Genetic, cellular and structural characterization of the membrane potential-dependent cell-penetrating peptide translocation pore

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Abstract

Cell-penetrating peptides (CPPs) allow intracellular delivery of bioactive cargo molecules. The mechanisms allowing CPPs to enter cells are ill-defined. Using a CRISPR/Cas9-based screening, we discovered that KCNQ5, KCNN4, and KCNK5 potassium channels positively modulate cationic CPP direct translocation into cells by decreasing the transmembrane potential ($V_m$). These findings provide the first unbiased genetic validation of the role of $V_m$ in CPP translocation in cells. In silico modeling and live cell experiments indicate that CPPs, by bringing positive charges on the outer surface of the plasma membrane, decrease the $V_m$ to very low values (-150 mV or less), a situation we have coined megapolarization that then triggers formation of water pores used by CPPs to enter cells. Megapolarization lowers the free energy barrier associated with CPP membrane translocation. Using dyes of varying dimensions in CPP co-entry experiments, the diameter of the water pores in living cells was estimated to be 2(-5) nm, in accordance with the structural characteristics of the pores predicted by in silico modeling. Pharmacological manipulation to lower transmembrane potential boosted CPPs cellular internalization in zebrafish and mouse models. Besides identifying the first proteins that regulate CPP translocation, this work characterized key mechanistic steps used by CPPs to cross cellular membrane. This opens the ground for strategies aimed at improving the ability of cells to capture CPP-linked cargos in vitro and in vivo.
Introduction

Cell penetrating peptides (CPPs) are short non-toxic sequences of 5-30 amino acids present in proteins able to cross membranes such as homeoproteins and some viral components. CPPs can also be used to deliver bioactive cargo (siRNAs, DNA, polypeptides, liposomes, nanoparticles and others) in cells for therapeutic or experimental purposes (Bechara and Sagan, 2013; Futaki et al., 2013; Guidotti et al., 2017; Illien et al., 2016; Jones and Sayers, 2012; Koren and Torchilin, 2012; Madani et al., 2011; Mueller et al., 2008; Ruseska and Zimmer, 2020; Trabulo et al., 2010; Vasconcelos et al., 2013).

Even though they differ in their origin (Frankel and Pabo, 1988; Green and Loewenstein, 1988; Joliot et al., 1991; Oehlke et al., 1998) and physico-chemical properties, the majority of CPPs carry positive charges in their sequence (Bechara and Sagan, 2013; Guidotti et al., 2017; Jones and Sayers, 2012; Madani et al., 2011). Poly-arginine (e.g. R9), HIV-1 TAT\textsubscript{47-57} and Penetratin (Antennapedia\textsubscript{43-58}) are among the most used and studied CPPs.

The mode of CPP cellular entry is still debated and no proteins have been identified that regulate this process. The CPP entry process starts after the initial electrostatic interactions between the positively charged CPP and the negatively charged components of the cell membrane (Bechara and Sagan, 2013; Futaki et al., 2013; Guidotti et al., 2017; Jones and Sayers, 2012; Koren and Torchilin, 2012; Madani et al., 2011; Ruseska and Zimmer, 2020; Trabulo et al., 2010; Vasconcelos et al., 2013). Interaction with acid sphingomyelinase (Verdurmen et al., 2010) and glycoaminoglycans (Åmand et al., 2012; Bechara et al., 2013; Butterfield et al., 2010; Fuchs and Raines, 2004; Futaki and Nakase, 2017; Ghibaudi et al., 2005; Gonçalves et al., 2005; Hakansson and Caffrey, 2003; Rullo et al., 2011; Rusnati et al., 1999; Ziegler, 2008; Ziegler and Seelig, 2004, 2011), local membrane deformation (Hirose et al., 2012), as well as calcium fluxes (Melikov et al., 2015) have been suggested to play a role in CPP internalization. CPPs enter cells through
a combination of two non-mutually exclusive mechanisms (Illien et al., 2016): endocytosis and direct translocation (Bechara and Sagan, 2013; Futaki et al., 2013; Guidotti et al., 2017; Jones and Sayers, 2012; Koren and Torchilin, 2012; Madani et al., 2011; Ruseska and Zimmer, 2020; Trabulo et al., 2010; Vasconcelos et al., 2013). The nature of these entry mechanisms is debated and not fully understood at the molecular level. The vesicular internalization of CPPs may occur through clathrin-dependent endocytosis, macropinocytosis, and caveolin-1-mediated endocytosis (Bechara and Sagan, 2013; Futaki et al., 2013; Guidotti et al., 2017; Jones and Sayers, 2012; Koren and Torchilin, 2012; Madani et al., 2011; Trabulo et al., 2010). In this case, access to the cytoplasm requires that the CPPs break out of endosomes through a poorly understood process called endosomal escape.

Direct translocation allows the CPPs to access the cytosol through their ability to cross the plasma membrane. There is currently no unifying model to explain mechanistically how direct translocation proceeds and no genes have yet been identified to modulate the manner by which CPPs cross cellular membranes. Direct translocation across the plasma membrane often seemed to originate from specific areas of the cells, suggesting discrete structures on the plasma membrane involved in CPP entry (Allolio et al., 2018; Duchardt et al., 2007; Hirose et al., 2012; Wallbrecher et al., 2017; Ziegler et al., 2005). There is a general consensus though that an adequate plasma membrane potential ($V_m$) is required for direct translocation to occur based on live cell experiments (Rothbard et al., 2004; Wallbrecher et al., 2017; Zhang et al., 2009), as well as in silico studies (Gao et al., 2019; Lin and Alexander-Katz, 2013; Moghal et al., 2020; Via et al., 2018). Electrophysiological and pharmacological $V_m$ modulations have revealed that depolarization blocks CPP internalization (Rothbard et al., 2004; Zhang et al., 2009) and hyperpolarization improves the internalization of cationic CPPs (Chaloin et al., 1998; Henriques et al., 2005; Moghal et
al., 2020; Rothbard et al., 2004; Wallbrecher et al., 2017). By itself, a sufficiently low $V_m$ (i.e. hyperpolarization) appears to trigger CPP direct translocation in live cells (Rothbard et al., 2004; Wallbrecher et al., 2017; Zhang et al., 2009). *In silico* modeling has provided evidence that membrane hyperpolarization leads to the formation of transient water pores, allowing CPP translocation into cells (Gao et al., 2019; Herce and Garcia, 2007; Herce et al., 2009; Lin and Alexander-Katz, 2013; Via et al., 2018) but the free energy landscape governing CPP translocation has not been determined. Moreover, the nature and the structural characteristics of the pores used by CPPs to cross the plasma membrane have not been investigated in live cells.

Here, we provide the first genetic evidence that validates the importance of $V_m$ for CPP direct translocation and we characterize the diameter of the water pores used by CPPs to enter live cells. We also determined the role of the $V_m$ in modulating the free energy barrier associated with membrane translocation and the impact of the $V_m$ on CPP translocation kinetics.
Results

Modes of TAT-RasGAP<sub>317-326</sub> cellular entry

In the present work, we have used TAT-RasGAP<sub>317-326</sub> as a model compound to investigate the molecular basis of CPP cellular internalization. This peptide is made up of the TAT<sub>48-57</sub> CPP and a 10 amino-acid sequence derived from the SH3 domain of p120 RasGAP (Michod et al., 2004). TAT-RasGAP<sub>317-326</sub> sensitizes cancer cells to chemo-, radio- and photodynamic therapies (Chevalier et al., 2015; Michod et al., 2009; Pittet et al., 2007; Tsoutsou et al., 2017) and prevents cell migration and invasion (Barras et al., 2014). This peptide also exhibits antimicrobial activity (Heulot et al., 2017). Some cancer cell lines, such as Raji (Burkitt’s lymphoma), SKW6.4 (transformed B-lymphocytes) and HeLa (cervix carcinoma), are directly killed by this peptide. The manner by which TAT-RasGAP<sub>317-326</sub> kills cells has recently been uncovered (Serulla et al., 2020). The peptide first accesses the cell’s cytosol by direct translocation through the plasma membrane. It then binds to specific phospholipids, such as phosphatidylserine and phosphatidylinositol-bisphosphate that are enriched in the inner leaflet of the plasma membrane. This binding allows the peptide to disrupt the cell’s membrane causing its death by necrosis.

Most CPPs can enter cells by direct translocation and by endocytosis (Bechara and Sagan, 2013; Futaki et al., 2013; Guidotti et al., 2017; Illien et al., 2016; Jones and Sayers, 2012; Koren and Torchilin, 2012; Madani et al., 2011; Mueller et al., 2008; Ruseska and Zimmer, 2020; Trabulo et al., 2010; Vasconcelos et al., 2013). This is also the case for TAT-RasGAP<sub>317-326</sub> (Figure 1A-B and Videos 1-3). Two types of staining were observed in cells incubated with this peptide: i) vesicular only or ii) vesicular and cytosolic (Figure 1A and Figure 1–figure supplement 1A). When the peptide cytosolic signal was strong, it masked the vesicular staining (Figure 1A). In our experimental settings, the cytosolic acquisition of TAT-RasGAP<sub>317-326</sub> occurred only through direct translocation and not...
through endosomal escape (Figure 1–figure supplement 2, Video 4) and was not due to phototoxicity (Dixit and Cyr, 2003; Ha and Tinnefeld, 2012; Levitus and Ranjit, 2011; Zheng et al., 2014) (Figure 1–figure supplement 3).

**Identification of potassium channels as mediators of TAT-cargo direct translocation into cells**

As TAT-RasGAP<sub>317-326</sub> needs to translocate through the plasma membrane to reach the cytosol, a prerequisite for the peptide to kill cells (Serulla et al., 2020), we used the killing ability of the peptide in a CRISPR/Cas9 screen to identify genes involved in CPP direct translocation in two different cell lines (Raji and SKW6.4 cells) (Figure 2–figure supplement 1A). The top candidate genes identified through this approach were specific potassium channels or genes coding for proteins known to regulate such channels indirectly (e.g. PIP5K1A (Suh and Hille, 2008)) (Figure 2A and Figure 2–figure supplement 1B). KCNQ5, identified in Raji cells, is a voltage-dependent potassium channel. KCNN4 and KCNK5, identified in SKW6.4 cells, are calcium-activated channels and belong to the two-pore (voltage-independent) potassium channel family (Shieh et al., 2000), respectively.

These potassium channels were pharmacologically or genetically inactivated (Figure 2B and Figure 2–figure supplement 2A-C) to validate their involvement in the direct translocation of TAT-RasGAP<sub>317-326</sub> through the plasma membrane and the resulting death induction. The KCNQ family inhibitor, XE-991 (Schroeder et al., 2000), as well as KCNQ5 genetic invalidation (Figure 2–figure supplement 2A), fully blocked peptide internalization in Raji cells and protected them from the killing activity of the peptide (Figure 2B and Figure 2–figure supplement 2D). SKW6.4 cells individually lacking KCNN4 or KCNK5
(Figure 2–figure supplement 2B), or SKW6.4 cells treated with TRAM-34, a KCNN4 inhibitor (Wulff et al., 2001; Wulff et al., 2000) were impaired in their ability to take up the peptide and were partially protected against its cytotoxic activity (Figure 2B and Figure 2–figure supplement 2D). Inhibition of KCNN4 activity with TRAM-34 in KCNK5 knock-out cells did not further protect the cells against TAT-RasGAP<sub>317-326</sub>-induced death. In HeLa cells, TRAM-34, but not XE-991, inhibited TAT-RasGAP<sub>317-326</sub> internalization and subsequent death (Figure 2B). Thus, in HeLa cells, KCNN4 channels regulate the membrane translocation of the peptide. This was confirmed by knocking out KCNN4 in these cells (Figure 2B and Figure 2–figure supplement 2C). Resistance to TAT-RasGAP<sub>317-326</sub>-induced death in KCNQ5 knock-out Raji cells and KCNN4 knock-out SKW6.4 or HeLa cells was restored through ectopic expression of the corresponding FLAG- or V5-tagged channels (Figure 2–figure supplement 2E-F and Figure 2–figure supplement 3), ruling out off-target effects.

We next determined whether vesicular internalization or direct translocation were affected in cells with impaired potassium channel activities. Compared to their respective wild-type controls, the percentage of cells with diffuse cytosolic location of FITC-TAT-RasGAP<sub>317-326</sub> was drastically diminished in cells lacking one of the CRISPR/Cas9 screen identified potassium channels in the respective cell lines (Figure 2C and Figure 2–figure supplement 3A-B). This was mirrored by an increase in the percentage of knock-out cells with vesicular staining. The invalidation of potassium channels did not affect transferrin or dextran internalization into cells (Figure 2–figure supplement 3C) or the infectivity of vesicular stomatitis virus (Torriani et al., 2019), substantiating the non-involvement of these channels in endocytosis pathways.

One possibility to explain the above-mentioned results is that the absence of potassium channels reduces peptide binding to cells, thereby hampering subsequent peptide cellular
uptake. At a 20 μM concentration, TAT-RasGAP<sub>317-326</sub> is readily taken up by wild-type Raji cells but not by KCNQ5 knock-out cells. At this concentration, peptide binding was slightly lower in knock-out than in wild-type cells (Figure 2–figure supplement 3D, upper graph). However, augmenting the peptide concentrations in the extracellular medium of KCNQ5 knock-out cells to reach surface binding signals equivalent or higher than what was obtained in wild-type cells, still did not result in peptide cellular internalization unless ≥80 mM of the peptides were used and even in this case, the uptake remained inefficient (Figure 2–figure supplement 3D). Differences in peptide binding is therefore not the cause of the inability of potassium channel knock-out cells to take up TAT-RasGAP<sub>317-326</sub>.

We then assessed whether the role of potassium channels in cellular internalization also applied to TAT cargos other than RasGAP<sub>317-326</sub>. TAT-PNA is an oligonucleotide covalently bound to TAT, which can correct a splicing mutation within the luciferase-coding sequence (Abes et al., 2007; Kang et al., 1998). This can only occur if TAT-PNA reaches the cytosol. The luciferase activity triggered by TAT-PNA was diminished in the presence of potassium channel inhibitors and in potassium channel knock-out cell lines (Figure 2–figure supplement 4A). Cytosolic access of TAT-Cre, which can recombine a loxP-RFP-STOP-loxP-GFP (D’Astolfo et al., 2015; Wadia et al., 2004) gene construct, was then assessed. Switch from red to green fluorescence occurs only when TAT-Cre reaches the nucleus. This took place in wild-type Raji cells but not in the KCNQ5 knock-out cells (Figure 2–figure supplement 4B). We finally tested a clinical phase III therapeutic D-JNKI1 compound (Guidotti et al., 2017; Vasconcelos et al., 2013) used in the context of hearing loss and intraocular inflammation. The internalization of this peptide was completely blocked in Raji cells lacking KCNQ5 (Figure 2–figure supplement 4C, left). D-JNKI1 internalization was also diminished in SKW6.4 cells lacking KCNN4 and KCNK5 channels, as well as in HeLa cells lacking KCNN4 potassium channel (Figure 2–figure supplement 4C, middle and right
These data demonstrate that the absence of specific potassium channels diminishes or even blocks the entry of various TAT-bound cargos.

Potassium channels maintain plasma membrane polarization that is required for cationic CPP entry into cells

Potassium is the main ion involved in setting the plasma membrane potential ($V_m$). The potassium channels identified in the CRISPR/Cas9 screen may therefore participate in the establishment of an adequate $V_m$ permissive for CPP direct translocation (Chaloin et al., 1998; Henriques et al., 2005; Moghal et al., 2020; Rothbard et al., 2004; Wallbrecher et al., 2017; Zhang et al., 2009). Figure 3A (left graph) shows that genetic disruption or pharmacological inhibition of KCNQ5 in Raji cells led to an increase in their $V_m$ (from -26 mV to -15 mV, validated with electrophysiological recordings; see Figure 3–figure supplement 1A). Surprisingly, such minimal increase in $V_m$ between the wild-type and the respective knock-out Raji cells practically abolished CPP internalization (Figure 3B, left graph), indicating that above a certain threshold, the $V_m$ is no longer permissive for CPP direct translocation. In SKW6.4 and HeLa cells, $V_m$ measurement was much more variable than in Raji cells. Nevertheless, a trend of increased $V_m$ was observed when KCNN4 or KCNK5 were invalidated genetically or pharmacologically (Figure 3A, middle and right graphs) and this was accompanied by reduced peptide uptake (Figure 3B, middle and right graphs). As the CRISPR/Cas9 screens performed in various cell lines identified a variety of potassium channels required for efficient CPP internalization, we conclude that it is the $V_m$ maintenance activity of these channels that is important for CPP direct translocation and not some specific features of the channels.

If the reason why invalidation of the KCNQ5, KCNN4, and KCNK5 potassium channels
inhibits TAT-RasGAP<sub>317-326</sub> cellular entry is cell depolarization, a similar response should be obtained by artificially depolarizing cells. Indeed, depolarizing cells with gramicidin (Eisenman et al., 1978) (making non-specific 0.4 nm pores (Kelkar and Chattopadhyay, 2007) within cell membranes) or by increasing the extracellular concentration of potassium (dissipating the potassium gradient) totally blocked cytosolic peptide acquisition into the three studied cell lines (Figure 3B) but not peptide endocytosis (Figure 3–figure supplement 1B). Hence, cellular depolarization in itself inhibits TAT-RasGAP<sub>317-326</sub> direct translocation into the cytosol.

Next, we determined whether hyperpolarization could reverse the inability of potassium channel knock-out cells to take up TAT-RasGAP<sub>317-326</sub>. Cells were either incubated in the presence of valinomycin (Rimmele and Chatton, 2014), which leads to formation of potassium-like channels, or transfected with KCNJ2 channel that also provokes potassium efflux and membrane hyperpolarization (Xue et al., 2014). Figure 3B shows that hyperpolarization of cells lacking CRISPR/Cas9-identified potassium channels fully restored peptide translocation. Moreover, hyperpolarization increased peptide cytosolic acquisition in wild-type cells (Figure 3B). Similar effect, albeit to a lesser extent, was observed by ectopically expressing the KCNQ5 CRISPR/Cas9-identified potassium channel in wild-type and KCNN4 knock-out SKW6.4 and HeLa and by ectopically expressing KCNN4 in wild-type and KCNQ5 knockout Raji cells (Figure 3 – figure supplement 1C). Additionally, cells such as primary rat cortical neurons that naturally have a low V<sub>m</sub> (-48 mV) take up the CPP in their cytosol more efficiently than cells with higher V<sub>m</sub> such as HeLa cells (-25 mV) (Figure 3–figure supplement 1D). Altogether, these results demonstrate that the V<sub>m</sub> modulates internalization of TAT-RasGAP<sub>317-326</sub> in various cell lines. This internalization can be manipulated through cellular depolarization to block it and through hyperpolarization to increase it, confirming earlier results obtained for the R8 CPP
in Jurkat cells (Rothbard et al., 2004).

We then assessed whether the entry of TAT, nanomeric arginine (R9), and Penetratin (Figure 3–figure supplement 2A), three commonly used cationic CPPs in biology and medicine, was regulated by the plasma membrane potential as shown above for TAT-RasGAP_{317-326}. Similarly, to TAT-RasGAP_{317-326}, these CPPs are taken up by HeLa cells by both direct translocation and endocytosis (Figure 3–figure supplement 2B-C).

Depolarization, induced by either gramicidin or high extracellular potassium concentrations (Figure 3D), led to decreased cytosolic fluorescence of these CPPs, while valinomycin-mediated hyperpolarization favored their translocation in the cytosol (Figure 3C, upper graphs, Figure 3E, and Figure 3–figure supplement 2D-E). Although the cellular membrane composition of neurons may differ from the other cell lines used in this study, the $V_m$ also controlled peptide translocation in non-transformed rat primary cortical neurons (Figure 3–figure supplement 3). In contrast, depolarization had no impact on the ability of the cells to endocytose these CPPs (Figure 3C, bottom graphs), further confirming that CPP endocytosis is not affected by $V_m$. Finally, we note that CPP membrane binding was only minimally affected by depolarization (Figure 3–figure supplement 4). Hence, the reason why depolarized cells do no take up CPPs is not a consequence of reduced CPP binding to cells, confirming our earlier observation obtained with TAT-RasGAP_{317-326} (Figure 2–figure supplement 3D). Altogether the data presented in Figure 3 show that direct translocation of cationic CPPs is modulated by the $V_m$ of cells and that specific potassium channels are involved in this modulation.

**CPP direct translocation modeling**

To further study the mechanism of CPP cellular entry through direct translocation, we took
advantage of coarse-grained molecular dynamics technique and MARTINI force field 2.2p
(Marrink et al., 2007; Marrink and Tieleman, 2013). In our simulations we have used TAT-
RasGAP_{317-326}, TAT, R9 and Penetratin in presence of a natural cell membrane-like
composition (for both inner and outer leaflets) while earlier studies have employed simpler
membrane composition (Gao et al., 2019; Herce et al., 2009; Lin and Alexander-Katz,
2013; Moghal et al., 2020; Via et al., 2018; Zhang et al., 2009). Membrane
hyperpolarization was achieved by setting an ion imbalance (Delemotte et al., 2008; Gao
et al., 2019; Gurtovenko and Vattulainen, 2007; Herrera and Pantano, 2009) through a net
charge difference of 30 positive ions (corresponding to a $V_m$ of $\sim$2V) between the
intracellular and extracellular space. The use of very high $V_m$ values, typically used in
computational studies, is required to capture nanosecond occurring events. This protocol
(Figure 4–figure supplement 1A) allowed us to observe CPP translocation across
membranes within a few tens of nanoseconds (Figure 4A and Video 5). In presence of
$\sim$2V $V_m$, the CPPs approached the membrane on the extracellular side and this led to the
formation of a water column within the membrane that the CPP then used to move to the
intracellular space (Video 5). The movement of the positive charges carried by the CPPs,
as well as extracellular cations, to the intracellular compartments via the water pore
induced membrane depolarization. This depolarization provoked the collapse of the water
pore and membrane resealing. Even though CPPs play an active role in their
internalization, the mere presence of the CPP in the absence of a sufficiently low $V_m$ was
not enough to trigger water pore formation (Figure 4B, right graph and Video 6). These
data confirm earlier work describing the role of the $V_m$ in CPP penetration into or through
bilipidic membranes (Gao et al., 2019; Herce and Garcia, 2007; Herce et al., 2009; Lin and
Alexander-Katz, 2013; Moghal et al., 2020; Via et al., 2018; Zhang et al., 2009). TAT, R9,
and Penetratin all translocated into the intracellular compartment but with different
propensities (Figure 4B, left graph) and with different kinetics (Figure 4–figure supplement
1B) that appeared to be related to the positive charges they carry (Figure 3–figure supplement 2A): the more positively charged a CPP, the higher probability to translocate across cell membranes and the faster kinetics of water pore formation at a given $V_m$.

We also applied a metadynamics protocol to estimate the impact of the $V_m$ on the free energy landscape of R9 translocation. The free energy barriers recorded in depolarized membranes ($V_m = 0$) and polarized membranes ($V_m = -80$ mV) were similar (Figure 4C-D). The obtained value of about 200 kJ/mol is in line with recent estimation of the free energy barrier associated with CPP tranlocation at a $V_m=0$ (Gao et al., 2019). Only at much lower $V_m$ values (-150 mV) was a marked decrease in free energy barrier recorded. This indicates that hyperpolarization values found in resting cells [down to about -80 mV in neurons and higher in many other cells types (Yang and Brackenbury, 2013)] are not more favorable than fully depolarized membranes to establish conditions for the formation of water pores. It appears therefore that cells need to decrease their $V_m$ to much lower values (e.g. -150 mV or lower) to reach conditions compatible with water pore formation. This *in silico* observation may appear contradictory with our results obtained in live cells showing direct translocation at -25 mV (Figure 2), as well as with the experiment demonstrating that CPP cytosolic internalization was more efficient in cortical neurons in comparison to less negatively charged HeLa cells (Figure 3–figure supplement 1D). We therefore postulate that the presence of CPPs on the cell surface induces locally a substantial voltage drop from the resting $V_m$. To test this assumption, we analyzed the electrostatic potential map in a molecular system composed of the R9 peptide in contact with the plasma membrane in the absence of an external electrostatic field (Figure 4E). This simulation indicated that the presence of CPPs at the cell surface is sufficient to decrease locally the transmembrane potential to about -150 mV (Figure 4E). This was not observed in the absence of the CPP. In conclusion, our data support a model where CPPs further...
decrease the Vm of resting cells to very low values (equal or less than -150 mV) that are compatible with spontaneous water pore formation and that we coin megapolarization.

Our model also predicts that the electric force exerted on CPPs when cells are megapolarized permit CPPs to accumulate in the cytosol and reach concentrations that are higher than in the extracellular milieu. Figure 4–figure supplement 1C shows indeed that cells can concentrate TAT-RasGAP<sub>317-326</sub> in the cytosol of Raji and Hela cells, up to 100 times in extremes cases.

**Structural characterization of the pore allowing CPP entry in live cells**

Propidium iodide (PI), with a diameter of 0.8-1.5 nm (Bowman et al., 2010) or fluorophore-labelled 3 kDa, 10 kDa, and 40 kDa dextrans, with diameters (provided by Thermofisher) of 2.3 ±0.38 nm (Thorne and Nicholson, 2006), 4.5 nm and 8.6 nm, respectively (Figure 5- figure supplement 1A), were used to estimate the size of the water pores formed in the presence of CPPs in live cells. These molecules by themselves did not translocate in the cytosol of cells (Figure 5A and Figure 5-figure supplement 1B). They were then co-incubated with different FITC-labelled CPPs and their uptake monitored by confocal microscopy. While PI and CPPs efficiently co-entered cells (Figure 5B and Figure 5-figure supplement 1C-D), there was only marginal co-entry of the dextrans with the CPPs (Figure 5B). The marginal dextran co-entry was inversely correlated with the dextran diameters (inset in Figure 5B): ~2.3 nm-wide dextrans entered cells better than ~4.5 nm-wide dextrans and ~8.6 nm-wide dextrans mostly remained outside cells. The entry of PI and CPPs in cells occurred with identical kinetics (Figure 5-figure supplement 1D), further supporting the notion that they enter cells together. The PI/CPP co-entry was prevented by cell depolarization (Figure 5-figure supplement 1B), which is expected if PI accesses the cytosol via the megapolarization-induced pores used by CPP to enter cells. CPPs do not need to be labelled with a fluorophore to allow PI co-entry into cells (Figure 5B, "unlabelled
TAT + PI” condition), ruling out phototoxicity as a confounding effect. Similar results were obtained in primary rat cortical neurons, where PI cytosolic signal was observed in cells that took up the selected CPPs through direct translocation (Figure 5C). These data are compatible with the notion that water pores triggered by CPPs allow molecules up to ~2 nm in diameter to efficiently enter cells. They are also in line with the in silico prediction of the water pore diameter of 1.6+/−0.26 nm obtained by analyzing the structure of the pore at the transition state (i.e. when the CPP is crossing the cell membrane; see Figure 4A).

Molecules in the 2-5 nm diameter range, such as 3- and 10 kDa dextrans, can still use this entry route to a limited extent. In this context, the Cre recombinase, with a diameter of 5 nm (estimated from its crystal structure; NDB:PD0003), can be transported by TAT into cells (Figure 2-figure supplement 4B), another indication that the pores used by cationic CPPs to enter cells can allow the passage of molecules up to 5 nm.

Despite identical net positive charges (Figure 3-figure supplement 2A), and as reported earlier (Mitchell et al., 2000), the K9 peptide made of 9 lysine residues was less capable of translocating into cells compared to R9 (Figure 5B and Figure 3-figure supplement 2C, right graph). This may be due to the deprotonation of K9 once in the plasma membrane (see discussion). However, in the few cases when cells have taken up K9, PI co-internalized as well (middle graph of Figure 5B). This indicates that K9 has a reduced capacity compared to R9 to trigger water pore formation but when they do, PI can efficiently translocate through the pores created by K9.

Modelling experiments indicate that water pores are created in membranes subjected to sufficiently high (absolute values) $V_m$. We therefore tested whether the mere hyperpolarization of cells (i.e. in the absence of CPPs) could trigger the translocation of PI into cells, indicative of water pore formation. Figure 5D (left) shows that the hyperpolarizing drug valinomycin significantly increased PI cell permeability. In contrast,
depolarization, mediated by gramicidin, reduced PI internalization (Figure 5D, left). Cells incubated with CPPs took up PI in their cytosol to a much greater extent than when cells were treated with valinomycin (Figure 5D, left), as expected if CPPs participate in setting plasma membrane megapolarization.

Figure 5E shows the correlation between cytosolic PI accumulation over time and $V_m$. Based on this correlation, we estimated the $V_m$ of cells incubated with a CPP to be in the order of -150 mV (Figure 5F). In accordance with the modelling experiments, these data further support the notion i) that water pore formation in cells is favored by cell hyperpolarization and inhibited by depolarization and ii) that CPPs themselves (Rao et al., 2014; Wallbrecher et al., 2017) further contribute to the establishment of local megapolarization in the plasma membrane.

**Megapolarization improves CPP internalization in vivo**

We investigated whether it was possible to experimentally manipulate the $V_m$ to favor CPP internalization in *in vivo* situations. Systemic exposure of zebrafish embryos to valinomycin in eggwater led to cell hyperpolarization (Figure 6-figure supplement 1A) and improved internalization of a TAT-based CPP (Figure 6-figure supplement 1B). This systemic treatment, while not acutely toxic, halted development (Figure 6-figure supplement C-E).

However, local valinomycin injection did not affect long-term viability (Figure 6-figure supplement 1F) and efficiently increased CPP cellular internalization (Figure 6A). Subcutaneous injections of valinomycin in mice induced tissue hyperpolarization (Figure 6-figure supplement 1G) and boosted the CPP delivery in skin cells (Figure 6B). These results demonstrate that hyperpolarizing drugs can be used to ameliorate CPP internalization in animal tissues.
Discussion

Multiple models, mostly inferred from artificial experimental paradigms, have been proposed to explain CPP direct translocation. These include the formation of pores made of the CPPs themselves that they use for their own entry, the formation of inverted micelles in the plasma membrane that translocate the CPPs, or diffusion of the CPPs across the plasma membrane (Bechara and Sagan, 2013; Futaki et al., 2013; Guidotti et al., 2017; Koren and Torchilin, 2012; Trabulo et al., 2010). Our simulation and cellular data, while providing no evidence for such models, demonstrate that CPP cellular internalization is potassium channel- and $V_m$-dependent in vitro and in vivo. Potassium channels are required to establish a basal low $V_m$, subsequently permissive for CPP direct translocation. Hyperpolarizing drugs, such as valinomycin, enhance permissiveness.

When CPPs come into contact with the plasma membrane, they decrease even more the $V_m$, resulting in a locally megapolarized membrane. This increases the likelihood of water pore formation that the CPPs then use to penetrate into cells according to their electrochemical gradient (Figure 7). Water pores are created by a combination of lipid head group reorientation coupled to intrusion of a column of water in the membrane bilayer. Water movement plays therefore an active role in the formation of the pore and is not merely occurring once the pores are formed. The movement of the positive charges carried by the CPPs into the cell, as well as the transport of extracellular cations (e.g. Na$^+$), dissipates the $V_m$, resulting in the collapse of the water pores and sealing of the plasma membrane. CPP-mediated formation of water pores is therefore transient and does not affect cell viability. Multiple rounds of CPP-driven water pore formation and CPP translocation into cells can lead to intracellular accumulation of the CPP to concentrations higher than found outside cells (Figure 4-figure supplement 1C).

It has not been possible to measure directly the precise values of the $V_m$ that allow the
formation of water pores used by CPPs to enter cells. Using an indirect calculation mode based on the uptake of PI alongside CPPs, we have estimated that a $V_m$ in the order of $-150 \text{ mV}$ is required for water pores to be formed (Figure 5F). This might be an underestimation however as modeling data indicate that, at $-150 \text{ mV}$, the free energy barrier, while being markedly diminished compared to those calculated at $-80 \text{ mV}$ or $0 \text{ mV}$, is not fully abrogated (Figure 4D). Possibly therefore, the local $V_m$ where CPPs interact with the plasma membranes is much lower than $-150 \text{ mV}$ and/or changes in CPP structures occur (e.g. refolding, aggregation) leading to further reduction in free energy barrier.

It is worth mentioning that the applied coarse-grained Martini forcefield, as any other model, has a number of known limitations (Marrink et al., 2019; Marrink and Tieleman, 2013) such as the chemical and spatial resolution, which are both limited compared to atomistic models. There is also a shifted balance between entropy and enthalpy due to the reduced number of degrees of freedom. Moreover, the secondary structure is an input parameter of the model, which implies that secondary structure elements remain fixed during the simulation (Monticelli et al., 2008). However, the coarse-grained approach has demonstrated to provide reliable results in the context of protein-membrane interactions and peptide translocation (Castillo et al., 2013; Koch et al., 2019; Marrink et al., 2004; Monticelli et al., 2008; Monticelli et al., 2010; Periole et al., 2009; Periole et al., 2007; Ramadurai et al., 2010; Yesylevskyy et al., 2010). Moreover, the ability of Martini Coarse-grained forcefield to model realistic and heterogeneous membranes was widely demonstrated in literature, as summarized in a recent review paper (Marrink et al., 2019).

Our model posits that the number of positively charged amino acids influence the ability of CPPs to hyperpolarize cells and hence to form water pores that they take to translocate into cells. CPP hydropathy strongly correlates with penetration of water molecules in the
lipid bilayer, thus supporting the hypothesis that the amount of water each CPP can route inside the membrane is modulated by the hydrophobic and hydrophilic character of the peptide (Grasso et al., 2018). The nature of cationic amino acids in peptides determines their translocation abilities. It is known for example that peptides made of 9 lysines (K9) poorly reaches the cytosol (Figure 5B and Figure 3-figure supplement 2C) and that replacing arginine by lysine in Penetratin significantly diminishes its internalization (Amand et al., 2012; Mitchell et al., 2000). According to our model, K9 should induce megapolarization and formation of water pores that should then allow their translocation into cells. However, it has been determined that, once embedded into membranes, lysine residues tend to lose protons (Armstrong et al., 2016; Li et al., 2013; MacCallum et al., 2008). This will thus dissipate the strong membrane potential required for the formation of water pores and prevent lysine-containing CPPs to cross the membrane. In contrast, arginine residues are not deprotonated in membranes and water pores can therefore be maintained allowing the arginine-rich CPPs to be taken up by cells. This phenomenon was not modeled in our coarse-grained in silico simulations because the protonation state was fixed at the beginning of the simulation runs and was not allowed to evolve. An additional potential explanation to the internalization differences observed between arginine- and lysine-rich peptides is that even though both arginine and lysine are basic amino acids, they differ in their ability to form hydrogen bonds, the guanidinium group of arginine being able to form two hydrogen bonds (Fromm et al., 1995) while the lysyl group of lysine can only form one. Compared to lysine, arginine would therefore form more stable electrostatic interactions with the plasma membrane. According to previously published studies (Kosuge et al., 2008; Mitchell et al., 2000), the optimal length of consecutive arginine residues appears to be between 9-16 amino acids, resulting in optimal CPP cytosolic acquisition. Shorter and longer peptides have decreased internalization efficiencies. The role of the Vm presented in our model is consistent with the reduced uptake of short
polyarginine peptides but the Vm parameter of our model cannot explain why longer polyarginine peptides are less efficiently taken up by cells. Our work however also indicates that the water pores created by megapolarization have a diameter of about $2(-5)$ nm. Molecules larger than 2 nm are therefore less efficiently transported through these water pores and if polyarginine peptides reach that size their internalization will be hindered. The efficiency of direct translocation of peptides is therefore likely modulated by their sizes, the secondary structures they adopt, and the number of positive charges they carry.

Cationic residues are not the only determinant in CPP direct translocation. The presence of tryptophan residues also plays important roles in the ability of CPPs to cross cellular membranes. This can be inferred from the observation that Penetratin, despite only bearing 3 arginine residues can penetrate cells with similar propensities compared to R9 or TAT that contain 9 and 8 arginine residues, respectively. The aromatic characteristics of tryptophan is not sufficient to explain how it favors direct translocation as replacing tryptophan residue with the aromatic amino acid phenylalanine decreases the translocation potency of the RW9 (RRWWRRWRR) CPP (Derossi et al., 1994). Rather, differences in the direct translocation promoting activities of tryptophan and phenylalanine residues may come from the higher lipid bilayer insertion capability of tryptophan compared to phenylalanine (Christiaens et al., 2002; Jobin et al., 2015; MacCallum et al., 2008). There is a certain degree of interchangeability between arginine and tryptophan residues as demonstrated by the fact that replacing up to 4 arginine residues with tryptophan amino acids in the R9 CPP preserves its ability to enter cells (Walrant et al., 2020). It appears that loss of positive charges that contribute to water pore formation can be compensated by acquisition of strengthened lipid interactions when arginine residues are replaced with tryptophan residues. This can explain why a limited number of
arginine/tryptophan substitutions does not compromise CPP translocation through membranes. Therefore, despite the importance of the membrane potential for CPP direct translocation into cells, other factors also appear to play a role in this process.

While the nature of the CPPs likely dictate their uptake efficiency as discussed in the previous paragraph, the composition of the plasma membrane could also modulate how CPPs translocate into cells. In the present work, we have recorded CPP direct translocation in transformed or cancerous cell lines as well as in primary cells. These cells display various abilities to take up CPPs by direct translocation and the present work indicates that this is modulated by their Vm. But as cancer cells display abnormal plasma membrane composition (Szlasa et al., 2020), it will be of interest in the future to determine how important this is on their capacity to take up CPPs.

CPPs can also enter cells through endocytosis, a mode of entry that is not inhibited by cell depolarization (Figure 3C and Figure 2-figure supplement 4C). The differences between CPPs in terms of how efficiently direct translocation is modulated by the Vm (Figure 3C-E and Figure 3-figure supplement 2E) could be explained by their relative dependence on direct translocation or endocytosis to penetrate cells. The positive charges in CPPs favor direct translocation and consequently the sensitivity to cell depolarization. On the other hand, when endocytosis is the predominant type of entry, CPP cytosolic uptake will be less affected by both hyperpolarization and depolarization.

We propose, based on the work described here, that hyperpolarization induced by drugs such as valinomycin represents a simple alternative or parallel approach to optimize CPP internalization. However hyperpolarizing drugs may be toxic when systemically applied. For example, valinomycin at the concentrations used to induce hyperpolarization (10 μM) would be lethal if systemically injected in mice (LD50 in the low micromolar (Daoud and Juliano, 1986)). On the other hand, local administration of valinomycin is far less toxic.
(Gad et al., 1985; Waksman, 1953) as confirmed here in zebrafish and mice. Hyperpolarizing agents may therefore be preferentially used for local or topical applications, which is incidentally the case for the clinically approved CPPs\textsuperscript{1,2}.

Strategies to improve CPP delivery are becoming increasingly elaborate through the use of nanoparticles (Bansal et al., 2018), double-coated nanoparticles (Khalil et al., 2018), liposome-polycation-DNA complexes (Wu et al., 2018), branched peptides (Jeong et al., 2016), etc. Our data provides a characterization at molecular level that can be taken advantage of to i) improve or optimize « old » CPPs, ii) design new CPPs, iii) help explain the behaviors of newly discovered CPPs (Du et al., 2011; Kauffman et al., 2018; Yin et al., 2009), iv) discriminate between target cells and cells that should be left unaffected based on $V_m$ and v) distinguish between direct translocation and endosomal escape. The present work indicates that the impact on megapolarization should be evaluated when chemical modifications are performed on cationic CPPs to augment their delivery capacities.
Materials and Methods

Chemicals

Puromycin 10 mg/ml (Thermo Fisher, ref. no. A11138-02) was aliquoted and stored at -20 °C. Blasticidin (Applichem, ref. no. A3784) was dissolved at 1 mg/mL in water and stored at -20 °C. XE-991 and TRAM-34 (Alomone labs, ref. no. X-100 and T-105 respectively) was dissolved in DMSO at 100 mM and stored at -20 °C. Cells were preincubated with 10 µM of these inhibitors for 30 minutes and then kept throughout the experiments. Pyrene butyrate (Sigma, ref. no. 257354) was dissolved in DMSO at 20 mM and stored at -20 °C. Live Hoechst 33342 (Sigma, ref. no. CDS023389) was aliquoted and stored at -20°C. Trypan Blue 0.4% (Life technologies, ref. no. 15250061) was stored at room temperature. AlexaFluor488-labeled human transferrin was dissolved in PBS 5 at mg/ml and stored at 4°C (Thermo Fisher, ref. no. 13342). TexasRed-labelled neutral 3’000 and 40’000 Da dextran was dissolved in PBS at 10 mg/ml and stored at -20°C (Thermo Fisher, ref. no. D3329 and D1829, respectively). TMR-labelled 10’000 neutral dextran was dissolved in PBS at 10 mg/ml and stored at -20°C (Thermo Fisher, ref. no. D1816).

Antibodies

The rabbit polyclonal anti-V5 (Bethyl, ref. no. A190-A120), mouse monoclonal anti-FLAG antibody was from Sigma-Aldrich (ref. no. F1804), rabbit monoclonal anti-actin (Cell signaling, ref. no. 4970) and rat monoclonal anti-γ-tubulin (Santa Cruz, ref. no. sc-51715) antibodies were used for Western blotting.

Cell lines
All cell lines were cultured in 5% CO2 at 37°C. Raji (kind gift from the laboratory of Aimable Nahimana, ATCC: CCL-86), SKW6.4 (kind gift from the laboratory of Pascal Schneider, ATCC: TIB-215) and HeLa (ATCC: CCL-2) cells were cultured in RPMI (Invitrogen, ref. no. 61870) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, ref. no. 10270-106). HEK293T (ATCC: CRL-3216) were cultured in DMEM supplemented with 10% FBS and were used only for lentiviral production. All cell lines were mycoplasma negative and authenticated via Microsynth cell authentication service. Unless otherwise indicated, experiments were performed in RPMI with 10% FBS.

**Zebrafish**

Zebrafish (Danio rerio) from AB line were bred and maintained in our animal facility under standard conditions, more specifically at 28.5°C and on a 14:10 hours light:dark cycle at the Zebrafish facility of the School of Biology and Medicine (cantalonal veterinary approval VD-H21). Zebrafish of 20 hours post fertilization were collected and treated with 0.2 mM phenylthiourea (PTU, Sigma, St. Louis, MO) to suppress pigmentation. Embryos were raised at 28.5°C in Eggwater (0.3 g sea salt/L reverse osmosis water) up to 4 days post fertilization.

**Mice**

C57BL/6NCrl were acquired from Charles River laboratories, which were then housed and bred in our animal facility. All experiments were performed according to the principles of laboratory animal care and Swiss legislation under ethical approval (Swiss Animal Protection Ordinance; permit number VD3374.a).
Primary cortical neuronal culture

Sprague-Dawley rat pups (from Janvier, France) were euthanized in accordance with the Swiss Laws for the protection of animals, and the procedures were approved by the Vaud Cantonal Veterinary Office. Primary neuronal cultures from cortices of 2-day-old rats were prepared and maintained at 37 °C with a 5% CO2-containing atmosphere in neurobasal medium (Life Technologies, 21103-049) supplemented with 2% B27 (Invitrogen, 17504044), 0.5 mM glutamine (Sigma, G7513) and 100 μg/ml penicillin-streptomycin (Invitrogen, 15140122) as described previously (Vaslin et al., 2007). Neurons were plated at a density of ~3 × 10^5 cells on 12-mm glass coverslips coated with 0.01% poly-L-lysine (Sigma, P4832). Half of the medium was changed every 3-4 days and experiments were performed at 12–13 days in vitro.

Confocal microscopy

Confocal microscopy experiments were done on live 300'000 cells. Cells were seeded for 16 hours onto glass bottom culture dishes (MatTek, corporation ref. no. P35G-1.5-14-C) in 2 mL RPMI, 10% FBS and treated as described in the Figures in 1 mL media, 10%FBS. For nuclear staining, 10 μg/ml live Hoechst 33342 (Molecular probes, ref. no. H21492) was added in the culture medium 5 minutes before washing cells twice with PBS. After washing, cells were examined with a plan Apochromat 63x oil immersion objective mounted on a Zeiss LSM 780 laser scanning fluorescence confocal microscope equipped with gallium arsenide phosphide detectors and three lasers (a 405 nm diode laser, a 458-476-488-514 nm argon laser, and a 561 nm diode-pumped solid-state laser). Time-lapse experiments were done using an incubation chamber set at 37°C, 5% CO2 and visualized
with a Zeiss LSM710 Quasar laser scanning fluorescence confocal microscope equipped with either Neofluar 63x, 1.2 numerical aperture (NA) or plan Neofluar 100x, 1.3 NA plan oil immersion objective (and the same lasers as above). Visual segregation of cells based on types of CPP entry, associated either with vesicular or diffuse cytosolic staining, was performed as shown in Figure 1-figure supplement 1A. Cell images were acquired at a focal plane near the middle of the cell making sure that nuclei were visible. Image acquisition was performed using the same settings for the data presented in the same panel or in the related supplementary panels, unless otherwise indicated.

Flow cytometry

Flow cytometry experiments were performed using a Beckman Coulter FC500 instrument. Cells were centrifuged and resuspended in PBS prior to flow cytometry. Data analysis was done with Kaluza Version 1.3 software (Beckman Coulter).

Cell death and CPP internalization measurements

With the exception of neurons, cell death was quantitated with 8 μg/ml propidium iodide (Sigma, ref. no. 81845). Unless otherwise indicated, cell death was assessed after 16 hours of continuous incubation in Raji and SKW6.4 cells and 24 hours in HeLa cells. Prior to treatment, 300'000 cells were seeded in 6-well plates for 16 hours in 2 mL media, 10% FBS. Treatment was done in 1 ml media with 10%FBS. Cell death and peptide internalization were analyzed by flow cytometry. Internalization measurements were done after one hour of incubation. Peptide internalization in primary cortical neurons was assessed by confocal microscopy with LSM780. Cell-associated fluorescence was
quantitated with ImageJ. When cytosolic fluorescent was recorded with ImageJ, the
regions of interest that were analyzed were chosen so as not to contain labelled
endosomes (Figure 1-figure supplement 2D, circle).

Lentivirus production

Recombinant lentiviruses were produced as described\(^{92,93}\) with the following modification:
the envelope plasmid pMD.G and the packaging vector pCMVΔR8.91 were replaced by
pMD2.G and psPAX2, respectively.

In vitro membrane potential measurements

Two methods were used to assess cellular membrane potential in vitro. With the first
method, the membrane potential was determined by incubating 300’000 cells for 40
minutes with 100 nM of the fluorescent probe DiBAC4(3) (Thermofisher, ref. no. B438) in
6-well plates in 1 mL media, 10% FBS. and the median fluorescence intensity was then
assessed by flow cytometry. Calculation of the actual membrane potential in mV based on
the DiBAC4(3) signals was performed as described earlier (Klapperstuck et al., 2009;
Krasznai et al., 1995). The second method relied on electrophysiology recordings. To
perform these, the bath solution composition was (in mM): 103.9 NaCl, 23.9 NaHCO3, 2
CaCl2, 1.2 MgCl2, 5.2 KCl, 1.2 NaH2PO4, 2 glucose and 1.7 ascorbic acid. The pipet
solution was composed of (in mM): 140 KMeSO4, 10 HEPES, 10 KCl, 0.1 EGTA, 10
phosphocreatine and 4 MgATP. The patch pipets had a resistance of 2.4-3.6 MΩ.
Perforated patch recordings were performed as previously described (Cueni et al., 2008).
Briefly, freshly prepared gramicidin D (Sigma, ref. no. G5002), at 2.8 μM final
concentration, was added to prefiltered patch pipet solution and then sonicated for three consecutive times during 10 seconds. Cell-attached configuration was achieved by applying negative pressure on patch pipet until seal resistance of over one Giga Ohm was reached. After gaining cell access through gramicidin created pores, membrane potential measurements were done in current clamp at 0 pA for at least three minutes. Since primary rat neurons are killed following full depolarization induced by gramicidin, the standard curve from membrane potential calculations (Klapperstuck et al., 2009; Krasznai et al., 1995) were performed using gramicidin-treated Raji cells incubated with increasing concentrations of DiBac4(3) for 40 minutes. Images of the cells were then taken using a LSM780 confocal microscope and the cell-associated fluorescence quantitated with ImageJ.

Relative membrane potential assessment in vivo

Zebrafish embryos in Egg water (see Zebrafish section) were incubated for 40 minutes in the presence or in the absence of various concentrations of valinomycin together with 950 nM DiBac4(3). The embryos were then fixed and visualized under a confocal LSM710 microscope (Adams and Levin, 2012). DiBac4(3)-associated fluorescence of a region of interest of about 0.0125 mm2 in the tail region was quantitated with ImageJ. The values were normalized to the control condition (i.e. in the absence of valinomycin). Mice were intradermally injected with 10 μl of a 950 nM DiBac4(3) PBS solution containing or not 10 μM valinomycin and sacrificed one hour later. The skin was excised, fixed in 4% formalin, paraffin-embedded and used to prepare serial histological slices. Pictures of the slices were taken with a CYTATION3 apparatus. The DiBac4(3)-associated fluorescence in the whole slice was quantitated with ImageJ. The slice in the series of slices prepared from a
given skin sample displaying the highest fluorescence signal was considered as the one nearest to the injection site. The signals from such slices are those reported in the Figures.

**Experimental modulation of the plasma membrane potential**

*Flow cytometry assessment of CPP internalization*

Raji, SKW6.4 or HeLa cells: three hundred thousand cells were plated on non-coated plates to avoid cell adherence in 500 µl RPMI, 10% FBS. Cellular depolarization was induced by preincubating the cells at 37°C with 2 µg/ml gramicidin for 5 minutes and/or by placing them in potassium-rich buffer (Hirose et al., 2012) for 30 minutes (40 mM KCl, 100 mM potassium glutamate, 1 mM MgCl2, 1 mM CaCl2, 5 mM glucose, 20 mM HEPES, pH7.4). Cells were then treated with the selected CPPs at the indicated concentrations for one hour when peptide internalization was recorded or with 100 nM DiBac4(3) for 40 minutes when membrane potential needed to be measured. Hyperpolarization in Raji cells in the presence of TAT-RasGAP317-326 was performed by treating the cells with 10 µM valinomycin for 20 minutes in RPMI without serum. Cells were then treated with 5 µM TAT-RasGAP317-326 for one hour or 100 nM DiBac4(3) for 40 minutes. In the case of SKW6.4 and HeLa cells, hyperpolarization was induced by infection with a viral construct expressing KCNJ2 (see “virus production” section). Cells were then treated with 40 µM of indicated CPP for one hour or 100 nM DiBac4(3) for 40 minutes.

*CPP cytosolic internalization quantitation based on confocal microscopy*

Three hundred thousand wild-type HeLa cells were plated overnight on glass-bottom dishes in 2 mL RPMI with 10% FBS. The next day, serum was removed and cells were preincubated at 37°C with 2 µg/ml gramicidin for 5 minutes, 10 µM valinomycin for 20...
minutes or were left untreated in 1 mL media with 10%FBS. The indicated CPPs were then added and cells were incubated for one hour at 37°C. Cells were then washed and visualized in RPMI without serum under a confocal microscope. CPP cytosolic internalization was quantitated within a cytosolic region devoid of endosomes using ImageJ. The number of CPP-positive vesicles was visually determined per cell in a given focal plane.

Neurons (12 days post-isolation) were preincubated 30 minutes with 5 mM TEA (tetraethylammonium, Sigma Aldrich, ref. no.T2265; gramicidin is toxic in these neurons; see section “Membrane potential measurements in vitro”) to induce depolarization or 10 μM valinomycin to induce hyperpolarization in bicarbonate-buffered saline solution (116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO₄, 1 mM NaH₂PO₄, 26.2 mM NaHCO₃, 0.01 mM glycine, 1.8 mM CaCl₂, 4.5 mg/mL glucose) in a 37°C, 5% CO2 incubator. The cells were then incubated one hour with 2 μM of FITC-labeled TAT-RasGAP₃₁₇₋₃₂₆. The cells were finally washed thrice with PBS and images were acquired using a LSM780 confocal microscope. Cell-associated peptide fluorescence was quantitated using ImageJ.

Setting membrane potential by changing potassium concentrations in the media

RPMI-like media made without potassium chloride and without sodium chloride was from Biowest (Supplementary file 1). Varying concentrations of potassium chloride were added to this medium containing 10% FBS. Sodium chloride was also added so that the sum of potassium and sodium chloride equaled 119 mM (considering the concentrations of sodium and potassium in FBS). Three hundred thousand cells were preincubated in 1 mL media, 10% FBS containing different concentrations of potassium for 20 minutes, then different CPPs at a 40 μM concentration were added and cells were incubated for one
hour at 37°C in 5% CO2. Cells were washed once in PBS and CPP internalization was measured by flow cytometry. The corresponding membrane potential was measured with DiBac4(3).

**In silico CPP translocation free energy through MARTINI coarse-grained simulations**

An asymmetric multi-component membrane was constructed and solvated using CHARMM-GUI (Jo et al., 2008; Qi et al., 2015). Each layer contained 100 lipids (Supplementary file 2), in a previously described composition (Ingolfsson et al., 2014). The membrane was solvated with 2700 water molecules, obtaining a molecular system of 10200 particles. The MARTINI force field 2.2p (Marrink et al., 2004; Wassenaar et al., 2015) was used to define phospholipids' topology through a coarse-grained (CG) approach. The polarizable water model has been used to assess the water topology (Yesylevskyy et al., 2010). Each peptide (R9, TAT, and TAT-RasGAP317-326) structural model has been obtained by PEPFOLD-3 server (Lamiable et al., 2016), as done in previous studies in the field (Grasso et al., 2015; Grasso et al., 2018; Serulla et al., 2020).

For each molecular system, one CPP was positioned 3 nm far from the membrane outer leaflet, in the water environment corresponding to the extracellular space. Then, the system was equilibrated through four MD simulations of 100ps, 200ps, 500 ps, and 100 ns under the NPT ensemble. Position restraints were applied during the first three MD simulations and gradually removed, from 200 kJ/mol*nm² to 10 kJ/mol*nm². Velocity rescaling (Bussi et al., 2007) temperature coupling algorithm and time constant of 1.0 ps were applied to keep the temperature at 310.00 K. Berendsen (Berendsen, 1984) semi-isotropic pressure coupling algorithm with reference pressure equal to 1 bar and time constant 5.0 ps was employed. Then, all systems were simulated for the production run in the NPT ensemble with the time step of 20 fs. Electrostatic interactions were calculated by
applying the particle-mesh Ewald (PME) (Darden, 1993) method and van der Waals
interactions were defined within a cut-off of 1.2 nm. Periodic boundary conditions were
applied in all directions. Trajectories were collected every 10 ps and the Visual Molecular
Dynamics (VMD) (Humphrey et al., 1996) package was employed to visually inspect the
simulated systems. Three different transmembrane potential values have been
considered: 0 mV, 80 mV, and 150 mV. In the MD simulations, an external electric field
$E_{\text{ext}}$ was applied parallel to the membrane normal $z$, i.e., perpendicular to the bilayer
surface. This was achieved by including additional forces $F_i = q_i E_{\text{ext}}$ acting on all charged
particles $i$. In order to determine the effective electric field in simulations, we applied a
computational procedure reported in literature (Gumbart et al., 2012). A well-tempered
metadynamics protocol (Barducci et al., 2008) was applied to estimate the free energy
landscape of CPP translocation. Two collective variables have been considered: the
lipid/water density index, and the CPP-membrane distance. Gaussian deposition rate of
2.4 kJ/mol every 5 ps was initially applied and gradually decreased on the basis of an
adaptive scheme. Gaussian widths of 0.5, and 0.2 nm were applied following a well-
established scheme (Deriu et al., 2016; Granata et al., 2013; Grasso et al., 2017; Laio and
Gervasio, 2008). In particular, the Gaussian width value was of the same order of
magnitude as the standard deviation of the distance CV, calculated during unbiased
simulations. The well-tempered metadynamics simulations were computed using
GROMACS 2019.4 package (Abraham et al., 2015) and the PLUMED 2.5 open-source
plug-in (Tribello et al., 2014). The reconstruction of the free-energy surface was performed
by the reweighting algorithm procedure (Tiwary and Parrinello, 2015) allowing the
estimation of the free energy landscape. The comparison between the water pore
formation free energy estimated by our MARTINI coarse-grained simulations and previous
estimations available in literature is reported in Supplementary file 3. Each system was
simulated (with a 20-fs time step) until convergence was reached. The electrostatic
potential maps were computed by the APBS package (Baker, 2004) on the molecular
system composed of R9 peptide in contact with the cell membrane, without any applied
external electrostatic field. In detail, the non-linear Poisson-Boltzmann equation was
applied using single Debye-Huckel sphere boundary conditions on a 97x97x127 grid with
a spacing of 1Å centered at the COM of the molecular system. The relative dielectric
constants of the solute and the solvent were set to 2.5 and 78.4, respectively. The ionic
strength was set to 150 mM and the temperature was fixed at 310K (Baker, 2004; Grasso
et al., 2017). The average and standard deviation values of the local transmembrane
potential have been computed considering ten different trajectory snapshots taken from
the molecular trajectory.

In silico pore formation kinetics though Martini coarse-grained simulations
The same molecular system previously constructed and equilibrated has been investigated
to estimate the water pore formation kinetics by applying a constant electrostatic potential
(Bockmann et al., 2008; Fernandez et al., 2010; Gao et al., 2019; Gumbart et al., 2012;
Gurtovenko and Lyulina, 2014; Kirsch and Bockmann, 2016; Tieleman, 2004; Ziegler and
Vernier, 2008). Each peptide (R9, TAT, and TAT-RasGAP_{317-326}) was positioned 3 nm far
from the membrane at the beginning of each simulation. The relatively small size of the
molecular system and the application of Coarse-Grained Martini forcefield, allowed us to
study the pore formation kinetics, requiring many simulations at varying field strengths. In
detail, 25 simulations were performed for each molecular system at different external
electric field strengths from 0.0055 V/nm to 0.090 V/nm. In the MD simulations, an external
electric field $E_{ext}$ was applied parallel to the membrane normal $z$, i.e., perpendicular to the
bilayer surface. This was achieved by including additional forces $F_i = q^*E_{ext}$ acting on all
charged particles $i$. All the MD simulations were performed until the water pore formation
event was observed. In order to determine the effective electric field in simulations, we applied a computational procedure reported in literature (Gumbart et al., 2012). The results are reported in Figure 4-figure supplement 1.

In silico cell membrane hyperpolarization modeling through ion-imbalance in Martini coarse-grained simulations

The translocation mechanism of each CPP has been studied by ion-imbalance in a double bilayer system (Delemotte et al., 2008; Gao et al., 2019; Gurtovenko and Vattulainen, 2007; Herrera and Pantano, 2009). The same asymmetric membrane considered to perform the single-bilayer simulations ( ) was used to build up the double-bilayer system. The double-membrane system was solvated with 4300 water molecules, obtaining a molecular system of 20000 particles. The MARTINI force field 2.2p (Marrink et al., 2004; Wassenaar et al., 2015) was used to define phospholipids' topology through a coarse-grained (CG) approach. The polarizable water model has been used to model the water topology (Yesylevskyy et al., 2010). The elastic network ELNEDYN (Periole et al., 2009) has been applied to reproduce the structural and dynamic properties of the CPPs.

For each molecular system, one CPP was positioned in the middle of the double bilayer system, 2nm far from the membrane outer leaflets, in the water environment corresponding to the extracellular space. Then, the system was equilibrated through four MD simulations of 100ps, 200ps, 500 ps, and 100 ns under the NPT ensemble. Position restraints were applied during the first three MD simulations ang gradually removed, from 200 kJ/mol*nm² to 10 kJ/mol*nm². Velocity rescaling (Bussi et al., 2007) temperature coupling algorithm and time constant of 1.0 ps were applied to keep the temperature at 310.00 K. Berendsen (Berendsen, 1984) semi-isotropic pressure coupling algorithm with
reference pressure equal to 1 bar and time constant 5.0 ps was employed. Then, all
systems were simulated for the production run in the NPT ensemble with the time step of

Membrane hyperpolarization was achieved through a net charge difference of 30 positive
ions between intracellular and extracellular space, considering all charged ions of the
system and fulfilling the full system electroneutrality. Ten different replicas of each
molecular simulation have been performed until the water pore formation and closure
events have been observed. The visual inspection of the simulated molecular systems is
reported in Figure 4-figure supplement 1A. To analyze whether the CPPs were able to
cross the membrane and reach the intracellular compartment, their trajectories were
studied in the last five nanoseconds of each simulation replica. Considering the CPP
position with respect the membrane bilayers and the CPP’s solvent accessible surface
area (SASA), three different compartments were defined: intracellular space, lipid bilayer
(cell membrane) and extracellular space. The radius of the water pores within the
membrane was calculated as previously done in literature (Gurtovenko and Vattulainen,
2007; Leontiadou et al., 2004). We assumed that the central part of the cylindrical water
pore contains N water molecules at the same density as outside of the water flux.

In vitro assessment of water pores

Three hundred thousand wild-type HeLa cells or primary rat cortical neurons were
incubated with 32 μg/ml PI (0.8-1.5 nm diameter 75) or 200 μg/ml dextran of different
molecular weight in the presence or in the absence of the indicated CPPs in normal,
depolarizing (2 μg/ml gramicidin) or hyperpolarizing (10 μM valinomycin) conditions in 1
mL media, 10% FBS. Time-lapse images were acquired by confocal microscopy every 10
seconds. The percentage of cells where direct CPP translocation has occurred, as well as
the percentage of cells positively stained for PI, were manually quantitated using ImageJ based on snap shot images taken after 30 minutes of incubation, as shown in Figure 5-figure supplement 1C. Quantitation of cell percentage was not selective in terms of fluorescence intensity. Cytosolic PI fluorescence was assessed with ImageJ, by selecting a region within the cell cytoplasm devoid of endosomes. Saponin 0.1% (Sigma, ref. no. 4706, diluted in PBS weight:volume) was used as a permeabilizing agent (30 minutes incubation at 37°C in a 5% CO₂ incubator) that leads to cell death to differentiate signal intensity between live cells with water pores and dead cells. As, the signal of PI internalization in saponin treated cells was saturated, lower laser settings were used to look at dead cells than at cells with water pores. Three fitting models were obtained:

- exponential decline: \( y = 2570 \cdot e^{(-x/54)} \)
- exponential: \( y = 2570 \cdot e^{-0.02 \cdot x} \)
- modified power: \( y = 2570 \cdot 0.98^x \)

These equations fitted equally well the PI uptake/Vm curve in Figure 5E. For the calculations used in Figure 5F, the exponential decline equation was used.

Zebrafish viability

FITC-TAT-RasGAP_{317-326(W317A)} internalization in zebrafish was assessed either by adding the peptide directly in Egg water or by injection. Experiments in which the peptide was added in the water were performed on fish between four and 24 hours post fertilization. Viability assays were done on embryos of four, six and 24 hours post fertilization to determine a maximal nonlethal dose of the peptide that can be used. Different concentrations of the peptide were added to 500 µl water per well in 24-well plate, with between eight and eleven embryo per well. Fish viability was visually assessed
at 20 hours post incubation with the peptide. Hyperpolarization associated viability was visually assessed at different time points in presence or in the absence of the peptide and in presence or in the absence of different concentration of valinomycin. Zebrafish were visualized with binocular microscope and CYTATION3 apparatus. Survival was visually assessed under a binocular microscope by taking into consideration the embryo transparency (as dead embryos appear opaque), general development characteristics and motility.

**CPP internalization in vivo**

To assess peptide internalization in zebrafish, two methods were used: 1) addition of the peptide directly in 500 µl of Egg water in 24-well plates containing between eight and twelve embryos per well or 2) intramuscular injections. In the case of the first method, after the indicated treatments, zebrafish were washed, fixed in 4% PFA/PBS for one hour at room temperature. Whole embryos were mounted on slides with Fluoromount-G (cBioscience, ref. no. 00-4958-02). Zebrafish were then visualized under a LSM710 confocal microscope. Experiments where the peptide was added directly to the water were performed on zebrafish at 18 hours post fertilization to limit cuticle development that would hinder peptide access to the cells. In the case of the second method, 8 nl injections (containing the various combinations of peptide and valinomycin and 0.05 % (vol:vol) phenol red as an injection site labelling agent) were done on 48 hours post fertilization embryos into the tail muscle around the extremity of yolk extension, after chorion removal and anesthesia with 0.02% (w:vol) tricaine \textsuperscript{122} buffered in sodium bicarbonate to pH7.3. At this age, zebrafish already have well developed tissues that can be easily visually distinguished. Injections were done with an Eppendorf Microinjections FemtoJet 4i apparatus. After the indicated treatments, embryos were fixed in 4% PFA/PBS and
visualized under a confocal microscope. Some embryos were kept alive for viability evaluation post injection until the age of 4 days.

Experiments with mice were performed in 10-14 weeks old C57BL/6NCrl mice anaesthetized with ketasol/xylasol (9.09 mg/ml ketasol and 1.82 mg/ml xylasol in water; injection: 10 µl per g of body weight). The back of the mice was shaved and intradermic injections were performed (a total of 10 µl was injected). Mice were kept under anesthesia for one hour and Artificial tears (Lacryvisc) were used to avoid eye dryness. Mice were then sacrificed by CO2 inhalation, skin was cut at injection sites, fixed in 4% formalin and paraffin embedded for histology analysis. For each sample, 10 to 15 slides were prepared and peptide internalization was visualized with a CYTATION3 apparatus. Fluorescence intensity was quantitated with ImageJ. The slices displaying the highest fluorescence signal were considered as those nearest to the injection site and the fluorescent values from these slides were used in Figure 6B.

Assessment of endosomal escape and direct translocation

Three hundred thousand cells were seeded onto glass-bottom dishes in 2 mL RPMI, 10% FBS for 16 hours. Quantitation of cytosolic fluorescence was performed within live HeLa cells pre-incubated with 80 µM TAT-RasGAP_{317-326} for 30 minutes at 37°C in 1 mL media, with 10% FBS and then incubated for the indicated periods of time in the presence (i.e. no wash after the pre-incubation) or in the absence (i.e. following three consecutive washes with RPMI supplemented with 10% FBS) of extracellular labelled peptide. Endosomal escape from lysosomes was induced in presence of 1 mM LLOME (L-Leucyl-L-Leucine methyl ester) (Repnik et al., 2017), added in the 1 mL media, 10% FBS 30 minutes after CPP was washed out and persisted throughout the experiment). Confocal images were
taken every five minutes after the 30-minute pre-incubation. For each cell, the fluorescence intensity of one region of interest (ROI) devoid of labelled endosomes throughout the experiment was quantitated over time using ImageJ Time Series Analyzer V3. The surface of the ROI was identical for all cells. Only cells displaying labelled endosomes after the 30-minute pre-incubation were analyzed. Note that the washing steps, for reasons unclear at this time, induced a slightly higher initial ROI intensity signal.

Transferrin internalization quantitation

Wild-type HeLa cells were plated in 12-well plates (200’000 cells per well) for 16 hours in 1 ml RPMI (Invitrogen, ref. no. 61870), supplemented with 10 % heat-inactivated fetal bovine serum (FBS; Invitrogen, ref. no. 10270-106). Cells were then incubated in presence of 20 µg/ml AlexaFluor-488 conjugated transferrin for 20 minutes at 37°C in 5% CO2. Cells were washed with PBS and pelleted after trypsinization. To quench membrane bound transferrin fluorescence, cells were resuspended in 0.2% trypan blue diluted in PBS. Transferrin internalization was quantified by flow cytometry using Beckman Coulter FC500 instrument. Data analysis was done with Kaluza Version 1.3 software (Beckman Coulter).

TAT-PNA-induced luciferase activity

The LeGOiG2-LUC705 lentiviral construct encodes a luciferase gene interrupted by a mutated human beta globin intron 2. This mutation creates a new aberrant splicing site at position 705 that when used produced an mRNA that encodes a truncated non-functional luciferase. In the presence of the TAT-peptide nucleic acid (TAT-PNA) CPP described below, the aberrant splice site is masked allowing the production of a functional luciferase enzyme. Lentiviruses produced using this construct were employed to infect cells. The
doses used resulted in >90% cells infected (based on GFP expression from the lentiviral vector). The infected cells (200'000 cells in 12-well plates containing 1 ml of RPMI, 10% FBS) were treated or not with 5 μM TAT-PNA (GRKKRRQRRR-CCTCCTACCTCAGTTACA). TAT-PNA is made of TAT$_{48-57}$ and an an oligonucleotide complimentary to a sequence containing the aberrant splice site. After 16 hours incubation, cells were washed twice in HKR buffer (119 mM NaCl, 2.5 mM KCl, 1 mM NaH$_2$PO$_4$, 2.5 mM CaCl$_2$, 1.3 mM MgCl$_2$, 20 mM HEPES, 11 mM dextrose, pH 7.4) and lysed in 40 μl HKR containing 0.1 % Triton X-100 for 15 minutes at room temperature.

Luciferase activity was measured with a GLOMAXTM 96 Microplate Luminometer (Promega) using a Dual-Luciferase Reporter Assay (Promega) and normalized to the protein content. Results are displayed as the ratio between the protein-normalized luciferase signal obtained in TAT-PNA-treated cells and the signal obtained in control untreated cells.

**TAT-Cre recombinase production, purification and recombination assay**

Raji cells were infected with a lentivirus encoding a Cre-reporter gene construct (D'Astolfo et al., 2015). TAT-Cre recombinase was produced as described (Wadia et al., 2004). Briefly, E. coli BL21 transformed with the pTAT-Cre plasmid (#917, Addgene plasmid #35619) were grown for 16 hours in LB containing 100 μg/mL kanamycin. Protein production was induced at OD600 of 0.6 with 500 μM IPTG (isopropyl β-D-1-thiogalactopyranoside) for three hours. Bacteria were collected by centrifugation at 5000 x g and kept at -20°C. Purification was performed on Äkta prime (GE, Healthcare, USA) equipped with a 1 ml HisTrap FF column equilibrated with binding buffer (20 mM sodium phosphate, 500 mM NaCl, 5 mM imidazole pH 7.4). The day of the purification, bacterial pellet was resuspended in lysis buffer (binding buffer with protease inhibitors (Roche, ref.
no. 4693132001; one tablet per 50 ml), 0.025 mg/ml DNase I (Roche, ref. no. 04716728001), and 2 mg/ml lysozyme (Roche, ref. no. 10 837 059 001) and sonicated six times for 30 seconds. After 20 minutes centrifugation at 5'000 x g, the supernatant was filtered through Steriflip 0.45µm and loaded on the column. Elution buffer (20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole pH 7.4) was used to detach His-tagged proteins from the column. Imidazole was removed from collected fractions by overnight dialysis using 10K MWCO cassette (Thermo Scientific, ref. no. 66807) in PBS. Raji cells encoding the Cre-reporter were treated for 48 hours with 20 µM TAT-Cre-recombinase. Fluorescence was imaged using a Nikon Eclipse TS100 microscope.

Assessment of CPP binding to plasma membranes

Three hundred thousand cells were incubated for 60 seconds in 1 mL RPMI supplemented with 10% FBS and 10 mM HEPES in Eppendorf tubes at 37°C in the presence of increasing concentrations of FITC-TAT-RasGAP_{317-326}. Half of the cells were then immediately placed on ice, pelleted at 4°C, and resuspended in one ml of ice-cold PBS and then split into two tubes, one of which receiving a final concentration of 0.2% (w:w) trypan blue to quench surface-associated FITC signals. The cells (still kept at 4°C) were then analyzed by flow cytometry. The surface associated peptide signal was calculated by subtracting total fluorescence measured in PBS and fluorescence measured after trypan blue quenching. The other half of the cells after the 60 second peptide incubation was incubated at 37°C for one hour at which time the cellular internalization of the labelled peptide was assessed by flow cytometry.

Transient calcium phosphate transfection in HeLa cells
Calcium phosphate-based transfection of HeLa cells was performed as previously described (Jordan et al., 1996). Briefly, cells were plated overnight in DMEM (Invitrogen, ref. no. 61965) medium supplemented with 10% heat-inactivated FBS (Invitrogen, ref. no. 10270-106). 2.5 µg of total plasmid DNA of interest was diluted in water, CaCl2 was added and the mixture was incubated in presence of HEPES 2x for 60 seconds before adding the total mixture drop by drop to the cells. Media was changed 10 hours after.

Isothermal titration calorimetry (ITC)

ITC was performed using MicroCal ITC200 (Malvern Panalytical) at 37°C with 600 nM FITC-R9 in the cell (total volume 300 µl) and consecutive injections (2.5 µl/injection, except for the first injection of 0.4 µl) of 6 mM PI from the syringe (total volume 40 µl) with 2 minutes delay between injections and 800 rotations/minute rotation speed. Differential power was set to 7, as we had no prior knowledge of the expected reaction thermodynamics. The results in Figure 5-figure supplement 2A are represented as a: thermogram (measurement of thermal power need to ensure that there is no temperature difference between reference and sample cells in the calorimeter as a function of time) and a binding isotherm (normalized heat per peak as a function of molar ratio).

Colony formation assay

Three hundred thousand wild-type HeLa cells were plated overnight in RPMI with 10% FBS in 6-well plates. Cells were then treated for one hour in the presence of indicated concentrations of CPP, PI and membrane potential modulating agents (gramicidin or valinomycin) in 1 mL RPMI. As control, cells were either left untreated or incubated in presence of DMSO used as vehicle for gramicidin and valinomycin. Cells were then...
washed, trypsinized and plated on 10 cm dishes at a density of 300 cells per condition. Colonies were counted at day 14 after 100% ethanol fixation for 10 minutes and Giemsa staining. Washes were done with PBS.

**Genome-scale CRISPR/Cas9 knockout screening**

The human GeCKO v2 library (2 plasmid system) (Addgene plasmid #1000000049) was amplified by electroporation using a Bio-Rad Gene Pulser II electroporation apparatus (Bio-Rad #165-2105) and the Lucigen Endura bacteria (Lucigen ref. no. 60242). Cells were plated on LB Agar plates containing 100 µg/mL ampicillin. After 14 hours at 32°C, colonies were scrapped and plasmids recovered with the Plasmid Maxi kit (Qiagen, ref. no. 12162). To produced lentivirus library, 12 T-225 flasks were seeded with 12x10^6 HEK293T cells per flask in 40 ml DMEM, 10% FBS. The day after, 10 µg pMD2.G, 30 µg psPAX2 and 25 µg GeCKO plasmid library in 1.8 ml H2O were mixed with 0.2 ml 2.5 M CaCl2 (final calcium concentration: 250 mM). This solution was mixed (v/v) with 2x HEPES buffer (280 mM NaCl, 10 mM KCl, 1.5 mM Na₂HPO₄, 12 mM D-glucose, 50 mM HEPES), incubated for one minute at room temperature, added to the culture medium, and the cells placed back in a 37°C, 5% CO₂ incubator for seven hours. The culture medium was then removed and replaced by DMEM supplemented with 10 % FBS containing 100 U/ml penicillin and 100 µg/ml streptomycin. Forty-eight hours later, the medium was collected and centrifuged 5 min at 2'000 g to pellet the cells. The remaining cell-free medium (12 x 40 ml) was then filtered through a 0.45 µm HV/PVDF (Millipore, ref. no. SE1M003M00) and concentrated ~100 times by resuspending the viral pellet obtained by ultracentrifugation at 70'000 g for two hours at 4°C in ~5 ml ice-cold PBS. The concentrated viruses were aliquoted in 500 µl samples and stored at -80°C.
To express the Cas9 endonuclease, cells (e.g. Raji or SKW6.4) were infected with Cas9 expressing viruses that were produced in HEK293T cells transfected with the lentiCas9-Blast (#849, Addgene plasmid #52962), pMD2.G, and psPAX2 plasmids as described in the main method under “Lentivirus production”. The infected cells were selected with 10 µg/mL blasticidin for a week. The multiplicity of infection (MOI) of the GeCKO virus library was determined as follow. Different volumes of the virus library were added to 3x10^6 Cas9-expressing cells plated in 12-well plates. Twenty-four hours later, the cells were split in two wells of 12-well plates. One well per pair was treated with 10 µg/mL puromycin for 3 days (the other cultured in normal medium). Cell viability was determined by trypan blue exclusion and MOI was calculated as the number of cells in the well treated with puromycin divided by the number of cells in the control well. The virus volume yielding to a MOI ~0.4 was chosen to perform large-scale infection of 12x10^7 cells that was carried out in 12-well plates with 3x10^6 cells per well. After 24 hours, the infected cells were collected and pooled in a T-225 flask and selected with 10 µg/mL puromycin for a week. Thirty millions of these were frozen (control untreated cells) and 60 million others were treated with 40 µM TAT-RasGAP_{317-326} for 8 days (Raji) or for 17 days (SKW6.4) with a medium and peptide renewal every 2-3 days. Thirty million of the peptide-treated cells were then also frozen. Genomic DNA was extracted from the control and the peptide-treated frozen cells using the Blood & Cell Culture DNA Midi Kit according to manufacturer’s instructions (Qiagen, ref. no. 13343). A first PCR was performed to amplify the lentiCRISPR sgRNA region using the following primers:

F1: 5’-AATGGACTATCATATGCTTACCGTAACTGAAAGTATTTCG-3’

R1: 5’-CTTTAGTTTGTATGTCTGTTGCTATTATGTCTACTATTCTTTCC-3’

A second PCR (see Supplementary file 4 for the primers used) was performed on 5 µl of the first PCR reaction to attach Illumina adaptors with barcodes (nucleotides highlighted in
green) and to increase library complexity (using the sequences highlighted in red) to prevent signal saturation when the sequencing is performed. The blue sequences are complementary to the extremities of the first PCR fragments.

Both PCRs were performed in 100 µl with the 2 µl of the Herculase II Fusion DNA Polymerase from Agilent (ref. no. 600675) according the manufacturer’s instructions. Amplicons were gel extracted, quantitated, mixed and sequenced with a MiSeq (Illumina). Raw FASTQ files were demultiplexed and processed to contain only unique sgRNA sequences. The number of reads of each sgRNA was normalized as described\(^\text{125}\). The MAGeCK algorithm\(^\text{126}\) was used to rank screening hits by the consistent enrichment among multiple sgRNAs targeting the same gene.

**CRISPR/Cas9-based genome editing**

Single guide RNAs targeting the early exon of the protein of interest were chosen in the sgRNA library\(^\text{133}\) and are listed in Supplementary file 5. LentiCRISPR plasmids specific for a gene were created according to the provided instructions\(^\text{127}\). Briefly, oligos were designed as follow: Forward 5’-CACCGnnnnnnnnnnnnnnnnnn-3’; Reverse-3’-CnnnnnnnnnnnnnnnnnnCAA-5’, where nnnnnnnnnnnnnnnnnnn in the forward oligo corresponds to the 20 bp sgRNA. Oligos were synthetized, then phosphorylated and annealed to form oligo complex. LentiCRISPR vector was BsmBI digested and dephosphorylated. Linearized vector was purified and gel extracted and ligated to oligo complex. The lentiCRISPR vector containing the sgRNA was then used for virus production. Recombinant lentiviruses were produced as described\(^\text{93}\) with the following modification: pMD.G and pCMVDR8.91 were replaced by pMD2.G and psPAX2 respectively. Cells were infected and selected with the appropriate dose of puromycin (2
µg/ml for HeLa). Clone isolation was performed by limiting dilution in 96 well-plate.

**TA cloning**

TA cloning is a subcloning technique that allows integration of a PCR-amplified product of choice into a PCR2.1 vector based on complementarity of deoxyadenosine added onto the PCR fragment by Taq polymerase. This approach is useful to distinguish between several alleles and to determine whether the cells are heterozygous or homozygous at a given locus. TA cloning kit (Life technologies, ref. no. K202020) was used according to manufacturer’s instructions to sequence DNA fragment containing the region targeted by a given sgRNA. Briefly, DNA was isolated and the fragment of interest was PCR-amplified using primers listed in Supplementary file 6, then ligated into PCR2.1 vector. *E. coli* competent cells were then transformed and at least 15 colonies were selected per condition for DNA isolation and sequencing.

**Plasmid constructs**

The hKCNN4-V5.lti (#953) lentiviral plasmid encoding a V5-labeled version of the KCNN4 potassium channel was from DNASU (ref. n° HsCD00441560). The hKCNK5-FLAG.dn3 (#979) plasmid encoding the human KCNK5 potassium channel (NCBI reference sequence NM_003740.3), Flag-tagged at the C-terminus, was purchased from GenScript (ref. n° OHu13506). The Myc-mKCNJ2-T2A-IRES-tdTomato.lti (#978) lentiviral vector encoding the mouse Kir2.1 (KCNJ2) potassium channel and tdTomato (separated by an IRES) was generated by subcloning myc-mKCNJ2-T2A-Tomato.pCAG plasmid (#974, Addgene plasmid #60598) into a lentiviral backbone LeGo-iT2 (#809), a gift from Boris Fehse (Addgene plasmid #27343), through ligation of both plasmids after digestion with
BamHI (NEB, reg. no. R313614). The pMD2.G plasmid (#554, Addgene plasmid #12259) encodes the envelope of lentivirus. The psPAX2 plasmid (#842, Addgene plasmid #12260) encodes the packaging system. Both pMD2.G and psPAX2 plasmids were used for lentiviral production. The Flag-hKCNQ5(G278S)-IRES-NeoR plasmid (#938) codes for the N-terminal Flag-tagged G278S human KCNQ5 inactive mutant and a neomycin resistant gene separated by an IRES sequence. It was generated by subcloning a BamHI/Xmal digested PCR fragment obtained by amplification of pShuttle-Flag-hKCNQ5(G278S)-IRES-hrGFP2 (#937, kind gift from Dr. Kenneth L. Byron) using forward primer #1397 (CAT CGG GAT CCG CTA TAC CGG CCA CCA TGG ATT ACA AGG A) and reverse primer #1398 (CAT CGC CCG GGG CTA TAC CGT ACC GTC GAC TGC AGA ATT C) into the lentiviral vector TRIP-PGK-IRES-Neo (#350) opened with the same enzyme. The Flag-hKCNQ5(SM,G278S)-IRES-Neo (#939) plasmid is identical to Flag-hKCNQ5(G278S)-IRES-NeoR except that the sequence targeted by the sgKCNQ5.1 sgRNA (Supplementary file 5) was mutated with the aim to decrease Cas9-mediated degradation. Silent mutations (SM), at the protein level, were introduced using the QuikChange II XL Site-Directed Mutagenesis Kit (ref. no. 200522) according to manufacturer’s instructions using forward primer #1460 (AAA TAA GAA CCA AAA ATC CTA TGT ACC ATG CCG TTA TCA GCT CCT TGC TGT GAG CAT AAA CCA CTG AAC CCA G) and reverse primer #1461 (CTG GGT TCA GTG GTT TAT GCT CAC AGC AAG GAG CTG ATA ACG GCA TGG TAC ATA GGA TTT TTG GTT CTT ATT T).

The Flag-hKCNQ5(SM)-IRES-NeoR (#940) lentiviral construct codes for a Flag-tagged wild-type version of human KCNQ5. It was made by reverting the G278S mutation in Flag-hKCNQ5(SM,G278S)-IRES-Neo (#939) using the QuikChange II XL Site-Directed Mutagenesis Kit with the #1462 forward primer (TTT TGT CTC CAT AGC CAA TAG TTG TCA ATG TAA TTG TGC CCC) and the #1463 reverse primer (GGG GCA CAA TTA CAT TCA ATG TAA TTG TGC CCC) and the #1463 reverse primer (GGG GCA CAA TTA CAT TCA ATG TAA TTG TGC CCC).
TGA CAA CTA TTG GCT ATG GAG ACA AAA). The pLUC705 (Kang et al., 1998) (#876, gift from Dr. Bing Yang) plasmid encodes a luciferase gene interrupted by a mutated human beta globin intron 2. This mutation creates a new aberrant splicing site at position 705 that when used produced an mRNA that encodes a truncated non-functional luciferase (Kang et al., 1998). To introduce this construct into a lentiviral vector, the pLUC705 plasmid was digested with HindIII/XhoI, blunted with T4 DNA polymerase, and ligated into Stul-digested and dephosphorylated LeGO-iG2 (#807, Addgene plasmid #27341), resulting in plasmid pLUC705.LeGO-iG2 (#875). The pTAT-Cre (#917, Addgene plasmid #35619) bacterial plasmid encodes a histidine-tagged TAT-Cre recombinase. The Cre reporter lentiviral vector (#918, Addgene plasmid #62732) encodes a LOXP-RFP-STOP-LOXP-GFP gene construct. Cells expressing this plasmid appear red but once recombination has occurred when TAT-Cre is translocated into cells the RFP-STOP fragment will be excised, GFP but not RFP will now be produced, and cells will appear green.

**Peptides**

TAT-RasGAP\textsubscript{317-326} is a retro-inverso peptide (i.e. synthesized with D-amino-acids in the opposite direction compared to the natural sequence) labeled or not with FITC or TMR. The TAT moiety corresponds to amino-acids 48–57 of the HIV TAT protein (RRRQRRKKRG) and the RasGAP\textsubscript{317-326} moiety corresponds to amino-acids from 317 to 326 of the human RasGAP protein (DTRLNTVWMW). These two moieties are separated by two glycine linker residues in the TAT-Ras-GAP317–326 peptide. FITC-bound peptides without cargo: TAT, Penetratin (RQIKWFQNRRMKWKK), R9 (RRRRRRRRR) and K9 (KKKKKKKKK) were synthesized in D-amino-acid conformation. All peptides were synthesized in retro-inverso conformation (over the years different suppliers were used
with routine checks for activity of TAT-RasGAP<sub>317-326</sub> derived peptides, Biochemistry Department of University of Lausanne, SBS Genetech, China and Creative Peptides, USA) and resuspended to 1 mM in water.

**Statistical analysis**

Statistical analysis was performed on non-normalized data, using GraphPad Prism 7. ANOVA multiple comparison analysis to wild-type condition was done using Dunnett’s correction (Figure 3A-C and Figure 3-figure supplement 1C B and Figure 3C, top panel, and PI internalization in Figure 5C, as well as TAT-PNA internalization in Figure 2-figure supplement 5A). ANOVA multiple comparison analysis between several conditions was done using Tuckey’s correction (Figure 3-figure supplement 2E). Comparison between two conditions was done using two-tailed paired t-test for the CPP internalization experiments described in Figure 3C (bottom panel), Figure 6A-B, Figure 1-figure supplement 1B, and Figure 3-figure supplement 4. All measurements were from biological replicates. Unless otherwise stated, the horizontal bars in the graph represent the median, the height of columns correspond to averages, and the dots in the Figures correspond to values derived from independent experiments.

**Data availability**

DNA sequencing data from the CRISPR/Cas9-based screens are available through the following link: https://www.ncbi.nlm.nih.gov/sra/SRP161445. Note: this link is currently blocked for public access, but will be released upon publication.
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Competing interests

Authors declare no competing financial and non-financial interests.

Materials & Correspondence

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Figure legends

Figure 1: TAT-RaGAP<sub>317-326</sub> cellular entry modes

(A) Depiction of the different modes of CPP entry into cells. Confocal microscopy was performed on the indicated cell lines incubated for one hour with 40 μM FITC-TAT-RasGAP<sub>317-326</sub> in RPMI, 10% FBS. Cells were washed with PBS prior to visualization. Vesicular staining is indicative of CPP endocytosis while diffuse cytosolic staining is a consequence of CPP direct translocation into cells. Scale bar: 10 μm. (B) Quantitation of the different modes of CPP entry as a function of time (FITC-TAT-RasGAP<sub>317-326</sub> continually present in the media) using the experimental conditions presented in panel A. Types of staining were visually quantitated as indicated in Figure 1-figure supplement 1 (n=157 cells per condition). There was no indication of fluorescence quenching, due to endosomal acidification, preventing the detection of CPP-containing endosomes (in at least during the first hour of CPP exposure) (Figure 1-figure supplement 1B). TAT-RasGAP<sub>317-326</sub> enters cells via endocytosis and direct translocation, but only direct translocation mediates its biological activity and leads to cell death (Figure 1-figure supplement 4). Results correspond to the average of three independent experiments.

Figure 1-figure supplement 1: TAT-RasGAP<sub>317-326</sub> internalization mode attribution and no impact of quenching for the detection of CPP-containing endosomes.

(A) Visual attribution of the various types of staining associated with FITC-CPP internalization in wild-type Hela cells. Cells 1-9 took up the CPP through both direct translocation and endocytosis (cells 1-4 and cells 5-9 displaying strong and weak cytosolic signal, respectively); cells 10-15 acquired FITC-TAT-RasGAP<sub>317-326</sub> in vesicles only, no cytosolic staining being detected. The experimental conditions are those of panel a. Scale bar: 20 μm. (B) Potential fluorophore quenching does not prevent detection of CPP-containing endosomes. Wild-type HeLa cells were incubated for one hour at 37°C in the presence of 40 μM TAT-RasGAP<sub>317-326</sub> labeled with either FITC (susceptible to quenching at low pH) or TMR (not quenched at low pH) fluorophores. The number of vesicles was visually determined based on confocal images. There were no fewer vesicles detected in cells when the peptide was labelled with FITC than when it was labelled with TMR.

Figure 1-figure supplement 2: No evidence of endosomal escape from TAT-RasGAP<sub>317-326</sub>-containing endosomes

To assess whether cytosolic peptide accumulation could be due to endosomal escape, we incubated cells with the indicated concentrations of FITC-TAT-RasGAP<sub>317-326</sub> for 30 minutes, washed the cells and then monitored the cytosolic CPP acquisition only in cells that had initially
taken up the peptide by endocytosis, i.e. cells that did not display, at the beginning of image
acquisition, a cytosolic signal indicative of direct translocation (examples of such cells are
indicated by red arrows in the scheme). The graphs show single cell quantitation of FITC-TAT-
RasGAP<sub>317-326</sub> cytosolic fluorescence (see panel D) according to the experimental setups shown
on the left. The cell traces correspond to acquisitions gathered from three independent
experiments (panel A, 19 cells, panel B, 16 cells, and panel C, 22 cells). As a control, when
cells were not washed (i.e. the peptide was present in the medium), cytosolic acquisition
increased over time (panel A; see also left panel in Movie 4). However, no increase in the
cytosolic signal was observed in washed out cells containing peptide-labelled endosomes
(p panel B and Movie 4, right panel). An increase in cytosolic fluorescence could be detected
when endosomal escape was artificially induced by the endosome/lysosome disruptor LLOME
(Repnik et al., 2017) (panel C). In live cells therefore diffuse cytosolic TAT-RasGAP<sub>317-326</sub> signal
originates from direct translocation and not from endosomal escape. (D) Representative
confocal image showing two FITC-TAT positive cells. Nucleus is labeled in blue, endosomes
with pink arrows and orange-circled zones correspond to cytosolic regions devoid of
endosomes. Such regions are those that are used to record CPP cytosolic fluorescence signals.
Scale bar: 10 μm.

Figure 1-figure supplement 3: Cytosolic acquisition of fluorophore-labelled CPPs is not a
consequence of laser-induced cellular damage
HeLa cells were incubated with 80 μM FITC-TAT-RasGAP<sub>317-326</sub> for 30 minutes with the
indicated frequencies of laser exposures (n=133 cells per condition). Quantitation of the
cytosolic fluorescence intensity after 30 minutes (n=235 cells per condition) is shown on the left
and the percentage of cells with cytosolic fluorescence is shown on the right. FITC-TAT-
RasGAP<sub>317-326</sub> translocation into cells occurred similarly after 30 minutes whether the cells were
illuminated once or 60 times over the 30 minutes period. Even if one argues that a single
illumination is sufficient to cause the internalization seen after a 30-minute incubation period
with the CPP, then the internalization should be the same whether the illumination is performed
at 1 minute or at 30 minutes. This is not what is observed: the internalization of the FITC-
labelled CPP increases overtime independently of the number of times the cells are illuminated.
The results correspond to three independent experiments.

Figure 1-figure supplement 4: TAT-RasGAP<sub>317-326</sub> enters cells via endocytosis and direct
translocation, but only direct translocation mediates its biological activity.
(A-E) HeLa cells were incubated with 80 μM FITC-TAT-RasGAP<sub>317-326</sub> in RPMI, 10% FBS for the
indicated periods of time. Peptide internalization and cell death was assessed by flow
cytometry. (A) Left: representative flow cytometry dot plot showing the gating strategy used in
flow cytometry experiments. Right: representative flow cytometry histogram of HeLa cells
incubated with FITC-TAT-RasGAP_{317-326} in RPMI, 10% FBS for 60 minutes. The dotted line represents cellular auto-fluorescence. (B) Variation over time of the low and high intensity peak values. Peptide internalization was assessed by flow cytometry. The results correspond to three independent experiments. Note the different scales used to plot the median values of the low and high intensity populations. (C) Quantitation of cells with low intensity peptide fluorescence and vesicular staining. Total peptide internalization was assessed by flow cytometry. The percentage of cells with low CPP cytosolic fluorescence was visually quantitated on confocal images (n=164 cells per condition). The results correspond to three independent experiments. (D) Quantitation of the pattern of appearance of cells with high intensity peptide fluorescence and strong diffuse cytosolic staining. Data assessment as in panel C. The results correspond to three independent experiments. (E) Kinetics of FITC-TAT-RasGAP_{317-326}-induced death in the two populations described in panel B. Cell death was assessed by propidium iodide (PI) staining. The results correspond to three independent experiments.

**Figure 2: Identification of potassium channels as mediators of direct translocation of CPPs into cells**

(A) Identification of genes implicated in TAT-RasGAP_{317-326} internalization in Raji and SKW6.4 cells. The graphs depict the p-value (calculated using the MAGeCK procedure; see Materials and Methods) for the difference in sgRNA expression between peptide-treated and control cells for the ~20'000 genes targeted by the CRISPR/Cas9 library. (B) Quantitation of TAT-RasGAP_{317-326} entry (top) and induced death (bottom) in wild-type (WT) and knock-out (KO) cells. The WT and the corresponding potassium channel KO versions of the indicated cell lines were pretreated or not for 30 minutes with 10 μM XE-991 or with TRAM-34 and then incubated (still in the presence of the inhibitors when initially added) with, or without 40 μM (Raji and SKW6.4 cells) or 80 μM (HeLa cells) TAT-RasGAP_{317-326}. Internalization was recorded after one hour and cell death after 16 hours (Raji and SKW6.4) or 24 hours (HeLa). Results correspond to the average of three independent experiments. TAT-RasGAP_{317-326} concentrations and time of incubation used were adjusted so that the CPP induced similar cell death (between 60% and 90%) in the wild-type versions of the different cell lines. (C) Quantitation of the modalities of TAT-RasGAP_{317-326} entry in wild-type and KCNQ5 knock-out Raji cells. Cells were incubated with FITC-TAT-RasGAP_{317-326} for various periods of time and peptide staining was visually quantitated on confocal images (n=165 cells for each time-point). The high percentage of cells with vesicular staining in the knock-out (KO) cells results from the absence of strong
diffuse staining masking endosomes. The results correspond to the average of three experiments.

**Figure 2-figure supplement 1: CRISPR/Cas9 screen and target gene identification**

(A) Scheme of the CRISPR/Cas9 screening strategy to identify genes involved in TAT-RasGAP
317-326 cellular entry. Raji and SKW6.4 cells were infected with a single guide RNA (sgRNA) CRISPR/Cas9 lentiviral library in conditions favoring the expression of only one specific sgRNA per cell (which is indicated by differentially colored cells). The infected cells were then incubated in the presence or in the absence of 40 μM TAT-RasGAP317-326 for 8 days (Raji) or 17 days (SKW6.4). The expression of sgRNAs in both populations was assessed by massive parallel sequencing to determine the enrichment or depletion in specific sgRNAs in the peptide-treated population. (B) Scatter-plot depicting the changes in sgRNA expression between the control and treated cell populations. The most significantly modulated sgRNA sets are color-coded.

**Figure 2-figure supplement 2: Validation of the targets identified through the CRISPR/Cas9 screening**

(A-C) Sequencing of the regions targeted by the sgRNAs disrupting KCNQ5, KCNK5 and KCNN4 potassium channels in Raji (panel A), SKW6.4 (panel B) or HeLa cells (panel C). Mutations, insertions and deletions induced by the CRISPR/Cas9 system are shown in red. Except the cases mentioned below, these mutations induce a frame shift and early termination of the open reading frame. The sequences targeted by the sgRNAs are highlighted in yellow. The sgRNA-induced mutations in the first allele of clone Q5-2, in the first allele of SKW6.4 cells clone N4-2, and in the first allele of clone K5-2 do not induce a frame-shift but are located in critical domains necessary for potassium channel activity, such as the pore-forming region of KCNK5 (uniprot accession number:O95279), region adjacent to the pore of KCNQ5 (uniprot accession number: Q9NR82) or calcium recognition domain of KCNN4 (uniprot accession number: O15554). Hence, these alleles presumably encode non-functional channels. Clones Q5-1 in Raji, N4-1 and K5-1 in SKW6.4, and N4-1 in HeLa cells were those that were used in subsequent experiments. The blue nucleotides correspond to silent changes, aimed at reducing sgRNA-targeting, introduced in the KCNQ5-encoding lentiviral vector used in panel e. PAM (protospacer adjacent motif) sequences are italicized. (D-F) The indicated wild-type (WT) and knock-out (KO) cells, infected or not with sgRNA-resistant FLAG-KCNQ5-encoding lentiviruses or KCNN4-expressing lentiviruses, were tested for their ability to be killed by TAT-RasGAP
317-326. Cell death was assessed by flow cytometry of PI-stained cells after 16 hours (Raji and SKW6.3 cells) or 24 hours (HeLa cells). The results correspond to the average of three independent experiments. Expression of the FLAG-KCNQ5 and KCNN4-V5 constructs was detected by western blotting using an anti-FLAG antibody and an anti-V5 antibody, respectively.
Note that the Raji, SKW6.4 and HeLa knock-out cell lines still express Cas9 and the sgRNAs targeting the potassium channels. Hence, the ectopically expressed wild-type potassium channel-encoding cDNAs can be targeted by the CRISPR/Cas9 system but this nevertheless allows for a detectable expression of the FLAG- or V5-tagged constructs although to much lower levels than in similarly infected wild-type cells lacking Cas9 and sgRNAs (Figure 2 - figure supplement 2E - source data 1 v95; compare last two lanes). In Raji cells, the re-expressed KCNQ5 channel contained 5 silent changes in the sgRNA recognition site (see panel A) but this proved insufficient to allow for a strong ectopic expression (compare lanes 2 and 4 in Figure 2 - figure supplement 2F - source data 1 and in Figure 2 - figure supplement 2F - source data 3).

**Figure 2-figure supplement 3:** Potassium channels modulate direct CPP translocation, but not endocytosis

(A) Same as Figure 2C, but for wild-type, KCNN4 and KCNK5 SKW6.4 knock-out cells. The results correspond to the average of three independent experiments. (B) As panel A, but for wild-type and KCNN4 knock-out HeLa cells. The results correspond to the average of three independent experiments. (C) Quantitation by flow cytometry of 20 µg/ml AlexaFluor488-transferrin (left) or 200 µg/ml 10 kDa FITC-Dextran (right) internalization in the indicated wild-type (WT) cell lines and their corresponding knock-out (KO) versions, pretreated or not for 30 minutes with the XE-991 (10 µM) or TRAM-34 (10 µM) potassium channel inhibitors. Transferrin and dextran internalization was allowed to proceed for 60 minutes (still in the presence of inhibitors when these were used in the 30 minute pre-incubation period). To quench membrane bound fluorescence, cells were incubated with 0.2% trypan blue prior to flow cytometry analysis. The independent experiment replicates are color-coded. (D) Assessment of FITC-TAT-RasGAP_{317-326} cell surface binding on wild-type and KCNQ5 knock-out Raji cells after 60 seconds of incubation (top), as well as associated peptide internalization after one hour of treatment (bottom). The results correspond to at least five independent experiments.

**Figure 2-figure supplement 4:** Potassium channels regulate the cellular internalization of various TAT-bound cargos

(A) TAT-PNA-induced luciferase activity in the indicated cell lines pretreated or not with potassium channel inhibitors (XE-991 or TRAM-34) or genetically invalidated for specific potassium channels. Results are normalized to non-stimulated cells (dashed lines). The independent experiment replicates are color-coded. The p-values correspond to the assessment of the significance of the differences with the control wild-type condition using ANOVA multiple comparison analysis with Dunnett’s correction. (B) Representative microscopy images of wild-type and KCNQ5 knock-out Raji cells expressing loxP-RFP-STOP-loxP-GFP and treated or not with 20 µM TAT-Cre for 48 hours. The results correspond to one of three independent experiments. (C) Internalization, recorded by flow cytometry, of FITC-D-JNK11 after
one hour of incubation in the indicated cell lines genetically invalidated (KO) or not (WT) for specific potassium channels. The results correspond to the median of three independent experiments.

**Figure 3: Potassium channels maintain plasma membrane polarization that is required for CPP entry into cells**

**(A) Assessment of the resting plasma membrane potential in the indicated wild-type cell lines and the corresponding potassium channel knock-out (KO) clones in the presence or in the absence 10 μM XE-991 or TRAM-34. The grey and white zones correspond to non-treated cells and inhibitor-treated cells, respectively. NT, not treated. The p-values correspond to the assessment of the significance of the differences with the control wild-type condition using ANOVA multiple comparison analysis with Dunnett’s correction. Each dot in a given condition represents an independent experiment.**

**(B) Effect of cellular depolarization (left of the grey zone) and hyperpolarization (right of the grey zone) on peptide internalization in the absence of serum. The indicated cell lines and the corresponding channel knock-out (KO) clones were pretreated or not with depolarization agents (2 μg/ml gramicidin for 5 minutes or high extracellular potassium buffer for 30 minutes) or with hyperpolarization inducer (10 μM valinomycin), followed by the addition of TAT-RasGAP_{317-326} for one hour. Alternatively, hyperpolarization was achieved by ectopic expression of the KCNJ2 potassium channel. Membrane potential and peptide internalization were then determined. Membrane potential was measured in the presence of DiBac4(3) by flow cytometry. Peptide internalization was measured by flow cytometry in the presence of 0.2% trypan blue. The p-values correspond to the assessment of the significance of the differences with the control wild-type condition using ANOVA multiple comparison analysis with Dunnett’s correction. Each dot in a given condition represents an independent experiment. Treatment with valinomycin was used in the absence of serum as the latter is expected to interfere with the drug (Rimmele and Chatton, 2014). As shown in Figure 3-figure supplement 5A, removing serum from the culture medium sensitized cells to TAT-RasGAP_{317-326} and consequently, the CPP concentration had to be adapted accordingly (Figure 3-figure supplement 5B). Serum withdrawal does not affect the $V_m$ (Figure 3-figure supplement 5C).**

**(C) Quantitation of cytosolic CPP signal (top) and the number of endocytic vesicles per cell (bottom) in wild-type HeLa cells (n=158 cells) incubated for one hour with 10 μM FITC-CPP in control, depolarizing (2 μg/ml gramicidin), or hyperpolarizing (10 μM valinomycin) conditions in the absence of serum based on
confocal microscopy images (Figure 3-figure supplement 2D). Comparison between different conditions to non-treated control was done using ANOVA test with Dunnett’s correction for multiple comparison. The number of endocytic vesicles per cell was quantitated based on confocal images. Statistical comparison was done using t-tests. Quantitation of vesicles was not performed in hyperpolarizing conditions due to masking from strong cytosolic signal. The confocal images were acquired in the middle of the cells based on Hoechst fluorescence. (D) Modulation of the \( V_m \) membrane potential by varying extracellular potassium concentrations. Assessment of membrane potential changes in Raji cells incubated in RPMI medium containing the indicated concentrations of potassium chloride (isotonicity was maintained by adapting the sodium chloride concentrations; see methods). Membrane potential was measured with DiBac4(3). The results correspond to the median of 6 independent experiments. (E) Internalization of various CPPs in the presence of different concentrations of potassium chloride in the media. Data for a given experiment are linked with thin blue lines.

**Figure 3-figure supplement 1: Importance of the \( V_m \) for TAT-RasGAP\(_{317-326}\) direct translocation**

(A) Membrane potential measurement validation. Membrane potential of wild-type and KCNQ5 knockout (KO) Raji cells was measured using DiBac4(3), a fluorescent membrane potential sensor, or by performing perforated patch electrophysiology recordings. Each dot in the figures reporting DiBac4(3) measurements corresponds to the median of 10'000 cell recording. For perforated patch, each dot in the figures corresponds to the membrane potential of one cell. (B) Representative confocal images of wild-type HeLa cells incubated with 80 \( \mu \)M FITC-TAT-RasGAP\(_{317-326}\) for one hour in the presence (depolarized) or in the absence (not treated) of 2 \( \mu \)g/ml gramicidin. Scale bar: 10 \( \mu \)m. (C) Effect of ectopic expression of the CRISPR/Cas9-identified potassium channels in wild type cells and the corresponding knockouts (right of the grey zone) on the cell membrane potential and FITC-TAT-RasGAP\(_{317-326}\) internalization in the absence of serum. Cells were incubated with TAT-RasGAP\(_{317-326}\) for one hour. Membrane potential was measured using DiBac4(3) by flow cytometry. Peptide internalization was measured by flow cytometry in the presence of 0.2% trypan blue. The p-values correspond to the assessment of the significance of the differences with the control wild-type condition using ANOVA multiple comparison analysis with Dunnett’s correction. (CD) Representative confocal images of primary rat cortical neurons (left) and wild-type HeLa cells (right) incubated for one hour at 37°C with FITC-TAT-RasGAP\(_{317-326}\) in the absence of serum. To highlight the differential capacity of these cells to take up the CPP in cytosol, a low concentration of TAT-RasGAP\(_{317-326}\) (2 \( \mu \)M) was used here. At higher concentrations, this CPP
readily enters HeLa cells (see panel B). Post incubation cells were washed and imaged with a confocal microscope using the same laser settings for both cell types. Nuclei of HeLa cells were labelled with Hoechst (blue staining). Scale bar: 20 μm.

**Figure 3-figure supplement 2: Importance of the V_m for the direct translocation of several cationic CPPs**

(A) Sequences of the indicated CPPs and their net charge. Positively charged amino acids (arginine and lysine) are color-coded. (B) Confocal microscopy quantitation of HeLa cells displaying the indicated types of CPP internalization at various concentrations after 30 minutes of incubation. The results correspond to the average of three independent experiments. We did not record cells with only cytosolic fluorescence (i.e. without fluorescent endosomes). (C) Confocal microscopy quantitation of HeLa cells displaying the indicated types of CPP internalization. The CPPs (40 μM) were continuously present in the media during the course of the experiment. The results correspond to the average of three independent experiments. (D) Left: Representative confocal images of wild-type HeLa cells incubated with 10 μM of the indicated CPP in the absence of serum in physiological, depolarized (2 μg/ml gramicidin) or hyperpolarized (10 μM valinomycin) conditions. Right: Quantitation of the cell percentage with the indicated FITC-CPP cytosolic signal intensity in depolarized, non-treated and hyperpolarized conditions. Refer to Figure 3C for the corresponding quantitation of cytosolic fluorescence intensity and number of CPP-positive endocytic vesicles per cell. Scale bar: 10 μm.

**Figure 3-figure supplement 3: Effect of V_m modulation on TAT-RasGAP<sub>317-326</sub> translocation in primary neuronal cells**

Assessment of membrane potential (top) and FITC-TAT-RasGAP<sub>317-326</sub> (2 μM) cytosolic uptake after one hour (bottom) in control, depolarized and hyperpolarized primary rat cortical neurons. Depolarization was induced by 30-minutes preincubation with 5 mM tetraethylammonium (TEA), a potassium channel blocker and hyperpolarization was induced by 30-minutes preincubation with 10 μM valinomycin. The membrane potential modulators we present throughout the experiment. Membrane potential measurements were performed with DiBac(4)3 (see Materials and Methods section). The results correspond to the median of at least three independent experiments based on confocal images (n=111 cells).

**Figure 3-figure supplement 4: CPP binding to cells is not affected by depolarization**

Assessment of FITC-CPP binding to cellular membrane of wild-type Raji cells in normal or depolarized conditions after 60 seconds of incubation (top), as well as associated peptide internalization after one hour of treatment (bottom). Cells were preincubated for 30 minutes in the presence of RPMI-media containing 5.2 or 91.9 mM potassium chloride and then treated with 40 μM of the indicated CPPs. The results correspond to at least six independent
experiments. Comparison between non-treated or depolarized conditions was done using two-tailed paired t-test.

**Figure 3-figure supplement 5: Effect of serum on the sensitivity to TAT-RasGAP<sub>317-326</sub> and CPP internalization**

(A) Quantitation of TAT-RasGAP<sub>317-326</sub>-induced death in Raji, SKW6.4 and HeLa cells in the presence or in the absence of serum. The results correspond to the median of three independent experiments. This panel shows that serum removal sensitizes cells to TAT-RasGAP<sub>317-326</sub>. This is in line with previous observations that cationic CPPs interact with proteins present in serum (Kosuge et al., 2008; Ziegler et al., 2005), which results in lower CPP availability in the media. (B) Quantitation of wild-type (WT) and knock-out (KO) cell death after incubation for 16 hours (Raji and SKW6.4) or 24 hours (HeLa) in serum-free RPMI media in the presence of increasing concentrations of TAT-RasGAP<sub>317-326</sub>. Arrows indicate the chosen peptide concentrations for experiments performed in the absence of serum. The results correspond to the median of three independent experiments. Cytosolic internalization results were very similar between the experiments performed in the presence or in the absence of serum, once the CPP concentrations were adjusted (Figure 3C and Figure 3-figure supplement 2E). (C) Membrane potential measurement in wild-type Raji cells performed in the presence or in the absence of 10% serum. Membrane potential was assessed using DiBac4(3).

**Figure 4: Hyperpolarization favors the formation of ~2 nm-wide water pores used by CPPs to translocate into cells**

(A) Visualization of in silico modelled, time-dependent, TAT-RasGAP<sub>317-326</sub> penetration and subsequent translocation across cellular membrane through a water pore. Water molecules within membranes are depicted by red spheres (and by red dots outside the membrane). (B) Quantitation of CPP localization in hyperpolarized or depolarized conditions based on coarse-grained molecular dynamics simulations. Membrane hyperpolarization was achieved through a net charge difference of 30 positive ions between intracellular and extracellular space in a double bilayer system obtaining a transmembrane potential of -2.2 V. Such low membrane potential was required to visualize translocation within the time frame of the simulations (100 nanoseconds). (C) Free energy landscape of R9 translocation reported as a function of CPP-membrane distance. The metadynamics simulations have been performed at transmembrane potential values of 0 mV, -80 mV, and -150 mV (black, brown, and green curve). (D) Free energy barrier for CPP translocation at different transmembrane potential values. (E) Electrostatic potential
maps of molecular systems that contain or not one R9 peptide in contact with the cell membrane, without any applied external electrostatic field.

**Figure 4-figure supplement 1: In silico modeling of CPP direct translocation**

(A) Schematic depiction of the molecular system used to estimate water pore formation kinetics. The static electric field ($E_{ext}$) has been highlighted with a green arrow. Water molecules are shown as red structures (small when outside membranes and large when found within membranes). (B) Assessment of the time necessary for water pore formation in the presence or in the absence of the indicated CPPs at 37°C based on in silico pore formation kinetics experiments. To estimate the time needed for CPPs to translocate through membranes at -150 mV, we established the relationship (see fitting function below) between the time of CPP translocation and the $V_m$ in the Volt range used during the simulation runs and extrapolated from this relationship the time needed for CPP translocation at a $V_m$ of -150 mV. Even though this extrapolation is likely to lack accuracy because of the well-known limitation of the MARTINI forcefield in describing the absolute kinetics of the molecular events, the values obtained are consistent with the kinetics of CPP direct translocation observed in living cells (Figures 1B and 2C and Figure 3-figure supplement 2C). Fitting function: $t = A_0 \times e^{(A_1 \times V)}$, where $t$ is the simulation time of water pore formation, $V$ is the transmembrane potential, $A_0$ and $A_1$ the fitting coefficients. The Pearson correlation coefficients of the fitting curves are $R_{R9} = 0.85$, $R_{Penetratin} = 0.95$, $R_{TATRasGAP} = 0.91$, and $R_{NoCPP} = 0.87$. (C) Cells can concentrate CPPs in their cytosol. Wild-type Raji and HeLa cells were incubated with 40 μM FITC-TAT-RasGAP$_{317-326}$ for one hour at 37°C in RPMI, 10% FBS. Images were acquired with a confocal microscope. TAT-RasGAP$_{317-326}$ fluorescence quantitation was performed using ImageJ using region of interest within the cytosol of cells that had acquired the peptide through direct translocation. The dotted line represents the fluorescence in the cell culture media (i.e. outside of the cells).

**Figure 5: Estimation of the size of the pore used by CPPs to enter cells**

(A-B) Quantitation of the percentage of cells with cytosolic staining after the indicated treatment. The indicated compounds (32 μg/ml PI, 200 μg/ml dextran, 40 μM CPP) were incubated for 30 minutes on HeLa cells. Depolarization, indicated by an asterisk, was induced with 2 μg/ml gramicidin. The percentage of cells displaying cytosolic internalization of the indicated molecules was then determined on confocal images (n=207 cells; see the methods and Figure 5-figure supplement 1C). Inset corresponds to an enlargement of the percentage of cells positive for dextran in the presence of R9. The results correspond to at least three independent experiments. CPPs such as R9 do not bind to PI (Figure 5-figure supplement 2A) and thus PI entry and accumulation within cells was not the result of CPP carry over. (C) Quantitation of the percentage of primary rat
cortical neurons with cytosolic staining incubated for 30 minutes with the indicated CPPs (2 μM) and PI (32 μg/ml). The percentage of cells displaying cytosolic internalization of the indicated molecules was then determined on confocal images (n=153 cells), as in panel B. (D) Left graph: quantitation of PI cytosolic internalization in wild type HeLa cells after 30 minutes of incubation in normal, depolarizing (2 μg/ml gramicidin) or hyperpolarizing (10 μM valinomycin) conditions in the presence or in the absence of 40 μM FITC-R9. Right graph: as in left graph, but using lower laser power to avoid saturation of the signal obtained in saponin-permeabilized cells. Cytosolic internalization was quantitated from confocal images using ImageJ (n=319 cells; see the methods). The p-values correspond to the assessment of the significance of the differences with the non- treated (NT) control condition using ANOVA multiple comparison analysis with Dunnett’s correction. The results correspond to three independent experiments. PI staining is commonly used to assess cell membrane integrity, frequently associated with cell death (see for example Figure 2B, lower graphs). This dye poorly fluoresces in solution (Figure 5-figure supplement 2B). However, the PI cytosolic intensity values in dead permeabilized cells are several orders of magnitude higher than those recorded after cell hyperpolarization (compare the left and right graphs in the present panel). (E) Relation between cytosolic PI cytosolic internalization and membrane potential measured with the DiBac4(3) sensor in HeLa cells. Each dot represents an independent experiment. (F) The relation between PI cytosolic internalization and membrane potential from panel E was used to calculate membrane potential based on PI fluorescence in HeLa cells and its corresponding KCNN4 KO. Measured membrane potential was acquired in the presence of DiBac4(3) based on its cytosolic fluorescence. Each dot in a given condition represents an independent experiment.

**Figure 5-figure supplement 1: Evidence for low molecular weight pore formation in living cells during CPP direct translocation**

(A) Structure and dimensions of the molecules used to probe the size of the pore used by CPPs to enter cells. Dextran molecules are fluorescently labelled with Texas Red or TMR. (B-D) HeLa cells were incubated with 32 μg/ml propidium iodide (PI) in the presence or in the absence of 40 μM FITC-R9, or left untreated for 30 minutes at 37°C in RPMI, 10% FBS. Depolarization was induced with 2 μg/ml gramicidin. Images were obtained by confocal microscopy. As shown in Figure 5-figure supplement 2B, the fluorescence produced by 32 μg/ml of PI in solution (i.e. without cells) is under our threshold of detection. The observed fluorescence dots and signal in cells incubated with this PI concentration correspond therefore to cell autofluorescence and not
PI fluorescence (compare images i and ii in panel B). Hence, when PI fluorescence is detected in cells (see the examples in panels C or D), the corresponding PI concentration is higher than 32 µg/ml (Figure 5-figure supplement 2B). In other words, cellular PI fluorescence is detected because cells are able to concentrate this cationic dye in their cytosol, as they are able to do with cationic CPPs (Figure 4-figure supplement 1C). (B) PI does neither enter live cells by itself (left images), nor does it enter depolarized cells, whether CPPs (here R9) are present or not (middle images). The fluorescent signal from FITC-labelled CPPs is not detected in the channel used to record PI fluorescence. In other words, the signal recorded as PI does not come from FITC fluorescence leaking into the PI channel (right images). (C) Criteria used for the visual scoring performed in Figure 5 in HeLa cells. The percentage of cells positive for PI and cells that have acquired FITC-R9 through direct translocation, have been assessed based on the following criteria. Cells 1, 3-6 are considered as positive for PI (left) and cells 1-6 are counted as those, where direct translocation has occurred (right). The rest of the cells were considered as negative. Scale bar: 20 µm. (D) Kinetics of FITC-CPP and PI uptake. Images were acquired over time with a confocal microscope and cytosolic fluorescence was quantitated using ImageJ similarly to what is shown in Figure 1-figure supplement 2D.

**Figure 5-figure supplement 2: Control experiments pertinent to the use of PI**

(A) PI does not interact with R9. The potential binding between PI (6 mM) and R9 (0.6 mM) at 37°C was assessed by isothermal titration calorimetry. Representative power and heat of injection are shown. This experiment was repeated three times with similar results. (B) PI fluorescence detection in media in the absence of cells. Wells containing the indicated PI concentrations in RPMI were illuminated using the same settings as in Figure 5B and Figure 5C, left panel. Fluorescence intensity within the full field of the acquired images was quantitated using ImageJ. The arrow indicates the PI concentration used for water pore-related experiments (32 µg/ml). (C) Assessment of colony formation potential after transient CPP and membrane potential treatments. DMSO was used as a vehicle for gramicidin and valinomycin. HeLa cells were incubated for one hour with the indicated treatment, washed and plated on 10 cm dishes. Number of colonies were counted after 14 days in culture after Giemsa staining. These data indicate that cell viability is not affected by transient treatment with the indicated CPPs, Vm modulating agents and PI. The results obtained in cells incubated with CPPs further show that water pore formation does not compromise cell survival.

**Figure 6: Hyperpolarization improves CPP internalization in vivo**

(A) CPP internalization in zebrafish embryos in normal and hyperpolarized conditions. Forty-eight-hour post fertilization, zebrafish embryos were injected with 3.12 µM FITC-TAT-RasGAP317-326(W317A) with or without 10 µM valinomycin. Scale bar: 200 µm. The results correspond to three independent experiments. (B) CPP internalization in C57BL/6N
mice in normal and hyperpolarized conditions. Mice were injected with 5 μM FITC-TAT-RasGAP_317-326(W317A) with or without 10 μM valinomycin (n=11 injections per condition). In both panels, the p values associated with the comparisons of the “CPP” and “CPP + valinomycin” conditions were calculated using two-tailed paired t-tests.

Figure 6-figure supplement 1: Zebrafish and mouse membrane potential modulation

(A) Eighteen hours post fertilization zebrafish embryos were incubated for 40 minutes with the indicated concentrations of valinomycin and 950 nM DiBac4(3). DiBac4(3) fluorescence was then recorded and normalized to the non-treated control. The decrease in DiBac4(3) fluorescence indicates membrane hyperpolarization. Membrane potential values could not be calculated, as a standard curve would have to be performed in zebrafish. DiBac4(3) internalization was assessed from confocal images of the fish tail region. (B) Eighteen hours post fertilization zebrafish embryos were incubated with or without 3.12 μM TAT-RasGAP_317-326 (W317A), a mutant version that is not toxic to cells, in the presence of the indicated concentrations of valinomycin. Peptide internalization was assessed from confocal images of the fish tail region. (C-D) Eighteen hours post fertilization zebrafish embryos were incubated 1 hour with the indicated concentrations of valinomycin, in the absence (panel C) or in the presence (panel D) of 3.12 μM TAT-RasGAP_317-326 (W317A), then washed, and incubated in Egg water. The viability of each fish was assessed over 52 hours at the indicated time points. (E) Representative images of zebrafish treated as described in panels C and D, washed, and further incubated in Egg water. Images were taken with a CYTATION3 apparatus at a 4x magnification at 70 hours post fertilization (hpf). (F) Survival of 48 hours post fertilization zebrafish embryos following intramuscular injection of 3.12 μM TAT-RasGAP_317-326 (W317A) peptide in the presence or in the absence of 10 μM valinomycin. Survival was visually assessed under a binocular microscope by taking into consideration the embryo transparency (as dead embryos appear opaque), development characteristics and motility. (G) Mice were intradermally injected with DiBac4(3) in the presence or in the absence of 10 μM valinomycin. DiBac4(3) fluorescence was then recorded and normalized to the mean of non-treated (NT) control. DiBac4(3) fluorescence was assessed as in panel A.

Figure 7: Model of CPP direct translocation through water pores.

Cationic CPP translocation across cellular membranes is favored by the opening of potassium channels or by hyperpolarizing drugs, such as valinomycin. This sets a sufficiently low membrane potential permissive for CPP direct translocation. When cationic CPPs bind to these already polarized membranes, they induce megapolarization (i.e. a
membrane potential estimated to be -150 mV or lower). This leads to the formation of water pores that are then used by CPPs to enter cells.
References


peptide binding to mimetic membranes. Proc Natl Acad Sci U S A 111, 12684-12688.


Source data legends

**Figure 2 - figure supplement 2E - source data 1**
Uncropped blot and original full raw unedited blot corresponding to the anti-FLAG blot shown in Figure 2 – figure supplement 2E.

**Figure 2 - figure supplement 2E - source data 2**
Uncropped blot and original full raw unedited blot corresponding to the anti-actin blot shown in Figure 2 – figure supplement 2E.

**Figure 2 - figure supplement 2F - source data 1**
Uncropped blot and original full raw unedited blot corresponding to the anti-V5 blot shown in Figure 2 – figure supplement 2F (left hand-side).

**Figure 2 - figure supplement 2F - source data 2**
Uncropped blot and original full raw unedited blot corresponding to the anti-α-tubulin blot shown in Figure 2 – figure supplement 2F (left hand-side).

**Figure 2 - figure supplement 2F - source data 3**
Uncropped blot and original full raw unedited blot corresponding to the anti-V5 blot shown in Figure 2 – figure supplement 2F (right hand-side).

**Figure 2 - figure supplement 2F - source data 4**
Uncropped blot and original full raw unedited blot corresponding to the anti-α-tubulin blot shown in Figure 2 – figure supplement 2F (right hand-side).
Supplementary file legends

**Supplementary file 1: Components of the culture medium lacking potassium chloride and sodium chloride**
This table lists the components found in the Biowest RPMI-like media that lacks potassium chloride and sodium chloride.

**Supplementary file 2. Membrane lipid composition considered in the present study**
This table lists the proportions of various lipids found in the inner and outer layers of the plasma membrane that we have used for our simulations.

**Supplementary file 3. Nascent water pore free energy estimations in various studies**
This table reports the free energy that needs to be overcome for the formation of water pores that was calculated in the indicated studies.

**Supplementary file 4. Primer sequences used in the second PCR performed to bar code the sgRNAs of the selected populations**
The green nucleotides correspond to the bar codes. The red nucleotides are added to increase library complexity to prevent signal saturation when the sequencing is performed. The blue sequences are complementary to the extremities of the first PCR fragments.

**Supplementary file 5. sgRNAs used to disrupt the indicated genes**
This table provides the sequences of the sgRNAs used to target the first exon of the indicated genes.

**Supplementary file 6. Forward and reverse primers used in the TA cloning procedure**
Videos

**Video 1: TAT-RasGAP<sub>317-326</sub> internalization in Raji cells over a 16-hour period.**

Representative confocal time-lapse recording of wild-type Raji cells incubated with 5 μM TAT-RasGAP<sub>317-326</sub> for 16 hours in RPMI in the absence of serum. For the first 30 minutes of the recording, images were taken every 30 seconds, then until the end of the recording, images were taken every five minutes. Peptide was present in the media throughout the recording. Yellow and pink arrows indicate cells taking up the peptide by direct translocation and by endocytosis, respectively. Cyan arrows point towards labelled endosomes and green asterisks to dead cells. Scale bar: 20 μm. Time is displayed in hours:minutes.

**Video 2: Early peptide entry in wild-type Raji cells.**

Time-lapse recording of Raji cells incubated with 40 μM TAT-RasGAP<sub>317-326</sub> for 30 minutes in RPMI, 10% FBS. Peptide was present in the media throughout the recording and images were taken for 30 minutes at 10-second intervals. Scale bar: 10 μm. Time is displayed in minutes:seconds.

**Video 3: Early peptide entry in wild-type HeLa cells.**

Time-lapse recording of HeLa cells incubated with 80 μM FITC-TAT-RasGAP<sub>317-326</sub> in RPMI, 10% FBS. Yellow and pink arrows indicate cells experiencing direct translocation and endocytosis, respectively. Images were taken for 30 minutes at 10 second intervals. Scale bar: 20 μm. Time is displayed in minutes:seconds.

**Video 4: Distinction between endosomal escape and direct translocation.**

Wild-type HeLa cells were preincubated with 80 μM FITC-TAT-RasGAP<sub>317-326</sub> for 30 minutes in RPMI, 10% FBS and then imaged every 5 minutes for 4 hours at 37°C, 5% CO₂. Movie on the left was recorded in the continuous presence of the peptide. Movie on the right was recorded after the peptide was washed three times with RPMI, 10% FBS. Scale bar: 10 μm. Time is displayed in hours:minutes.
Video 5: *In silico* visualization of water pore formation in the presence of the indicated CPPs across a polarized membrane bilayer.

This movie shows the translocation of the indicated CPPs across a plasma membrane in the presence of a membrane potential of -2.2 V. This simulation was performed by molecular dynamics MARTINI coarse-grained approach using an asymmetric multi-component bilayer in the presence of ion-imbalance to polarize the membrane.

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Video 6: *In silico* visualization of water pore formation in the presence of the indicated CPPs across a non-polarized membrane bilayer.

This movie shows the lack of translocation of the indicated CPPs across a plasma membrane in the absence of a membrane potential (0 V). This simulation was performed by molecular dynamics MARTINI coarse-grained approach using an asymmetric multi-component bilayer in the absence of ion-imbalance.
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Figure 1

HeLa SKW6.4

A

No CPP uptake | Vesicular | Vesicular + cytosolic
---|---|---
Raji | | |
SKW6.4 | | |
HeLa | | |

B

- Negative
- Vesicular
- Vesicular + cytosolic

% cells with indicated staining

Time (min)
Figure 1-figure supplement 1

Fluorophore carried by the CPP
Figure 1-figure supplement 2

A

B

C

D

Nucleus and nucleoli
Endosomes
Cytosolic regions devoid of endosomes
Figure 1-figure supplement 3
A

Lentivirus CRISPR library

WT cells

TAT-RasGAP<sub>317-326</sub> - treated population

Un-treated population

PCR amplification of sgRNA region

High-throughput sequencing and comparison of sgRNA abundance

B

2 4 6 8 10 12

1 2 3 4 5 6 7 8

CPP treated

(log2 normalized sgRNA counts)

3 8 13 18

1 3 5 7 9 11

Non-treated control

(log2 normalized sgRNA counts)

CPP treated

(log2 normalized sgRNA counts)

Raji

KCNQ5

ARRDC3

MAGEB5

FRMD7

PIP5K1A

SKW6.4

KCNN4

KCNK5

ATP1B3

SLC39A14

Figure 2 - figure supplement 1
Figure 2-figure supplement 2

A

Raji clone # Q5-1, KCNQ5 sequence

WT
Allele 1
Allele 2

Raji clone # Q5-2, KCNQ5 sequence

WT
Allele 1
Allele 2

HeLa clone # N4-1, KCNN4 sequence

WT
Allele 1
Allele 2

HeLa clone # N4-2, KCNN4 sequence

WT
Allele 1
Allele 2

B

SKW6.4 clone # N4-1, KCNN4 sequence

WT
Allele 1
Allele 2

SKW6.4 clone # N4-2, KCNN4 sequence

WT
Allele 1
Allele 2

SKW6.4 clone # K5-1, KCNK5 sequence

WT
Allele 1
Allele 2

SKW6.4 clone # K5-2, KCNK5 sequence

WT
Allele 1
Allele 2

D

Raji

WT
KCNQ5 KO (clone Q5-1)
KCNQ5 KO (clone Q5-2)

SKW6.4

WT
KCN4 KO (clone N4-1)
KCN4 KO (clone N4-2)
KCN5 KO (clone K5-1)
KCN5 KO (clone K5-2)

E

Raji

KCNQ5 KO
KCNQ5 KO + Flag-KCNQ5

SKW6.4

KCN4 KO
KCN4 KO + KCNN4-V5

HeLa

KCN4 KO
KCN4 KO + KCNN4-V5

F

Cell death (% PI-positive cells)

Cell death (% PI-positive cells)

TAT-RasGAP\_17.326 [µM]

TAT-RasGAP\_17.326 [µM]
Figure 2-figure supplement 3
**Figure 2-figure supplement 4**

**A**

Luciferase activity in Raji, HeLaSKW6.4, and Raji WT cells. The graphs show the luciferase activity for WT and KO (KCNQ5 KO and KCN4 KO) cells with or without Cre reporter and Cre reporter + TAT-Cre.

**B**

Immunofluorescence images showing the internalization of FITC-D-JNKI1 in Raji WT and KCNQ5 KO cells, and Cre reporter and Cre reporter + TAT-Cre conditions.

**C**

Graphs showing the internalization of FITC-D-JNKI1 in Raji, SKW6.4, and HeLa cells with WT and KO (KCNQ5 KO and KCNN4 KO) conditions. The data points indicate a trend of increased internalization with higher concentrations of FITC-D-JNKI1.
A

Membrane potential [mV]

Wild-type

KCNQ5 KO

DiBac4(3)

Perforated patch

B

Figure 3-figure supplement 1

C

Raji

SKW6.4

HeLa

NT

KCNQ5 KO

NT

KCNN4 KO

NT

KCNQ5 KO

p<0.001

p=0.001

p=0.058

p=0.007

p=0.002

p=0.013

p=0.225

p=0.022

p=0.001

p<0.001

p>0.999

p=0.001

D

2 µM FITC-TAT-RasGAP317-326

2 µM FITC-TAT-RasGAP317-326

Hoechst 33342

Rat cortical neurons

HeLa

Figure 3-figure supplement 1
A

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B

![Graphs showing cell percentage with indicated staining over time for CPP concentrations ranging from 10 to 1000 µM.](image)

C

![Graphs showing percentage of cells with indicated staining for different CPPs over time.](image)

D

![Images showing cellular staining under different conditions: Not treated, Depolarized, and Hyperpolarized for TAT, R9, and Penetratin.](image)

E

![Bar graphs showing cytosolic fluorescence (a.u.) for different concentrations of TAT, R9, and Penetratin in Raji and HeLa cells.](image)

Figure 3-figure supplement 2
Figure 3-figure supplement 3
Figure 3-figure supplement 4
Figure 3-figure supplement 5

A

Raji

SKW6.4

HeLa

Cell death [% PI-positive cells]

0 5 10 15 20

0 5 10 15 20 25 30 35 40

WT

KCNN4 KO

KCNK5 KO

B

Raji

SKW6.4

HeLa

Cell death [% PI-positive cells]

0 5 10 15 20

0 5 10 15 20

WT

KCNN4 KO

KCNQ5 KO

TAT-RasGAP317-326 [µM]

C

Membrane potential [mV]

Wild-type Raji

Figure 3-figure supplement 5
Figure 4
Figure 4-figure supplement 1

A

Outside membrane

Inside membrane

Water molecules

CPP

Hydrophilic phospholipid head

Hydrophobic phospholipid tail

Phospholipids

E_ext

Intracellular space

Inner leaflet

Outer leaflet

Extracellular space

B

Time required for water pore formation at $V_m = -150 \text{ mV}$

- No CPP - days
- TAT - 20 minutes
- TAT-RasGAP<sub>317-326</sub> < 10 minutes
- Penetratin < 10 minutes
- R9 < 10 minutes

C

Fluorescence [a.u.]

0.1

Raji

HeLa

Figure 4-figure supplement 1
Figure 5

A. No CPP added

B. Cells incubated with the indicated CPPs

C. Primary rat cortical neurons

D. Cytosolic fluorescence [a.u.]

E. Cytosolic fluorescence [a.u.]

F. Membrane potential [mV]

$y = 2570 \cdot e^{-x/54}$
**Figure 5-figure supplement 1**

**A**

Propidium Iodide

- 0.8-1.5 nm

**B**

Non-depolarized cells

![Image of non-depolarized cells with PI and FITC-R9](image)

Depolarized cells

![Image of depolarized cells with PI and FITC-R9](image)

Non-depolarized cells

![Image of non-depolarized cells with FITC-R9](image)

**C**

![Images showing PI and FITC-R9 in cells](image)

**D**

![Images showing normalized cytosolic uptake](image)

**Normalized cytosolic uptake**

- FITC-CPP
- PI

**Time (min)**

0 5 10 15 20 25 30

**Normalized cytosolic uptake**

0.0 0.2 0.4 0.6 0.8 1.0 1.2
Figure 5-figure supplement 2
Figure 6

A. 56 hpf

- Not treated
- Valinomycin 10 µM

B. C57BL/6N

- Not treated
- Valinomycin 10 µM

- Internalization [a.u.]
- Time [hours post injection]
- CPP
- CPP + valinomycin

- Internalization [a.u.]
- FITC-CPP
- valinomycin

p<0.0001
p=0.0021
p=0.0084
p=0.0010
p=0.0001

Epidermis
Dermis
Hypodermis
Figure 6-figure supplement 1
Figure 7

Potassium ions

Cationic CPP

Potassium channel

Water pore

~2 nm