as compared with control cells treated with phosphate-buffered saline (PBS). The Beclin 1-Lz(K)/Lz(E)-R11 hybrid also increased the number of LC3-II dots while the Beclin(scr)-Lz(K)/Lz(E)-R11 did not, indicating Beclin 1-Lz(K) peptide-specific autophagy induction. It was demonstrated in the previous report[7] that the transduced Beclin 1-Tat PTD peptide inhibits the interaction of endogenous Beclin 1 protein with GAPR1 on the Golgi apparatus, resulting in the release of free Beclin 1 proteins to the cytosol, which enhances autophagy. The ability of the hybrid peptides to exhibit an identical function was tested. As seen in Figure 3B and 3C, Beclin 1-R11 treatment released endogenous Beclin 1 proteins into the cytoplasm. Interestingly, the released Beclin 1 proteins are detected as dot-like structures. The number of Beclin 1-positive dots in Beclin 1(scr)-R11-treated cells is lower (1.22 per cell cytoplasm) than in Beclin 1-R11-treated cells (3.62 per cell cytoplasm). Similarly, treatment of cells with Beclin 1-Lz(K)/Lz(E)-R11 hybrid peptides clearly increased the amount of Beclin 1-positive dots in the cytoplasm (5.74 per cell cytoplasm). On the other hand, treatment of cells with Beclin(scr)-Lz(K)/Lz(E)-R11, Beclin 1-Lz(K), Lz(E)-R11, and Beclin(scr)-Lz(K) did not lead to significant increases in the number of Beclin 1-positive dots (0.35, 0.26, 0.89, and 0.71 per cell cytoplasm, respectively) as compared with PBS treatment (0.79 per cell cytoplasm). To further confirm the autophagy induction with the hybrid peptide we investigated the amount of p62/SQSTM1 protein in cells, which is a substrate of autophagy (Fig. 3D). It is well known that p62/SQSTM1 is degraded when autophagy is promoted. The Beclin 1-Lz(K)/Lz(E)-R11 hybrid significantly reduced the amount of p62/SQSTM1, suggesting the enhancement of autophagy with the hybrid. These results suggest that Beclin 1-Lz(K)/Lz(E)-R11 was successfully delivered into the cytosol through formation of the hybrid between Lz motifs. Furthermore, internalized Beclin 1-Lz(K) peptide induced autophagy, indicating that the Lz motif-based transduction does not interfere with the physiological function of the peptide cargo in the cells.

Beclin 1-Lz(K)/Lz(E)-R11 hybrid peptides were then compared with Beclin 1-R11 with respect to their abilities to induce autophagy. The peptide concentration to be added to the cells (Fig. 4A) was titrated. The augmented LC3-II production, a marker for autophagy induction, was observed by addition of 5 μM Beclin 1-Lz(K)/Lz(E)-R11 hybrid (LC3-II/actin ratio 0.37). It was more evident at 10 μM peptide concentration (LC3-II/actin ratio 0.82), while no obvious increase of LC3-II was detected by Beclin(scr)-Lz(K)/Lz(E)-R11 (LC3-II/actin ratio 0.09 and 0.13 at 5 μM and 10 μM, respectively). A strong increase of LC3-II was observed when Beclin 1-R11 was added, even at 5 μM concentration (LC3-II/actin ratio 0.54). Addition of Beclin(scr)-R11 did not change the amount of LC3-II. Next, a time-course experiment was conducted using a constant peptide concentration (10 μM) (Fig. 4B). Increased LC3-II production was detected as early as 0.5 h after Beclin 1-R11 peptide addition but the Beclin 1-Lz(K)/Lz(E)-R11 hybrid required a 1-hour incubation. Interestingly, in the case of Beclin 1-R11, another lower molecular weight band other than that attributable to LC3-II emerged at 2 hours' incubation, and this band appeared more strongly at 3 hours' incubation. By contrast, such a band was not observed following Beclin 1-Lz(K)/Lz(E)-R11 addition. These results indicate that Beclin 1-R11 induces autophagy at a lower concentration and faster than the Beclin 1-Lz(K)/Lz(E)-R11 hybrid peptide, but Beclin 1-R11 may have an undesired side effect represented by emergence of the lower molecular weight band. This band may signal a degraded product of the LC3 protein.

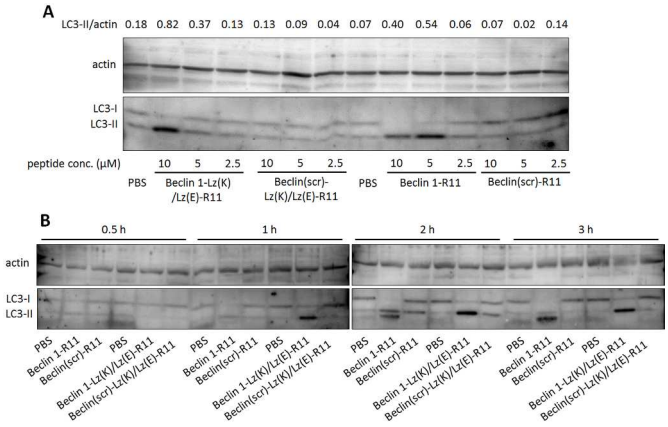


Fig. 4 Comparison of the Lz-based hybrid peptide with the R11 directly conjugated-peptide. (A) Cells were incubated with the indicated concentration of peptides for 1 h. The cell lysates were separated by SDS-PAGE and analyzed by western blot. An autophagy marker LC3 and actin were detected on immunoblots. The band intensity was quantified and the ratio of LC3-II/actin was calculated. (B) Cells were incubated with 10 μM of peptide for the indicated time.

Since the unexpected lower molecular weight band was detected in the Beclin 1-R11-treated cells, it was hypothesized that Beclin 1-R11 may have cytotoxicity and induce apoptosis, resulting in degradation of LC3-II. To assess this, the possibility of apoptosis being induced by the addition of Beclin 1-R11 and Beclin 1-Lz(K)/Lz(E)-R11 was tested. HeLa cells were cultivated in the absence or presence of the peptides for 3 h and fixed. After fixation, cell death was evaluated with Annexin V-FITC and ethidium homodimer III (EH III) in apoptotic & necrotic cell detection kits (PromoKine, Heidelberg, Germany). The fluorescence images of the cells are shown in Figure 5. Beclin 1-R11 treatment made cells positive for both Annexin V-FITC and EH III fluorescence, which shows progression of apoptosis. On the other hand, neither significant Annexin V-FITC nor EH III fluorescent signals were detected in cells treated with Beclin 1-Lz(K)/Lz(E)-R11 hybrid peptide. The cells, which were treated with the hybrid for 3 h, washed with PBS, and further incubated in complete medium for an additional 21 h, still showed no noticeable cell death (data not shown). It was also obvious that the cell shape of Beclin 1-R11-treated cells was different from that of PBS-treated cells and hybrid-treated cells. These results indicate that Beclin 1-R11 causes not only autophagy but also undesired cell death, while the hybrid peptide induces autophagy without apoptosis. It remains unclear why Beclin 1-R11 has such the severe cytotoxicity. Although R11 is known to have relatively higher cytotoxicity than Tat PTD in certain assays, R11 itself may not be the determinant for the cytotoxicity in our system since Beclin 1-Lz(K)/Lz(E)-R11 hybrid treatment showed no significant cell death. In addition, treatment of cells with Lz(E)-R11 alone also did not result in severe cell death (data not shown). The cytotoxicity of R11-containing peptides could be due to sequence and/or orientation of the peptide fused to R11, but not R11 itself. The cytotoxicity of CPP component is sometimes an obstacle for the present transduction systems but

the Lz-based transduction system allows us to utilize Lz(E)-R11 for all cargos without any consideration about cytotoxicity.

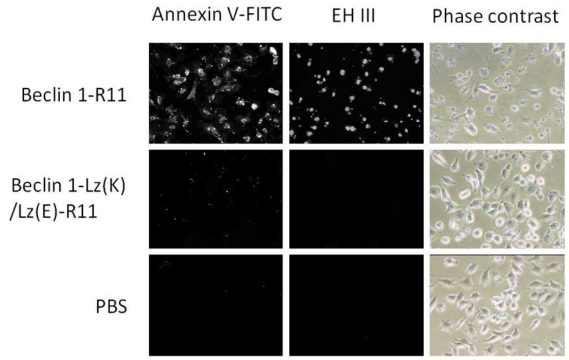


Fig. 5 Beclin 1-R11 evoked cell death. HeLa cells treated with peptide or PBS are stained with Annexin V-FITC (left) and EH III (center). The right panels show their phase contrast images.

Conclusions

In order to connect a CPP carrier with a cargo of interest without a direct covalent bond, heterodimeric leucine zippers were used. Similar, although not identical, Lz sequences were used in a previous study[9] to deliver green fluorescent protein (GFP). However, whether a cargo executes the desired physiological function after delivery into cells remained largely unknown. The previously characterized autophagy-inducing peptide[7] was used to investigate whether the peptide transduced by the Lz-based method described in this study works normally inside cells. Here, the results clearly showed the peptide-dependent induction of autophagy in cells treated with Lz-based hybrid peptides, as expected. This suggests that the Lz-based transduction method can deliver a functional cargo. In comparison of the hybrid peptides with the peptide directly conjugated with R11 CPP (Beclin 1-R11), it was noticed that the latter induced severe cell death, while both induce autophagy with different features. We observed the protein with lower molecular weight than LC3-II on immunoblots. LC3-II recruted in the inner membrane of autolysosome is known to be degraded in general autophagy process. However, to our knowledge, there has been no report showing that the degraded products of LC3-II emerged as the lower molecular weight band detected in our results. Also, if the observed lower weight molecule was a cleavage product in the normal autophagy process, it would not have accumulated over incubation time and treatment of cells with the Beclin 1-Lz(K)/Lz(E)-R11 hybrid should have shown the identical band. From these points of view, it may be unlikely that it was generated in the normal autophagy process. We observed severe cell death when the cells were terated with Beclin 1-R11, but not the hybrid peptides. Thus, it is likely that the lower molecular weight band would result from the undesired cytotoxicity of Beclin 1-R11 which may activate cellular proteases to raise it although a responsible protease(s) remains unknown.

In this study, it was first demonstrated that the Lz-based hybrid system expands the present methodology for functional biomolecules transduction and offers a suitable way to change the physiological function of cells.

Financial support from two Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT) (No. 25410181 and 25460573) is acknowledged. The authors are grateful for assistance from Mami Nakashima with the measurement of fluorescence spectra and from Feifei Wang with fluorescence microscopy.

Notes and references

[a] Department of Immunology, Faculty of Medicine, Kinki University, 377-2 Ohno-Higashi, Sayama, Osaka 589-8511, Japan. *E-mail: hakata@med.kindai.ac.jp; Tel: +81-72-366-0221.

[b] Department of Applied Chemistry, Faculty of Science and Engineering Kinki University, 3-4-1 Kowakae, Higashi-Osaka, Osaka 577-8502, Japan. *E-mail: kitamatu@apch.kindai.ac.jp; Tel: +81-6-6721-2332.

[c] Department of Physiology, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University, 2-5-1 Shikata-cho, kita-ku, Okayama 700-8558, Japan

[d] Department of Biotechnology, Graduate School of Natural Science and Technology, Okayama University, 3-1-1 Tsushimanaka, kita-ku, Okayama 700-8530, Japan

† Electronic Supplementary Information (ESI) available: [structures of fluorescent amino acids, matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectra of synthesized peptides and a fluorescence image of Fam-Lz(K)]. See DOI: 10.1039/c000000x/

1 S. Futaki, *Adv. Drug Deliver. Rev.*, 2005, **57**, 547.

2 E. Vivès, P. Brodin and B. Lebleu, *J. Biol. Chem.*, 1997, **272**, 16010; S. R. Schwarze, A. Ho, A. Vocero-Akbani and S. F. Dowdy, *Science*, 1999, **285**, 1569.

3 I. Nakase, M. Niwa, T. Takeuchi, K. Sonomura, N. Kawabata, Y. Koike, M. Takehashi, S. Tanaka, K. Ueda, J. C. Simpson, A. T. Jones, Y. Sugiura and S. Futaki, *Mol. Ther.*, 2004, **10**, 1011.

4 T. Hitsuda, H. Michiue, M. Kitamatsu, A. Fujimura, F. Wang, T. Yamamoto, X.-J. Han, H. Tazawa, A. Uneda, I. Ohmori, T. Nishiki, K. Tomizawa and H. Matsui, *Biomaterials*, 2012, **33**, 4665.

5 (a) N. Bendifallah, F. W. Rasmussen, V. Zachar, P. Ebbesen, P. E. Nielsen and U. Koppelhus, *Bioconjugate Chem*., 2006, **17**, 750; (b) R. K. June, K. Gogoi, K. A. Eguchi, X.-S. Cui and S. F. Dowdy, *J. Am. Chem. Soc.*, 2010, **132**, 10680.

6 H. Wendt, L. Leder, H. Härmä, I. Jelesarov, A. Baici and H. R. Bosshard, *Biochemistry*, 1997, **36**, 204.

7 S. Shoji-Kawata, R. Sumpter Jr, M. Leveno, G. R. Campbell, Z. Zou, L. Kinch, A. D. Wilkins, Q. Sun, K. Pallauf, D. MacDuff, C. Huerta, H. W. Virgin, J. B. Helms, R. Eerland, S. A. Tooze, R. Xavier, D. J. Lenschow, A. Yamamoto, D. King, O. Lichtarge, N. V. Grishin, S. A. Spector, D. V. Kaloyanova and B. Levine, *Nature*, 2013, **494**, 201.

8 E. Kondo, K Saito, Y. Tashiro, K. Kamide, S. Uno, T. Furuya, M. Mashita, K. Nakajima, T. Tsumuraya, N. Kobayashi, M. Nishibori, M. Tanimoto and M. Matsushita, *Nat. Commun.*, 2012, **3**, 951.

9 M. B. Hansen, W. P. R. Verdurmen, E. H. P. Leunissen, I. Minten, J. C. M. van Hest, R. Brock and D. W. P. M. Löwik, *ChemBioChem*, 2011, **12**, 2294.

