

Letters

A Novel Cell-Penetrating Peptide Sequence Derived by Structural Minimization of a Snake Toxin Exhibits Preferential Nucleolar Localization

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Abstract: Structural simplification of a 42-residue venom peptide by N-to-C-terminal splicing led to two sequences [YKQCHKKG-GXKKGS, where X = nil (**1**) or 6-aminohexanoyl (**2**)], both efficiently taken up by HeLa cells and, most interestingly, specifically localized at the nucleolus. Retro-**2** was taken up less efficiently, but a single (His → Ile) replacement recovered the translocation ability. None of the peptides were cytotoxic up to 100 μ M. Enantio-**1** did not translocate, suggesting that peptide uptake was receptor-mediated.

Cell penetrating peptides (CPPs^a) are short cationic amphipathic sequences with high affinity for lipid membranes and cell-surface proteoglycans.^{1,2} CPPs can translocate membrane systems, localize in different cell compartments, and often mediate the intracellular delivery of assorted cargos, including drugs, imaging agents, biopolymers (nucleic acids, polypeptides), liposomes, and nanoparticles^{3–5} and have for this reason raised considerable interest as therapeutic tools. The first recognized CPP sequences were the transactivator of transcription from HIV-1, named Tat protein,⁶ and the antennapedia (Antp) homeodomain from *Drosophila*.⁷ Later, short fragments of Tat [e.g., Tat(47–57) or Tat(48–60)]⁸ and a 16-amino acid segment of Antp [Antp(43–58)], named “penetratin”⁹ were demonstrated to be sufficient to translocate through the plasma membranes. Since then, an increasing number of natural and synthetic CPPs have been identified and utilized to translocate and deliver into the cell cytoplasm and nucleus distinct types of cargos both in vitro and in vivo.^{10–13} Transduction peptides, containing or not containing homing nuclear domains, have also been identified in other proteins like VP22 from *Herpes simplex* virus type I,¹⁴ fibroblast growth factors 1 and 2,¹⁵ or the opioid neuropeptide dynorphin.¹⁶ Some eukaryotic antimicrobial peptides also display cell-penetrating behavior, somehow expected

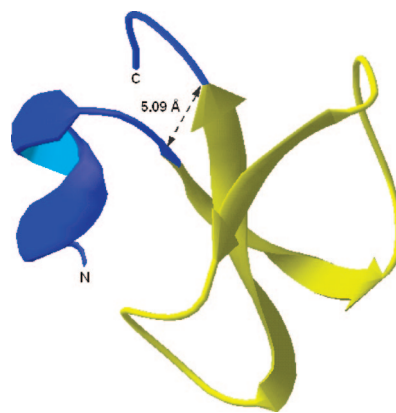


Figure 1. 3D structure of crotoxin (PDB entry no. 1h5o)^{29,30} showing proximity between the N- (α -helix, residues 1–9, blue) and C-terminal (random, residues 28–32) regions (both highlighted in blue), which is the basis for the design of peptides **1** and **2** and retro versions **3** and **4**.

from the structural similarities (cationic character, amphipathic nature) that underlie their ability to interact with lipid bilayer components, and eventually penetrate cells and target specific organelles.¹⁷ Examples along these lines include the amphibian, histone 2A-derived peptide buforin II¹⁸ or salivary histatin 5, which translocates into the cytoplasm of *Leishmania* and accumulates into the mitochondrion.¹⁹ Hybrid buforin 2-magainin 2 sequences have been shown to translocate efficiently across the bacterial membrane and deliver into the cytoplasm the antimicrobial α -helical portion of magainin 2.²⁰

Animal venoms are a very rich source of bioactive polypeptides that in several cases have been turned into actual or candidate drugs. Snakes,²¹ scorpions,²² spiders,²³ or sea snails,²⁴ to name just a few, produce and secrete a valuable diversity of toxins capable of interacting and interfering with distinct molecular targets in the cell. Hitherto, a very limited number of animal toxins able to translocate the cytoplasmic membrane and localize into distinct cell compartments are known. Two examples are maurocalcine,²⁵ a scorpion toxin of 33 amino acids and three disulfide bonds that localizes in the cytoplasm and release calcium ions, and crotoxin,²⁶ a rattlesnake toxin of 42 amino acids and also three disulfide bonds that selectively translocates into actively proliferating cells, both in vivo and in vitro, during the G1/S phase of the cell cycle and localizes to the nucleus. For both peptides, the uptake mechanism appears to involve initial binding to heparan sulfate proteoglycans, followed by endocytosis for crotoxin²⁷ and macropinocytosis for maurocalcine.²⁸ In a solution NMR study, crotoxin was shown to adopt an $\alpha\beta\beta\beta$ fold^{29,30} (Figure 1), similar to that of the human antimicrobial peptide β -defensin 2 or to scorpion toxins targeting sodium channels.

Structure-guided deconstruction of complex, highly folded bioactive peptides has been advantageously used to identify the structural traits essential for their biological activity,^{31,32} i.e., their pharmacophore, and in turn to design minimalist, therapeutically useful versions of such peptides.³³ Although identification of which particular section of a protein will translocate still relies more on experimental than on predictive approaches,³⁴ it seemed worthwhile to perform a structural dissection of

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^a Abbreviations: Ahx, 6-aminohexanoic acid; CPP, cell-penetrating peptide; DIC, differential interference contrast; FITC, fluorescein isothiocyanate; Fmoc, 9-fluorenylmethoxycarbonyl; HPLC, high performance liquid chromatography; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

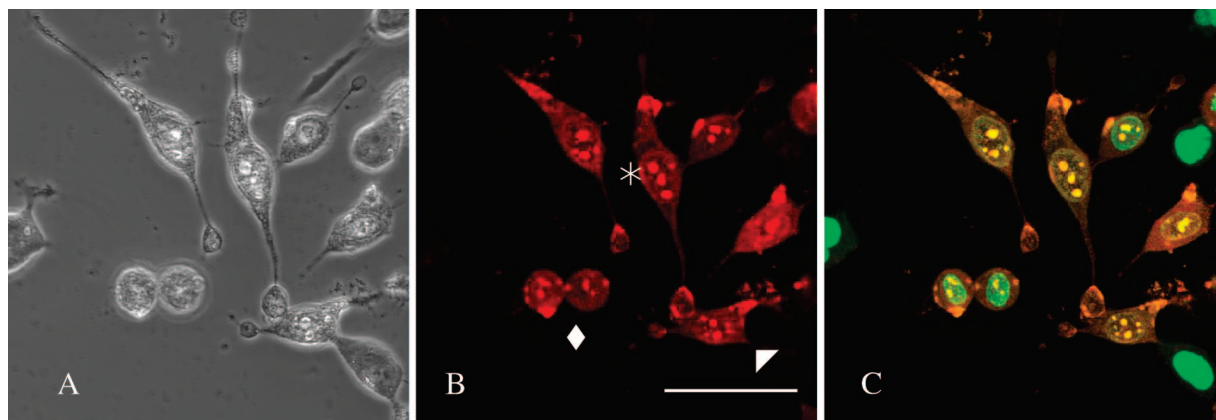


Figure 2. Nucleolar localization of peptide **R1** with evidence of preserved nuclear envelope integrity. Slide-adhered live HeLa cells (see Supporting Information for details) were incubated with 15 μ M peptide, and cell uptake was monitored for 60 min. Then the vital dye DRAQ5 was added for live nuclear visualization: (A) DIC image; (B) confocal microscopy image; (C) overlay of panel B image with DRAQ5 fluorescence. All panels are at the same magnification, bar = 50 μ m. In panel B, symbols indicate cytokinesis (\blacklozenge), reassociating nucleoli (triangle), and interphasic nucleoli (*). In panel C, the nucleus, the nuclear envelope and metaphasic chromosomes are clearly visible.

Table 1. Peptides Representing N-to-C-Terminal Splicing of Crotonamine

peptide ^a	amino acid sequence ^b	residues spliced
1	YKQCHKKGKKGSG	(1–9)–(38–42)
2	YKQCHKKGKXKKGSG	(1–9)–Ahx–(38–42)
3	GSGKKGGKKHCQKY	(42–38)–(9–1)
4	GSGKKGGKKJCQKY	as 3 , with His \rightarrow Ile replacement
5	ykqchkkGGkkGsG	D-enantiomer of 1

^a Derivatives with an N-terminal rhodamine B fluorophore (labeled **R1–R5**) were also prepared for each peptide. ^b Non-native residues are underlined. X = 6-amino hexanoic acid. Lower case describes D-amino acid residues.

crotonamine along the lines of the aforesaid strategies in an attempt to define a minimal structural motif capable of membrane translocation and internalization. Herein, we discuss some relevant findings in this direction.

Our attention has focused particularly on the two ends of crotonamine, which are brought spatially rather close (5.09 Å between the C α of Gly-9 and Lys-38, Figure 1) by the specific $\alpha\beta\beta\beta$ folding of the peptide. As both these regions are quite rich in the basic (His, Lys) residues commonly found in CPPs, we hypothesized that their splicing into a linear sequence might produce a peptide with translocating properties. Thus, peptide **1** (YKQCHKKGKKGSG), spanning residues 1–9 and 38–42 of crotonamine, and its analogue **2**, where the N- and C-terminal segments are spliced through a 6-amino hexanoic acid (Ahx) flexible spacer residue (Table 1), were initially prepared.

Peptides **1** and **2** were readily synthesized by Fmoc solid phase methods, purified by HPLC, and satisfactorily characterized by MALDI-TOF MS (see Supporting Information). To examine their cell uptake, fluorescent derivatives with a rhodamine B dye at the N-terminus were also prepared (**R1** and **R2**, respectively).

The ability of **R1** and **R2** to translocate through eukaryotic membranes was next investigated. HeLa cells were cultured to 60% confluence in standard conditions, then incubated with **R1**, and the time course of peptide uptake was monitored by in vivo time-lapsed imaging confocal microscopy. At 15 min incubation time (data not shown), discrete fluorescent spots were visualized on the membranes; then, between 45 and 60 min, the peptide was seen to translocate through the membrane and to concentrate preferentially in the nucleus and specifically in the nucleolus (Supporting Information Figure SF1). The internalization process was concentration-dependent and observable at peptide concentrations above 15 μ M. We also ascertained that at pH 7.2

the peptide remained essentially in monomeric (free thiol) form, with $\leq 1\%$ of dimer detectable by HPLC.

In Figure 2 it can be seen that uptake of **1** and subsequent localization at the nucleolus do not affect the integrity of the nuclear envelope, as assessed by the DRAQ5 live nuclear dye. For **2**, a similar pattern of cell penetration and nucleolar localization was observed (Supporting Information Figure SF2), indicating that the additional flexibility provided by Ahx at the splicing sites did not significantly alter the translocation efficacy. By use of an FITC-labeled Tat(48–60) peptide as positive control (i.e., 100% translocation), **1** and **2** were found to penetrate about 50% of the live HeLa cells (compare panels A and B in Supporting Information Figure SF2) in the 15–50 μ M interval. The peptides are seen (Figure 2 and Supporting Information Figures SF1 and SF2) to bind various stages of nucleolar organization, including premitotic and reassociating nucleoli, self-organizing nucleoli, nucleoli of cytokinetic cells, and also metaphasic chromosomes.

The partial chemical similarity of the two crotonamine end regions spliced into **1** and **2**, namely, the presence of positively charged residues at each moiety, made it worth exploring whether sequence inversion would preserve the cell-penetrating properties. Peptide **3**, the retro version of **2** (Table 1), was thus synthesized and shown to translocate to a much lower extent than the previous two, though the nucleolar homing preference remained (Supporting Information Figure SF3). A His \rightarrow Ile point mutation (analogue **4**, Table 1) caused a relative increase in uptake and nucleolar localization (Supporting Information Figure SF4), albeit to a lesser degree than **1** or **2**. Thus, for both retro analogues a minimum 50 μ M peptide concentration was required for translocation to be observed, in contrast with 15 μ M for **1** and **2**.

In all cases, the fluorescent signal of the internalized CPP (**R1–R4**) vanished after 24 h of incubation of HeLa cells with peptide-free culture medium, indicating relatively fast degradation of the internalized species. Last but not least, in an MTT cytotoxicity assay, all four peptides were found to be harmless to HeLa cells up to 100 μ M.

As an initial exploration of the mechanism of translocation of these CPPs, peptide **5**, the all-D enantiomer of **1**, was tested. In contrast to the above results, on incubation of HeLa cells with **5** no significant uptake was observed, the peptide localizing exclusively in a diffuse fashion at the cytoplasmic membrane (Supporting Information Figure SF5). While mechanisms used

by CPPs to traverse membranes are not fully elucidated, there is now sufficient evidence of an endosomal pathway of internalization for peptides such as Tat or Antp,^{17,35–37} involving not only caveolae,³⁸ clathrin-coated pit and vesicles,³⁹ and macropinocytosis⁴⁰ but also lipid raft-dependent and clathrin-independent endocytosis.⁴¹ We therefore interpret the failure of peptide **5** to penetrate HeLa cells as suggestive that its L-amino acid congeners (**1–4**) gain access to intracellular compartments by receptor-mediated endocytosis.

Taken together, the above results clearly demonstrate that the spliced crotamine peptides **1** and **2**, and to a lesser extent **4** and **3**, not only comprise a new group of CPPs but, more fittingly, outline a novel nucleolar localization signature with potentially promising applications in therapeutics. Aside from the Cajal bodies and the splicing speckles, the nucleolus is the most prominent functional structure in the nucleus of eukaryotic cells. In addition to its conventional role in transcription of rRNAs and ribosome biogenesis,⁴² other nucleolar functions have been unveiled in recent years that may be more attuned to therapeutic developments. These include roles in viral infection, regulation of tumor suppression and oncogenesis, stress sensing, control of aging, and modulation of telomerase activity. These functions are all related to the movement of nucleolar components, sequestration of regulatory cell-cycle-dependent proteins, and transit of proteins that leave the nucleolus after their respective transactions.⁴³ Given their ability to localize in assembled and dissociated nucleoli, one might suggest a role for peptides **1–4** in the investigation of biomolecular traffic in subnuclear bodies.

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Supporting Information Available: Details of solid phase synthesis, purification, and characterization of **1–5** and their rhodamine B-labeled derivatives, including instrumentation, tabulated data, and copies of HPLC chromatograms. This material is available free of charge via the Internet at <http://pubs.acs.org>.

References

- (1) Langel, U., Ed. *Cell-Penetrating Peptides*; CRC Press: Boca Raton, FL, 2002.
- (2) Poon, G. M.; Gariépy, J. Cell-surface proteoglycans as molecular portals for cationic peptide and polymer entry into cells. *Biochem. Soc. Trans.* **2007**, *35*, 788–793.
- (3) Martin, M. E.; Rice, K. G. Peptide-guided gene delivery. *AAPS J.* **2007**, *9*, E18–E29.
- (4) Patel, L. N.; Zaro, J. L.; Shen, W. C. Cell penetrating peptides: intracellular pathways and pharmaceutical perspectives. *Pharm. Res.* **2007**, *24*, 1977–1992.
- (5) Foged, C.; Nielsen, H. M. Cell-penetrating peptides for drug delivery across membrane barriers. *Expert Opin. Drug Delivery* **2008**, *5*, 105–117.
- (6) Frankel, A. D.; Pabo, C. O. Cellular uptake of the tat protein from human immunodeficiency virus. *Cell* **1988**, *55*, 1189–1193.
- (7) Joliot, A.; Pernelle, C.; Deagostini-Bazin, H.; Prochiantz, A. Antennapedia homeobox peptide regulates neural morphogenesis. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 1864–1868.
- (8) Vivès, E.; Brodin, P.; Lebleu, B. A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. *J. Biol. Chem.* **1997**, *272*, 16010–16017.
- (9) Derossi, D.; Joliot, A. H.; Chassaing, G.; Prochiantz, A. The third helix of the antennapedia homeodomain translocates through biological membranes. *J. Biol. Chem.* **1994**, *269*, 10444–10450.
- (10) Kabouridis, P. S. Biological applications of protein transduction technology. *Trends Biotechnol.* **2003**, *21*, 498–503.
- (11) Joliot, A.; Prochiantz, A. Transduction peptides: from technology to physiology. *Nat. Cell Biol.* **2004**, *6*, 189–196.
- (12) Mäe, M.; Langel, U. Cell-penetrating peptides as vectors for peptide, protein and oligonucleotide delivery. *Curr. Opin. Pharmacol.* **2006**, *6*, 509–514.
- (13) Futaki, S. Membrane permeable peptide vectors: chemistry and functional design for the therapeutic applications. *Adv. Drug Delivery Rev.* **2008**, *60*, 447.
- (14) Elliott, G.; O'Hare, P. Intercellular trafficking and protein delivery by a herpesvirus structural protein. *Cell* **1997**, *88*, 223–233.
- (15) Wiedlocha, A.; Falnes, P. O.; Rapak, A.; Klingenberg, O.; Muñoz, R.; Olsnes, S. Translocation of cytosol of exogenous, CAAX-tagged acidic fibroblast growth factor. *J. Biol. Chem.* **1995**, *270*, 30680–30685.
- (16) Marinova, Z.; Vukojevic, V.; Surcheva, S.; Yakovleva, T.; Cebers, G.; Pasikova, N.; Usynin, I.; Hugonin, L.; Fang, W.; Hallberg, M.; Hirschberg, D.; Bergman, T.; Langel, U.; Hauser, K. F.; Pramanik, A.; Aldrich, J. V.; Gräslund, A.; Terenius, L.; Bakalkin, G. Translocation of dynorphin neuropeptides across the plasma membrane. A putative mechanism of signal transmission. *J. Biol. Chem.* **2005**, *280*, 26360–26370.
- (17) Henriques, S. T.; Melo, M. N.; Castanho, M. A. Cell-penetrating peptides and antimicrobial peptides: how different are they? *Biochem. J.* **2006**, *399*, 1–7.
- (18) Park, C. B.; Yi, K. S.; Matsuzaki, K.; Kim, M. S.; Kim, S. C. Structure–activity analysis of buforin II, a histone H2A-derived antimicrobial peptide: the proline hinge is responsible for the cell-penetrating ability of buforin II. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 8245–8250.
- (19) Luque-Ortega, J. R.; Van't Hof, W.; Veerman, E. C.; Saugar, J. M.; Rivas, L. Human antimicrobial peptide histatin 5 is a cell-penetrating peptide targeting mitochondrial ATP synthesis in Leishmania. *FASEB J.* **2008**, *22*, 1817–1828.
- (20) Takeshima, K.; Chikushi, A.; Lee, K. K.; Yonehara, S.; Matsuzaki, K. Translocation of analogues of the antimicrobial peptides magainin and buforin across human cell membranes. *J. Biol. Chem.* **2003**, *278*, 1310–1315.
- (21) Birrell, G. W.; Earl, S. T.; Wallis, T. P.; Masci, P. P.; de Jersey, J.; Gorman, J. J.; Lavin, M. F. The diversity of bioactive proteins in Australian snake venoms. *Mol. Cell. Proteomics* **2007**, *6*, 973–986.
- (22) Bringans, S.; Eriksen, S.; Kendrick, T.; Gopalakrishnakone, P.; Livk, A.; Lock, R.; Lipscombe, R. Proteomic analysis of the venom of *Heterometrus longimanus* (Asian black scorpion). *Proteomics* **2008**, *8*, 1081–1096.
- (23) Estrada, G.; Villegas, E.; Corzo, G. Spider venoms: a rich source of acylpolyamines and peptides as new leads for CNS drugs. *Nat. Prod. Rep.* **2007**, *24*, 145–161.
- (24) Becker, S.; Terlau, H. Toxins from cone snails: properties, applications and biotechnological production. *Appl. Microbiol. Biotechnol.* **2008**, *79*, 1–9.
- (25) Boisseau, S.; Mabrouk, K.; Ram, N.; Garmy, N.; Collin, V.; Tadmouri, A.; Mikati, M.; Sabatier, J. M.; Ronjat, M.; Fantini, J.; De Waard, M. Cell penetration properties of maurocalcine, a natural venom peptide active on the intracellular ryanodine receptor. *Biochim. Biophys. Acta* **2006**, *1758*, 308–319.
- (26) Kerkis, A.; Kerkis, I.; Rádis-Baptista, G.; Oliveira, E. B.; Vianna-Morgante, A. M.; Pereira, L. V.; Yamane, T. Crotamine is a novel cell-penetrating protein from the venom of rattlesnake *Crotalus durissus terrificus*. *FASEB J.* **2004**, *18*, 1407–1409.
- (27) Nascimento, F. D.; Hayashi, M. A.; Kerkis, A.; Oliveira, V.; Oliveira, E. B.; Rádis-Baptista, G.; Nader, H. B.; Yamane, T.; Tersariol, I. L.; Kerkis, I. Crotamine mediates gene delivery into cells through the binding to heparan sulfate proteoglycans. *J. Biol. Chem.* **2007**, *282*, 21349–21360.
- (28) Ram, N.; Aroui, S.; Jaumain, E.; Bichraoui, H.; Sadoul, R.; Mabrouk, K.; Ronjat, M.; Lortat-Jacob, H.; De Waard, M. Direct peptide interaction with surface glycosaminoglycans contribute to the cell penetration of maurocalcine. *J. Biol. Chem.* **2008**, *283*, 24274–24284.
- (29) Nicastro, G.; Franzoni, L.; Chiara, C.; Mancin, A. C.; Giglio, J. R.; Spisni, A. Solution structure of crotamine, a Na⁺ channel-affecting toxin from *Crotalus durissus terrificus* venom. *Eur. J. Biochem.* **2003**, *270*, 1969–1979.
- (30) Fadel, V.; Bettendorff, P.; Herrmann, T., Jr.; Oliveira, E. B.; Yamane, T.; Wüthrich, K. Automated NMR structure determination and disulfide bond identification of the myotoxin crotamine from *Crotalus durissus terrificus*. *Toxicon* **2005**, *46*, 759–767.
- (31) Romestand, B.; Molina, F.; Richard, V.; Roch, P.; Granier, C. Key role of the loop connecting the two beta strands of mussel defensin in its antimicrobial activity. *Eur. J. Biochem.* **2003**, *270*, 2805–2813.
- (32) Vila-Perelló, M.; Sánchez-Vallet, A.; García-Olmedo, F.; Molina, A.; Andreu, D. Structural dissection of a highly knotted peptide reveals

- minimal motif with antimicrobial activity. *J. Biol. Chem.* **2005**, *280*, 1661–1668.
- (33) Vila-Perelló, M.; Tognon, S.; Sánchez-Vallet, A.; García-Olmedo, F.; Molina, A.; Andreu, D. A minimalist design approach to antimicrobial agents based on a thionin template. *J. Med. Chem.* **2006**, *49*, 448–451.
- (34) Hansen, M.; Kilk, K.; Langel, U. Predicting cell-penetrating peptides. *Adv. Drug Delivery Rev.* **2008**, *60*, 572–579.
- (35) Richard, J. P.; Melikov, K.; Vives, E.; Ramos, C.; Verbeure, B.; Gait, M. J.; Chernomordik, L. V.; Lebleu, B. Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake. *J. Biol. Chem.* **2003**, *278*, 585–590.
- (36) Console, S.; Marty, C.; García-Echeverría, C.; Schwendener, R.; Ballmer-Hofer, K. Antennapedia and HIV transactivator of transcription (TAT) “protein transduction domains” promote endocytosis of high molecular weight cargo upon binding to cell surface glycosaminoglycans. *J. Biol. Chem.* **2003**, *278*, 35109–35114.
- (37) Potocky, T. B.; Menon, A. K.; Gellman, S. H. Cytoplasmic and nuclear delivery of a TAT-derived peptide and a beta-peptide after endocytic uptake into HeLa cells. *J. Biol. Chem.* **2003**, *278*, 50188–50194.
- (38) Ferrari, A.; Pellegrini, V.; Arcangeli, C.; Fittipaldi, A.; Giacca, M.; Beltram, F. Caveolae-mediated internalization of extracellular HIV-1 tat fusion proteins visualized in real time. *Mol. Ther.* **2003**, *8*, 284–294.
- (39) Vendeville, A.; Rayne, F.; Bonhoure, A.; Bettache, N.; Montcourrier, P.; Beaumelle, B. HIV-1 Tat enters T cells using coated pits before translocating from acidified endosomes and eliciting biological responses. *Mol. Biol. Cell* **2004**, *15*, 2347–2360.
- (40) Wadia, J. S.; Stan, R. V.; Dowdy, S. F. Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nat. Med.* **2004**, *10*, 310–315.
- (41) Jones, S. W.; Christison, R.; Bundell, K.; Joyce, C. J.; Brockbank, S. M.; Newham, P.; Lindsay, M. A. Characterisation of cell-penetrating peptide-mediated peptide delivery. *Br. J. Pharmacol.* **2005**, *145*, 1093–1102.
- (42) Shaw, P.; Doonan, J. The nucleolus. Playing by different rules. *Cell Cycle* **2005**, *4*, 102–105.
- (43) Olson, M. O.; Dundr, M. The moving parts of the nucleolus. *Histochem. Cell Biol.* **2005**, *123*, 203–216.

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