Homeotic Transformation Induced by Protein Transduction

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Abbreviation

Antp	*Antennapedia*
A/P	antero-posterior
abd-A	*abdominal-A*
Abd-B	*Abdominal-B*
ANT-C	*Antennapedia* Complex
ARH	autosomal recessive hypercholesterolaemia
BX-C	*Bithorax* Complex
Myc	v-myc avian myelocytomatosis viral oncogene homolog
cad	*caudal*
CI	clathrin-independent
CLIC	clathrin- and dynamine- independent carriers
CLIO	cross-linked iron oxide nanoparticles
CME	clathrin-mediated endocytosis
COL	Colinearity rule
CPPs	Cell Penetrating Peptides
Cre	Cre recombinase
CtxB	cholera toxin B
da	*dachshund*
DAB2	*Disabled homologue 2*
Dfd	*Deformed*
Dll	*Distalless*
dpp	*decapentaplegic*
EIPA	5-(N-Ethyl-N-isopropyl) amiloride
EM	electron microscopy
EMSA	electrophoresis mobility shift assay

en	*engrailed*
FACS	Fluorescence activated cell sorting
FITC	fluorescein isothiocyanate
FSNPs	FITC-doped silica nanoparticles
ftz	*fushi tarazu*
GPCR	G protein-coupled receptor
GPI	Glycosylphosphatidylinositol
GSLs	Glycosphingolipids
GUVs	giant unilamellar vesicles
HD	homeodomain
HDAC	histone deacetylase
HD*Antp*	*Antennapedia* homeodomain
hh	*hedgehog*
hiPSC	Human Induced Pluripotent Stem Cells
His	Histidine
HIV-1	human immunodeficiency virus 1
HNFs	human newborn fibroblasts
HP	high performance
HS	heparan sulfate
HSPGs	heparan sulfate proteoglycans
Hth/Exd	*Homothorax*/*Extradentical*
IMAC	immobilized metal affinity chromatography
iPSCs	induced pluripotent stem cells
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Klf-4	*Kruppel-like factor 4*
Lab	*Labial*
LDL	Low-density lipoprotein
LDLR	low-density lipoprotein receptor
MAP	model amphipathic peptide

mESC	mouse embryonic stem cell
MRI	magnetic resonance imaging
MTS	membrane translocation sequences
NECAP	adaptin ear-binding coat-associated protein
NLS	nuclear localisation sequence
Oct-4	*Octamer-binding transcription factor 4*
pb	*proboscidea*
PBS	Phosphate buffered saline
Phe	Phenylalanine
piPSCs	protein-induced pluripotent stem cells
PTD	protein transduction domains
PtdIns(4,5)P2	phosphatidylinositol (4,5) – bisphosphate
SCK	Shell cross-linked
Scr	*Sex combs reduced*
Sox2	*SRY (sex determining region Y)-box 2*
Ss	*Spineless*
SV40	simian virus-40
T2	mesothoracic segment
Tat	Trans-activator of transcription
TFs	transcription factors
Trp	Tryptophan
Ubs	*Ultrabithorax*
vg	*vestigial*
VPA	valproic acid
X-Gal	5-bromo-4-chloro-indolyl-β-D-galactopyranoside

Introduction

About two decades ago, the homeodomain of *Drosophila* transcription factor *Antennapedia* was shown to be able to translocate across the plasma membrane from the extracellular space into the cytoplasm (Joliot *et al.,* 1991), in a receptor-independent manner (Derossi *et al.,* 1996). This discovery potentially opens an avenue for intracellular delivery of functional proteins, such as transcription factors (TFs) to modify the gene expression pattern and ultimately to change cell identity. In an effort to induce specific antenna to leg transformations (Schneuwly *et al.,* 1986) in *Drosophila* imaginal disc tissue without using conventional genetic manipulation, we have been trying to achieve this phenotypic readout by the method of protein transduction, namely imposing biological effects on the cells solely by introducing exogenous protein into them (Hadorn 1965; Prochiantz and Joliot 2003). In contrast to the traditional way to trigger the action of TFs via genetic tools, the approach of protein transduction requires the comprehensive understanding of the architecture of the plasma membrane, and the phenomena of macromolecular cellular internalization and the mechanisms underlying the translocation of the *Antennapedia* homeodomian, to prepare the road map for the journey of exogenous protein intracellular trafficking and transduction.

1. PERMEABILITY OF THE PLASMA MEMBRANE

Dating back to the middle of the 17th Century, the term "Cell" was first used to identify microscopic structures by the English physicist Robert Hooke (Hooke 1665) when he was describing a sliver of cork. Subsequent studies started to recognize that there was an additional boundary layer surrounding the cell, in addition to the wall-like structure in plant cells. But little was known about its chemical nature until late in the 19th century. By investigating permeable substances on this boundary layer, Overton from Zurich University demonstrated that the layer was permeable to solutes such as lower aliphatic alcohols, ether, acetone, chloroform, but not to various physiologically important solutes such as amino acids, sugars, or electrolytes (Overton 1895; 1896; 1899). Overton's findings led to the hypothesis that the thin membranes surrounding cells have the properties of oil. In his book

on anesthesia he claimed the layers surrounding cells "lipoids" were made of lipids and cholesterol (Overton 1901; Kleinzeller 1997). Later on, using red blood cell as model material, the Dutch scientists Gorter and Grendel found that the amount of erythrocyte lipid they had extracted could cover twice the area needed to enclose all the cells (Gorter 1925). This indicated that the plasma membrane consists of two layers of lipid, namely lipid bilayer. The direct visualization was achieved by using the transmission electron microscopy (EM) in the late 1950s (Sjostrand 1953). The plasma membrane appears by EM as two dense lines separated by an intervening space—often referred to as "railroad track" (Robertson 1958; 1958; 1960).

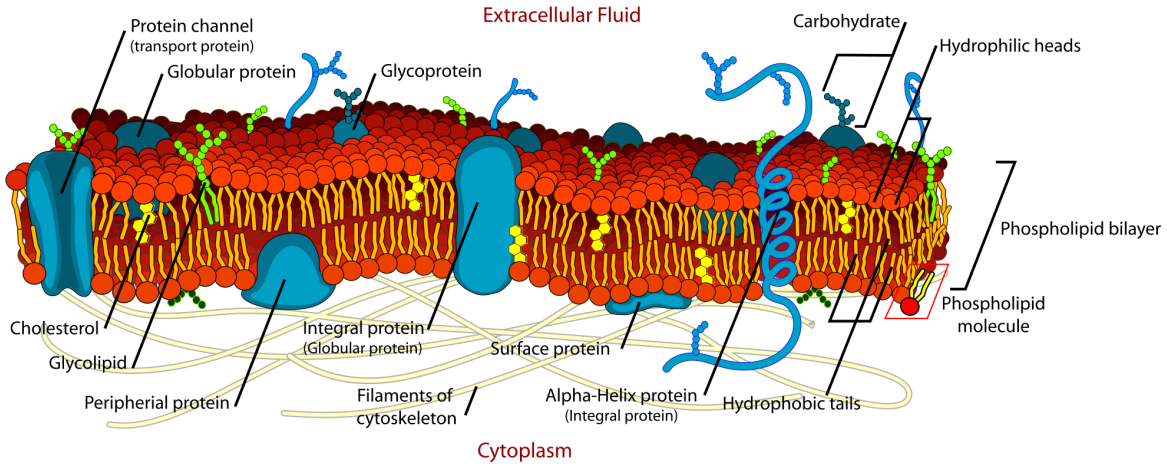


Fig. 1 Diagram of the plasma membrane representing the fluid-mosaic-Model proposed by Singer and Nicolson's (1972). Amphipathic phospholipids form self-sealing bilayer with hydrophobic tails pointing toward the center of two layers and hydrophilic head exposured to water. Integral membrane proteins and peripheral membrane proteins contribute to the fluid mosaic. http://en.wikipedia.org/wiki/File:Cell_membrane_detailed_diagram_en.svg

For cells, the lipid bilayer is an ideal barrier preventing most substances from diffusing either from the outside environment into the interior of cells, or vice versa. Even though the bilayer is only a few (*ca*. 5nm) nanometers in width (Fig.1) (Lewis and Engelman 1983), it is impermeable to most molecules other than small hydrophobic ones (Singer and Nicolson 1972). The ability of small molecules to diffuse through a lipid bilayer is related to their lipid solubility. Strongly polar molecules such as ions passing through the bilayer extremely slowly, due to the fact that inorganic ions are surrounded by a shell of water molecules, which needs to be stripped off for ions to pass through the hydrocarbon center of the lipid bilayer, but this process is energetically unfavorable. Small, more weakly polar molecules such as ethanol or glycerol can diffuse relatively readily, but larger molecule such as glucose penetrate very slowly (Elliott 2009).

The impermeability of the plasma membrane raises the question of how cells can take up the essential nutrients, such as sugars, amino acids, ions, and so on. For instance, glucose can slowly diffuse through the plasma membrane, but it is by large not sufficient for the energy consumption in cells, therefore some particular transporters are designed for facilitating glucose uptake. These transporters are integral membrane proteins that form a small passageway through the membrane (Fig. 1), enabling selected molecules to cross the plasma membrane. Based on the manner of energy consumption, there are two kinds of transportation systems, an active one and a passive one. In the active system, fueled directly or indirectly by the hydrolysis of ATP, substances can be transported against their concentration gradients by active transporters, namely pumps. Otherwise in the passive transport mode, which is energy-independent, simply by opening of a channel, substances can move along a concentration gradient. However specificity is vital in both systems for pumps and channels to function properly. They handle only highly selected molecules. These transporters are found in large numbers and of varying specificities, thus contributing to the identity of the plasma membrane of each cell type (Elliott 2009).

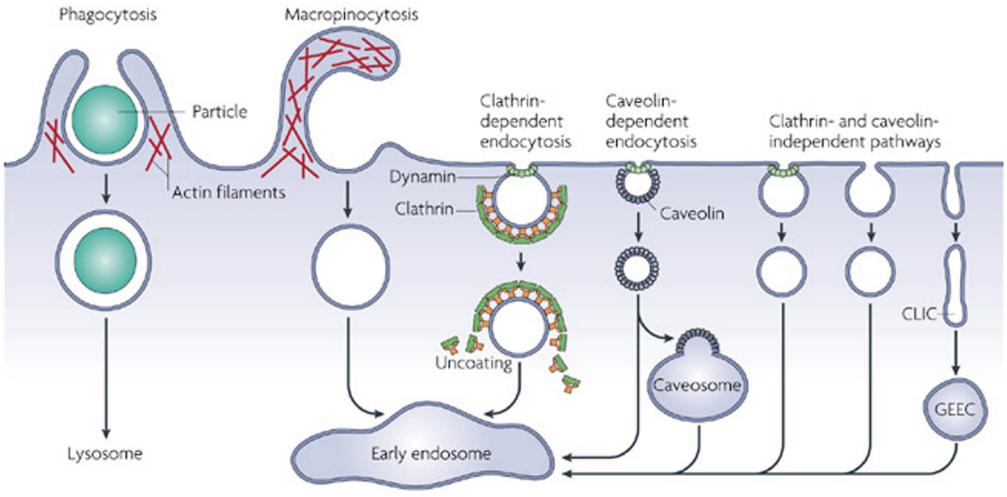


Fig. 2 Endocytosis. Phagocytosis exists only in specialized cells, whereas macropinocytosis can be stimulated in various cells and pinching off vesicles are fused into early endosome rather than lysosome. Clathrin- or caveolin-dependent endocytic pathway produce coated vesicles and their cargos selection are receptor-mediated. There are clathrin- and caveolin-independent entries newly emerging, such as clathrin- and dynamine- independent carriers (CLIC), whose components need to be further investigated. **Modified from S. Mayor & E. Pagano 2007.**

2. ENDOCYTOSIS

In addition to the transporting systems, endocytosis is another essential mode for cells taking up substances, particularly for macromolecules. Unlike transporters sitting in the membrane bilayer, opening or closing their passageways, endocytic entries involve dramatic changes in membrane morphology and multi-protein machineries in action. Endocytosis is a generic term for all the internalization entries associated with the plasma membrane inward folding and subsequent pinching off of vesicles (Doherty and McMahon 2009).

The phagocytosis and macropinocytosis were the first two endocytic entries that have been described during the period between the late of 19th and early 20th century. Even before any knowledge of the chemical properties of the plasma membrane, the Russian scientist Metchnikoff could vividly describe the movement of cells when they battling invading pathogens and formulate the initial definition of phagocytosis in 1883 (Metchnikoff 1883; Tauber 2003). Inspired by this "cell eating" behavior and its theory, the American physician Meltzer hypothesized that cells should be able to "drink" as well (Meltzer 1904). Experimental evidence came up nearly 30 years later when Lewis described a process during which "waving" cell surface ruffles close back on the plasma membrane to form vesicles (>1μm in diameter) appearing bright by phase contrast microscopy, and named it as "Pinocytosis", later renamed as macropinocytosis (Lewis 1931).

Following the introduction of glutaraldehyde fixation in EM technique, in the 1960s, Roth and Porter were able to reveal a new endocytic entry (Roth and Porter 1964). While they tried to depict the process of how yolk proteins were internalized into mosquito ovaries, they found the "bristle-coated" vesicles forming on the cell surface and pinching off subsequently. These vesicles were usually 250nM in diameter, much smaller than the macropinotic vesicles. It took another decade before Pearse developed a method for isolating coated vesicles and purifying the "bristle-coats" (Pearse 1976). Clathrin was then identified as the major component of protein that forming the "bristle" like coat around the vesicles. Afterwards, clathrin-mediated endocytosis, which is responsible for the internalization of nutrients, low-density lipoprotein (LDL), pathogens, antigens, and growth factors receptors, became the most well-characterized pathway of internalization of macromolecules into cells (Tab. 1) (Hopkins *et al.,* 1985; Morris *et al.,* 1985; Garcia *et al.,* 2001; Dong *et al.,* 2003; Keyel *et al.,* 2006).

As the budding structure from the plasma membrane is a prerequisite for any

endocytic pathway, and clathrin coats are visible around spherical membrane pits as well as pinching off vesicles by EM, it was possible to identify clathrin-independent budding events by the absence of such a coat (Hinshaw and Schmid 1995; Doherty and McMahon 2009). Meanwhile, more evidence had been accumulated that perturbation of clathrin-dependent uptake could not block all endocytosis. Therefore, the existence of clathrin-independent (CI) endocytic pathways has subsequently been revealed. Caveolin-dependent endocytosis was the first described CI endocytic pathway (Anderson *et al.,* 1992; Rothberg *et al.,* 1992), and then came clathrin- and caveolin-independent endocytosis, which has been further dissected into distinct pathways, based on their differential drug sensitivities and the reliance on certain proteins and lipids (Mayor and Pagano 2007; Doherty and McMahon 2009). Below is the detailed summary of three endocytic pathways including clathrin-dependent endocytosis, caveolae-dependent endocytosis and macropinocytosis, with the focus on vesicle architecture, the mechanisms underlying cargo selection and relevant physiological implications (Fujimoto *et al.,* 2000; Dong *et al.,* 2003; Takamori *et al.,* 2006).

2.1 Clathrin-mediated endocytois

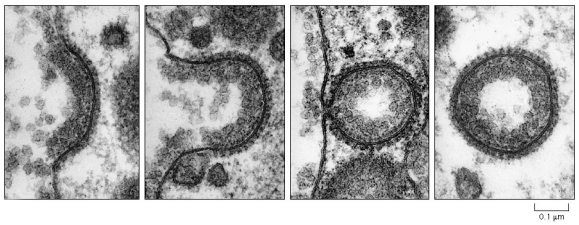


Fig. 3 Clathrin-coated vesicle forming. Initially, the plasma membrane start to invaginate to form a nacent pit, with a coat surrounding the intracellular surface of membrane. The coated pit rounds up and then pinches off at the neck of pit. **Courtesy of Perry and Gilbert 1979.**

Clathrin-mediated endocytosis has been implicated in many key cellular functions, including keeping cellular homeostasis by internalization of particular receptors, sampling the cell's environment for growth guidance and, bringing nutrients into cells (Keyel *et al.,* 2006; Eto *et al.,* 2008; McMahon and Boucrot 2011). This is a specific-receptor regulated endocytic entry, and cargos enclosed inside the vesicles are highly selected and concentrated. The three-limb-shaped clathrin molecule, called triskelion, is capable to polymerize and thus assemble a cage-like structure upon a nascent pit invaginated from the plasma membrane (Pearse 1976; Cheng *et al.,* 2007). Clathrin does not bind directly to the cellular membrane or to cargo receptors, therefore adaptor and accessory proteins are required to form a scaffold to recruit triskelia from the cytosol. Even before any morphological changes in the membrane can be observed, a putative nucleation zone with preference for lipid phosphatidylinositol (4,5)-bisphosphate starts to recruit the ubiquitous

adaptor protein AP2 (Collins *et al.,* 2002; Blondeau *et al.,* 2004; Robinson 2004), which in turn can recruit different cargo-specific adaptor proteins for the purpose of cargo selection (Tab. 1). Meanwhile triskelia are being recruited by the AP2 hub. The plasma membrane starts to invaginate to form a nascent pit, with a clathrin coat forming intracellularly. The coated pit continues to grow and becomes rounded up, then it pinches off by membrane fission at the neck of the pit (Fig. 3) (Perry and Gilbert 1979). Vesicle budding depends on a enzyme called dynamin (Kosaka and Ikeda 1983), which polymerizes around the neck of the nascent vesicle and generates membrane fission by GTP hydrolysis (Hinshaw and Schmid 1995; Sweitzer and Hinshaw 1998; Blondeau *et al.,* 2004; Ehrlich *et al.,* 2004; McMahon and Boucrot 2011).

Tab. 1 Clathrin-dependent endocytosis cargo selection (McMahon and Boucrot 2011)

Cargo-specific adaptors:	Functions
ARH	Coupling LDLR to AP2
DAB2	Coupling megalin and LDLR to AP2
Stonin	Coupling synaptotagmin to AP2
HRB	Coupling the SNARE protein VAMP7 to AP2
NECAP	Potential cargo-specific adaptor
Numb	Coupling Notch to AP2
β-arrestin	Coupling GPCRs to AP2

ARH, autosomal recessive hypercholesterolaemia

DAB2, Disabled homologue 2

LDLR, low-density lipoprotein receptor

NECAP, adaptin ear-binding coat-associated protein

GPCR, G protein-coupled receptor

Clathrin-mediated endocytosis is also exploited by viruses and bacteria to gain entry into cells (Rust *et al.,* 2004; Moreno-Ruiz *et al.,* 2009). These huge pathogenic cargoes require larger vesicles by changing the normal geometry of clathrin-coated vesicles. For instance, transferrin bound to its receptor has a diameter of ~13 nm, and one LDL particle has a diameter of ~25 nm (Anderson *et al.,* 1977); these cargoes can easily be accommodated in clathrin-coated vesicles with average internal diameters of ~40 nm without changing their

normal geometry (Takamori *et al.,* 2006; Singh *et al.,* 2007). In contrast, larger spherical viruses, such as reovirus (~85 nm in diameter) and influenza A (~120 nm in diameter) must increase vesicle size to be accommodated (Ehrlich *et al.,* 2004; Rust *et al.,* 2004). Bacteria and fungi are even larger than viruses, and they need to trigger local actin assembly to enable the plasma membrane inward folding and recruit clathrin to their sites of entry (Veiga and Cossart 2005; Eto *et al.,* 2008; Moreno-Ruiz *et al.,* 2009). The fact that all these pathogens gain the entry of entering the cells via clathrin-medicated endocytosis, complicates the issue of how cargoes have been selected in this type of endocytosis.

2.2 Caveolin-mediated endocytois

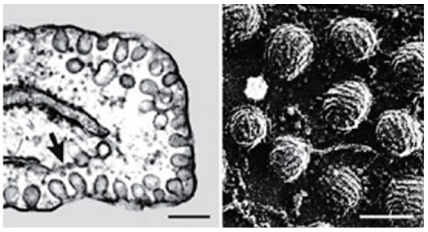


Fig. 4 Caveolin-mediated endocytois. Thin-section (left panel) and rapid freeze, deep-etch images (right panel) of caveolae in endothelial cells. The arrow in the left panel points to the endoplasmic reticulum near deeply invaginated caveolae.The scale bar is 100 nm. **Modified from R. W. Anderson 1998.**

Caveolae are small (approx. 50 nm in diameter) flask-shaped pits localized within a specialized plasma membrane zone called the lipid raft (Anderson *et al.,* 1992). Immunocytochemistry demonstrated that caveolin-1 is a specific component of the ridges that make the caveolar coat (Rothberg *et al.,* 1992). Caveolin-1 is a transmembrane protein adopting a hairpin conformation into the membrane, leaving both the N- and the C-termini of the protein facing the cytoplasm (Fig. 2). Caveolae have a specific lipid composition such as cholesterol, glycosphingolipids (GSLs) and other lipids with long straight acyl chains enriched in lipid-modified proteins (Anderson 1998; Singh *et al.,* 2003).

Any endocytic pathway that mediates the transport of a specific cargo will first require mechanisms for selection at the cell surface. However, in the absence of protein adaptors, little is known about how the cargo is selected for caveolae mediated pathways (Doherty and McMahon 2009). Lipid- or protein-based mechanisms have been proposed for deciphering cargo selection, but the potential components within each type of mechanism are only beginning to be analyzed.

Nevertheless, caveolar cargos are indeed diverse. Pharmacological depletion of cholesterol disrupts the integrity of caveolae and inhibits caveolar internalization of several cargos, including albumins, glycosphingolipid analogues, viruses like simian virus-40 (SV40) and echo virus, some integrins, toxins like cholera toxin B (CtxB), and antibodies (Shin and

Abraham 2001; 2001; Ferrari *et al.,* 2003; Fittipaldi *et al.,* 2003; Tsai *et al.,* 2003; Sharma *et al.,* 2005; Singh *et al.,* 2007). However, while interpreting cholesterol depletion, it should be taken into the consideration that other endocytic mechanisms are also cholesterol sensitive, depending on the cell type and the extent of depletion. In contrast, the depletion of glycosphingolipids alone blocks caveolar endocytosis and the effects of such depletion can be restored by specifically incubation with exogenous glycosphingolipids. Therefore pharmacological depletion of glycosphingolipids could be applied as a complementary approach of that of cholesterol (Cheng *et al.,* 2006).

2.3 Macropinocytosis

Macropinocytosis occurs spontaneously in some cells or is triggered in response to growth factor stimulation, such as epidermal growth factor, tumor-promoting factor and macrophage colony stimulating factor (Haigler *et al.,* 1979; Racoosin and Swanson 1989; Swanson 1989; Amyere *et al.,* 2002; Lim and Gleeson 2011). Following the initiation of the reaction, such as receptor binding of growth factors, the actin cytoskeleton underneath the plasma membrane rearranges to drive the formation of large size uncoated macropinosomes. As a consequence, macropinocytosis provides cells with a mode to non-selectively internalize large quantities of solute and membrane (Swanson 2008). In addition to its size, macropinosomes can also easily be identified through the use of fluid phase markers, such as Lucifer Yellow, horseradish peroxidase and dextran (Lim and Gleeson 2011).

Numerous infectious pathogens such as the protozoa, bacteria, viruses and prions exploit macropinocytosis to invade cells (Mercer and Helenius 2008; Swanson 2008; Laliberte and Moss 2009). Pathogens have developed a variety of strategies to modify the host's normal macropinocytic pathway to gain access to the interior of cells. For example, the vaccinia virus mimics apoptotic material and stimulates the formation of large, transient plasma membrane blebs rather than membrane ruffles (Mercer and Helenius 2008; Laliberte and Moss 2009). Prions contain a conserved N- terminal cationic domain which stimulates cellular uptake by lipid raft-dependent macropinocytosis (Magzoub *et al.,* 2006; Wadia *et al.,* 2008).

The field of endocytosis has undergone enormous growth in recent years on the understanding of their roles in the regulation of cell growth and development and the

implications on diseases and pathogens. The challenges remain for the field, such as to get highly specific defined marker in each particular pathway, to effectively evaluate the cross-talks and compensations among pathways, to understand the functions of specific lipids such as cholesterol in each pathway and, to comprehensively interpret how cargoes are selected on physiological as well as pathological conditions. Uniform terminology and well-established methodology for the identification of each pathway are expected to be accomplished in the coming future.

3. UNCOVENTIAL MODE OF CELL PENETRATING PEPTIDES INTERNALIZATION

In the late 1980s, an unexpected phenomenon of protein transduction was reported by Frankel *et al.*. *In vitro* purified Tat protein derived from human immunodeficiency virus 1 (HIV-1) could be taken up by Hela cells and subsequently activate the expression of a downstream reporter gene (Frankel and Pabo 1988; Vives *et al.,* 1997). Two years later, a similar observation was reported by Joliot *et al,* a recombinant peptide corresponding to a sequence of the homeodomain of *Antennapedia* (HD*Antp*) from *Drosophila* could penetrate into mammalian nerve cells and provoke a morphological differentiation of the neuronal cultures (Joliot *et al.,* 1991). Subsequently, analyzing the mechanism of cellular translocation by using mutant versions of HD*Antp*, Le Roux *et al.* claimed that removing two hydrophobic residues Phe48 and Trp49 of the third α-helix could abolish HD*Antp* cellular internalization, which led to the assumption that the third α-helix contains the responsible sequence (Leroux *et al.,* 1993). Further, Derossi *et al.* demonstrated that an inverted sequence or a sequence composed of D-amino acids could still pass through plasma membrane, which strongly suggested that it is internalized by a receptor-independent manner. The amino acid sequence of the third α-helix, RQIKIWFQNRRMKWKK, has then been designated as Penetratin (Derossi *et al.,* 1994; Derossi *et al.,* 1996). Meanwhile, the further investigation on Tat protein showed that truncated Tat protein could deliver beta-galactosidase as a fusion construct into the cytoplasm of multiple tissues after intraperitoneal injection in mice (Fig. 5) (Fawell *et al.,* 1994; Jo *et al.,* 2001). With a systematic screen for translocation ability of Tat protein, it was revealed that the sequence YGRKKRRQRRR is responsible for protein transduction and was designated as TAT (Vives *et al.,* 1997; Brooks *et al.,* 2005).

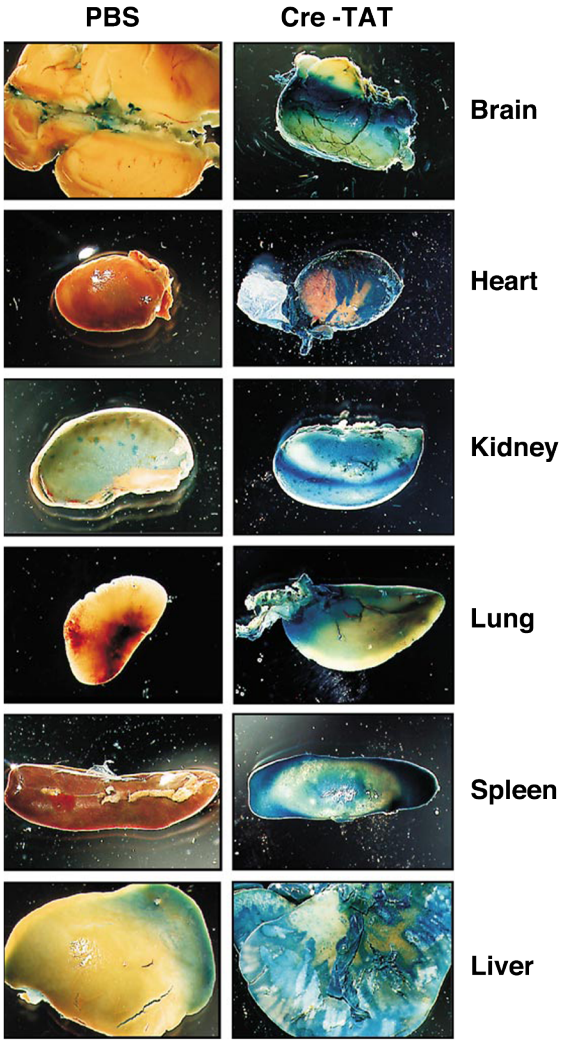


Fig. 5 Transduction of Cre recombinase via TAT in mice. Transgenic mice line in which Cre-mediated recombination activates expression of a -galactosidase reporter gene were injected intraperitoneally on three consecutive days with Cre-TAT or with PBS. Three days after the last injection, the mice were killed, and the organs from control and Cre-injected mice were stained with X-Gal and examined by dark-field microscopy. **Modified from D. Jo 2001.**

3.1 Classification of Cell Penetrating Peptides

TAT and Penetratin show some common properties: they are water soluble, short peptides, highly positively charged at physiological pH, being able to pass through the plasma membrane in a receptor-independent manner (Fischer *et al.,* 2001; Lindsay 2002; Drin *et al.,* 2003; Lundberg and Langel 2003; Deshayes *et al.,* 2005; Kerkis *et al.,* 2006; Heitz *et al.,* 2009; Sawant and Torchilin 2010; Madani *et al.,* 2011). Subsequently, the number of natural or synthetic peptides with cell-penetrating capabilities has continued to grow, and collectively the term Cell Penetrating Peptides (CPPs) has been adopted. A unified terminology and classification of CPPs has not yet been developed, nevertheless two ways of classification had been proposed (Kerkis *et al.,* 2006; Sawant and Torchilin 2010; Madani *et al.,* 2011).

- One of the classifications is based on the origin of the peptide. It includes protein derived CPPs, model peptides, designed CPPs.
  - Protein derived CPPs usually consist of the minimal effective sequence of their parent translocation proteins, and are known also as protein transduction domains (PTD) or membrane translocation sequences (MTS), such as TAT, Penetratin.

- Model peptides are CPPs that imitate the translocation features of known CPPs (Oehlke *et al.,* 1998). Such as model amphipathic peptide (MAP) (KLALKLAL-KALKAALKLA), or the highly positively charged polyarginine (RRRRRRRRRR).
- Designed CPPs are basically mosaic peptides composed of a hydrophilic and hydrophobic domain from different origin, including transportan (GWTLNSAG-YLLGKINLKALAALAKKIL), MPG (GALFLGFLGAAGSTMGAWSQPKKKRK-V) derived from the fusion peptide domain of HIV-1 gp41 protein and the nuclear localization sequence (NLS) of SV40 large T antigen (Simeoni *et al.,* 2003).

❖ The second classification is based on the interaction with the membrane lipids. This class includes primary amphipathic, secondary amphipathic and nonamphipathic.

- Primary amphipathic CPPs, such as transportan, about 20 residues in length, contain sequentially hydrophobic and hydrophilic residues along their entire length.
- Secondary amphipathic CPPs, such as Penetratin, contain few hydrophobic residues, and are shorter than primary amphiphatic CPPs
- Nonamphipathic peptides are rather short CPPs, with a high content of cationic residues such as polyarginine and TAT.

3.2 Applications of Cell Penetrating Peptides

Since the earliest report that truncated Tat protein was able to transport 120 KDa beta-galactosidase protein into the cytoplasm after intraperitoneal injection in mice (Fig. 5) (Fawell *et al.,* 1994; Schwarze *et al.,* 1999), more studies have focused on exploring this property of intracellular delivery (Bogoyevitch *et al.,* 2002). Despite the lack of a complete understanding of the underlying mechanisms, CPPs are currently being manufactured for using as transduction tools in laboratory studies (Lindsay 2002; Khalil *et al.,* 2006). It seems that CPPs as compared to other approaches, such as viruses, liposomes, electroporation and microinjection techniques, have particular advantages as delivery agents (Foged and Nielsen 2008; Veldhoen *et al.,* 2008; Sawant and Torchilin 2010). For instance, CPPs have yielded successful delivery to numerous cell types, including human Jurkat and Hela cell lines where liposomes perform poorly; unlike viral vectors, CPPs do not have the capacity of the genomic integration of the genetic material (Amsellem *et al.,* 2003; Krosl *et al.,* 2003). In contrast to the membrane rupturing techniques of electroporation and microinjection, there is no

evidence for significant membrane perturbation caused by CPPs internalization. Various cargoes have been successfully delivered, including small molecules, peptides and proteins, nanoparticles, polynucleotide, liposomes, and micelles (Kueltzo and Middaugh 2003; Lundberg and Langel 2003; Jarver and Langel 2004; Joliot and Prochiantz 2004; Krosl *et al.,* 2004; Magzoub and Graslund 2004; Trehin and Merkle 2004; Fischer 2007; Yagi *et al.,* 2007). A variety of cell lines and tissues have been targeted *in vitro* and *in vivo*. In the following section, I will discuss each of these applications in further detail.

- **Small-molecule delivery**: Intracellular delivery of small molecules can be boosted by conjugation to CPPs. For instance, an anticancer agent methotrexate demonstrated considerably enhanced cytotoxicity to a breast cancer cell line after conjugation with the peptide YTA2 (Lindgren *et al.,* 2006). Coupling antibiotic doxorubicin to Penetratin enhanced the ability of this anti-cancer drug to bypass the blood-brain barrier (Rousselle *et al.,* 2000).

- **Delivery of protein and peptides**: CPPs have been shown to be effective for delivery of a wide range of peptides and proteins, exhibiting great potential for protein-based therapeutics. TAT has been used for transporting various functional proteins in pathological conditions, such as cancer, inflammatory diseases, oxidative stress-related disorders, diabetes and brain injury. These proteins include anti-apoptotic protein Bcl-xL, anti-inflammatory Ik-Ba mutant protein, neuroprotection protein Hsp70 after cerebral ischemia (Moy *et al.,* 1996; Rousselle *et al.,* 2000; Cao *et al.,* 2002; Kabouridis *et al.,* 2002; Shibagaki and Udey 2002; Ono *et al.,* 2005; Lindgren *et al.,* 2006).
  - CPPs can dramatically increase insulin absorption. Co-administration of insulin with various CPPs enhanced intestinal uptake, as compared to no absorption with insulin alone. Penetratin has been shown to be the most effective promoter of insulin absorption as compared with other CPPs (Kamei *et al.,* 2008; Khafagy *et al.,* 2009).
  - PolyArginine has been fused to four reprogramming factors: Oct4, Sox2, Klf4, and c-Myc, generating protein-induced pluripotent stem cells (piPSCs) from fibroblasts (Kim *et al.,* 2009; Zhou *et al.,* 2009). With this protein transduction method, no concern would be generated by introducing viral vector as carrier comparing with the technique used in the first generation of induced pluripotent stem cells (iPSCs) by

Shinya Yamanaka's team (Takahashi and Yamanaka 2006). In Zhou's protocol, they designed a strategy of treating the cells in four cycles. In each cycle the mouse fibroblasts were first treated overnight with the recombinant reprogramming proteins purified from *E. coli* (Oct4-11R, Sox2-11R, Klf4-11R and c-Myc-11R) with or without 1 mM valproic acid (VPA), a histone deacetylase (HDAC) inhibitor that can significantly improve reprogramming efficiency, then followed by culturing for additional 36 hr without protein feeding and VPA treatment before the next cycle of the treatment. After imposing four repeated protein transductions of reprogramming proteins, the treated cells were kept in mouse embryonic stem cell media (mESC) until colonies emerged around day 30–35. Three positive colonies were obtained per 5×10^4 cells when treated with four proteins in the presence of VPA; and one positive colony obtained per 5×10^4 cells when treated with only three proteins (i.e., Oct4-11R, Sox2-11R, and Klf4-11R) in the presence of VPA; and no colony was obtained when only feeding with reprogramming proteins. In Kim's protocol, they generated stable HEK293 cell lines that could express each of the four human reprogramming factors fused with 9R and the myc tag. Using un-purified cell extracts from HEK293 to feed human newborn fibroblasts (HNFs), after six repeated cycles of 16 hr protein feeding treatment followed by 6 day incubation in ES media, five hiPSC like colonies were obtained per 5×10^5. Compared to virus-based protocols (about 0.01% of input cells), the efficiency of hiPSC generation is significantly lower using this protein based protocol (about 0.001% of input cells).

- **Nanocarrier:** CPPs-mediated delivery of different nanocarriers such as nanoparticles, liposomes, micelles, dendrimers, has been reported in the literature (Liu *et al.,* 2000). Dextran-coated cross-linked iron oxide nanoparticles (CLIO) (41 nm) could be coupled to TAT and efficiently delivered into cells, therefore could serve as a tool for tracking progenitor cells by magnetic resonance imaging (MRI) *in vivo* (Josephson *et al.,* 1999; Lewin *et al.,* 2000). Shell cross-linked (SCK) nanoparticles can also be conjugated to TAT to be used as a scaffold for the preparation of antigens for immunization (Liu *et al.,* 2000; Liu *et al.,* 2001; Becker *et al.,* 2004; Torchilin 2007). TAT conjugated FITC-doped silica nanoparticles (FSNPs) can be used for bioimaging purposes (Santra *et al.,* 2004; Santra *et al.,* 2005). Both liposomes and micelles can be

easily modified with CPPs to enhance their delivery efficiency (Torchilin *et al.,* 2003; Becker *et al.,* 2004; Santra *et al.,* 2005; Cryan *et al.,* 2006; Yoo *et al.,* 2006; Yagi *et al.,* 2007).

- **Delivery of nucleic acids:** CPPs have also been successfully used to enhance the delivery of nuclei acids including microRNA, siRNAs for gene regulation at mRNA level. CPPs-mediated delivery achieved both increased efficiency of delivery and of the sustained function of the cargo, when compared with liposomal delivery of the same cargo (Torchilin *et al.,* 2003; Chiu *et al.,* 2004; Muratovska and Eccles 2004; Davidson *et al.,* 2005; Veldhoen *et al.,* 2008).

3.3 Mechanisms underlying internalization of Cell Penetrating Peptides

Despite the common cationic feature of CPPs, it is believed that the translocation mechanism is not the same for different groups of CPPs. Nevertheless, two mechanisms of internalization have been proposed: direct penetrating through the lipid bilayer, and endocytosis (Richard *et al.,* 2003; Vives 2003; Fischer *et al.,* 2004; Zhang and Smith 2005; Zorko and Langel 2005; Henriques *et al.,* 2006; Duchardt *et al.,* 2007; El-Andaloussi *et al.,* 2007; Amand *et al.,* 2008; Yesylevskyy *et al.,* 2009).

3.3.1 Direct penetration

Direct penetration is an energy-independent mode, which may consist of distinct underlying physical mechanisms, including inverted micelle formation, pore formation, and the carpet-like model. The first step in all these mechanisms may require the interaction of the positively charged CPPs with the negatively charged components of the plasma membrane such as heparan sulfate, resulting in the transient destabilization of the membrane (Gerbal-Chaloin *et al.,* 2007; Payne *et al.,* 2007). The subsequent mechanism of internalization depends highly on the peptide concentration, peptide sequence, and lipid composition in the various model membrane studies. Generally, direct penetration occurs at high CPPs concentrations (Fischer *et al.,* 2000; Thoren *et al.,* 2000; Fretz *et al.,* 2007; Futaki *et al.,* 2007).

- **Pore formation**: Using giant unilamellar vesicles (GUVs) as model membrane, CPPs

permeabilize membranes by generating topologically active saddle-shaped membrane curvature, the type of curvature necessary for "hole" formation in a membrane (Mishra *et al.,* 2011). Pores appear when the concentration of the peptide exceeds a certain threshold, which may be different for different peptides.

- **Inverted micelle formation**: when the attractive potential between the peptide and lipid heads is strong, an inverted micelle is formed to minimize potential energy of the peptide, causing the peptide move from the outer layer to the inner layer across inverted micelle membrane (Kawamoto *et al.,* 2011).
- **Carpet-like model:** interactions between acidic phospholipids and the cationic CPPs result in a thinning of the membrane thus facilitating CPPs passing through plasma membrane (Pouny *et al.,* 1992; Salay *et al.,* 2011). Subsequent translocation of the CPPs is achieved when the CPPs concentration is above a certain threshold concentration (Alves *et al.,* 2010).

3.3.2 Endocytosis

Earlier studies had suggested that direct penetration was the uptake mechanism for the most CPPs. This conclusion was based on the observation that peptides enter the cell even at 4◦C, therefore, by an energy-independent route. Later studies showed that experimental artifacts were responsible for this conclusion. Using methanol or formaldehyde to fix the investigated cells for microscopy causes artificial dye penetration during fixation by damaging the integrity of the plasma membrane (Richard *et al.,* 2003). Nowadays by using trypsine to remove peptides associated with outside of the plasma membrane for fluorescence-activated cell sorting (FACS), we can overcome this problem. Now it is generally concluded that endocytosis is involved in the internalization process (Vendeville *et al.,* 2004; Wadia *et al.,* 2004). Direct penetration is more probable for primary hydrophobic CPPs at high concentrations, whereas endocytosis is the main uptake mechanism at low concentrations (Zaro *et al.,* 2006). The concentration threshold for direct penetration varies between different CPPs, different cell lines, and the presence of and the type of cargo (Jiao *et al.,* 2009; Khafagy *et al.,* 2009).

- **In clathrin-mediated endocytosis (CME),** a specific ligand-receptor interaction is

required for regulating cargo uptake through clathrin-coated pits and vesicles. Several cationic CPPs can facilitate the internalization of CME associated receptors on the cell surface without displaying specificity, and subsequent co-transfer of CPPs in the same vesicles can be observed. Fotin-Mleczek thus claimed that these cationic CPPs mediate receptor internalization through clathrin-coated vesicles (Fotin-Mleczek *et al.,* 2005). Direct evidence of the interaction between these receptors and CPPs as cargo requires further investigation. Along the same line, negatively charged heparan sulfate have been suggested to act as unspecific membrane receptors for positively charged CPPs and facilitate their internalization via clathrin-mediated endocytosis (Richard *et al.,* 2005).

- **Regarding caveolae-mediated endocytosis**, little is known about how the cargo is selected for the internalization due to lacking specific adaptor proteins and accessory proteins. A few papers showed that Tat-fusion proteins could co-localize with caveolin-1 in living cells (Ferrari *et al.,* 2003). Alternatively, the use of lipid raft disrupting agents could also provide the valuable information to assess how CPPs gain access to the cytoplasm of cells via caveolae-mediated endocytosis. By this approach, Fittipaldi claimed that the Tat-fusion protein takes the advantage of this endocytic pathway to a certain extent (Fittipaldi *et al.,* 2003).
- **Macropinocytosis** has also been implicated in the uptake process of different CPPs-cargo constructs. The interaction between CPPs-protein complexes and the heparan sulfate on the plasma membrane activates Rac1 and causes underlying actin remodeling, thus inducing macropinocytosis (Wadia *et al.,* 2008). The role of macropinocytosis in the uptake process of CPPs-protein complexes can be assessed by the use of amiloride or 5-(N-Ethyl-N-isopropyl) amiloride (EIPA), i.e. the Na^+/H^+ pump inhibitors, which are widely used as selective inhibitors of macropinocytosis (Vercauteren *et al.,* 2010). Macropinosomes can also easily be identified through the use of fluid phase markers, such as Lucifer Yellow, horseradish peroxidase and dextran (Lim and Gleeson 2011). TAT and polyarginine fused proteins have been repeatedly reported to be internalized *via* macropinocytosis (Magzoub *et al.,* 2006).

Identifying the specific internalization pathways used by CPPs or CPPs-cargo complexes has proven to be a difficult task, and contradictory or inconsistent results have

been reported regarding it. This is partially due to several observations such as varied threshold concentration for each peptide to induce direct penetration, utilizing multiple endocytic routes in parallel by some of peptides, and compensation pathways induced by down-regulation or inhibition of specific entries (Duchardt *et al.,* 2007; El-Andaloussi *et al.,* 2007). However, as underlying mechanisms confer the potential of the development CPPs for therapeutic purpose in the perspective of security and efficiency in the long term, great efforts need to put into the mechanistic investigations.

4. IMAGINAL DISC DEVELOPMENT IN *DROSOPHILA*

The *Drosophila* life cycle consists of three distinct phases, during each of which its body structure is specialized for a particular behavior. The larva hatches from an egg with little physical resemblance to the adult that it will produce (Fig. 6). While the larva spends most of its time crawling in the food, the highly mobile adult fly can enjoy the freedom in the air. The question of how these contrasting behaviors have been achieved during metamorphosis seems very puzzling at a glance, but taking a close look at the histology of *Drosophila* larva can reveal this myth. This physical transformation is practically solved by allowing the co-organization and co-development of larval and adult structures simultaneously during embryogenesis. The primordia of the adult structures will be segregated and undergo a distinct growth from the larval structures, in a way, the larva functions like a “host” for the adult tissue.

The adult appendages, such as antennae, wings, legs and so on, are derived from sac-like clusters of primordial cells known as imaginal discs. The adult insect is called “imago”, therefore the precursors of the adult structures are referred to as ”imaginal” tissue (Lewis and Engelman 1983). The imaginal discs develop into the adult appendages and the portion of the body wall where they are located. Different imaginal discs have their particular size and shape, and are named after the adult structure they form: wing disc, leg disc, eye-antennal disc, genital disc and so forth. The location of the 19 imaginal discs in the third instar larva is illustrated in Fig. 7A. *Drosophila* as one of the most widely studied model organism, the development of its appendages provides a stunning subject for studying the molecular mechanisms underlying organogenesis (Cohen *et al.,* 1991).

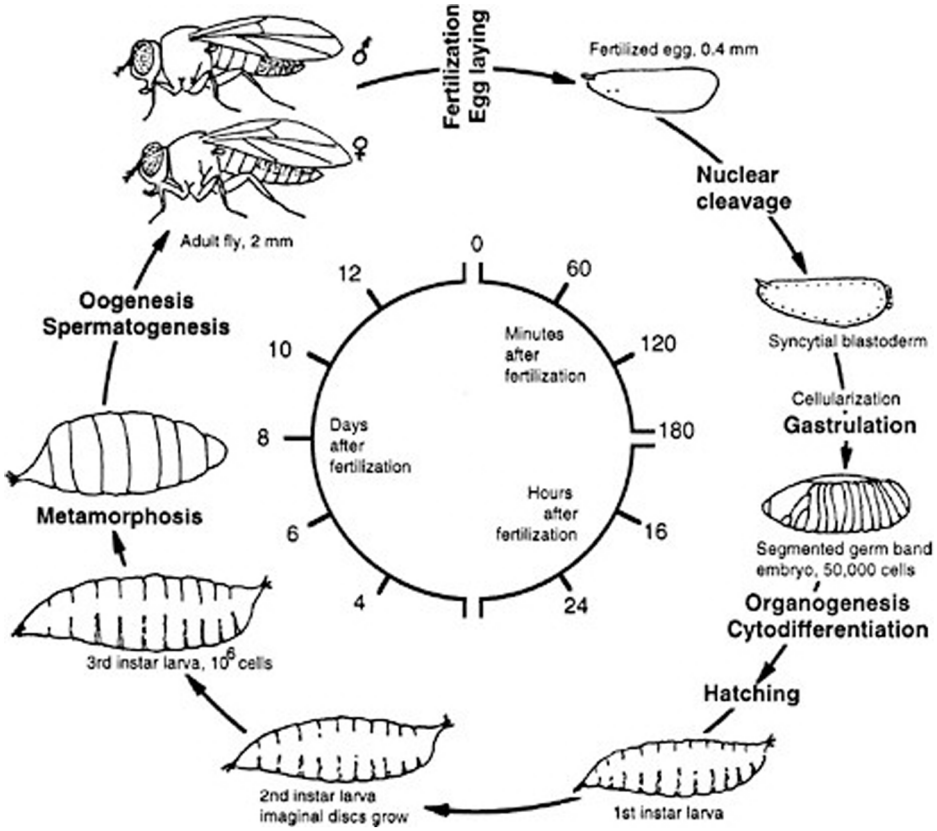


Fig. 6 Life cycle of *Drosophila melanogaster*. The larva hatches 1 day after the egg is fertilized. First, second, and third instar are larval stages, each ending with a molt. During pupation most of the larval tissues are destroyed and replaced by adult tissues derived from the imaginal discs that were growing in the larva. Times are given for the life cycle at 25°C. **Adapted from L. Wolpert 1998.**

4.1 Specifying imaginal discs along the body plan

In *Drosophila*, the body plan along the antero-posterior (A/P) axis is specified by ordered homeotic gene clusters. These genes are arranged along the chromosome in the same sequence as they are expressed along the A/P axis. It is a universal principle underlying animal development, which is designated as the "colinearity rule" (COL) (Lewis 1978). There are two separated clusters of tightly linked genes located on the 3rd chromosome in fly genome: the *Antennapedia* Complex (ANT-C) which contains five homeotic genes (*labial/lab, proboscidea/pb, Deformed/Dfd, Sex combs reduced/Scr, Antennapedia/Antp*), and the *Bithorax* Complex (BX-C) which contains three (*Ultrabithorax/Ubs, abdominal-A/abd-A, Abdominal-B/Abd-B*). The only homeotic gene that does not fall into one of these clusters is *caudal* (*cad*), which is responsible for analia development. ANT-C names after an *Antp* mutation which converts antennae to legs, and BX-C named after a mutation which converts

halteres to wings. In the absence of homeotic gene activity, all segments convert to the mesothoracic segment (T2), a sort of 'ground' state; no morphological diversity is generated along the A/P body axis (Gehring *et al.,* 2009). The ANT-C and BX-C genes encode transcription factors that contain a 180-base-pair homeobox encoding for a helix-turn-helix DNA-binding domain (Mcginnis *et al.,* 1984; Billeter *et al.,* 1993). *Drosophila* also has homeobox-containing genes outside the homeotic gene clusters. There are more than 100 homeobox-containing genes in the *Drosophila* genome (Richard *et al.,* 2003), and some of these play an essential role in specifying appendage identity (Morata 2001; Vercauteren *et al.,* 2010).

Drosophila appendages and antennae fall into two categories anatomically: the ventral ones, exemplified by the leg, and the dorsal ones, exemplified by the wing. Programming each appendage development, it requires the combination of a segment-specific function from homeotic genes and a ventral or dorsal property provided by the homeobox gene *Distalless* (*Dll*) and the regulatory gene *vestigial* (*vg*), respectively (Fig. 7B,C).

Dll and its homologues have a basic function in the formation of body outgrowths throughout the animal kingdom. In principle, all segments have the potential to develop appendages: in the absence of the BX-C genes, which repress *Dll* transcription, *Dll* is activated in an equivalent site in all abdominal segments and appendage primordia are formed. This fits nicely with the idea that insects derive from multilegged ancestors, and subsequently lost legs in their abdominal segments. Ectopic expression of *Dll* in dorsal discs induces the formation of distinct ventral appendage structures depending on the pattern of homeotic gene expression (Fig. 7C) (Gorfinkiel *et al.,* 1997).

Specifying the identity of the dorsal appendages seems to follow similar rules. *vg* seems to be an analogue to that of *Dll* in the ventral appendages. Its activity is necessary for the formation of wings and halteres and its ectopic expression induces wing or haltere tissue depending on the combination with homeotic genes (Fig. 7C) (Morata 2001).

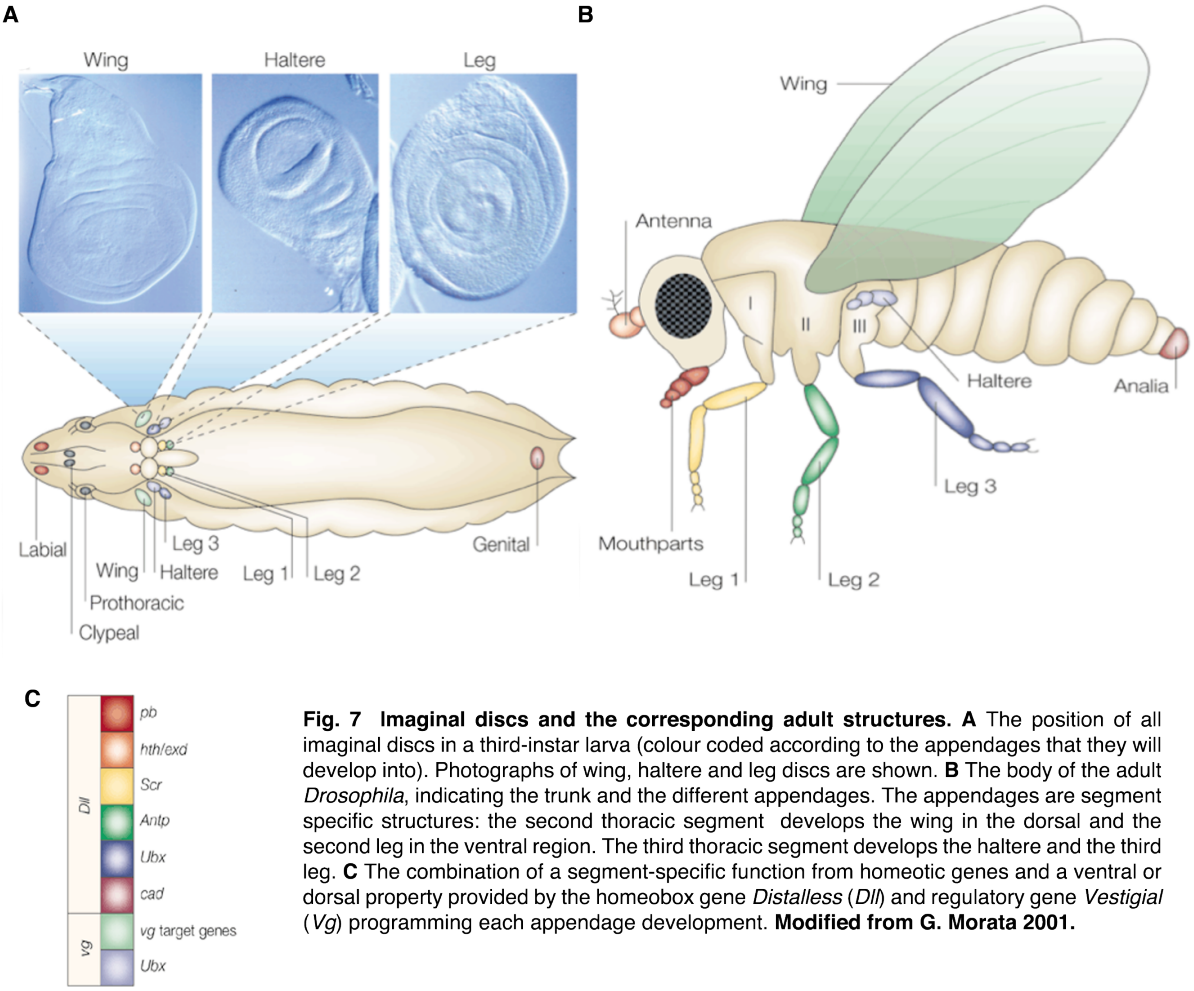


Fig. 7 Imaginal discs and the corresponding adult structures. A The position of all imaginal discs in a third-instar larva (colour coded according to the appendages that they will develop into). Photographs of wing, haltere and leg discs are shown. **B** The body of the adult *Drosophila*, indicating the trunk and the different appendages. The appendages are segment specific structures: the second thoracic segment develops the wing in the dorsal and the second leg in the ventral region. The third thoracic segment develops the haltere and the third leg. **C** The combination of a segment-specific function from homeotic genes and a ventral or dorsal property provided by the homeobox gene *Distalless* (*Dll*) and regulatory gene *Vestigial* (*Vg*) programming each appendage development. **Modified from G. Morata 2001.**

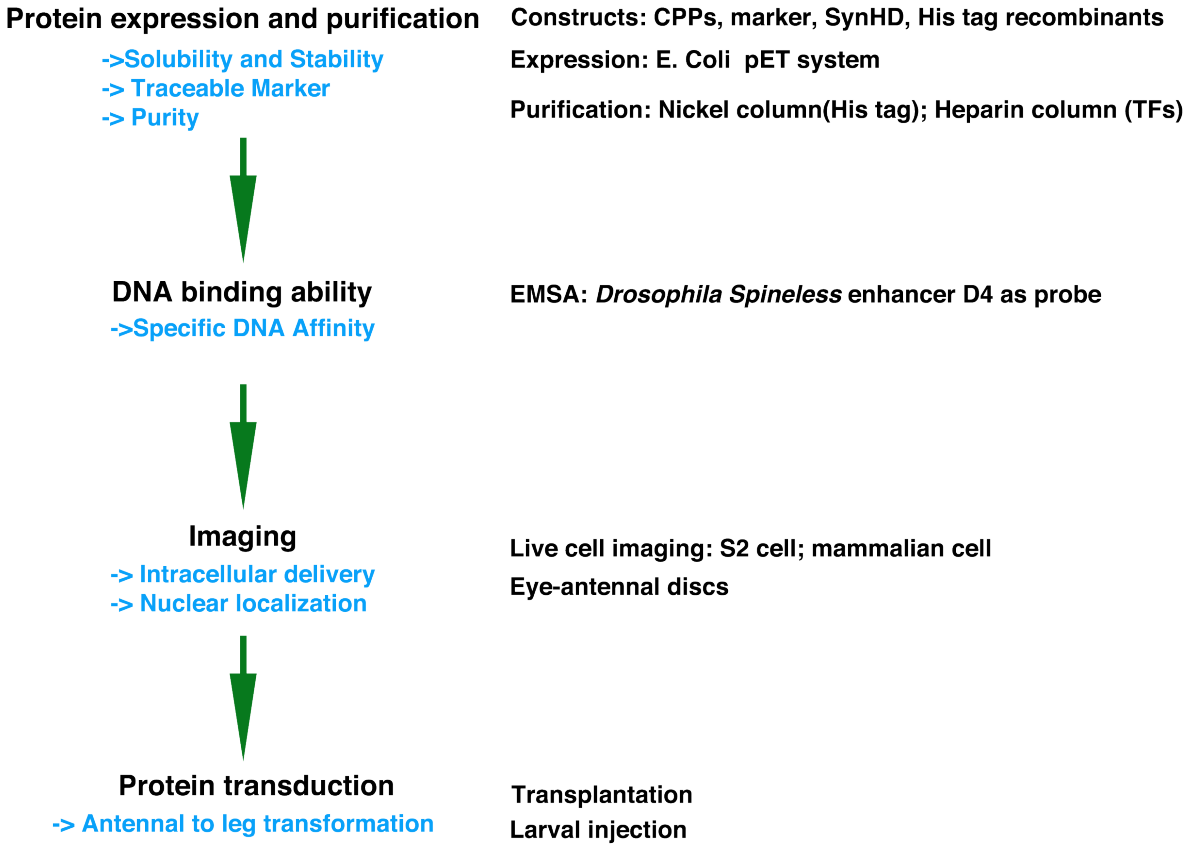
4.2 Molecular mechanisms underlying leg discs development

- **A/P compartment:** To appreciate how imaginal discs develop in the perspective of three dimensional structure, it is very important to establish the concept of compartmentalization. The concept of compartments was first discovered in the wing disc (Garcia-Bellido *et al.,* 1973), but it is the basic mechanism for all imaginal discs development. The leg disc is a cluster of about 30 cells at blastoderm stage but it grows to over 10,000 cells forming a sack-like structure by the third instar. At the first step in patterning, the anterior and posterior compartment will be developed from two distinct cell lineages. *engrailed* (*en*) as a classical selector gene specifies the identity of the posterior compartment cells and induces the expression of *hedgehog* (*hh*). The *Hh* protein induces a signaling region at the antero-posterior compartment boundary. In the dorsal region of the leg disc, the expression of *decapentaplegic* (*dpp*) is induced

along the antero-posterior boundary by *Hh*; in the ventral region, however, *Hh* induces expression of *wingless* (*wg*) instead of *dpp* along the boundary (Minami *et al.,* 1999).

- **Proximo-distal axis:** *Drosophila* legs are essentially jointed tubes of epidermis arranged like an old-fashioned telescope, one can imagine it as a series of concentric rings with the outmost ring giving rise to the base of the leg, and those nearer the center forming the more distal structures. *Dll* is expressed in the most distal region, while *homothroax* (*hth*), is expressed in the peripheral region surrounding *Dll*. *Hth* needs to bind to another protein *Extradentical* (*Exd*) as dimmer to play the function as transcription factor. Their actions lead to the expression of *dachshund* (*dac*), which also encodes a transcription factor, in a ring between *Dll* and *hth*, overlapping with *Dll* and *hth* expression's area, respectively. Each of these genes is required for the formation of particular leg regions, but the expression domains do not correspond to femur, tibia, and proximal tarsus.
- **Antenna to leg transformation**: Some mutations of several genes in *Drosophila* cause transformations of antenna toward second leg (Schneuwly *et al.,* 1986; Schneuwly *et al.,* 1987), and the best known of these mutations are dominant gain-of-function alleles of *Antp*; alternatively, ectopic expression *Antp* in antennal disc during the later 2nd instar or early 3rd instar can induce antenna to leg transformation (Gehring and Hiromi 1986). Underlying molecular mechanisms of this specific transformation can be easily explained by the regulation of *spineless* (*ss*), which is a determinant of antenna development. The *ss* enhancer, which has been identified by Duncan *et al.* recently, could be activated either by *Dll*, or by dimer *Hth*/*Exd*, or by trimmer *Hth*/*Exd*/*Dll*, but be repressed by *Antp* (Fig. 9). When *Antp* ectopically is expressed in antennal disc, it turns off the program of antennal development by repressing *ss*, therefore the ground state of leg program will subsequently dominate the development of targeted antennal discs, resulting in antenna-to-leg transformation.

5. WORK FLOW SHEET OF PROTEIN TRANSDUCTION



Regarding work flow, there are four experimental phases: protein expression and purification, *in vitro* binding functional assay, living imaging and *ex vivo* functional assay or protein transduction.

❖ Protein expression system: pET/Rosetta™ 2(DE3)*pLysS*

The pET System is the most powerful system developed for the cloning and expression of recombinant proteins in *E. coli*. Target genes are cloned in pET plasmids under control of strong bacteriophage T7 transcription and translation signals, and the expression of target genes is induced by providing a source of T7 RNA polymerase in the host cell. T7 RNA polymerase is so selective and active that almost all of the cell's resources are converted to target gene expression; the desired product can comprise more than 50% of the total cell protein a few hours after

induction. In the un-induced state, this system has the ability to maintain target genes transcriptionally silent, which is a big advantage to express potentially toxic proteins to the host cell. DE3 indicates that the host cell is a lysogen of λDE3, carrying a chromosomal copy of the T7 RNA polymerase gene under control of the lacUV5 promoter. The lacUV5 promoter is a mutated version of the lac promoter whose basal activity is dramatically less sensitive to intracellular levels of cyclic AMP molecules. Cyclic AMP is known to participate in activation of the lac promoter, and its level correlates with increasing densities of bacterial culture. Therefore, the lacUV5 promoter is less sensitive or less "leaky¨ at higher culture densities, which significantly enhance repression at this promoter and improve expression yields of particularly toxic target genes. *pLysS* strains express T7 lysozyme, which further suppresses basal expression of T7 RNA polymerase prior to induction, thus stabilizing pET recombinants encoding target proteins that affect cell growth and viability. Rosetta™ 2 host strains are BL21 derivatives designed to enhance the expression of eukaryotic proteins that contain codons rarely used in *E. coli*. These strains supply tRNAs for 7 rare codons (AGA, AGG, AUA, CUA, GGA, CCC, and CGG) on a compatible chloramphenicol-resistant plasmid. And the tRNA genes are driven by their native promoters. Rosetta™ 2(DE3)*pLysS* is one of the host strains with the highest stringent regulation on protein expression. Such strain is suitable for production of protein from target genes cloned in pET vectors by induction with IPTG. Genotype of Rosetta™ 2(DE3)*pLysS*: F- ompT hsdSB(rB- mB-) gal dcm (DE3) *pLysS* RARE2 (CamR).

❖ Protein purification I : Immobilized metal ion affinity chromatography

Histidine-tagged proteins have a high selective affinity for Ni^{2+} and several other metal ions, which can be immobilized on chromatographic media via chelation. Proteins containing histidine tag bing to metal-ion-charged media strongly while other cellular proteins do not bind or bind weakly. Practically, the binding buffer contains high concentration salt (e.g. 0.5 M NaCl), which can largely reduce unspecific affinities from electrostatic interactions and hydrophobic interactions. This chromatographic technique is often termed immobilized metal ion affinity chromatography (IMAC). Imidazole is used to elute the proteins bound to metal-ion-

charged media. An excess of imidazole is passed through the media, which displaces the his-tagged protein from beads or column. Moreover, his-tag is small and generally less disruptive than other tags to the properties of the proteins on which they are attached.

- Protein purification II: Heparin column

  The native glycosaminoglycan of porcine intestinal mucosa are extracted to covalently link to agrose beads for making heparin sepharose. The nature of negative charged glycosaminoglycan makes it a very versatile tool for the separation of many proteins e.g. DNA binding proteins, growth factors and steroid receptors. We apply heparin column purification after IMAC to remove imidazole and for polishing of the recombinant protein containing homeodomain.

- Traceable marker and live imaging: mCherry fluorescent protein tagging

  Fluorescent proteins are genetically encoded, easily imaged markers that are widely used in biology and biotechnology. mCherry is the second generation of monomer version of "red" fluorescent protein derived from tetrameric ''DsRed'' discovered in *Discosoma sp*. (Gross *et al.,* 2000). mCherry matures extremely rapidly, making it possible to visualize it very soon after activating transcription, with several other advantages such as being highly photostable and resistant to photobleaching (Shaner *et al.,* 2004).

- *In vitro* binding property of recombinant transcription factors: EMSA

  An electrophoretic mobility shift assay (EMSA) or mobility shift electrophoresis, also referred to as a gel shift assay, gel mobility shift assay, or gel retardation assay, is a common affinity electrophoresis technique used to study protein-DNA or protein-RNA interactions. In *Drosophila,* the homeobox-containing protein *fushi tarazu* (*ftz*) bings to cis-regulatory elements upstream of *engrailed* (*en*) gene to regulate its expression, during the establishment of the segment polarity. EMSA and DNase I protection assays have shown that an *EcoR* I - *Cla* I restriction fragment that maps -2.3 kb to -0.95 kb upstream of the *en* transcription initiation site contains three *ftz* binding sites, which are designated BS1, BS2 and BS3 (Muller *et al.,* 1988). BS2 has

been the most widely used probe to inspect the binding property of *Antp* homeodomain in EMSA assay ever since. However, recently, Duncan *et al.* has isolated *spineless* (*ss*) enhancer in *Drosophila* (Duncan *et al.,* 2010). Compared to *en* enhancer, *ss* enhancer is the *in vivo* binding site of *Antp*, and the interaction between *Antp* and other transcription co-factors on *ss* enhancer specify leg or antenna programming. *ss* enhancer contains a very conserved domain called D4, consisted of 62 base pairs of DNA, whic can be specifically recognized by full-length *Antp* and other transcription factors such as *Homothorax*/*Extradentical* (*Hth/Exd*) (Fig. 9). Therefore, D4 is the probe which has been used in the EMSA in this thesis.

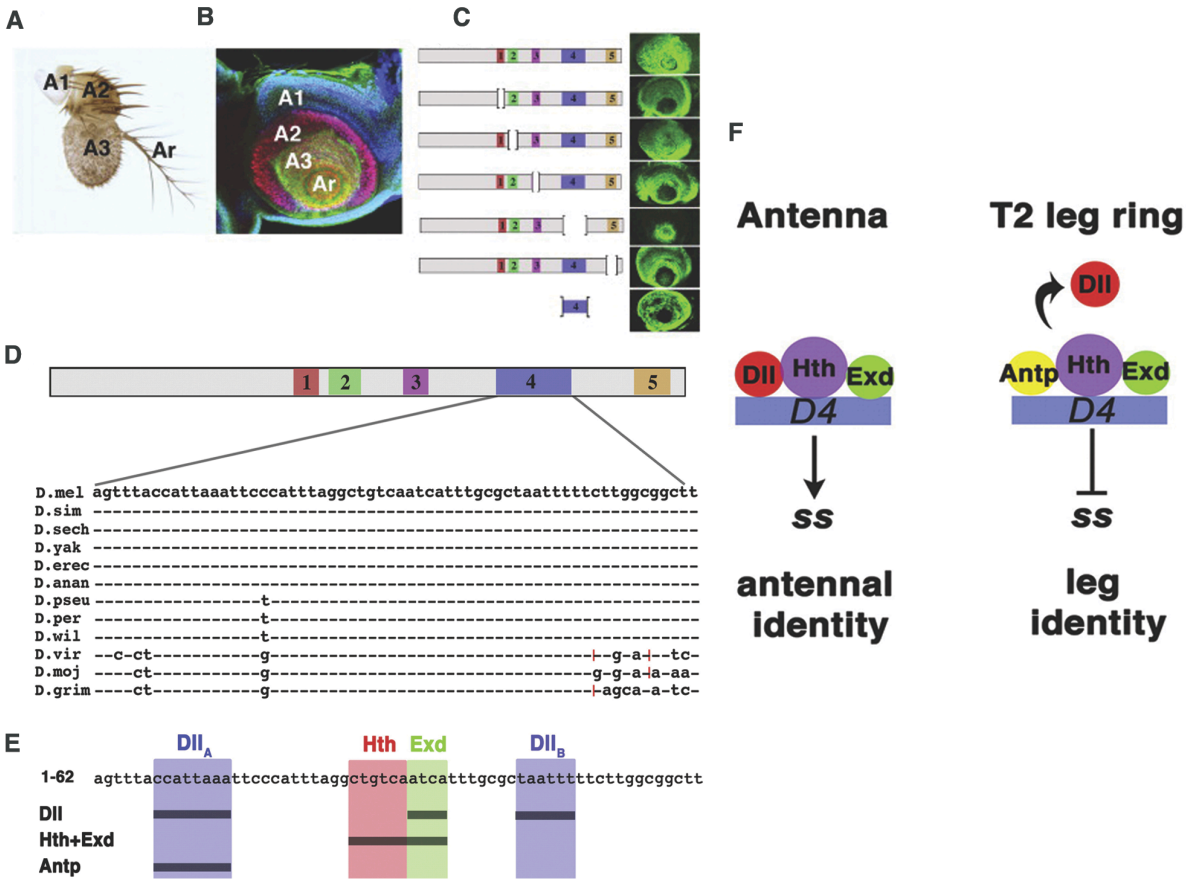


Fig. 9 Characteristics of D4 enhancer. A In wild-type adult antenna, the first (A1), second (A2), and third (A3) antennal segments and the arista (Ar) are indicated. **B** An antennal disc stained for *Hth* (blue), *Dll* (red), and *Ss* (green). *Hth* is expressed in the primordia of A1, A2, and A3; *Dll* is expressed in A2, A3, and the arista; and Ss is expressed in A3 and the arista. **C** Five conserved domains within *Ss* enhancer, and the expression pattern in each deletion derivatives are indicated. **D** Conservation of the sequence of D4 in 12 *Drosophila* species. **E** Binding sites of *Hth/Exd*, Dll and *Antp* on D4. **F** Model summarizing the control of D4 in the antenna and leg. Left: in the antenna, D4 is activated by binding of a *Dll/Hth/Exd* complex. Right: in the proximal ring of the leg, *Antp* displaces *Dll* and prevents activation of the enhancer. **Modified from Duncan et al., 2010.**

❖ Method for transplanting *Drosophila* imaginal discs

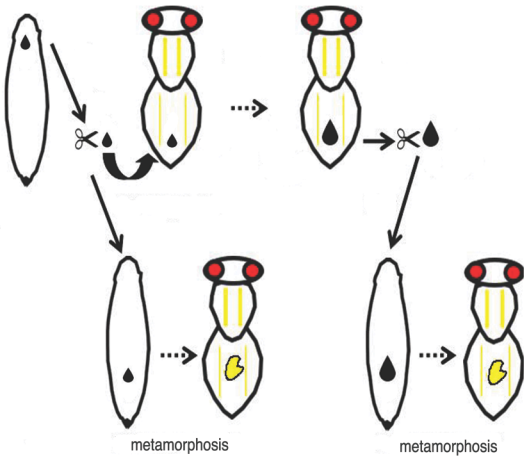


Fig. 10 Transplantation in *Drosophila* The disc after protein feeding can be transplanted either into the body cavity of a larval host where it differentiate into adult structure during metaphorphosis, or into the abdominal cavity of a fertilized adult female where the disc cells continue to proliferate. For the latter, disc implant will be isolated and transplanted into the body cavities of larva host where it undergoes metaphosis and differentiate into adult structure. **Modified from Hadorn, 1965.**

Eye-antennal discs are dissected from a later second instar larva and incubated with Chan-Gehring medium containing transduction proteins. One route is followed as the disc is transplanted into the body cavity of a larval host where it will undergo metamorphosis with host larva and differentiate adult structures (Fig. 10). The other route is that the disc is transplanted into the abdominal cavity of a fertilized adult female host to continue to proliferate in an undifferentiated state, and then the disc implant will be isolated and transplanted into the body cavities of larva host, where they will metamorphose and differentiate into adult structure (Fig. 10).

Manuscript I

Macropinocytic stimulation of Antennapedia homeodomain: a comparative study revealing its internalization differing from Penetratin

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Introduction

One of the most fundamental features of living organisms is that cells are separated from their external environment by a thin, but highly complex plasma membrane constituted of a lipid bilayer (Overton 1899). Although, the lipid bilayer is only a few nanometers in width (5 - 10 nm), it is impermeable to most molecules apart from small hydrophobic ones (Lewis and Engelman 1983). The ability of small molecules to diffuse through a lipid bilayer is related to their lipid solubility. Surprisingly, some recombinant proteins generated from E. *coli*, such as Tat derived from human immunodeficiency virus-1 (HIV-1) and the homeodomain of *Antennapedia* (HD*Antp*) from *Drosophila*, can gain free access to the cytoplasm of mammalian cells, in a receptor-independent manner. This phenomenon has attracted a lot of attention and it has been intensively investigated since its discovery more than two decades ago (Frankel and Pabo 1988; Joliot *et al.,* 1991).

While the underlying cellular internalization mechanism remains controversial, the most widely accepted hypothesis proposed the existence of a specific transduction domain (TD) within each of these naturally occurring proteins that is responsible for transporting its parent cargo protein across the plasma membrane (Derossi *et al.,* 1994). For instance, the third α-helix of the HD*Antp* corresponding to residues 43-58, was suggested to be a TD, and its capability of plasma membrane translocation could not be abolished by using the inverted

sequence or introducing D-amino acids into the peptide (Derossi *et al.,* 1996). Thus, the third α-helix of HD*Antp* has been designated as Penetratin.

Penetratin and other TDs share some common features; they are short peptides of less than 20 amino acids, highly positively charged, have the ability of intracellular delivery of various cargos when conjugated or even in the unconjugated mode (Sawant and Torchilin 2010; Madani *et al.,* 2011). Based on these characteristics, more sequences have been designed and tested for translocation ability, resulting in a quickly expanding family of peptides. Collectively, the term Cell-Penetrating Peptides (CPPs) has been coined to refer to those transduction domains and to the newly emerging peptides capable of penetrating cells (Heitz *et al.,* 2009).

Recent reports show that engineered supercharged GFP (Cronican *et al.,* 2010) and other naturally supercharged human proteins (Cronican *et al.,* 2011) with a ratio of charge units per kDa greater than 0.75 could deliver mCherry and other functional proteins with significantly higher efficiency than the conventional CPPs including TAT (TD of Tat protein), Penetratin and oligoarginine both in *vitro* and *in vivo*. Since HD*Antp* has a remarkably high ratio of positive charges per kDa i.e. 1.54 (Fig. 1A), this raises the question of whether the translocation capacity of Penetratin could truly represent that of its parent HD*Antp* protein.

Early studies based on Penetratin proposed that it enters cells by an energy-independent mode, directly transversing through the lipid bilayer (Derossi *et al.,* 1996). However these finding were contradicted by other studies suggesting the involvement of endocytosis in cellular translocation (Jiao *et al.,* 2009; Alves *et al.,* 2010). Here, we demonstrate that the *Antennapedia* homeodomain embraces much higher capacity of cellular translocation than its TD Penetratin, moreover, macropinocytosis plays a major role in the internalization of both macromolecules.

By re-evaluating the internalizations of both HD*Antp* and Penetratin, we found that HD*Antp* delivered mCherry with up to 5-fold greater efficiency than Penetratin into several mammalian cell lines, as well as into *Drosophila* S2 cells. We found that endocytic pathways play a major role in HD*Antp* internalization since cold treatment severely inhibited most cellular uptake. Based on the finding that EIPA, an inhibitor of the Na^+/H^+ exchanger, could decrease the cellular uptake most efficiently among the various pharmacological inhibitors

tested, and that intracellular HD*Antp* co-localizes with fluid phase dyes, we conclude that macropinocytosis, which is a form of non-coated endocytosis, plays a major role in both HD*Antp* and Penetratin internalization. We propose that the *Antennapedia* homeodomain stimulates macropinocytic entry compared to its TD Penetratin via the same endocytic route, but with higher efficiency. Macropinocytosis is an efficient route for nonselective endocytosis of solute macromolecules (Swanson 2008) and, therefore has important therapeutic implications for intracellular delivery of hydrophilic drugs (Lim and Gleeson 2011). Hence, our findings may have important implications for medical applications of protein-based therapeutics.

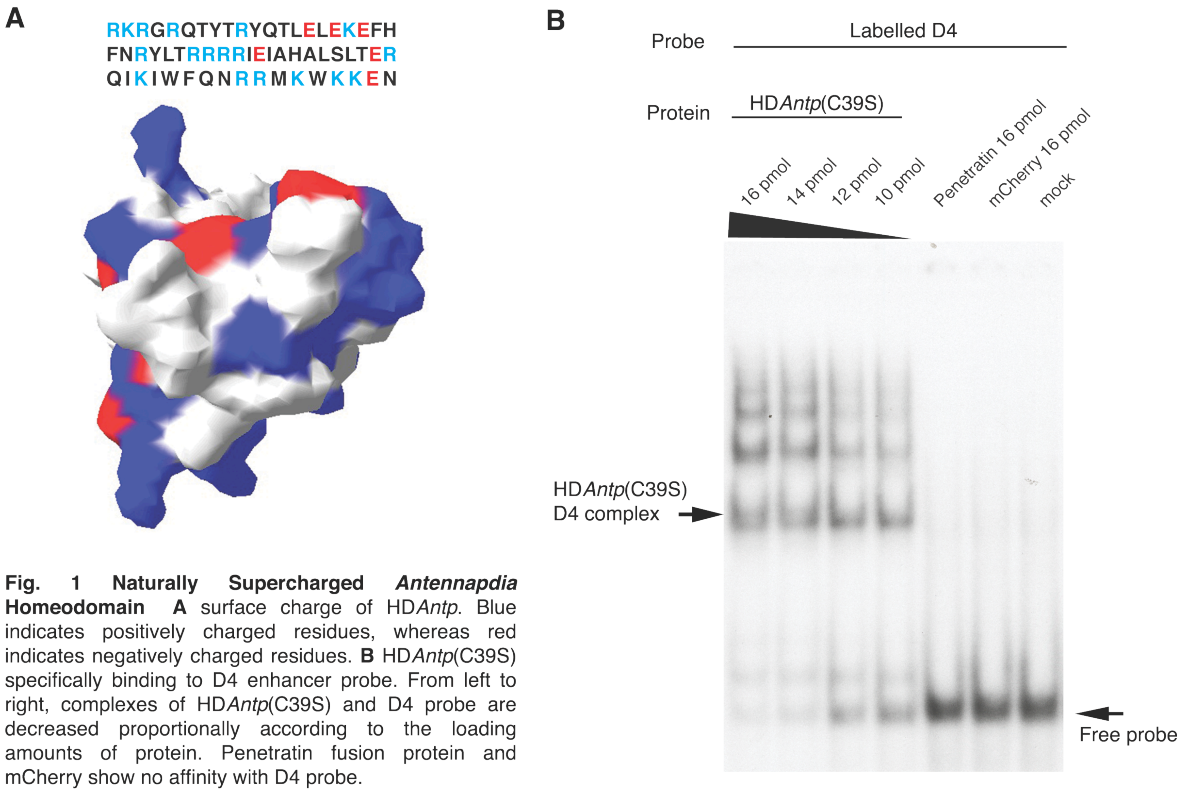


Fig. 1 Naturally Supercharged *Antennapdia* Homeodomain **A** surface charge of HD*Antp*. Blue indicates positively charged residues, whereas red indicates negatively charged residues. **B** HD*Antp*(C39S) specifically binding to D4 enhancer probe. From left to right, complexes of HD*Antp*(C39S) and D4 probe are decreased proportionally according to the loading amounts of protein. Penetratin fusion protein and mCherry show no affinity with D4 probe.

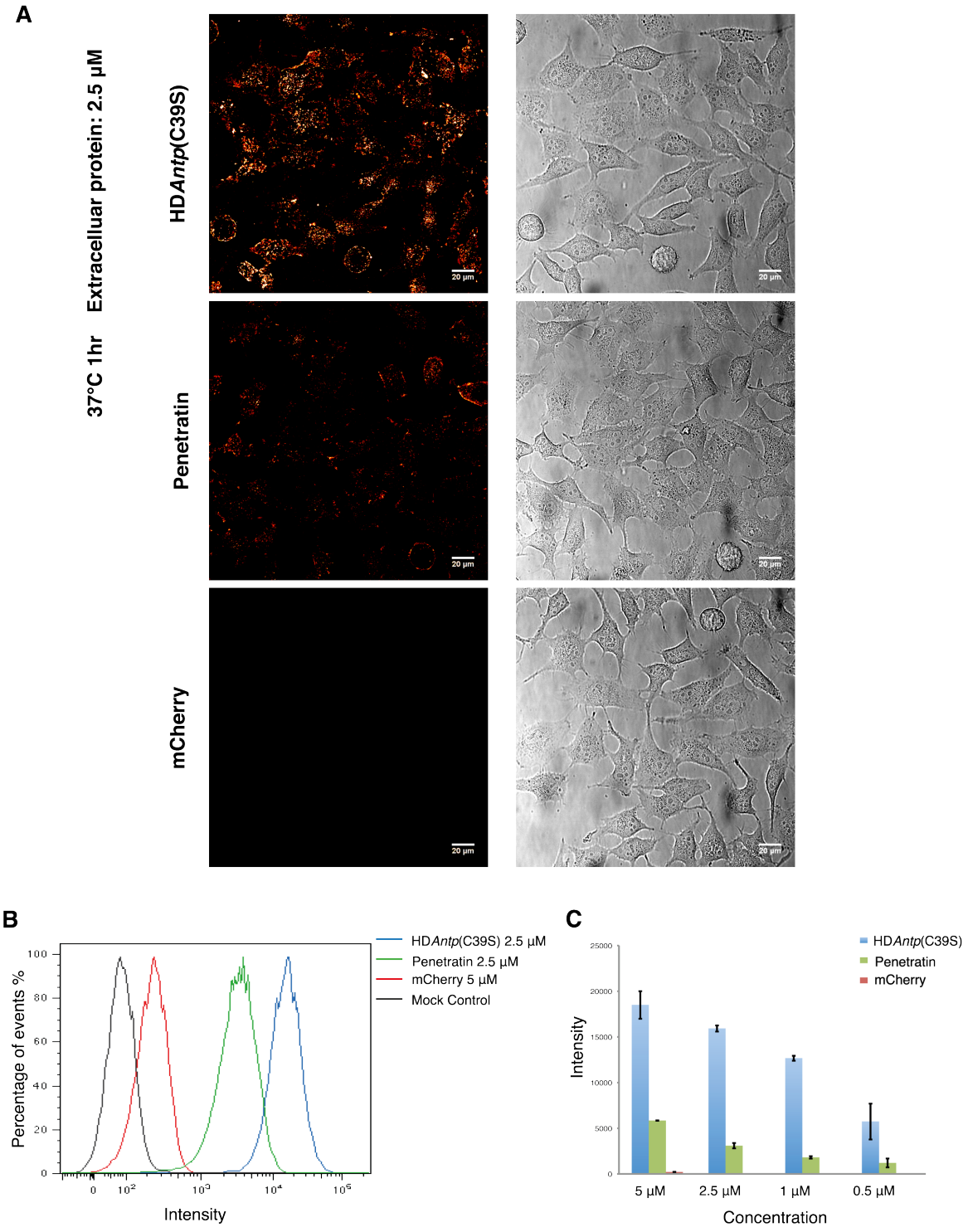


Fig. 2 HD*Antp*(C39S) compared with Penetratin on translocation efficiency. A Confocal microsocopy of live Hela cells. Cells incubated with the extracelluar protein concentration of 2.5 µM for 1hr at 37°C, with each recombinant protein HD*Antp*(C39S), Penetratin and mCherry. Fluorescent signal is displayed by the colour of hot red, and images are displayed as a maximum projected z stack of 15 confocal slices. **B** Flow cytometry of Hela cells. Cells incubated with the same condition as A, with the exception of mCherry at the concentration of 5 µM. **C** Quantification of the cellular uptaking at various concentrations was determined by fluorescence-activated cell sorting (FACS). Data are means of triplicate experiments ± SD.

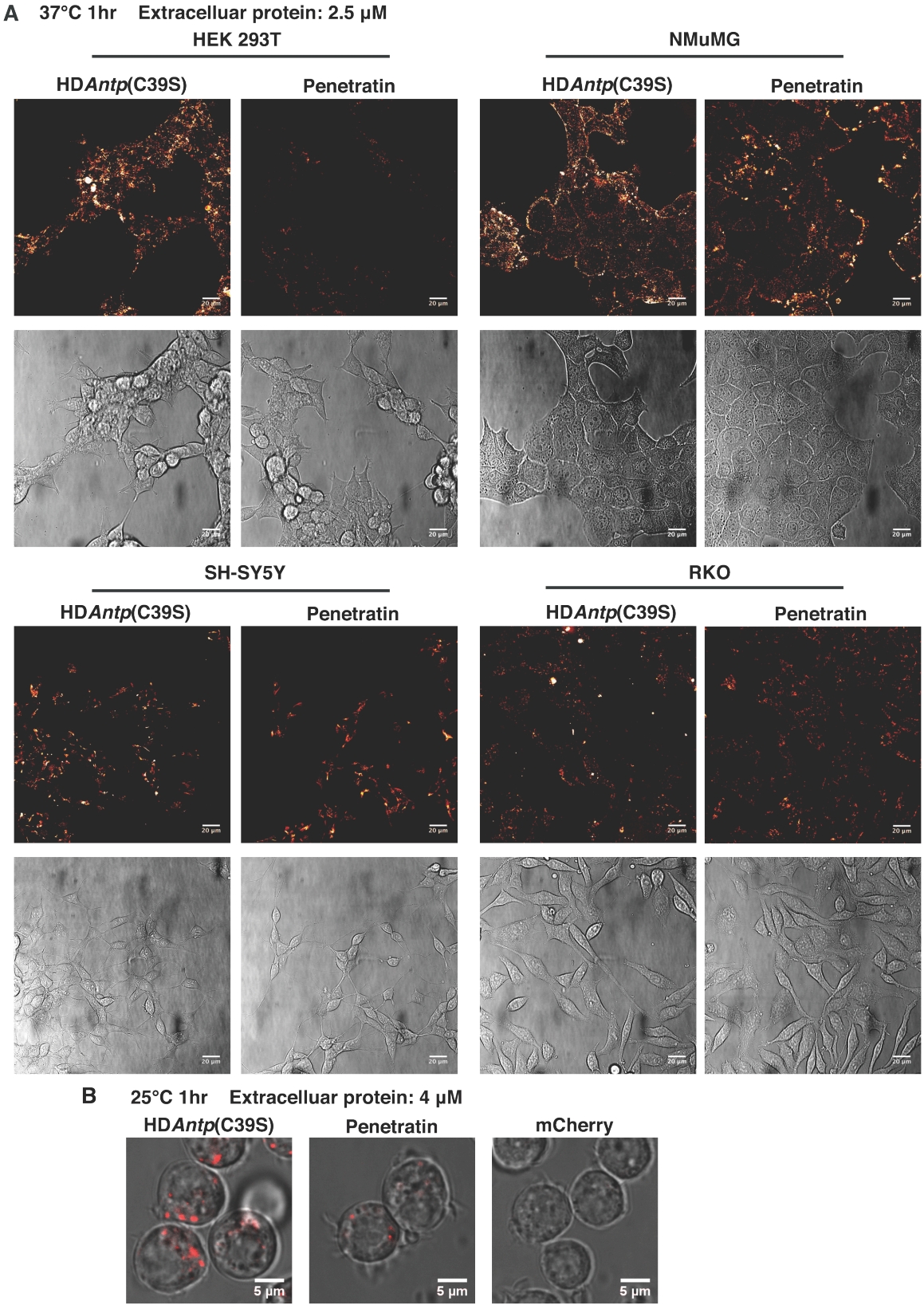


Fig. 3 Consistent difference between HD*Antp*(C39S) and Penetratin observed in various cell lines. A. Higher translocation efficiency of HD*Antp*(C39S) compared to that of Penetratin was observed in other mammalian cell lines including HEK 293T, NMuMG, SH-SY5Y, and RKO. Cells were incubated with the extracelluar protein concentration at 2.5 μM for 1hr at 37°C. Fluorescent signal is displayed by the colour of hot red, and images are displayed as a maximum projected z stack of 20 confocal slices. **B.** Co-focal microscopy on semi-adhesion *Drosophila* S2 cell.Cells were incubated with the extracelluar protein concentration at 4 μM for 1hr at 25°C. Fluorescent signal is displayed by colour of red, and images are displayed as one confocal slice.

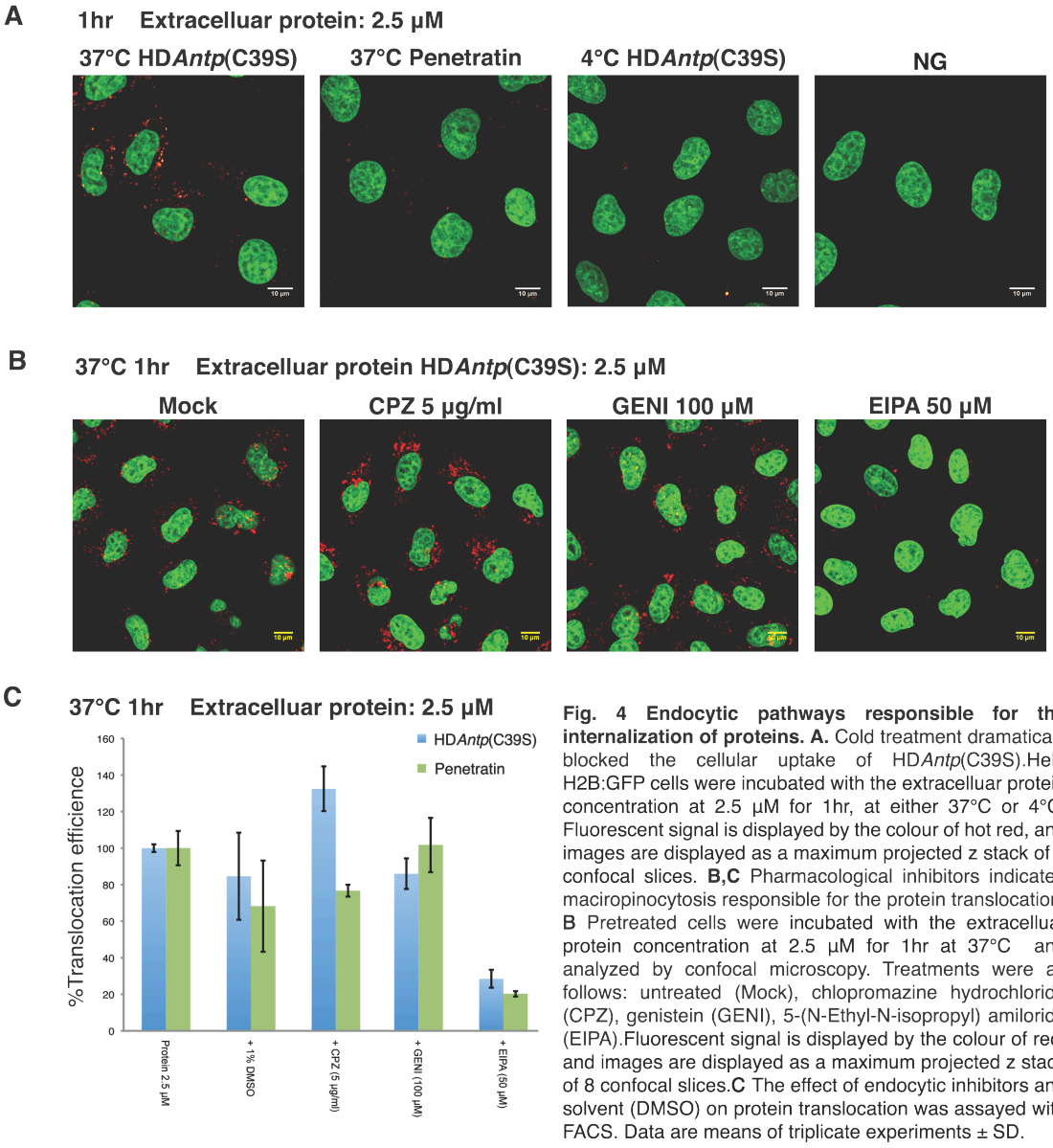


Fig. 4 Endocytic pathways responsible for the internalization of proteins. A. Cold treatment dramaticaly blocked the cellular uptake of HD*Antp*(C39S).Hela H2B:GFP cells were incubated with the extracelluar protein concentration at 2.5 µM for 1hr, at either 37°C or 4°C. Fluorescent signal is displayed by the colour of hot red, and images are displayed as a maximum projected z stack of 8 confocal slices. **B,C** Pharmacological inhibitors indicated maciropinocytosis responsible for the protein translocation. **B** Pretreated cells were incubated with the extracelluar protein concentration at 2.5 µM for 1hr at 37°C and analyzed by confocal microscopy. Treatments were as follows: untreated (Mock), chlopromazine hydrochloride (CPZ), genistein (GENI), 5-(N-Ethyl-N-isopropyl) amiloride (EIPA).Fluorescent signal is displayed by the colour of red, and images are displayed as a maximum projected z stack of 8 confocal slices.**C** The effect of endocytic inhibitors and solvent (DMSO) on protein translocation was assayed with FACS. Data are means of triplicate experiments ± SD.

Results

Characteristics of recombinant proteins

In order to be able to compare the penetration efficiency of HDAntp and its TD Penetratin, we fused them to flurorescent proteins. The mCherry fluorescent protein was fused as cargo thus avoiding the artifacts associated with fluorophore dye-dependent intracellular trafficking (Shaner *et al.,* 2004). The cysteine39 in wild type HD*Antp* was replaced by serine to avoid the formation of artificial intermolecular dimers by disulfide bridges (Qian *et al.,* 1994). Then, we generated mCherry-HD*Antp*(C39S), mCherry-Penetratin and, mCherry alone with

identical linkers and fusion orientations (Sup. Fig. 1A). After purification, each protein was further calibrated by fluorescence spectroscopy in addition to Bradford assay. Florescent standard curve was generated by serial dilution of mCherry and each testing sample was diluted into the range of the standard curve for quantification (Sup. Fig. 1B). These fusion proteins retained their ability of rapid intracellular translocation from the medium without cytotoxicity (Sup. Fig. 1C, D). To ascertain whether the recombinant proteins maintained their specific DNA binding ability, $^{a\text{-}32}$PdATP labeled probe D4 was applied for electrophoretic mobility shift assays (EMSA). D4 consists of 62 base pairs of DNA corresponding to a fragment within the enhancer of *Spineless* (*Ss*) in *Drosophila* (Duncan *et al.,* 2010), which can be specifically recognized by full-length *Antp* which serves as a transcriptional repressor and other co-transcription factors *Homothorax*/*Extradentical* (*Hth/Exd*) during leg development. As shown in Fig. 1B, HD*Antp*(C39S) specifically binds to the 62-bp D4 probe, forming protein/DNA complex proportionally to the amount of D4, whereas Penetratin and mCherry showed no affinity for the probes. We conclude that mCherry-HD*Antp*(C39S) maintains the specific binding ability with the consensus DNA sequence, while the cationic Penetratin peptide has no specific affinity for negatively charged DNA oligos.

HDAntp delivers mCherry with up to 5-fold greater efficiency than Penetratin into Hela cells

Since fixation of cells causes artificial redistribution of CPPs (Richard *et al.,* 2003), we monitored all the cellular uptake in live cells. Each recombinant protein was added to cell culture medium followed by 60 minutes of incubation. After washing with heparin containing PBS to remove proteins sticking to the cell, protein internalization was visualized by confocal fluorescence microscopy. HD*Anp*(C39S) fusion protein showed stronger intracellular signals than Penetratin (Fig. 2A), whereas mCherry alone barely gave any detectable signal under the same microscope settings. To further quantify translocation efficiency of each protein, we applied fluorescence-assisted cell sorting (FACS) to the Hela cells with the same treatment and an additional trypsinization step prior to FACS analysis. The forward and side scatter gate setting of FACS ruled out the signal from the dead cells that usually give strong false signals. At the extracellular protein concentration of 2.5 μM, HD*Antp*(C39S) was able to deliver mCherry with up to 5-fold greater efficiency than Penetratin into Hela cells (Fig. 2B,

C). The same pattern was seen with incubation conditions as low as 0.5 μM and as high as 5 μM, although the difference between HD*Antp*(C39S) and Penetratin narrowed down to 3 fold for the later (Fig. 2C). Overall, HDAntp has a greater ability to penetrate Hela cells than Penetratin alone.

Translocation difference between HDAntp(C39S) and Penetration is consistent in various cell lines, with slight variations

To rule out the possibility that the difference is Hela specific, various cell lines were used to compare the efficiency of transduction between HD*Antp*(C39S) and Penetratin. A higher translocation efficiency of HD*Antp*(C39S) as compared to Penetratin was observed in a range of mammalian cell lines including mouse mammary gland derived the normal epithelial cell line NMuMG, human colorectal adenocarcinoma cells derived RKO, human epithelial kidney HEK 293T cells, and the human neuroblastoma derived SH-SY5Y cell line (Fig. 3A), as well as in *Drosophila* S2 cells (Fig. 3B), suggesting the difference in penetration efficiency between the HD*Antp*(C39S) and Penetratin are independent of the cell-line used. Among these cell lines, HEK 293T presented the largestest difference between the HD*Antp*(C39S) and Penetration, whereas RKO displayed the smallest; NMuMG cells took up both HD*Antp*(C39S) and Penetration with high efficiency, but HD*Antp*(C39S) still showed higher internalization efficiency.

Endocytosis is involved in protein translocation

Previously, based on the assumption that Penetratin is the transduction domain of HD*Antp* and therefore fully representing the translocation capacity of its parent cargo protein, the study of the mechanism underlying the cellular uptake of HD*Antp* has been focused on that of Penetratin. Herein, we find that there is significant differences between them in terms of internalization efficiency, therefore the underlying mechanisms of HD*Antp*(C39S) transduction deserves re-evaluation. Since all endocytotic pathways are inhibited at low temperature, consequently, internalization likely reflects energy dependence or a direct translocation mechanism. To assess the role of endocytosis during internalization, Hela cells were incubated with HD*Antp*(C39S) at 4˚C and compared with the cells incubated at 37˚C. Confocal microcopy revealed that cold treatment dramatically inhibited the internalization of

HD*Antp*(C39S) (Fig. 4A), suggesting endocytic uptake was mainly responsible for protein translocation, at least at the respective extracellular protein concentration examined.

The endocytic uptake is sensitive to macropinocytosis inhibitor

Endocytic uptake involves dramatic morphological changes of the plasma membrane and multiple pathways of internalization. Endocytosis is a generic term for all mechanisms of cellular uptake associated with inward folding of the plasma membrane and pinching off of vesicles. In order to screen rapidly which endocytic pathway was involved during internalization, we first applied pharmacological inhibitors that interfere with individual endocytic pathways. Chlopromazine hydrochloride (CPZ) is a cationic amphiphilic drug that, inhibits clathrin-coated pit formation by a reversible translocation of clathrin and its adapter proteins from the plasma membrane to intracellular vesicles (Vercauteren *et al.,* 2010). 5 µg/ml CPZ treatment of Hela cells could boost the uptake of HD*Antp*(C39S) up to 50% (Fig. 4B, C), but has no significant effects on that of Penetratin (Fig. 4C), suggesting that clathrin-mediated endocytosis was not the major pathway for the entry of either protein. Genistein (GENI) is a tyrosine-kinase inhibitor that has been used to inhibit the caveolae-mediated uptake. 100 µM GENI had no effect, neither on HD*Antp*(C39S) nor on Penetratin translocation, which suggests that the caveolae-dependent pathway is not used by these proteins. 5-(N-Ethyl-N-isopropyl) amiloride (EIPA) is the derivative of amiloride which has been found to block Na^+/H^+ exchange and therefore inhibit macropinocytosis (Mercer and Helenius 2008). Treatment with 50 µM EIPA decreases the uptake of both HD*Antp*(C39S) and Penetratin by 70% (Fig. 4C). Sensitivity to EIPA is a characteristic of macropinocytic pathway. With 70% inhibition of internalization, EIPA was the most efficient inhibitor tested, indicating that both HD*Antp*(C39S) and Penetratin recombinant proteins are using macropinocytosis.

Co-localization with fluid phase dye after HDAntp(C39S) internalization

Macropinocytosis provides cells with a general, non-selective mode of uptake. To further validate macropinocytosis as a mechanism of uptake, we used the fluid phase marker 647-dextran to identify macropinosomes. Microscopy showed that HD*Antp*(C39S) co-internalized with fluid phase, but not with clathrin-mediated uptake of transferrin(Fig. 5). Compared with

cells only treated with Dextran, the uptake of Dextran from cells co-treated with HD*Antp*(C39S) was considerably elevated (Fig. 5).

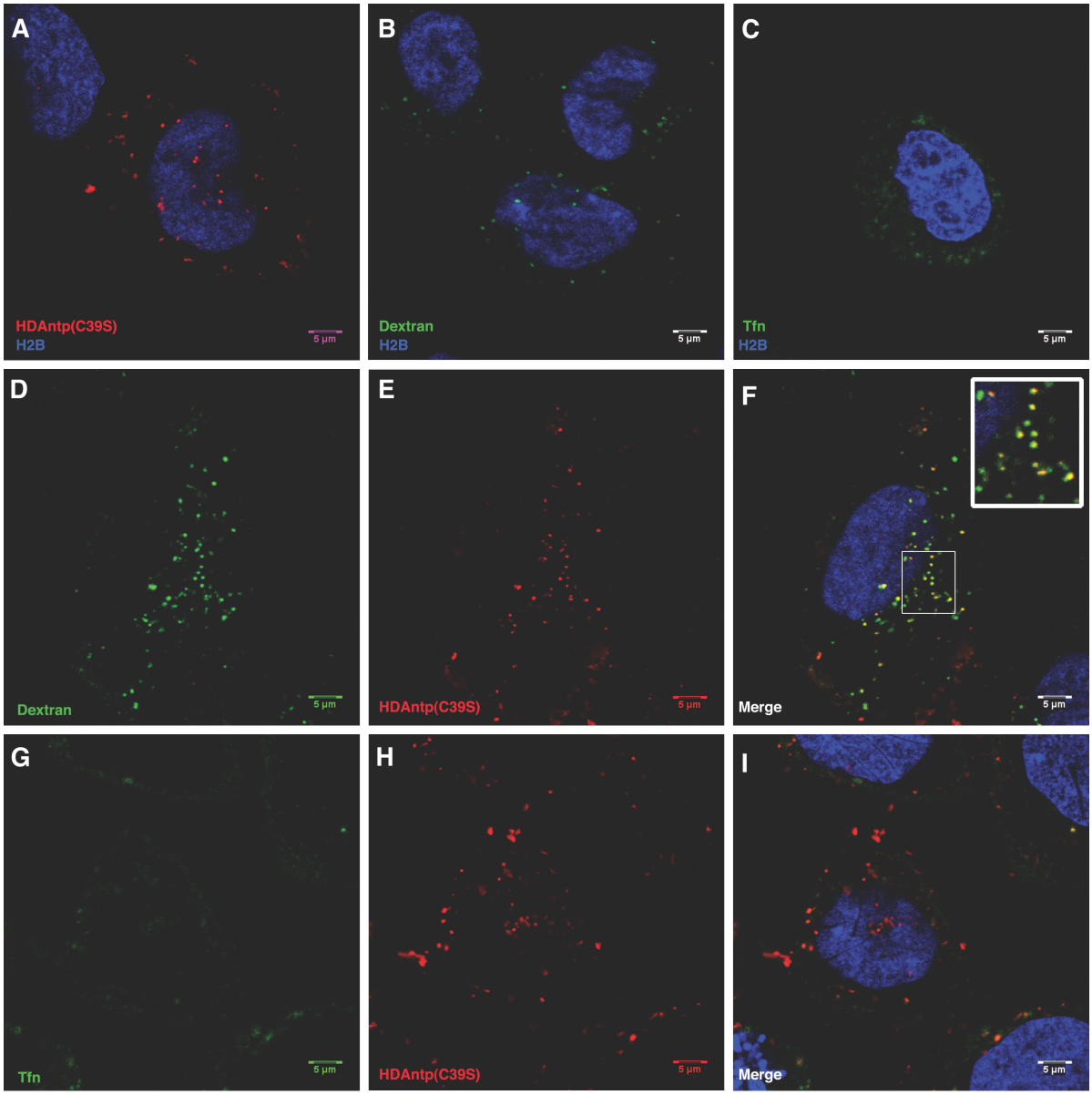


Fig. 5 Co-localization with fluid phase Dextran dye after HD*Antp*(C39S) internalization. Hela H2B:GFP were incubated in the presence of HD*Antp*(C39S)(**A**), or Dextran Alex-647(**B**), or Transferrin (Tfn) Alex-647 (**C**), seperatedly at 37 °C for 1hr; Hela H2B:GFP were incubated in the presence of HD*Antp*(C39S) and Dextran at 37°C for 1hr. Mono-channel for Dextran(**D**) and HD*Antp*(C39S) (**E**), and emerged(**F**) of D and E;Hela H2B:GFP were incubated in the presence of HD*Antp*(C39S) and Tfn at 37°C for 1hr.Mono-channel for Tfn(**G**) and HD*Antp*(C39S) (**H**), and emerged(**I**) of G and H.Nuclei are visualized as blue, and dextran or Tfn as green, and HD*Antp*(C39S) as red. All images on live cells are displayed from one confocal slice.

Discussion

Using fluorescence microscopy of living cells and FACS we found that the *Antennapedia* homeodomain which is highly positively charged is taken up more efficiently that Penetratin peptide. Similarly, despite of their diverse structures and known functions, some naturally supercharged human proteins (NSHPs) and engineered supercharged GFP have been shown to be able to penetrate mammalian cells with much higher efficiency than conventional CPPs, including Penetratin. Cationic CPPs have been reported to reach maximal penetrating potency with 8–15 positively charged amino acids and are inhibited by additional positively charged amino acids (Futaki *et al.,* 2001; Hansen *et al.,* 2008; Cronican *et al.,* 2011). HD*Antp* (7.8 kDa) that contains up to 18 positively charged amino acids and performs better than its Penetratin peptides in the term of translocation efficiency, suggesting charge distribution, molecular size, and density may also contribute to translocation efficiency.

Following the finding that internalization was temperature and therefore energy dependent suggesting an endocytotic mechanism of uptake, the internalization mechanism was dissected using pharmacological inhibitors. With 70% inhibition of translocation, the Na^+/H^+ exchanger inhibitor EIPA was the most efficient drug tested both for HD*Antp* and for Penetratin. As sensitivity of inhibition to EIPA is a characteristic of macropinocytosis, our results indicate that both of HD*Antp* and Penetratin are taken up by macropinocytosis, at least at low concentration e.g. 2.5 µM. Accordingly, co-localization with fluid phase dye, a marker of macropinosomes could be observed for intracellular HD*Antp* protein. Futhermore, a stronger signal of the fluid phase dye could be detected after co-incubation with HD*Antp* as compared to the cells treated with the dye only, indicating that macropinocytic entry is stimulated in the presence of HD*Antp*. However, the uptake of HD*Antp* could be a multimechanism process, as cold treatment could not block all the internalization, suggesting direct penetration through the plasma membrane. Furthermore, pharmacological depletion by CPZ could considerably boost HD*Antp* internalization, presumably via some compensation pathways which have been induced by CPZ inhibition, while slightly reducing that of Penetratin.

Macropinocytosis occurs constitutively only in some specialized cells, otherwise it is a signal-dependent process that normally occurs in response to growth factor stimulation. Pathogens can also stimulate macropinocytosis by mimicking apoptotic material resulting in the formation of plasma membrane blebs rather than ruffles. Recently, polyarginine, TAT and

prion protein containing a cationic domain, have been reported to deliver peptides and proteins into cells via macropinocytosis (Swanson 2008). Herein, we provide evidence that the *Antennapedia* homeodomain also uses macropinocytosis to gain access into the cells. As signaling from plasma membrane is required for actin remodeling to generate mechanical deformation forces, the interaction between positively charged CPPs and negatively charged extracellular heparan sulfate could initially trigger the signaling cascade for fluid phase endocytosis. This would presumably explain why positively charged peptides, polymers, and liposomes are able to penetrate cells.

Macropinocytic vesicles have no coat structures and are generally considered to be larger than 0.2 mm in diameter. As fluid phase endocytosis, macropinocytosis provides cells with a way to non-selectively internalize large quantities of solute. Thus, macropinocytosis may represent an effective approach for drug delivery into cells, particularly for hydrophilic macromolecules. The comparison between the famous CPP Penetratin and its parent *Antennapedia* homeodomain can give us a clue to understand the mechanisms of stimulation of macropinocytosis. Moreover, as CPPs including Penetratin are being utilized as intracellular delivery reagents at increasing frequency (Khafagy *et al.,* 2009), understanding the underlying mechanisms of its cellular internalization has profound implications for the therapeutic applications in the long term.

Materials and methods

Reagents-Methyl- β -cyclodextrin,5-(N-Ethyl-N-isopropyl) amiloride, poly(deoxyinosinic-deoxycytidylic) acid sodium salt, genistein, dichloro diphenyl trichloroethane, Chlopromazine, imidazole, heparin were purchased from Sigma. [alpha-32P]Deoxyadenosine 5'-triphosphate (dATP) isotope was obtained from Hartman-analytic. Transferrin Alex-647 and Dextran Alex-647 were purchased from Invitrogen.

Cell Lines and Cell Culture- Hela, Hela H2B::GFP, NMuMG, HEK 293T, RKO, SH-SY5Y cell lines were cultured in Dulbecco's Modified Eagle's medium DMEM with high W/Glutamax-I (Invitrogen) containing 10% heat-inactivated fetal bovine serum (FBS, Invitrogen), at 37 °C with 5% CO2. Schneider 2(S2) cells were cultured in Schneider's *Drosophila* Medium (Invitrogen) with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen) at 25 °C without requiring CO2 supply.

Live-Cell Imaging-Hela were seeded onto 8-well μ-Slide (ibidi) in 220 μl medium (1×10^4 per well). After 22 h, cells were washed once with PBS and incubated in serum-free DMEM for 30 min, or pretreated in the presence of drugs, followed by incubation in proteins solution for another 60 min. After incubation, cells were washed three times with cold 20 U mL^{-1} heparin in PBS to remove membrane-bound protein, and imaged in prewarmed growth medium. Cells were imaged on a Leica SP5 MP confocal microscope on a heated stage with either a 63× or 100× objective lens. Images were prepared using ImageJ software.

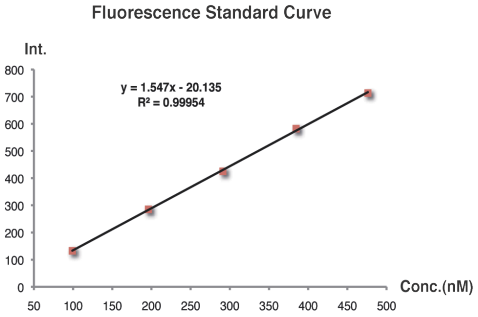
Flow Cytometry- Hela cells were plated onto a 24-well plate at a density of 6×10^4 cells per well. After 22 h, cells were incubated as above described in live imaging, then trypsinized, resuspended in 200 μL of growth medium and placed on ice. Cells were analyzed on an LSRII flow cytometer (BD Biosciences) mCherry internalization (ex, 561 nm). Cells were gated for live cells and at least 10,000 live cells were analyzed for each treatment. Data was analyzed with FlowJo software (Tree Star, Inc.)

EMSA-According to the sequence of D4, D4 EMSA F primer was designed as: 5'-agtttaccattaaattcccatttaggctgtcaatcatttgcgct-3' and D4 EMSA R as : 5'-aagccgccaagaaaaattag-cgcaaatgattgacagcctaaatggg-3'. Oligonucleotides were synthesized by Microsynth (Switzerland) and then labeled with [a-32P]dATP (Hartman) using the Klenow fragment of DNA pol I (New England Biolabs). 10 ng of annealed oligonucleotide was used per reaction. Unincorporated label was removed with illustra MicroSpin G-25 Columns (GE). Poly (dI.dC)(Sigma) was used to reduce nonspecific binding. The resulting solution was analyzed by non-denaturing electrophoresis using a 6% acrylamide gel, and visualized with X-ray film.

Supplementary information:

Supplementary Figure 1

A



Em. WL:	603.0 nm
Ex. WL:	589.0 nm
Response:	0.1 sec
Ex.BandWidth:	3 nm
Em.BandWidth:	3 nm
Sensitive:	Manual
PMT Voltage:	410 volt
No. of cycles:	3
Calib Curve:	Proportional
Expression:	Int = A * Conc.
Factor:	A = 1.547
Create Date:	17.01.2012 19:18
Standard blank:	2.8157
Blank correct:	On

B

$$\text{LDH Cytotoxicity} = \frac{[\text{Exp. Value } (\lambda 490) - \text{Exp. Value } (\lambda 650)] - [\text{Low CONT. Value } (\lambda 490) - \text{Low CONT. Value } (\lambda 650)]}{[\text{High CONT. Value } (\lambda 490) - \text{High CONT. Value } (\lambda 650)] - [\text{Low CONT. Value } (\lambda 490) - \text{Low CONT. Value } (\lambda 650)]} \times 100\ \%$$

Membrane integrity = 1 - LDH Cytotoxicity (%)

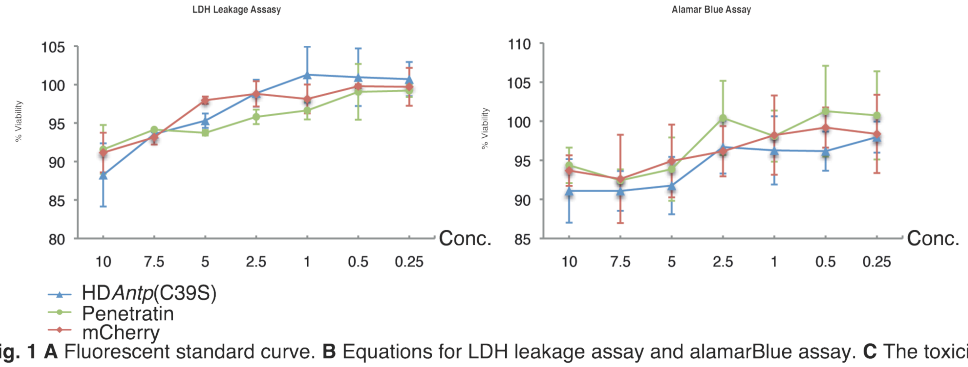
Low CONT. Value: Untreated normal cells
High CONT. Value: Lysis treated normal cells (maxium LDH release)

$$\text{AlamarBlue Cytotoxicity} = \frac{\text{Exp. Value } (\lambda 570) * \partial 1(\lambda 570) - \text{Exp. Value } (\lambda 600) * \partial 2(\lambda 600)}{\text{CONT. Value } (\lambda 570) * \partial 1(\lambda 570) - \text{CONT. Value } (\lambda 600) * \partial 2(\lambda 600)} \times 100\ \%$$

Cellular Viability = 1 - AlamarBlue Cytotoxicity (%)

CONT. Value: Untreated normal cells
∂1(λ570) = 117.216 / Molar extinction coefficients for alamarBlue at wavelength 570nM
∂1(λ600) = 80.586 / Molar extinction coefficients for alamarBlue at wavelength 600nM

C



Sup. Fig. 1 A Fluorescent standard curve. **B** Equations for LDH leakage assay and alamarBlue assay. **C** The toxicity of the recombinant fusions proteins was assessed in Hela cells. Their effect on plasma membrane integrity and cellular viability was addressed by LDH leakage assay and alamarBlue assay measuring the intracellular redox environment.

Protein Sequences

mCherry:

MHHHHHHHHHHIEGRVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGRP
YEGTQTAKLKVTKGGPLPFAWDILSPQFMYGSKAYVKHPADIPDYLKLSFPEGFKW
ERVMNFEDGGVVTVTQDSSLQDGEFIYKVKLRGTNFPSDGPVMQKKTMGWEASSE
RMYPEDGALKGEIKQRLKLKDGGHYDAEVKTTYKAKKPVQLPGAYNVNIKLDITSH
NEDYTIVEQYERAEGRHSTGGMDELYKG

mCherryPenetratin:

MHHHHHHHHHHIEGRVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGRP
YEGTQTAKLKVTKGGPLPFAWDILSPQFMYGSKAYVKHPADIPDYLKLSFPEGFKW
ERVMNFEDGGVVTVTQDSSLQDGEFIYKVKLRGTNFPSDGPVMQKKTMGWEASSE
RMYPEDGALKGEIKQRLKLKDGGHYDAEVKTTYKAKKPVQLPGAYNVNIKLDITSH
NEDYTIVEQYERAEGRHSTGGMDELYKGGDSLEFIASKLA

mCherryHD*Antp*(C39S):

MHHHHHHHHHHIEGRVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGRP
YEGTQTAKLKVTKGGPLPFAWDILSPQFMYGSKAYVKHPADIPDYLKLSFPEGFKW
ERVMNFEDGGVVTVTQDSSLQDGEFIYKVKLRGTNFPSDGPVMQKKTMGWEASSE
RMYPEDGALKGEIKQRLKLKDGGHYDAEVKTTYKAKKPVQLPGAYNVNIKLDITSH
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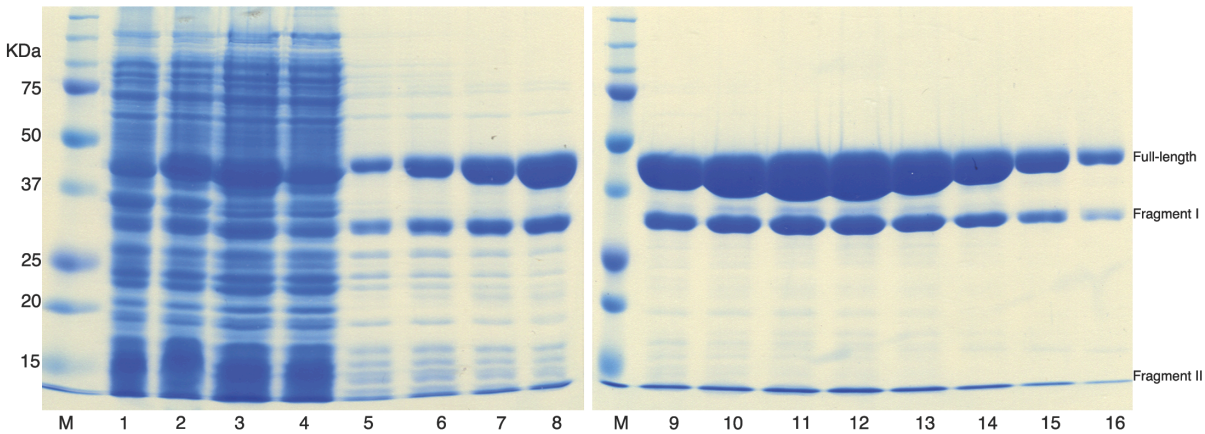
Supplementary Figure 2



Sup. Fig. 2 SDS-PAGE analysis of the expression and purification of HD*Antp*(C39S) by immobilized metal affinity chromatography Lane 1 and 2 indicate before and after induction,and a band of approximately 38 kDa is observed in lane 2. ; lane 3 and 4 indicate supernatant after centrifugation and flow through; lane 5-16 indicate each fractions eluated by imidazole gradients generated by ÄKTA prime™. mCherry fragmentation at the site of the MYG amino acid sequence during SDS-PAGE analysis, which produces two fragments with the size at 27.56 KDa (Fragment I) and at 9.68 KDa (Fragment II) along with 37.2 KDa (Full-length).

Protein induction and chromatography purification

Rosetta 2(DE3)*pLysS* (Novagen) cells were grown in 4 L LB cultures at 37° C to OD600 = ~0.6 and induced with 1 mM IPTG at 18° C for overnight. Cells were harvested by centrifugation (6000 rpm, 10min) and stored at -80° C. Frozen pellets were thawed in 80 ml HisTrap column buffer with 50mM Sodium phosphate, 1 M NaCl, 20mM imidazole, 1 mM DTT, pH 7.8 and lysed by sonication. The lysate was cleared by centrifugation (12,000 rpm, 40 min) and the supernatant was kept on ice as pretreated sample for further purification procedure.

A HisTrap HP 5ml column (GE Healthcare) was washed with 5 column volumes of distilled water, and equilibrated with 5 column volumes of column buffer. The supernatant was applied onto the column using a peristaltic pump. The flow through was collected for SDS-PAGE analysis. The column was mounted onto ÄKTA prime™, and washed with binding buffer until the absorbance reached a steady baseline (generally, at least 10-15 column volumes). The column was eluted using linear gradient as below. Elution buffer contains 50mM Sodium phosphate, 1 M NaCl, 1.5 M imidazole, 1 mM DTT, pH 7.8. The flow rate was kept at 2ml/min, running from 0% to 100% elution buffer in an 80ml volume. One ml fractions were collected and kept for SDS-PAGE analysis. The fractions enriched in target proteins, were diluted with 10 volumes of distilled water for further purification by Heparin column.

A HiTrap Heparin HP 5ml column (GE Healthcare) was washed with 5 column volumes of distilled water, and equilibrated with 5 column volumes of Heparin column buffer (20 mM Sodium phosphate, 20 mM NaCl, 1 mM DTT, 5% Glycerol, pH 7.0). The combined fractions from HisTrap HP column were loaded onto the Heparin column using a syringe or peristaltic pump. The flow through was collected for SDS-PAGE analysis. The column was mounted onto ÄKTA prime™, and washed with binding buffer until the absorbance reached a steady baseline. Then the column was eluted using linear gradient of elution buffer (20 mM Sodium phosphate, 1 M NaCl, 1 mM DTT, 5% Glycerol, pH 7.0). Flow rate was adjusted to 2ml/min, running from 0% to 100% elution buffer in a volume of 80 ml. One ml fractions were collected and kept for SDS-PAGE analysis.

The recombinant protein was desalted on a Hiprep 26/10 Desalting column with 1xPBS buffer containing 10% Glycerol. The protein samples were kept at 4° C.

LDH leakage assay-The assay was performed using the Cytotoxicity Detection KitPLUS (LDH) (Roche). In the assay, HeLa cells were seeded in 96-well plates in 100 µl medium (5 × 10^3 per well) and used for experiments performed in triplicates 22hrs after seeding. After 1 hr incubation with the recombinant protein, samples of 100 µl were added to 100 µl of an equal mixture of LDH Assay Substrate, Cofactor and Dye solution in a 96-well-plate and incubated at 20 ° C. After 10 min the reaction was quenched with 50 µl of stop solution. The absorbance was measured at 490 nm and the background absorbance was measured at 650 nm and subtracted. LDH leakage relevant cytotoxicity was calculated by the formula in Sup. Fig. 1.

AlamarBlue Assay-The assay was performed using the alamarBlue Cell Viability Reagent (Invitrogen). In the assay, Hela cells were seeded in 96-well plates in 100 µl medium (5 × 10^3 per well) and used for experiments performed in triplicates 22hrs after seeding. After 1 hr incubation with recombinant protein, samples of 100 µl were added to 10 µl of an equal mixture of alamarBlue reagent. After another 2hrs incubation at 37 ° C, monitor the absorbance of alamarBlue at 570 nm, using 600 nm as reference wavelength (normalize to the 600 nm value). Redox environment relevant cytotoxicity was calculated by the formula in Sup. Fig. 1.

Characterization of the cytotoxic properties of protein incubation

The toxicity of the recombinant fusions proteins was assessed in Hela cells. Their effect on plasma membrane integrity and cellular viability was addressed by a LDH leakage assay and an alamarBlue assay measuring the intracellular redox environment, respectively. The calculation equations are shown in Sup. Fig. 1. Hela cells were first incubated with serum free medium for 30 min, then followed by incubation for another 60 min for each assay as described above. As shown in Sup. Fig. 1 C neither HD*Antp*(C39S) nor Penetratin fused protein affected membrane integrity and cell viability at concentrations up to 7.5 µM, but slightly toxicity was detected at a concentration of 10 µM, particular in LDH assasy (approx. 10%). Additional observations by microscopy showed that 5 % of the cell population presents morphological changes and detaches from the growing vessel at concentration of 5 µM. In order to avoid artifacts related to the eventual cellular toxicity of the recombinant proteins we generally used a concentration of 2.5 µM for most experiments.

Manuscript II

Supercharged GFP fused to synthetic *Antennapedia* protein generated from *Escherichia* coli for protein transduction analysis

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***Abstract*:** Recently engineered supercharged GFP protein has been demonstrated to have a high capacity of intracellular delivering of cargo proteins that are fused to it. Here we report the expression, preparation and characterization of histidine tagged recombinant protein comprising supercharged GFP and synthetic *Antennapedia* protein. Using the Rosseta^2(DE3)*PlySs* as expression strain, we generated recombinant protein encoding 355 amino acid residue. The further purification steps were carried our by using immobilized nickel ion affinity chromatography and heparin affinity chromatography, subsequently. *In vitro* experiments revealed that purified recombinant protein could specifically bind to the DNA probe corresponding to the *Spineless* D4 enhancer from *Drosophila,* indicating that the fusion protein contains a functional homeotic domain. It can translocate rapidly into the cytoplasm of *Drosophila* S2 cells. In *ex vivo* assay, this protein can penetrate the basal membrane of eye-antennal discs and gain access to the nuclei of some of disc cells within short time. Our results demonstrate that supercharged GFP can facilitate the intracellular delivery of synthetic *Antennapedia* protein and potentially boost protein transduction of such transcription factors.

Highlights

► We expressed and purified recombinant Synthetic *Antennapedia* protein fused to supercharged GFP from an *Escherichia* coli expression system. ► The process is easy, fast

and cost effective. ► The product is of high purity and low level of toxicity. ► The product retains highly specific binding activity with DNA probe corresponding to the *Spineless* D4 enhancer of *Drosophila,* and is able to interact with other transcription factors. ► The product can penetrate the plasma membrane of *Drosophila* S2 cells rapidly, and gain access to the nuclei of eye-antennal disc cells in an *ex vivo* assay.

Keywords

Drosophila Antennapedia; Supercharged GFP; Expression & Purification; Intracellular delivery

Introduction

Antennapedia is homeotic gene of the *Antennapedia* Complex (*ANTP*-C), and codes transcription factor which plays an essential role in embryonic patterning and the development of the mesothoracic segment (Gehring and Hiromi 1986; Gehring *et al.,* 2009). The synthetic version of *Antennapedia* (SynHD*Antp*), which is comprised of the homeodomain of *Antennapedia* and YPWM protein-protein interaction motif only, can be functionally equivalent to the full-length *Antennapedia* for specifying cell identity (Papadopoulos *et al.,* 2011). About two decades ago, the homeodomain of *Antennapedia* was shown to have the capability of translocation through the plasma membrane from the intercellular space into the cytoplasm (Joliot *et al.,* 1991), in a receptor-independent manner (Derossi *et al.,* 1996). In an attempt to induce specific antenna to leg transformations in *Drosophila* imaginal disc by protein transduction, we have used a synthetic *Antennapedia* peptide without applying any genetic manipulation.

Recently a new version of GFP has been described, which has been extensively mutated at its surface-exposed residues resulting in extremely high net charge (GFP 36+). This "super-charged GFP" can deliver functional cargo proteins into the cytosol of a variety of cells *in vitro* and *in vivo,* with a remarkably high efficiency and no apparent cytotoxicity (Cronican *et al.,* 2010). We hypothesized that supercharged GFP may also be utilized as a potent tool to circumvent the endocytic entry for intracellular delivery of synthetic *Antennapedia*. For producing the peptide, we used a highly effective *E. coli* Rosseta^2(DE3)*PlySs* as expression strain, and HisTrap and Heparin columns for purification. The *in vitro* DNA binding properties of the recombinant protein product was analyzed by electrophoretic mobility shift

assays (EMSA) using the *Spineless* D4 enhancer as probe. Previously, the *engrailed* enhancer derived BS2 has been the most widely used as a probe to determine the binding specificity of purified *Antennapedia* homeodomain in EMSA assay, but *in vivo* BS2 is the putative binding site of *fushi tarazu* while also binds *Antennapedia* protein (Muller *et al.,* 1988). Recently, Duncan *et al.* has isolated D4 fragment within the enhancer of the antenna determination gene *Spineless* in *Drosophila* (Duncan et al., 2010). In leg development, *Antennapedia* is endogenously expressed in the leg discs where it represses *Spineless* by binding to its enhancer. By ectopically expressing *Antennapedia* in antennal disc *Spineless* is repressed, resulting in antenna-to-leg transformation. Therefore, D4 has been used as probe in the EMSA in this study. Finally, fluorescence microscopy was used to visualize uptake of the recombinant protein by S2 cells and live eye-antennal imaginal discs after *ex vivo* treatment with the recombinant protein.

Materials and methods

Construction of the GFP36+SynHDAntp(C39S) fusion expressing vector

The cysteine residue 39 in wild type *Antennapedia* homeodomain was replaced by serine to avoid the formation of artificial intermolecular dimers via disulfide bridges**.** PCR primers (GFP36+ F forward primer: 5'-gggcatatggctagcaaaggtgaacgtctg-3' and GFP36+ R reverse fusion primer: 5'-cgcatccagggatacagtggagacggggagccgccgctgccacc-3') were designed in accordance to the sequence of GFP36+ (Cronican *et al.,* 2010). PCR primers for synthetic *Antennapedia* (SynHD*Antp*(C39S) F forward fusion primer: 5′-ggtggcagcggcggctccccgtctc-cactgtatccctggatgcg-3′; SynHD*Antp* R reverse primer: 5′-gggctcgagggatcccggctcgcccttcgtc-3′.) were designed in accordance to the sequence of SynHD*Antp*(C39S). All primers were synthesized by Microsynth (Switzerland). The plasmid containing the GFP36+ sequence which was used as template was a generous gift of Dr. Liu. For the first round of PCR, PCR amplification of GFP36+ fragment was performed for 30 cycles (94 °C for 30 sec, 58 °C for 30 sec, 72 °C for 45 sec) followed by denaturation of DNA for 2 min at 94 °C. The same program for SynHD*Antp*(C39S) was used except for 30 sec of elongation. In the second round of PCR, after gel-extraction and purification the amplicons from the first round of PCR were used for the second round of PCR amplification. The GFP36+ F as a forward primer and SynHD*Antp*(C39S) R as a reverse primer were added into reaction mixture for 30 cylces

(94 °C for 30 sec, 58 °C for 30 sec, 72 °C for 90 sec) followed by denaturation of DNA for 2 min at 94 °C. The GFP36+ fused SynHD*Antp*(C39S) fragment was ligated between the Ndel and Xhol restriction sits of pET21b (Novagen). The recombinant plasmids were transformed into the competent E. coli Rosetta^2(DE3)pLysS cells to generate the stable expressing cell line.

Sequence of GFP36*+SynHDAntp(C39S) recombinant *protein

MASKGERLFRGKVPILVELKGDVNGHKFSVRGKGKGDATRGKLTLKFICTTGKLPV
PWPTLVTTLTYGVQCFSRYPKHMKRHDFFKSAMPKGYVQERTISFKKDGKYKTRAE
VKFEGRTLVNRIKLKGRDFKEKGNILGHKLRYNFNSHKVYITADKRKNGIKAKFKIR
HNVKDGSVQLADHYQQNTPIGRGPVLLPRNHYLSTRSKLSKDPKEKRDHMVLLEFV
TAAGIKHGRDERYKGGSGGSGGSGGSGGSGGSGGSGGSPSPLYPWMRSQFERK
RGRQTYTRYQTLELEKEFHFNRYLTRRRRIEIAHALSLTERQIKIWFQNRRMKWKKE
NKTKGEPGSLEHHHHHH

Protein induction and chromatography purification

Rosetta^2(DE3)pLysS (Novagen) cells were grown in 2 L LB cultures at 37° C to OD600 = ~0.6 and induced with 1 mM IPTG at 18° C overnight. Cells were harvested by centrifugation (6000 rpm, 10min) and stored at -80° C. Frozen pellets were thawed in 80 ml HisTrap column buffer with 50mM Sodium phosphate, 0.5 M NaCl, 20mM imidazole, 1 mM DTT, pH 7.8 and lysed by sonication. The lysate was cleared by centrifugation (12,000 rpm, 40 min) and the supernatant was kept on ice for further purification.

A HisTrap HP 5ml column (GE Healthcare) was washed with 5 column volumes of distilled water, and equilibrated with 5 column volumes of column buffer. The supernatant was applied onto the column using a peristaltic pump. The flow through was collected for SDS-PAGE analysis. The column was mounted onto ÄKTA prime™, and washed with binding buffer until the absorbance reached a steady baseline (generally, at least 10-15 column volumes). The column was eluted using linear gradient as below. Elution buffer contains 50mM Sodium phosphate, 0.5 M NaCl, 1.5 M imidazole, 1 mM DTT, pH 7.8. The flow rate was kept at 2ml/min, running from 0% to 100% elution buffer in an 80ml volume. One ml fractions were collected and kept for SDS-PAGE analysis. The fractions enriched in target proteins, were diluted with 10 volumes of distilled water for further purification by Heparin column.

A HiTrap Heparin HP 5ml column (GE Healthcare) was washed with 5 column volumes of distilled water, and equilibrated with 5 column volumes of Heparin column buffer (20 mM Sodium phosphate, 20 mM NaCl, 1 mM DTT, 5% Glycerol, pH 7.0). The combined fractions from HisTrap HP column were loaded onto the Heparin column using a syringe or peristaltic pump. The flow through was collected for SDS-PAGE analysis. The column was mounted onto ÄKTA prime™, and washed with binding buffer until the absorbance reached a steady baseline. Then the column was eluted using linear gradient of elution buffer (20 mM Sodium phosphate, 1 M NaCl, 1 mM DTT, 5% Glycerol, pH 7.0). Flow rate was adjusted to 2ml/min, running from 0% to 100% elution buffer in a volume of 80 ml. One ml fractions were collected and kept for SDS-PAGE analysis.

The recombinant protein was desalted on a Hiprep 26/10 Desalting column with 1xPBS buffer containing10% Glycerol. The protein samples were kept at 4° C.

EMSA

According to the sequence of D4, forward primer was designed as 5'-agtttaccattaaattcccatttaggctgtcaatcatttgcgct-3' and reverse primer as 5'-aagccgccaag-aaaaattagcgcaaatgattgacagcctaaatggg-3'. Oligonucleotides were synthesized by Microsynth (Switzerland) and then labeled with [a-32P]dATP (Hartman) using the Klenow fragment of DNA pol I (New England Biolabs). 10 ng of annealed oligonucleotide was used per reaction. Unincorporated label was removed with illustra MicroSpin G-25 Columns (GE). All proteins were produced by *in vitro* translation using the TnT T7 Rabbit Reticulocyte Lysate kit (Promega). 1 µg of circular plasmid DNA was used per reaction. The products were used without further purification. 2 to 10 µl of in vitro translation product were used per reaction. Poly (dI.dC) (Sigma) was used to reduce nonspecific binding. The plasmids used for in vitro translation were as follows: *Distalless*, *Homothorax*, *Extradentical* and *Antennapedia* constructs contain the full-length coding regions of the respective genes cloned into pT7βplink (Dalton and Treisman, 1992), a generous gift of Dr. Duncan.

Cell Culture

Drosophila S2 cells were cultured in Schneider's medium (Invitrogen) containing 10% heat-inactivated fetal bovine serum, at 25 °C without CO2 supply. Cells were split every 4-5 days to maintain by 1:5 dilution.

Ex vivo culture eye-antennal discs

Dissect third or the late second instar larvae by inverting the head section and removing the trachea and fat, leaving the eye-antennal imaginal discs for further treatment. Apply Chan-Gehring buffer as the incubation medium. Chan-Gehring buffer consists of 55 mM NaCl, 40 mM KCl, 5mM $CaCl_2$, 2mM $MnCl_2$, 40 mM glucose, 67.5 mM sucrose, 10 mM HEPES, pH 6.9.

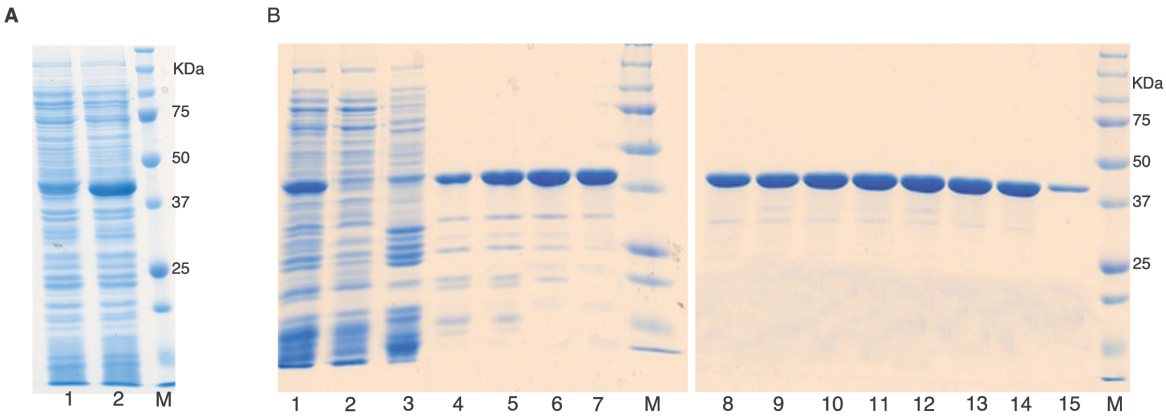


Fig. 1 SDS-PAGE analysis of the expression and purification of GFP36+SynHD*Antp*(C39S) A IPTG induction. lane 1 and 2 indicate before and after induction. A band of approximately 40.5 kDa is observed in lane 2. **B** His-tagged GFP36+ SynHDAntp(C39S) purified by immobilized metal affinity chromatography (IMAC). Lane 1 indicates supernatant, lane 2 is the flow through, lane 3 is the washing fraction using the binding buffer containing 100 mM imidazole, lane 4-15 indicate successive fractions eluated by imidazole gradients generated by ÄKTA prime™.

Results

Expression and purification of GFP36+SynHDAntp(C39S)

E. coli transformed by pET21b plasmid were cultured and induced with IPTG to express recombinant protein. Bacteria were collected both prior to and after IPTG induction for analysis with SDS–PAGE. A band of approximately 40.5 kDa was observed in IPTG-induced bacteria (Fig. 1A) which is close to the calculated molecular weight of recombinant protein. After lysis and centrifugation, recombinant protein was applied to an immobilized nickel affinity chromatography (IMAC) column at 4 °C. Most of the contaminating *E*. coli proteins were removed by this washing step. The fusion protein was eluted with gradients of imidazole and the purity of protein each fraction was analyzed by SDS–PAGE (Fig. 1B). Fractions from 7 to 15 were collected, and diluted 10 fold with distilled water before loading

on a Heparin column for removing the imidazole. Addition buffer exchange step is as described in material and method. The recombinant protein can be kept for 4° C for up to half year without losing specific binding property and fluorescence intensity.

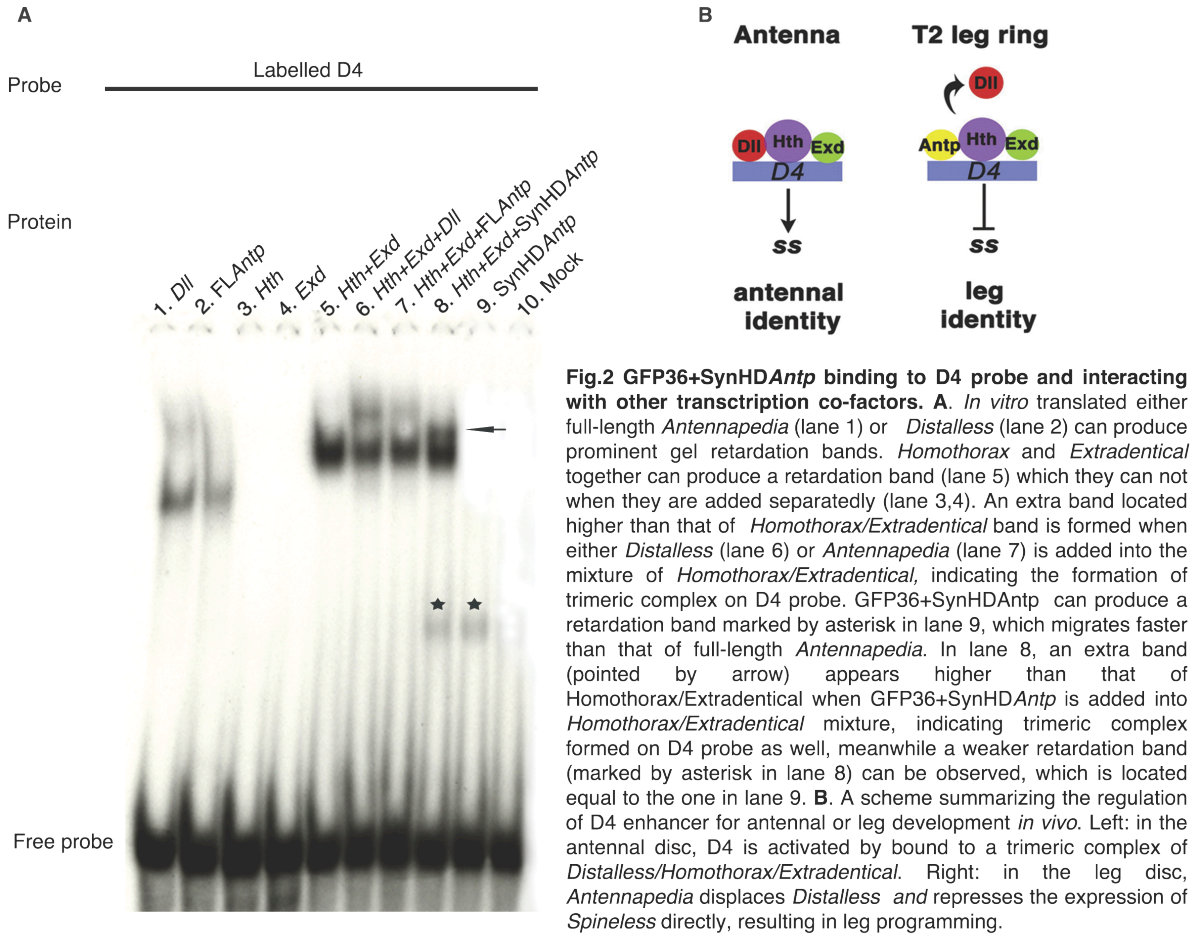


Fig.2 GFP36+SynHD*Antp* binding to D4 probe and interacting with other transctription co-factors. A. *In vitro* translated either full-length *Antennapedia* (lane 1) or *Distalless* (lane 2) can produce prominent gel retardation bands. *Homothorax* and *Extradentical* together can produce a retardation band (lane 5) which they can not when they are added separatedly (lane 3,4). An extra band located higher than that of *Homothorax/Extradentical* band is formed when either *Distalless* (lane 6) or *Antennapedia* (lane 7) is added into the mixture of *Homothorax/Extradentical,* indicating the formation of trimeric complex on D4 probe. GFP36+SynHDAntp can produce a retardation band marked by asterisk in lane 9, which migrates faster than that of full-length *Antennapedia*. In lane 8, an extra band (pointed by arrow) appears higher than that of Homothorax/Extradentical when GFP36+SynHD*Antp* is added into *Homothorax/Extradentical* mixture, indicating trimeric complex formed on D4 probe as well, meanwhile a weaker retardation band (marked by asterisk in lane 8) can be observed, which is located equal to the one in lane 9. **B**. A scheme summarizing the regulation of D4 enhancer for antennal or leg development *in vivo*. Left: in the antennal disc, D4 is activated by bound to a trimeric complex of *Distalless/Homothorax/Extradentical*. Right: in the leg disc, *Antennapedia* displaces *Distalless and* represses the expression of *Spineless* directly, resulting in leg programming.

In vitro binding property of recombinant GFP36+SynHDAntp(C39S)

To ascertain whether the recombinant proteins maintained their specific DNA binding ability, [a-32P]dATP labeled D4 probe was applied for electrophoretic mobility shift assays (EMSA). D4 consists of 62 base pairs of DNA corresponding to a fragment within the enhancer of *Spineless* in *Drosophila* (Duncan *et al.,* 2010). *In vitro* translated *Distalless* and full-length *Antennapedia* both produce prominent gel retardation bands in gel-shift assays. Although *Homothorax* and *Extradentical* do not produce retardation bands on their own, when mixed they bind cooperatively to produce a prominent retardation complex. When either *Distalless* or full-length *Antennapedia* are added to the *Homothorax*/*Extradentical* mixture, an extra band migrating more slowly than the *Homothorax*/*Extradentical* band in the

gel emerges, while the bands corresponding to binding of individual *Distalless* or *Antennapedia* with D4 is significantly reduced or totally replaced, hardly visible on the gel, indicating a trimetric protein complex of *Homothorax*/*Extradentical/Distalless* or *Homothorax*/*Extradentical/Antennapedia* is formed on D4 probe. To find out whether recombinant proteins GFP36+*SynHDAntp* behaves the same as full-length *Antennapedia* in *in vitro* gel-shift assay or be cable to form complex with the other two transcription co-factors, two additional lanes were used for investigation. As GFP36+*Syn*HD*Antp* has a lower molecular weight and higher positive charge than the full-length *Antennapedia*, it produces a gel retardation band lower in the gel than that of *in vitro* translated full-length *Antennapedia*. Correspondently, when GFP36+*SynHDAntp* is mixed with *Homothorax*/*Extradentical*, an extra band located higher than the *Homothorax*/*Extradentical* band in the gel emerges, but lower than the extra band from *Homothorax*/*Extradentical*/*Antennapedia,* indicating that GFP36+*SynHDAntp* is cable to bind to D4 probe and form trimetric complex with *Homothorax*/*Extradentical in vitro.*

Intracellular translocation of recombinant GFP36+SynHDAntp(C39S) in S2 cells and in imaginal discs

We first examined the uptake of recombinant GFP36+SynHD*Antp*(C39S) by *Drosophila* S2 cells. Within 1 hour of incubation at extracellular protein concentration of 1 μM at 25 ˚C, strong fluorescent intensity in the cytoplasm of S2 cells can be detected by microscopy, suggesting the GFP36+SynHD*Antp*(C39S) is able to penetrate the plasma membrane of S2 cells rapidly. Since S2 is semi-adherent cell line, only one confocal slice is displayed in Fig. 3. Furthermore, in order to investigate the translocation ability of GFP36+SynHD*Antp*(C39S) into imaginal disc tissue, we applied the recombinant proteins to eye-antennal disc dissected from the early 3rd instar larvae. Eye-antennal disc were dissected as described in material and method, and incubated in Chan-Gehring buffer containing 2 μM GFP36+SynHD*Antp*(C39S) for 4 hours at room temperature, then washed with Chan-Gehring buffer containing 20U Heparin to remove proteins sticking to the discs. Subsequently, the discs were stained with 1 μg/ml Hoechst 33342 for 10 minutes and washed three times with Chan-Gehring buffer and subjected to confocal fluorescence microscopy. GFP36+SynHD*Antp*(C39S) has clearly accumulated in some of the nuclei around the wounded area at the anterior of antennal disc,

but not the whole disc. Interestingly, the cells that have up taken GFP36+SynHD*Antp*(C39S) intend to exclude Hoechst 33342 staining as shown in Fig. 4 CE where few cells in the overlaid picture are yellow. Most of the cells are either green or red.

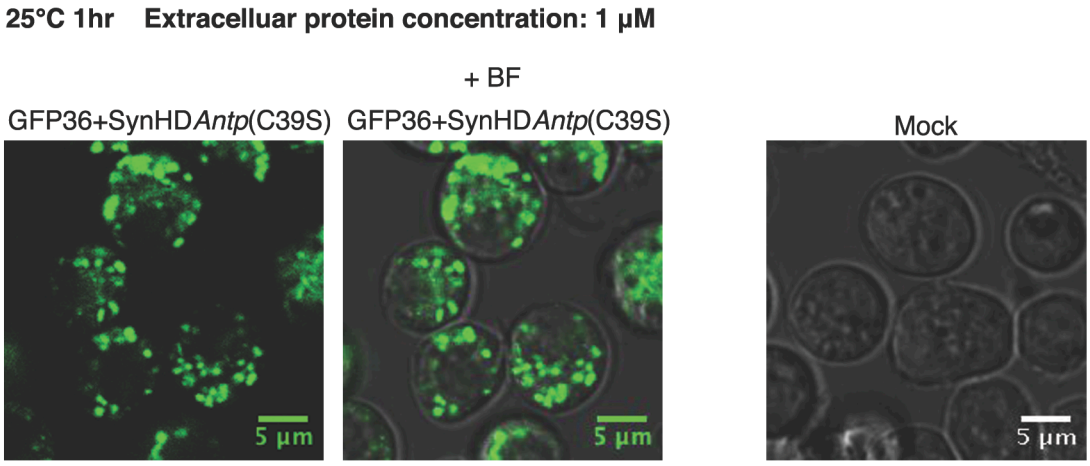


Fig. 3 Uptake of recombinant GFP36+SynHD*Antp*(C39S) into S2 cells. Within 1 hour of incubation at 25 ˚C, the fluorescent intensity can be detected by fluorescent microscopy. Since S2 is semi-adherent cell line, only one confocal slice is displayed.

Discussion

Because of their hydrophilic nature, most proteins can not penetrate into the cells, which preventing them from being useful agents for intracellular targeting. Over the past decade, a family of peptides has been developed for the delivery of proteins into cells. Cell penetrating peptides (CPPs) has been designated to these peptides, which including the HIV-1 transactivator of transcription derived TAT, oligoarginine, and the *Drosophila Antennapedia* homeodomain derived Penetratin. Based on the assumption that the *Antennapedia* homeodomain could be transported into the cytoplasm in a receptor-independent manner due to its "Penetratin" transduction domain, and subsequently translocate into the nuclei of cells playing its role as a transcription factor, we applied the synthetic version of the *Antennapedia* protein, which composed of the homeodomain and a YPWM motif, to change gene expression pattern of target cells by so called protein transduction. However, either by depending on the internal "Penetratin" or by fusing an additional CPPs inculding TAT and oligoarginine to the recombinant synthetic *Antennapedia* proteins, failed to reach the nuclei of target cell after translocation and internalized proteins were associated with intracellular vesicles, presumably endosome (Wu and Gehring, in Prep.). Systematic investigation of the mechanisms underlying the cellular uptake of Penetratin and *Antennapedia* homeodomain,

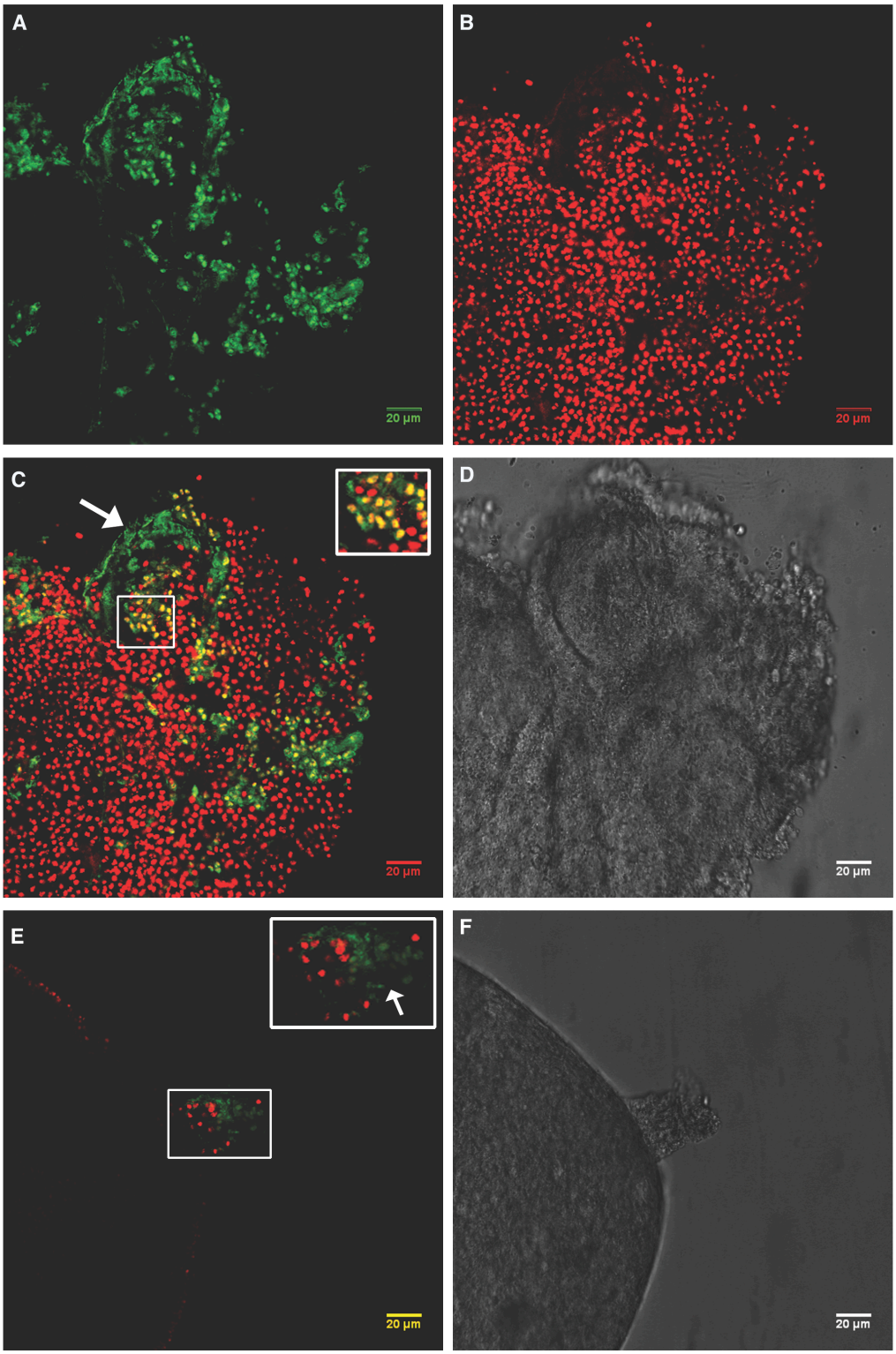


Fig. 4 Intracellular translocation of recombinant GFP36+SynHD*Antp*(C39S) in eye-antennal disc. Feed eye-antennal discs with 2 µM GFP36+SynHD*Antp*(C39S) for 4 hours at room temperature and then stained with 1 µg/ml Hoechst 33342 for 10 minutes before for confocal fluorescence microscopy. **A** GFP36+SynHD*Antp*(C39S) presented by green cellular internalization, one confocal slice is displayed. **B** Hoechst 33342 staining indicated by red, one confocal slice is displayed. **C** merged with A and B, GFP36+SynHD*Antp*(C39S) has accumulated in some of the nuclei around the wounded antero antennal disc, but not the whole disc. **D** bright field of A, B, C. **E** nueron optical, both GFP36+SynHD*Antp*(C39S) uptake and Hoechst 33342 staining are displayed. one confocal slice is displayed. The cells that have taken up GFP36+SynHD*Antp*(C39S) intend to exclude Hoechst 33342 staining, indicated by white arrows. **F** bright field of D.

unraveled that the internalization predominantly resulted from macropinocytosis. Recently, the report that modified supercharged GFP protein embraces the high capability of intracellular delivering cargo protein raised the question of whether it can facilitate accessing the cytosol delivery of the synthetic *Antennapedia* protein.

Following expression, the recombinant protein comprosed of supercharged GFP and synthetic version of the *Antennapedia* were purified by nickel and heparin column. Subsequent *in vitro* assay was shown that the purified protein can specifically bind to the DNA probe corresponding to the *Spineless* D4 enhancer, indicating the fusion protein containing functional homeodomain, and it quickly penetrate the plasma membrane of *Drosophila* S2 cells. In an *ex vivo* assay, this protein can pass through the basal membrane of eye-antennal discs and gain access to the nuclei of some of disc cells within a short period of time. Compared to various conventional CPPs such as Penetratin, TAT and polyarginine, supercharged GFP was shown for the first time to be able to accumulate in the nuclei of imaginal discs cells after short time incubation, exhibiting great potential for facilitating protein transduction by circumvent endoctyic entries.

Discussion

1. Homeotic transformation induced by protein transduction

Protein transduction is a concept which has been introduced into the scientific community since Frankel *et al.* and Joliot *et al.* reported respectively that *in vitro* purified proteins or polypeptides could pass through the plasma membrane and impose some biological impact on target cells. Subsequent mechanistic investigation suggested that this type of macromolecule translocation was receptor-independent, and a sort of protein transduction domains (PTDs) within these naturally occurring proteins were responsible for the internalization of their parent protein like cargos. Furthermore, these PTDs were found to be transplantable, namely they are able to deliver various proteins into cells and display no specificity on cargo selection. This transporting feature of PTDs makes them very intriguing as they may practically open an avenue of cellular transduction using protein or protein based therapeutics. Compared to conventional agents such as nucleic acids, compounds and lipids, proteins as agent can exhibit maximum functional specificity on intracellular targets and have no risk of integration into the genome. Therefore, PTDs have been intensively studied ever since, and more short sequences have been designed and tested for translocation ability, resulting in a quickly expanding family of peptides. Collectively, the term Cell-Penetrating Peptides (CPPs) has been coined to refer to those transduction domains and to the newly emerging peptides capable of penetrating cells. Although, the abilities of CPPs transporting are versatile in terms of cargoes selection as various small molecules, nanoparticles, liposome, nuclei acids including DNA, siRNAs and macroRNA have been documented to be successfully delivered into cells by CPPs, nevertheless, protein transduction is still the most interesting application of CPPs. On the one hand, there is no other effective approach for intracellular delivery protein; on the other hand, as mentioned above, proteins have unique advantages as agent. Therefore, our efforts have been focused on protein transduction in this thesis.

Rather than a unified term, protein transduction is more like a developing concept reflecting the application of CPPs associated with the functions of their cargo proteins. Nevertheless, protein transduction involves three indispensable steps: extracellular

application, plasma membrane translocation, and imposing some biological impact on the targets cells after internalization. Depending on the characteristics of cargo proteins, the impacts are very divers indeed, such as interfering pathological proteins by introducing dominant negative mutants, or providing increased amount of protective proteins in disordered conditions. Regarding specifically functioning as transcription factor and changing cell identity, the reports from Zhou *et al.* (Zhou *et al.,* 2009) and Kim *et al.* (Kim *et al.,* 2009) on protein-induced pluripotent stem cells (piPSCs) are informative. Polyarginine was fused to four reprogramming factors: Oct4, Sox2, Klf4, and c-Myc, and could generate piPSCs from fibroblasts. No concerns can be raised about introduction viral vectors as compared to the technique used in the first generation of induced pluripotent stem cells (iPSCs) by Shinya Yamanaka's team. However, the efficiency of transforming from a fibroblast to a pluripotent stem cell is significantly lower than viral based methods. While, no colony was obtained when only feeding with reprogramming proteins without the chemical boosting in Zhou's protocol; in Kim's protocol, five iPSC like colonies were obtained from 5 $\times 10^5$ input cells (about 0.001% of input cells) after 8 weeks establishment, approximately double the time with viral transduction and an efficiency which is 10 times lower (about 0.01% of input cells by viral transduction).

In the effort to induce specific antenna to leg transformations in *Drosophila* imaginal disc tissue without genetic manipulation, we have tried protein transduction. Tow decades ago, Joliot *et al.* reported that the homeodomain of *Antennapedia* embraces the capability of translocation across the plasma membrane from the intercellular space into the cytoplasm in a receptor-independent manner. In our investigation, *in vitro* generated synthetic *Antennapedia* composed of *Antennapedia* homeodomain and YPWM motif failed to reach the nuclei of target cells such as *Drosophila* S2 cells and imaginal disc cells. Instead they ended up in intracellular vesicle, presumably endosomes. Moreover, it could not facilitate the escape from these vesicles by fusing to extra CPPs inculding TAT or polyarginine, in addition to the endogenous Penetratin located in *Antennapedia* homeodomain. These observations motivated us to explore the mechanism underlying *Antennapedia* homeodomain cellular translocation. Systematic investigation of the mechanisms underlying the cellular uptake of *Antennapedia* homeodomain and its PTD Penetratin, we found macropinocytosis predominates protein internalization, which results in the association with vesicles and being inaccessible to the cytosol.

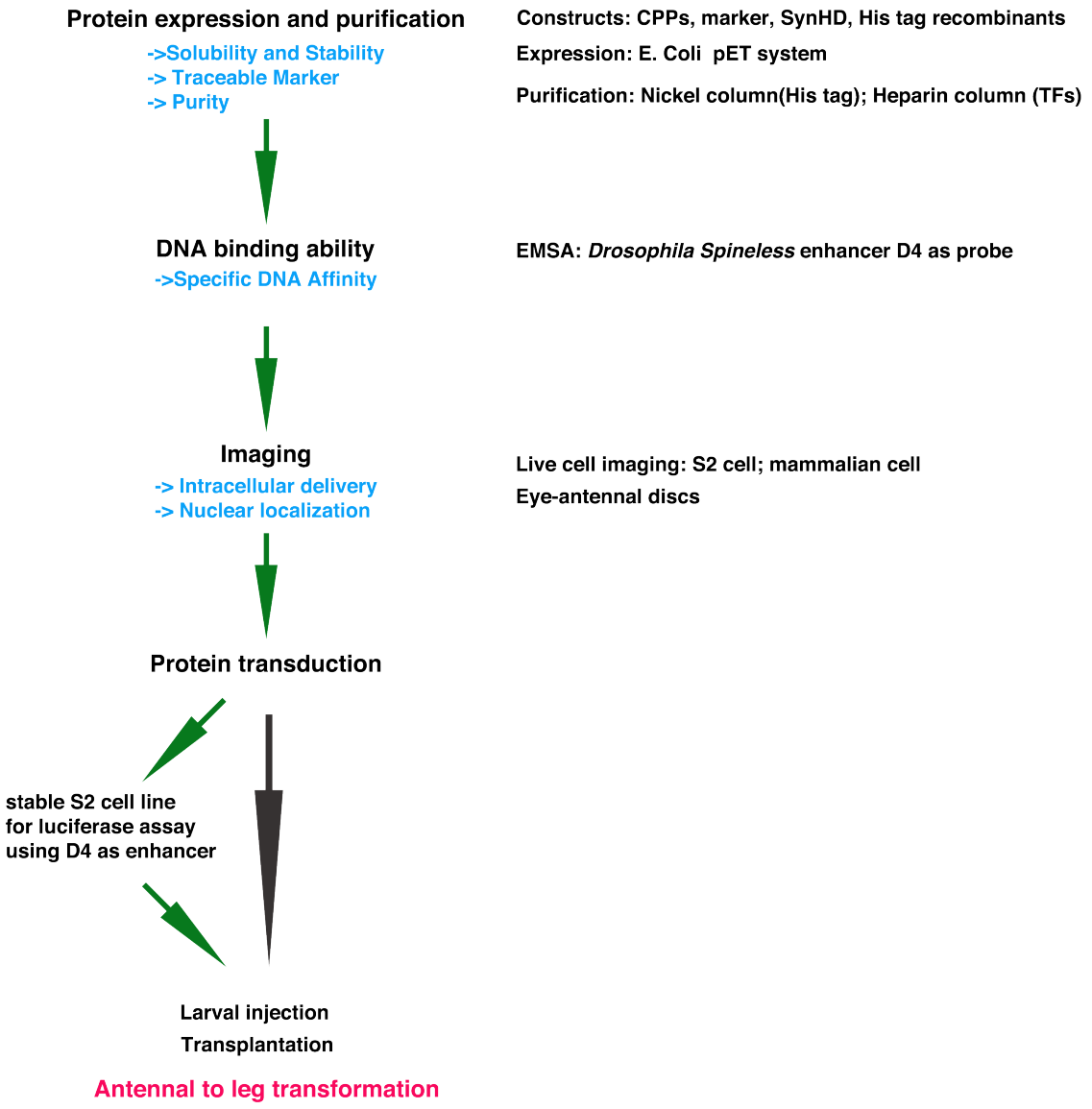
Recently engineered "supercharged" GFP, which has been extensively mutated at their surface-exposed residues resulting in extremely high net charge (GFP 36+), can deliver functional cargo proteins into the cytosol of a variety of cells *in vitro* and *in vivo,* with remarkable high efficiency and no apparent cytotoxicity. We hypothesized that supercharged GFP may also be utilized as a vector to circumvent the endocytic entry for intracellular delivery of synthetic *Antennapedia*. Then we purified the recombinant protein with GFP36+ fused to synthetic *Antennapedia* (homeodomain + YPWM motif) from *E. coli*, and demonstrated that it retains its specific binding properties like *in vitro* translated full-length *Antennapedia*. *Ex vivo* application the recombinant protein to imaginal discs reveals that GFP36+ fused synthetic *Antennapedia* can reach some nuclei of discs cells within a few hours, even though vesicle-associated intracellular distribution can be observed in S2 cell after very short time protein treatment, suggesting that GFP36+ might boost vesicle escape significantly.

However, homeotic transformation as the readout of protein transduction might be insensitive for accessing the performance of transduction proteins, given the inefficiency of piPSC obtained by protein transduction. One line of reporter at upstream should be established in the future to help the quantification of each protein as transduction reagent candidate, which I summarize in the modified work flow sheet as below:

- Luciferase assay in S2 cell line

  Genetic reporters are commonly used in cell biology to study gene expression and other cellular events coupled to gene expression, such as protein-DNA binding consensus interaction, receptor activity, intracellular signal transduction, mRNA processing, protein folding and protein-protein interactions. Firefly luciferase is widely used as a reporter for the following reasons:

  - Reporter activity is available immediately upon translation since the protein does not require post-translational processing.
  - The assay is very sensitive because its light production has the highest quantum efficiency known for any chemiluminescent reaction, and no background luminescence is found in the host cells or the assay chemistry.
  - The assay is rapid, requiring only a few seconds per sample.



The Luciferase Assay System is substantially improved over conventional assay methods in both sensitivity and simplicity. Light is produced by converting the chemical energy of luciferin oxidation through an electron transition, forming the oxyluciferin as product. Firefly luciferase, a monomeric 61kDa protein, catalyzes luciferin oxidation using ATP•Mg2+ as a co-substrate. The Luciferase Assay System yields linear results over at least eight orders of magnitude. Less than 10–20 molecules of luciferase have been detected under optimal conditions. Generally, 100-fold greater sensitivity can be achieved over the chloramphenicol acetyltransferase (CAT) assay.

In all, using D4 enhancer tandem as regulatory element for the luciferase reporter, we can detect how many functional protein molecules could reach nuclei and trans-activate downstream gene expression, quantify the performance of each protein as agent and quickly optimize the protein transduction condition. Compared to fluorescence microscopy, luciferase reporter system can provide delicate and functional guidance information before coming to *ex vivo* transplantation.

2. Direct penetration or endocytosis underlying the *Antennapedia* homeodomain translocation?

The plasma membrane is impermeable to most molecules other than small hydrophobic ones. The ability of small molecules to diffuse through a lipid bilayer is related to their lipid solubility. Strongly polar molecules such as ions traverse the bilayer extremely slowly, if at all; Small, more weakly polar molecules such as ethanol or glycerol can diffuse across relatively readily, but large molecule such as glucose penetrate very slowly. This explains why the phenomenon protein transduction has attracted considerable attention since its discovery, when it was found that the recombinant proteins Tat (hydrophilic macromolecule approximately 10 KDa) derived from human immunodeficiency virus-1 could gain access into the interior of the cells and trans-activate reporter gene expression like an endogenous transcription factor. Following Tat, a similar report came from a recombinant peptide corresponding to the sequence of the homeodomain of *Antennapedia* from *Drosophila,* which could penetrate into mammalian nerve cells and provoke a dramatic morphological differentiation of the neuronal cultures.

Two mechanisms of internalization have been proposed for cellular translocation: direct penetration through the lipid bilayer, and endocytosis. Direct penetration is an energy-independent mode, potentially via transient destabilization of the plasma membrane in the presence of these translocation polypeptides. Based on the observation that peptides enter the cell even at 4◦C, earlier studies had suggested that direct penetration was the mechanism for uptake of most peptides. Later studies, however, showed that experimental artifacts were responsible for this conclusion. Using methanol or formaldehyde to fix the investigated cells for microscopy causes artificial dye penetration during fixation by damaging the integrity of the plasma membrane. Applying trypsin to remove peptides associated with outside of the plasma membrane for fluorescence-activated cell sorting (FACS), and live cell imaging

became standard methodology of investigation.

As mentioned above, *in vitro* purified recombinant protein composed of the *Antennapedia* homeodomain and the YPWM motif did not reach the nuclei of the target cells including *Drosophila* S2 cells and imaginal disc cells and became associated with intracellular vesciles. The problem which we encountered contradicted to the theory that *Antennapedia* homeodomain embraces the ability of cellular translocation by direct penetration across the plasma membrane. Therefore, re-evaluating the internalization of *Antennapedia* homeodomain become indispensable or unavoidable for finding the solution to circumvent invalid intracellular delivery.

To assess the role of endocytosis for internalization, Hela cells, which are the most widely used cells for studying uptake mechanisms, were incubated at 4˚C and compared with the cells incubated at 37˚C. Confocal microcopy reveals that cold treatment can dramatically inhibit the internalization of *Antennapedia* homeodomain. At 37˚C the recombinant protein become localize in vesicles, strongly suggesting endocytosis plays an essential role underlying protein internalizaiton. Since endocytosis is a generic term for all mechanisms of cellular uptake associated with inward folding of the plasma membrane and pinching off of vesicles, we need to find out which type of endocytic entry is responsible for uptake of the *Antennapedia* homeodomain. The major endocytic pathways are usually distinguished on the basis of their differential sensitivity to pharmacological inhibitors, which can equally affect all cells in a population over a short period of time, and the effects can be easily quantified. In our study, with 70% inhibition of internalization, macropinocytosis inhibitor is the most efficient inhibitor tested, indicating *Antennapedia* homeodomain penetrates by macropinocytosis. Macropinocytic vesicles have no coat structures and are generally considered to be larger than 1 μm in diameter. As fluid phase of endocytosis, macropinocytosis provides cells with a way to internalize large quantities of solute non-selectively. Fluid phase dyes, serving as macropinosome markers, co-internalize with intracellular *Antennapedia* homeodomain, consistent with the observation of pharmacological inhibition by macropinocytosis inhibitor.

As macropinocytosis mediates *Antennapedia* homeodomain internalization, how the protein can escape from the macropinosome or more mature stages like early endosomes, multivesicular bodies, late endosomes and lysosomes, is the key challenge for protein

transduction. Since, utilizing supercharged GFP described above can facilitate the intracellular and nuclear delivery and largely solve the trafficking concerns, our effort for protein transduction can be more focused on the functional aspects in the future.

Constructs[1]	**Size**(KDa)	**Comments**[2]**:**
pET21b-(NotI)-SynHD*Antp*-(NotI)-ybbR[3]	/	Strong secondary structure forming on DNA template
pET21b-(NotI)-TAT-SynHD*Antp*-(NotI)-ybbR	/	
pET21b-(NotI)-Arg9-SynHD*Antp*-(NotI)-ybbR	/	
pET21b-(NotI)-Penetratin-SynHD*Antp*-(NotI)-ybbR	/	
pET21b-SynHD*Antp*-ybbR	11.42	Low expression efficiency
pET21b-TAT-SynHD*Antp*-ybbR	13.09	
pET21b-Arg9-SynHD*Antp*-ybbR	12.96	
pET21b-Penetratin-SynHD*Antp*-ybbR	13.48	
pET21b-SynHD*Antp*-ybbR-$(His)_6$	12.49	Expression and purification efficiency varied; ybbR conjugated dyes are plasma membrane permeable, which generating high signal background
pET21b-TAT-SynHD*Antp*-ybbR-$(His)_6$	14.16	
pET21b-Arg9-SynHD*Antp*-ybbR-$(His)_6$	14.03	
pET21b-Penetratin-SynHD*Antp*-ybbR-$(His)_6$	14.55	
pET21b-Citrine-SynHD*SCR*(WT)-$(His)_6$	41.53	High purity can be achieved by one step Nickel column purification; but proteins are of low solubility and instability.
pET21b-TAT-Citrine-SynHD*SCR*(WT)-$(His)_6$	44.63	
pET21b-Arg9-Citrine-SynHD*SCR*(WT)-$(His)_6$	44.81	
pET21b-Penetratin-Citrine-SynHD*SCR*(WT)-$(His)_6$	45.32	
pET21b-Citrine-SynHD*SCR*(AA)-$(His)_6$	41.53	
pET21b-TAT-Citrine-SynHD*SCR*(AA)-(-$(His)_6$	44.63	
pET21b-Arg9-Citrine-SynHD*SCR*(AA)-$(His)_6$	44.81	
pET21b-Penetratin-Citrine-SynHD*SCR*(AA)-(-$(His)_6$	45.32	
pET21b-Citrine-SynHD*SCR*(DD)-$(His)_6$	41.53	
pET21b-TAT-Citrine-SynHD*SCR*(DD)-(-$(His)_6$	44.63	
pET21b-Arg9-Citrine-SynHD*SCR*(DD)-$(His)_6$	44.81	
pET21b-Penetratin-Citrine-SynHD*SCR*(DD)-(-$(His)_6$	45.32	
pMal-MBP-IEGR-SynHD*Antp*-mCherry	39.45	Insufficient purity
pMal-MBP-IEGR-SynHD*Antp*-mCherry-Arg9	41.13	
pMal-MBP-IEGR-NLS-SynHD*Antp*-mCherry-Arg9	41.94	
pMal-MBP-IEGR-mCherry-Arg9	30.18	
pMal-MBP-IEGR-mCherry	28.77	
pET21b-$(His)_6$-SynHD*Antp*-mCherry	38.33	Improved purity compared to MBP constructs; but purity remain insufficient; artificial dimmer between HD*Antp*
pET21b-$(His)_6$-SynHD*Antp*-mCherry-Arg9	39.73	
pET21b-$(His)_6$-NLS-SynHD*Antp*-mCherry-Arg9	40.54	
pET21b-$(His)_6$-SynHD*Antp*-mCherry-TAT	39.97	
pET21b-$(His)_6$-mCherry-Arg9	29.57	
pET21b-$(His)_6$-mCherry	28.07	
pET21b-$(His)_{10}$-SynHD*Antp*(C39S)-mCherry	38.86	High purity can be achieved by one step Nickel column purification; proteins are soluble and stable.
pET21b-$(His)_{10}$-SynHD*Antp*(C39S)-mCherry-Arg9	40.26	
pET21b-$(His)_{10}$-SynHD*Antp*(C39S)-mCherry-TAT	40.40	
pET21b-$(His)_{10}$-HD*Antp*(C39S)-mCherry	37.24	
pET21b-$(His)_{10}$-mCherry-Penetratin	29.83	
pET21b-$(His)_{10}$-mCherry	28.60	

[1] The constructs have been generated for the project of this thesis.
[2] The protein product from each construct has been tested and screened for optimizing protein as transduction reagents.
[3] A short peptide which can be conjugated to fluorescence dye by *in vitro* reaction.

Reference

Alves, I. D., C. Y. Jiao, S. Aubry, B. Aussedat, F. Burlina, G. Chassaing, et al. (2010). "Cell biology meets biophysics to unveil the different mechanisms of penetratin internalization in cells." Biochimica Et Biophysica Acta-Biomembranes **1798**(12): 2231-2239.

Amand, H. L., K. Fant, B. Norden and E. K. Esbjorner (2008). "Stimulated endocytosis in penetratin uptake: Effect of arginine and lysine." Biochemical and Biophysical Research Communications **371**(4): 621-625.

Amsellem, S., F. Pflumio, D. Bardinet, B. Izac, P. Charneau, P. H. Romeo, et al. (2003). "Ex vivo expansion of human hematopoietic stem cells by direct delivery of the HOXB4 homeoprotein." Nature Medicine **9**(11): 1423-1427.

Amyere, M., M. Mettlen, P. Van der Smissen, A. Platek, B. Payrastre, A. Veithen, et al. (2002). "Origin, originality, functions, subversions and molecular signalling of macropinocytosis." International Journal of Medical Microbiology **291**(6-7): 487-494.

Anderson, R. G., M. S. Brown and J. L. Goldstein (1977). "Role of the coated endocytic vesicle in the uptake of receptor-bound low density lipoprotein in human fibroblasts." Cell **10**(3): 351-364.

Anderson, R. G., B. A. Kamen, K. G. Rothberg and S. W. Lacey (1992). "Potocytosis: sequestration and transport of small molecules by caveolae." Science **255**(5043): 410-411.

Anderson, R. G. W. (1998). "The caveolae membrane system." Annual Review of Biochemistry **67**: 199-225.

Becker, M. L., E. E. Remsen, D. Pan and K. L. Wooley (2004). "Peptide-derivatized shell-cross-linked nanoparticles. 1. Synthesis and characterization." Bioconjugate Chemistry **15**(4): 699-709.

Billeter, M., Y. Q. Qian, G. Otting, M. Muller, W. Gehring and K. Wuthrich (1993). "Determination of the Nuclear-Magnetic-Resonance Solution Structure of an Antennapedia Homeodomain-DNA Complex." Journal of Molecular Biology **234**(4): 1084-1094.

Blondeau, F., B. Ritter, P. D. Allaire, S. Wasiak, M. Girard, N. K. Hussain, et al. (2004). "Tandem MS analysis of brain clathrin-coated vesicles reveals their critical involvement in synaptic vesicle recycling." Proc Natl Acad Sci U S A **101**(11): 3833-3838.

Bogoyevitch, M. A., T. S. Kendrick, D. C. H. Ng and R. K. Barr (2002). "Taking the cell by stealth or storm? Protein Transduction Domains (PTDs) as versatile vectors for delivery." DNA and Cell Biology **21**(12): 879-894.

Brooks, H., B. Lebleu and E. Vives (2005). "Tat peptide-mediated cellular delivery: back to basics." Advanced Drug Delivery Reviews **57**(4): 559-577.

Cao, G. D., W. Pei, H. L. Ge, Q. H. Liang, Y. M. Luo, F. R. Sharp, et al. (2002). "In vivo delivery of a Bcl-xL fusion protein containing the TAT protein transduction domain protects against ischemic brain injury and neuronal apoptosis." Journal of Neuroscience **22**(13): 5423-5431.

Cheng, Y., W. Boll, T. Kirchhausen, S. C. Harrison and T. Walz (2007). "Cryo-electron tomography of clathrin-coated vesicles: structural implications for coat assembly." Journal of Molecular Biology **365**(3): 892-899.

Cheng, Z. J., R. D. Singh, D. K. Sharma, E. L. Holicky, K. Hanada, D. L. Marks, et al. (2006). "Distinct mechanisms of clathrin-independent endocytosis have unique sphingolipid requirements." Molecular Biology of the Cell **17**(7): 3197-3210.

Chiu, Y. L., A. Ali, C. Y. Chu, H. Cao and T. M. Rana (2004). "Visualizing a correlation between siRNA localization, cellular uptake, and RNAi in living cells." Chemistry & Biology **11**(8): 1165-1175.

Cohen, B., E. A. Wimmer and S. M. Cohen (1991). "Early Development of Leg and Wing Primordia in the Drosophila Embryo." Mechanisms of Development **33**(3): 229-240.

Collins, B. M., A. J. McCoy, H. M. Kent, P. R. Evans and D. J. Owen (2002). "Molecular architecture and functional model of the endocytic AP2 complex." Cell **109**(4): 523-535.

Cronican, J. J., K. T. Beier, T. N. Davis, J. C. Tseng, W. D. Li, D. B. Thompson, et al. (2011). "A Class of Human Proteins that Deliver Functional Proteins into Mammalian Cells In Vitro and In Vivo." Chemistry & Biology **18**(7): 833-838.

Cronican, J. J., D. B. Thompson, K. T. Beier, B. R. McNaughton, C. L. Cepko and D. R. Liu (2010). "Potent Delivery of Functional Proteins into Mammalian Cells in Vitro and in Vivo Using a Supercharged Protein." Acs Chemical Biology **5**(8): 747-752.

Cryan, S. A., M. Devocelle, P. J. Moran, A. J. Hickey and J. G. Kelly (2006). "Increased intracellular targeting to airway cells using octaarginine-coated liposomes: In vitro assessment of their suitability for inhalation." Molecular Pharmaceutics **3**(2): 104-112.

Davidson, T. J., S. Harel, V. A. Arboleda, G. F. Prunell, M. L. Shelanski, L. A. Greene, et al. (2005). "Highly efficient small interfering RNA delivery to primary mammalian neurons induces microRNA-like effects before mRNA degradation (vol 25, pg 10040, 2004)." Journal of Neuroscience **25**(5): 1311-1311.

Derossi, D., S. Calvet, A. Trembleau, A. Brunissen, G. Chassaing and A. Prochiantz (1996). "Cell internalization of the third helix of the antennapedia homeodomain is receptor-independent." Journal of Biological Chemistry **271**(30): 18188-18193.

Derossi, D., A. H. Joliot, G. Chassaing and A. Prochiantz (1994). "The 3rd Helix of the Antennapedia Homeodomain Translocates through Biological-Membranes." Journal of Biological Chemistry **269**(14): 10444-10450.

Deshayes, S., M. C. Morris, G. Divita and F. Heitz (2005). "Cell-penetrating peptides: tools for intracellular delivery of therapeutics." Cellular and Molecular Life Sciences **62**(16): 1839-1849.

Doherty, G. J. and H. T. McMahon (2009). "Mechanisms of Endocytosis." Annual Review of Biochemistry **78**: 857-902.

Dong, M., D. A. Richards, M. C. Goodnough, W. H. Tepp, E. A. Johnson and E. R. Chapman (2003). "Synaptotagmins I and II mediate entry of botulinum neurotoxin B into cells." Journal of Cell Biology **162**(7): 1293-1303.

Drin, G., S. Cottin, E. Blanc, A. R. Rees and J. Temsamani (2003). "Studies on the internalization mechanism of cationic cell-penetrating peptides." Journal of Biological Chemistry **278**(33): 31192-31201.

Duchardt, F., M. Fotin-Mleczek, H. Schwarz, R. Fischer and R. Brock (2007). "A comprehensive model for the cellular uptake of cationic cell-penetrating peptides." Traffic **8**(7): 848-866.

Duncan, D., P. Kiefel and I. Duncan (2010). "Control of the spineless antennal enhancer: Direct repression of antennal target genes by Antennapedia." Developmental Biology **347**(1): 82-91.

Ehrlich, M., W. Boll, A. van Oijen, R. Hariharan, K. Chandran, M. L. Nibert, et al. (2004). "Endocytosis by random initiation and stabilization of clathrin-coated pits." Cell **118**(5): 591-605.

El-Andaloussi, S., P. Jarver, H. J. Johansson and U. Langel (2007). "Cargo-dependent cytotoxicity and delivery efficacy of cell-penetrating peptides: a comparative study." Biochemical Journal **407**: 285-292.

Elliott, W. H. E., D. C. (2009). "Biochemistry and Molecular Biology." Oxford Univeristy Press **7**: 102-107.

Eto, D. S., H. B. Gordon, B. K. Dhakal, T. A. Jones, M. A. Mulvey, H. B. Gordon, et al. (2008). "Clathrin, AP-2, and the NPXY-binding subset of alternate endocytic adaptors facilitate FimH-mediated bacterial invasion of host cells." Cellular Microbiology **10**(12): 2553-2567.

Fawell, S., J. Seery, Y. Daikh, C. Moore, L. L. Chen, B. Pepinsky, et al. (1994). "Tat-Mediated Delivery of Heterologous Proteins into Cells." Proceedings of the National Academy of Sciences of the United States of America **91**(2): 664-668.

Ferrari, A., V. Pellegrini, C. Arcangeli, A. Fittipaldi, M. Giacca and F. Beltram (2003). "Caveolae-mediated internalization of extracellular HIV-1 tat fusion proteins visualized in real time." Mol Ther **8**(2): 284-294.

Fischer, P. M. (2007). "Cellular uptake mechanisms and potential therapeutic utility of peptidic cell delivery vectors: Progress 2001-2006." Medicinal Research Reviews **27**(6): 755-795.

Fischer, P. M., E. Krausz and D. P. Lane (2001). "Cellular delivery of impermeable effector molecules in the form of conjugates with peptides capable of mediating membrane translocation." Bioconjugate Chemistry **12**(6): 825-841.

Fischer, P. M., N. Z. Zhelev, S. Wang, J. E. Melville, R. Fahraeus and D. P. Lane (2000). "Structure-activity relationship of truncated and substituted analogues of the intracellular delivery vector Penetratin." Journal of Peptide Research **55**(2): 163-172.

Fischer, R., K. Kohler, M. Fotin-Mleczek and R. Brock (2004). "A stepwise dissection of the intracellular fate of cationic cell-penetrating peptides." J Biol Chem **279**(13): 12625-12635.

Fittipaldi, A., A. Ferrari, M. Zoppe, C. Arcangeli, V. Pellegrini, F. Beltram, et al. (2003). "Cell membrane lipid rafts mediate caveolar endocytosis of HIV-1 Tat fusion proteins." J Biol Chem **278**(36): 34141-34149.

Foged, C. and H. M. Nielsen (2008). "Cell-penetrating peptides for drug delivery across membrane barriers." Expert Opinion on Drug Delivery **5**(1): 105-117.

Fotin-Mleczek, M., S. Welte, O. Mader, F. Duchardt, R. Fischer, H. Hufnagel, et al. (2005). "Cationic cell-penetrating peptides interfere with TNF signalling by induction of TNF receptor internalization." J Cell Sci **118**(Pt 15): 3339-3351.

Frankel, A. D. and C. O. Pabo (1988). "Cellular Uptake of the Tat Protein from Human Immunodeficiency Virus." Cell **55**(6): 1189-1193.

Fretz, M. M., N. A. Penning, S. Al-Taei, S. Futaki, T. Takeuchi, I. Nakase, et al. (2007). "Temperature-, concentration- and cholesterol-dependent translocation of L- and D-octa-arginine across the plasma and nuclear membrane of CD34(+) leukaemia cells." Biochemical Journal **403**: 335-342.

Fujimoto, L. M., R. Roth, J. E. Heuser and S. L. Schmid (2000). "Actin assembly plays a variable, but not obligatory role in receptor-mediated endocytosis in mammalian cells." Traffic **1**(2): 161-171.

Futaki, S., I. Nakase, A. Taclokoro, T. Takeuchi and A. T. Jones (2007). "Arginine-rich peptides and their internalization mechanisms." Biochemical Society Transactions **35**: 784-787.

Futaki, S., T. Suzuki, W. Ohashi, T. Yagami, S. Tanaka, K. Ueda, et al. (2001). "Arginine-rich peptides - An abundant source of membrane-permeable peptides having potential

as carriers for intracellular protein delivery." Journal of Biological Chemistry **276**(8): 5836-5840.

Garcia, C. K., K. Wilund, M. Arca, G. Zuliani, R. Fellin, M. Maioli, et al. (2001). "Autosomal recessive hypercholesterolemia caused by mutations in a putative LDL receptor adaptor protein." Science **292**(5520): 1394-1398.

Garcia-Bellido, A., P. Ripoll and G. Morata (1973). "Developmental compartmentalisation of the wing disk of Drosophila." Nature: New biology **245**(147): 251-253.

Gehring, W. J. and Y. Hiromi (1986). "Homeotic Genes and the Homeobox." Annual Review of Genetics **20**: 147-173.

Gehring, W. J., U. Kloter and H. Suga (2009). "Evolution of the Hox gene complex from an evolutionary ground state." Current topics in developmental biology **88**: 35-61.

Gerbal-Chaloin, S., C. Gondeau, G. Aldrian-Herrada, F. Heitz, C. Gauthier-Rouviere and G. Divita (2007). "First step of the cell-penetrating peptide mechanism involves Rac1 GTPase-dependent actin-network remodelling." Biology of the Cell **99**(4): 223-238.

Gorfinkiel, N., G. Morata and I. Guerrero (1997). "The homeobox gene Distal-less induces ventral appendage development in Drosophila." Genes Dev **11**(17): 2259-2271.

Gorter, E. G., F. (1925). "On bimolecular layers of lipids on the chromocytes of the blood." Journal of Experimental Medicine **41**(4): 439-443.

Gross, L. A., G. S. Baird, R. C. Hoffman, K. K. Baldridge and R. Y. Tsien (2000). "The structure of the chromophore within DsRed, a red fluorescent protein from coral." Proceedings of the National Academy of Sciences of the United States of America **97**(22): 11990-11995.

Hadorn, E. (1965). "[Loss of Potency for Bristle Formation as a "Hereditary Feature" of a Cell Culture of Drosophila Melanogaster]." Z Naturforsch B **20**: 290-292.

Haigler, H. T., J. A. Mckanna and S. Cohen (1979). "Rapid Stimulation of Pinocytosis in Human Carcinoma-Cells a-431 by Epidermal Growth-Factor." Journal of Cell Biology **83**(1): 82-90.

Hansen, M., K. Kilk and U. Langel (2008). "Predicting cell-penetrating peptides." Advanced Drug Delivery Reviews **60**(4-5): 572-579.

Heitz, F., M. C. Morris and G. Divita (2009). "Twenty years of cell-penetrating peptides: from molecular mechanisms to therapeutics." British Journal of Pharmacology **157**(2): 195-206.

Henriques, S. T., M. N. Melo and M. Castanho (2006). "Cell-penetrating peptides and antimicrobial peptides: how different are they?" Biochemical Journal **399**: 1-7.

Hinshaw, J. E. and S. L. Schmid (1995). "Dynamin Self-Assembles into Rings Suggesting a Mechanism for Coated Vesicle Budding." Nature **374**(6518): 190-192.

Hooke, R. C. (1665). " Micrographia: or Some Physiological Descriptions of Miniature Bodies Made by Magnifying Glasses." Jo. Martyn, and Ja. Allestry.

Hopkins, C. R., K. Miller and J. M. Beardmore (1985). "Receptor-mediated endocytosis of transferrin and epidermal growth factor receptors: a comparison of constitutive and ligand-induced uptake." J Cell Sci Suppl **3**: 173-186.

Jarver, P. and U. Langel (2004). "The use of cell-penetrating peptides as a tool for gene regulation." Drug Discovery Today **9**(9): 395-402.

Jiao, C. Y., D. Delaroche, F. Burlina, I. D. Alves, G. Chassaing and S. Sagan (2009). "Translocation and Endocytosis for Cell-penetrating Peptide Internalization." Journal of Biological Chemistry **284**(49): 33957-33965.

Jo, D. W., A. Nashabi, C. Doxsee, Q. Lin, D. Unutmaz, J. Chen, et al. (2001). "Epigenetic regulation of gene structure and function with a cell-permeable Cre recombinase." Nature Biotechnology **19**(10): 929-933.

Joliot, A., C. Pernelle, H. Deagostinibazin and A. Prochiantz (1991). "Antennapedia Homeobox Peptide Regulates Neural Morphogenesis." Proceedings of the National Academy of Sciences of the United States of America **88**(5): 1864-1868.

Joliot, A. and A. Prochiantz (2004). "Transduction peptides: from technology to physiology." Nature Cell Biology **6**(3): 189-196.

Josephson, L., C. H. Tung, A. Moore and R. Weissleder (1999). "High-efficiency intracellular magnetic labeling with novel superparamagnetic-tat peptide conjugates." Bioconjugate Chemistry **10**(2): 186-191.

Kabouridis, P. S., M. Hasan, J. Newson, D. W. Gilroy and T. Lawrence (2002). "Inhibition of NF-kappa B activity by a membrane-transducing mutant of I kappa B alpha." Journal of Immunology **169**(5): 2587-2593.

Kamei, N., M. Morishita, Y. Eda, N. Ida, R. Nishio and K. Takayama (2008). "Usefulness of cell-penetrating peptides to improve intestinal insulin absorption." Journal of Controlled Release **132**(1): 21-25.

Kawamoto, S., M. Takasu, T. Miyakawa, R. Morikawa, T. Oda, S. Futaki, et al. (2011). "Inverted micelle formation of cell-penetrating peptide studied by coarse-grained simulation: Importance of attractive force between cell-penetrating peptides and lipid head group." Journal of Chemical Physics **134**(9).

Kerkis, A., M. A. F. Hayashi, T. Yamane and I. Kerkis (2006). "Properties of cell penetrating peptides (CPPs)." Iubmb Life **58**(1): 7-13.

Keyel, P. A., S. K. Mishra, R. Roth, J. E. Heuser, S. C. Watkins and L. M. Traub (2006). "A single common portal for clathrin-mediated endocytosis of distinct cargo governed by cargo-selective adaptors." Molecular Biology of the Cell **17**(10): 4300-4317.

Khafagy, E. S., M. Morishita, K. Isowa, J. Imai and K. Takayama (2009). "Effect of cell-penetrating peptides on the nasal absorption of insulin." Journal of Controlled Release **133**(2): 103-108.

Khalil, I. A., K. Kogure, H. Akita and H. Harashima (2006). "Uptake pathways and subsequent intracellular trafficking in nonviral gene delivery." Pharmacological Reviews **58**(1): 32-45.

Kim, D., C. H. Kim, J. I. Moon, Y. G. Chung, M. Y. Chang, B. S. Han, et al. (2009). "Generation of Human Induced Pluripotent Stem Cells by Direct Delivery of Reprogramming Proteins." Cell Stem Cell **4**(6): 472-476.

Kleinzeller, A. (1997). "Ernest Overton's Contribution to the Cell Membrane Concept: A Centennial Appreciation." Physiology **12**(1): 49-53.

Kosaka, T. and K. Ikeda (1983). "Reversible Blockage of Membrane Retrieval and Endocytosis in the Garland Cell of the Temperature-Sensitive Mutant of Drosophila-Melanogaster, Shibirets1." Journal of Cell Biology **97**(2): 499-507.

Krosl, G., M. P. Giard, J. Krosl, R. K. Humphries, G. Sauvageau and D. C. Roy (2004). "Recombinant TAT-HOXB4 protein promotes ex vivo expansion of primitive human hematopoietic cells." Blood **104**(11): 780A-781A.

Krosl, J., P. Austin, N. Beslu, E. Kroon, R. K. Humphries and G. Sauvageau (2003). "In vitro expansion of hematopoietic stem cells by recombinant TAT-HOXB4 protein." Nature Medicine **9**(11): 1428-1432.

Kueltzo, L. A. and C. R. Middaugh (2003). "Nonclassical transport proteins and peptides: An alternative to classical macromolecule delivery systems." Journal of Pharmaceutical Sciences **92**(9): 1754-1772.

Laliberte, J. P. and B. Moss (2009). "Appraising the apoptotic mimicry model and the role of phospholipids for poxvirus entry." Proc Natl Acad Sci U S A **106**(41): 17517-17521.

Leroux, I., A. H. Joliot, E. Blochgallego, A. Prochiantz and M. Volovitch (1993). "Neurotrophic Activity of the Antennapedia Homeodomain Depends on Its Specific DNA-Binding Properties." Proceedings of the National Academy of Sciences of the United States of America **90**(19): 9120-9124.

Lewin, M., N. Carlesso, C. H. Tung, X. W. Tang, D. Cory, D. T. Scadden, et al. (2000). "Tat peptide-derivatized magnetic nanoparticles allow in vivo tracking and recovery of progenitor cells." Nature Biotechnology **18**(4): 410-414.

Lewis, B. A. and D. M. Engelman (1983). "Lipid Bilayer Thickness Varies Linearly with Acyl Chain-Length in Fluid Phosphatidylcholine Vesicles." Journal of Molecular Biology **166**(2): 211-217.

Lewis, E. B. (1978). "A gene complex controlling segmentation in Drosophila." Nature **276**(5688): 565-570.

Lewis, H. (1931). "Pinocytosis." John Hopkins Hosp. Bull. **47**: 666-679.

Lim, J. P. and P. A. Gleeson (2011). "Macropinocytosis: an endocytic pathway for internalising large gulps." Immunology and Cell Biology **89**(8): 836-843.

Lindgren, M., K. Rosenthal-Aizman, K. Saar, E. Eiriksdottir, Y. Jiang, M. Sassian, et al. (2006). "Overcoming methotrexate resistance in breast cancer tumour cells by the use of a new cell-penetrating peptide." Biochemical Pharmacology **71**(4): 416-425.

Lindsay, M. A. (2002). "Peptide-mediated cell delivery: application in protein target validation." Current Opinion in Pharmacology **2**(5): 587-594.

Liu, J. Q., Q. Zhang, E. E. Remsen and K. L. Wooley (2001). "Nanostructured materials designed for cell binding and transduction." Biomacromolecules **2**(2): 362-368.

Liu, J. Q., Q. Zhang and K. L. Wooley (2000). "Bioconjugates of protein transduction domain (PTD) and shell crosslinked nanoparticles: Nanostructured materials designed for cell delivery." Abstracts of Papers of the American Chemical Society **220**: U289-U289.

Lundberg, P. and U. Langel (2003). "A brief introduction to cell-penetrating peptides." Journal of Molecular Recognition **16**(5): 227-233.

Madani, F., S. Lindberg, U. Langel, S. Futaki and A. Graslund (2011). "Mechanisms of cellular uptake of cell-penetrating peptides." J Biophys **2011**: 414729.

Magzoub, M. and A. Graslund (2004). "Cell-penetrating peptides: from inception to application (vol 37, pg 147, 2004)." Quarterly Reviews of Biophysics **37**(3-4): 315-315.

Magzoub, M., S. Sandgren, P. Lundberg, K. Oglecka, J. Lilja, A. Wittrup, et al. (2006). "N-terminal peptides from unprocessed prion proteins enter cells by macropinocytosis." Biochem Biophys Res Commun **348**(2): 379-385.

Mayor, S. and R. E. Pagano (2007). "Pathways of clathrin-independent endocytosis." Nature Reviews Molecular Cell Biology **8**(8): 603-612.

Mcginnis, W., C. P. Hart, W. J. Gehring and F. H. Ruddle (1984). "Molecular-Cloning and Chromosome Mapping of a Mouse DNA-Sequence Homologous to Homeotic Genes of Drosophila." Cell **38**(3): 675-680.

McMahon, H. T. and E. Boucrot (2011). "Molecular mechanism and physiological functions of clathrin-mediated endocytosis." Nature Reviews Molecular Cell Biology **12**(8): 517-533.

Meltzer, J. (1904). "Edema: A Consideration of Physiologic and Pathologic Factors Concerned in Its Formation."

Mercer, J. and A. Helenius (2008). "Vaccinia virus uses macropinocytosis and apoptotic mimicry to enter host cells." Science **320**(5875): 531-535.

Metchnikoff, É. (1883). "Untersuchungen über die Mesodermalen Phagozyten einiger Wirbeltiere." Biol. Zent. bI. **3**: 560-565.

Minami, M., M. Kinoshita, Y. Kamoshida, H. Tanimoto and T. Tabata (1999). "Brinker is a target of Dpp in Drosophila that negatively regulates Dpp-dependent genes." Nature **398**(6724): 242-246.

Mishra, A., G. H. Lai, N. W. Schmidt, V. Z. Sun, A. R. Rodriguez, R. Tong, et al. (2011). "Translocation of HIV TAT peptide and analogues induced by multiplexed membrane and cytoskeletal interactions." Proceedings of the National Academy of Sciences of the United States of America **108**(41): 16883-16888.

Morata, G. (2001). "How Drosophila appendages develop." Nature Reviews Molecular Cell Biology **2**(2): 89-97.

Moreno-Ruiz, E., M. Galan-Diez, W. D. Zhu, E. Fernandez-Ruiz, C. d'Enfert, S. G. Filler, et al. (2009). "Candida albicans internalization by host cells is mediated by a clathrin-dependent mechanism." Cellular Microbiology **11**(8): 1179-1189.

Morris, R. E., A. S. Gerstein, P. F. Bonventre and C. B. Saelinger (1985). "Receptor-mediated entry of diphtheria toxin into monkey kidney (Vero) cells: electron microscopic evaluation." Infection and immunity **50**(3): 721-727.

Moy, P., Y. Daikh, B. Pepinsky, D. Thomas, S. Fawell and J. Barsoum (1996). "Tat-mediated protein delivery can facilitate MHC class I presentation of antigens." Molecular Biotechnology **6**(2): 105-113.

Muller, M., M. Affolter, W. Leupin, G. Otting, K. Wuthrich and W. J. Gehring (1988). "Isolation and sequence-specific DNA binding of the Antennapedia homeodomain." The EMBO journal **7**(13): 4299-4304.

Muratovska, A. and M. R. Eccles (2004). "Conjugate for efficient delivery of short interfering RNA (siRNA) into mammalian cells." Febs Letters **558**(1-3): 63-68.

Oehlke, J., A. Scheller, B. Wiesner, E. Krause, M. Beyermann, E. Klauschenz, et al. (1998). "Cellular uptake of an alpha-helical amphipathic model peptide with the potential to deliver polar compounds into the cell interior non-endocytically." Biochimica Et Biophysica Acta-Biomembranes **1414**(1-2): 127-139.

Ono, M., Y. Sawa, M. Ryugo, A. N. Alechine, S. Shimizu, R. Sugioka, et al. (2005). "BH4 peptide derivative from Bcl-xL attenuates ischemia/reperfusion injury thorough anti-apoptotic mechanism in rat hearts." European Journal of Cardio-Thoracic Surgery **27**(1): 117-121.

Overton, E. (1895). "Über die osmotischen Eigenschaften der lebenden Pflanzen und Thierzelle." Vierteljahrsschr.Naturforsch. Ges. Zürich **40**: 159-201.

Overton, E. (1896). "Über die osmotischen Eigenschaften der Zelle und ihre Bedeutung für die Toxikologie und Pharmakologie." Vierteljahrsschr. Naturforsch. Ges. Zürich **41**: 383-406.

Overton, E. (1899). "Über die allgemeinen osmotischen Eigenschaften der Zelle, ihre vermutliche Ursachen und ihre Bedeutung für die Physiologie." Vierteljahrsschr. Naturforsch. Ges. Zürich **44**: 88-114.

Overton, E. (1901). "Studien über die Narkose, zugleich ein Beitrag zur allgemeinen Pharmakologie." Jena, Germany: Fischer Verlag.

Papadopoulos, D. K., D. Resendez-Perez, D. L. Cardenas-Chavez, K. Villanueva-Segura, R. Canales-del-Castillo, D. A. Felix, et al. (2011). "Functional synthetic Antennapedia genes and the dual roles of YPWM motif and linker size in transcriptional activation and repression." Proceedings of the National Academy of Sciences of the United States of America **108**(29): 11959-11964.

Payne, C. K., S. A. Jones, C. Chen and X. Zhuang (2007). "Internalization and trafficking of cell surface proteoglycans and proteoglycan-binding ligands." Traffic **8**(4): 389-401.

Pearse, B. M. F. (1976). "Clathrin - Unique Protein Associated with Intracellular Transfer of Membrane by Coated Vesicles." Proceedings of the National Academy of Sciences of the United States of America **73**(4): 1255-1259.

Perry, M. M. and A. B. Gilbert (1979). "Yolk transport in the ovarian follicle of the hen (Gallus domesticus): lipoprotein-like particles at the periphery of the oocyte in the rapid growth phase." Journal of cell science **39**: 257-272.

Pouny, Y., D. Rapaport, A. Mor, P. Nicolas and Y. Shai (1992). "Interaction of Antimicrobial Dermaseptin and Its Fluorescently Labeled Analogs with Phospholipid-Membranes." Biochemistry **31**(49): 12416-12423.

Prochiantz, A. and A. Joliot (2003). "Can transcription factors function as cell-cell signalling molecules?" Nature Reviews Molecular Cell Biology **4**(10): 814-819.

Qian, Y. Q., D. Resendezperez, W. J. Gehring and K. Wuthrich (1994). "The Des(1-6)Antennapedia Homeodomain - Comparison of the Nmr Solution Structure and the DNA-Binding Affinity with the Intact Antennapedia Homeodomain." Proceedings of the National Academy of Sciences of the United States of America **91**(9): 4091-4095.

Racoosin, E. L. and J. A. Swanson (1989). "Macrophage Colony-Stimulating Factor (Rm-Csf) Stimulates Pinocytosis in Bone Marrow-Derived Macrophages." Journal of Experimental Medicine **170**(5): 1635-1648.

Richard, J. P., K. Melikov, H. Brooks, P. Prevot, B. Lebleu and L. V. Chernomordik (2005). "Cellular uptake of unconjugated TAT peptide involves clathrin-dependent endocytosis and heparan sulfate receptors." J Biol Chem **280**(15): 15300-15306.

Richard, J. P., K. Melikov, E. Vives, C. Ramos, B. Verbeure, M. J. Gait, et al. (2003). "Cell-penetrating peptides - A reevaluation of the mechanism of cellular uptake." Journal of Biological Chemistry **278**(1): 585-590.

Robertson, J. D. (1958). "The Cell Membrane Concept." Journal of Physiology-London **140**(3): P58-P59.

Robertson, J. D. (1958). "The Ultrastructure of Cell Membranes." Anatomical Record **130**(2): 440-440.

Robertson, J. D. (1960). "The Molecular Structure and Contact Relationships of Cell Membranes." Progress in Biophysics & Molecular Biology **10**: 343-418.

Robinson, M. S. (2004). "Adaptable adaptors for coated vesicles." Trends in Cell Biology **14**(4): 167-174.

Roth, T. F. and K. R. Porter (1964). "Yolk Protein Uptake in Oocyte of Mosquito Aedes Aegypti L." Journal of Cell Biology **20**(2): 313-&.

Rothberg, K. G., J. E. Heuser, W. C. Donzell, Y. S. Ying, J. R. Glenney and R. G. Anderson (1992). "Caveolin, a protein component of caveolae membrane coats." Cell **68**(4): 673-682.

Rousselle, C., P. Clair, J. M. Lefauconnier, M. Kaczorek, J. M. Scherrmann and J. Temsamani (2000). "New advances in the transport of doxorubicin through the blood-brain barrier by a peptide vector-mediated strategy." Molecular Pharmacology **57**(4): 679-686.

Rust, M. J., M. Lakadamyali, F. Zhang and X. W. Zhuang (2004). "Assembly of endocytic machinery around individual influenza viruses during viral entry." Nature Structural & Molecular Biology **11**(6): 567-573.

Salay, L. C., T. M. Nobre, M. C. Colhone, M. E. D. Zaniquelli, P. Ciancaglini, R. G. Stabeli, et al. (2011). "Dermaseptin 01 as antimicrobial peptide with rich biotechnological potential: study of peptide interaction with membranes containing Leishmania amazonensis lipid-rich extract and membrane models." Journal of Peptide Science **17**(10): 700-707.

Santra, S., B. Liesenfeld, D. Dutta, D. Chatel, C. D. Batich, W. H. Tan, et al. (2005). "Folate conjugated fluorescent silica nanoparticles for labeling neoplastic cells." Journal of Nanoscience and Nanotechnology **5**(6): 899-904.

Santra, S., H. Yang, D. Dutta, J. T. Stanley, P. H. Holloway, W. H. Tan, et al. (2004). "TAT conjugated, FITC doped silica nanoparticles for bioimaging applications." Chemical Communications(24): 2810-2811.

Sawant, R. and V. Torchilin (2010). "Intracellular transduction using cell-penetrating peptides." Molecular Biosystems **6**(4): 628-640.

Schneuwly, S., R. Klemenz and W. J. Gehring (1987). "Redesigning the body plan of Drosophila by ectopic expression of the homoeotic gene Antennapedia." Nature **325**(6107): 816-818.

Schneuwly, S., A. Kuroiwa, P. Baumgartner and W. J. Gehring (1986). "Structural organization and sequence of the homeotic gene Antennapedia of Drosophila melanogaster." The EMBO journal **5**(4): 733-739.

Schwarze, S. R., A. Ho, A. Vocero-Akbani and S. F. Dowdy (1999). "In vivo protein transduction: Delivery of a biologically active protein into the mouse." Science **285**(5433): 1569-1572.

Shaner, N. C., R. E. Campbell, P. A. Steinbach, B. N. G. Giepmans, A. E. Palmer and R. Y. Tsien (2004). "Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp red fluorescent protein." Nature Biotechnology **22**(12): 1567-1572.

Sharma, D. K., J. C. Brown, Z. J. Cheng, E. L. Holicky, D. L. Marks and R. E. Pagano (2005). "The glycosphingolipid, lactosylceramide, regulates beta(1)-integrin clustering and endocytosis." Cancer Research **65**(18): 8233-8241.

Shibagaki, N. and M. C. Udey (2002). "Dendritic cells transduced with protein antigens induce cytotoxic lymphocytes and elicit antitumor immunity." Journal of Immunology **168**(5): 2393-2401.

Shin, J. S. and S. N. Abraham (2001). "Caveolae as portals of entry for microbes." Microbes and Infection **3**(9): 755-761.

Shin, J. S. and S. N. Abraham (2001). "Co-option of endocytic functions of cellular caveolae by pathogens." Immunology **102**(1): 2-7.

Simeoni, F., M. C. Morris, F. Heitz and G. Divita (2003). "Insight into the mechanism of the peptide-based gene delivery system MPG: implications for delivery of siRNA into mammalian cells." Nucleic Acids Research **31**(11): 2717-2724.

Singer, S. J. and G. L. Nicolson (1972). "The fluid mosaic model of the structure of cell membranes." Science **175**(4023): 720-731.

Singh, R. D., E. L. Holicky, Z. J. Cheng, S. Y. Kim, C. L. Wheatley, D. L. Marks, et al. (2007). "Inhibition of caveolar uptake, SV40 infection, and beta 1-integrin signaling by a nonnatural glycosphingolipid stereoisomer." Journal of Cell Biology **176**(7): 895-901.

Singh, R. D., V. Puri, J. T. Valiyaveettil, D. L. Marks, R. Bittman and R. E. Pagano (2003). "Selective caveolin-1-dependent endocytosis of glycosphingolipids." Molecular Biology of the Cell **14**(8): 3254-3265.

Sjostrand, F. S. (1953). "Electron Microscopy of Mitochondria and Cytoplasmic Double Membranes." Nature **171**(4340): 30-31.

Swanson, J. A. (1989). "Phorbol Esters Stimulate Macropinocytosis and Solute Flow through Macrophages." Journal of Cell Science **94**: 135-142.

Swanson, J. A. (2008). "Shaping cups into phagosomes and macropinosomes." Nature Reviews Molecular Cell Biology **9**(8): 639-649.

Sweitzer, S. M. and J. E. Hinshaw (1998). "Dynamin undergoes a GTP-dependent conformational change causing vesiculation." Cell **93**(6): 1021-1029.

Takahashi, K. and S. Yamanaka (2006). "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors." Cell **126**(4): 663-676.

Takamori, S., M. Holt, K. Stenius, E. A. Lemke, M. Gronborg, D. Riedel, et al. (2006). "Molecular anatomy of a trafficking organelle." Cell **127**(4): 831-846.

Tauber, A. I. (2003). "Metchnikoff and the phagocytosis theory." Nat Rev Mol Cell Biol **4**(11): 897-901.

Thoren, P. E. G., D. Persson, M. Karlsson and B. Norden (2000). "The Antennapedia peptide penetratin translocates across lipid bilayers - the first direct observation." Febs Letters **482**(3): 265-268.

Torchilin, V. P. (2007). "Tatp-mediated intracellular delivery of pharmaceutical nanocarriers." Biochemical Society Transactions **35**: 816-820.

Torchilin, V. P., T. S. Levchenko, R. Rammohan, N. Volodina, B. Papahadjopoulos-Sternberg and G. G. M. D'Souza (2003). "Cell transfection in vitro and in vivo with nontoxic TAT peptide-liposome-DNA complexes." Proceedings of the National Academy of Sciences of the United States of America **100**(4): 1972-1977.

Trehin, R. and H. P. Merkle (2004). "Chances and pitfalls of cell penetrating peptides for cellular drug delivery." European Journal of Pharmaceutics and Biopharmaceutics **58**(2): 209-223.

Tsai, B., J. M. Gilbert, T. Stehle, W. Lencer, T. L. Benjamin and T. A. Rapoport (2003). "Gangliosides are receptors for murine polyoma virus and SV40." Embo Journal **22**(17): 4346-4355.

Veiga, E. and P. Cossart (2005). "Listeria hijacks the clathrin-dependent endocytic machinery to invade mammalian cells." Nature Cell Biology **7**(9): 894-U846.

Veldhoen, S., S. D. Laufer and T. Restle (2008). "Recent developments in peptide-based nucleic acid delivery." International Journal of Molecular Sciences **9**(7): 1276-1320.

Vendeville, A., F. Rayne, A. Bonhoure, N. Bettache, P. Montcourrier and B. Beaumelle (2004). "HIV-1 Tat enters T cells using coated pits before translocating from acidified endosomes and eliciting biological responses." Molecular Biology of the Cell **15**(5): 2347-2360.

Vercauteren, D., R. E. Vandenbroucke, A. T. Jones, J. Rejman, J. Demeester, S. C. De Smedt, et al. (2010). "The Use of Inhibitors to Study Endocytic Pathways of Gene Carriers: Optimization and Pitfalls." Molecular Therapy **18**(3): 561-569.

Vives, E. (2003). "Cellular uptake of the Tat peptide: an endocytosis mechanism following ionic interactions." Journal of Molecular Recognition **16**(5): 265-271.

Vives, E., P. Brodin and B. Lebleu (1997). "A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus." Journal of Biological Chemistry **272**(25): 16010-16017.

Wadia, J. S., M. Schaller, R. A. Williamson and S. F. Dowdy (2008). "Pathologic prion protein infects cells by lipid-raft dependent macropinocytosis." PLoS One **3**(10): e3314.

Wadia, J. S., R. V. Stan and S. F. Dowdy (2004). "Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis." Nature Medicine **10**(3): 310-315.

Yagi, N., Y. Yano, K. Hatanaka, Y. Yokoyama and H. Okuno (2007). "Synthesis and evaluation of a novel lipid-peptide conjugate for functionalized liposome." Bioorganic & Medicinal Chemistry Letters **17**(9): 2590-2593.

Yesylevskyy, S., S. J. Marrink and A. E. Mark (2009). "Alternative mechanisms for the interaction of the cell-penetrating peptides penetratin and the TAT peptide with lipid bilayers." Biophys J **97**(1): 40-49.

Yoo, H., B. S. Nagaralli and D. S. Chung (2006). "ANYL 164-Folate-PEG conjugated fluorescent silica nanoparticles for labeling cancer cells." Abstracts of Papers of the American Chemical Society **232**.

Zaro, J. L., T. E. Rajapaksa, C. T. Okamoto and W. C. Shen (2006). "Membrane transduction of oligoarginine in HeLa cells is not mediated by macropinocytosis." Molecular Pharmaceutics **3**(2): 181-186.

Zhang, W. Y. and S. O. Smith (2005). "Mechanism of Antp(43-58), penetration of membrane bilayers." Biophysical Journal **88**(1): 141A-141A.

Zhou, H. Y., S. L. Wu, J. Y. Joo, S. Y. Zhu, D. W. Han, T. X. Lin, et al. (2009). "Generation of Induced Pluripotent Stem Cells Using Recombinant Proteins." Cell Stem Cell **4**(5): 381-384.

Zorko, M. and U. Langel (2005). "Cell-penetrating peptides: mechanism and kinetics of cargo delivery." Advanced Drug Delivery Reviews **57**(4): 529-545.

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